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Aspectos relevantes sobre a etiofisiopatologia da candidíase vulvovaginal e ação imunomoduladora de β-glucana

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Orientador: Profª. Drª. Terezinha Inez Estivalet Svidzinski

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Bonfim-Mendonça, P.S.; **Aspectos relevantes sobre a etiofisiopatologia da candidíase vulvovaginal e ação imunomoduladora de β-glucana**. 2014, Tese (doutorado) – Universidade Estadual de Maringá.

A alta incidência da candidíase vulvovaginal (CVV), e a respectiva importância clínica dessa doença, vêm impulsionando pesquisas que visam esclarecer os mecanismos de patogenicidade e identificação dos fatores de virulência de *Candida* spp. Entretanto, poucos estudos tem sido objetivados no âmbito da avaliação interação fungo-hospedeiro, mecanismos protetores envolvidos (modulação da resposta imune) ou fatores associados à susceptibilidade à infecção, principalmente nas pacientes com CVVR (candidíase vulvovaginal recorrente). Portanto, a proposta deste trabalho foi avaliar aspectos relevantes sobre a etiofisiopatologia da candidíase vulvovaginal e ação imunomoduladora de β-glucana. Como modelo experimental usamos isolados clínicos de *C. albicans* e *C. glabrata* oriundos de mulheres com diferentes sintomatologias para CVV: ASS (sem sintomas), CVV (um único episódio) e CVVR (vários episódios/ano). O resumo dos nossos resultados mostraram que o isolado CVVR de *C. albicans* provocou alterações bioquímicas na resposta inflamatória desencadeada por neutrófilos, especialmente na produção de espécies reativas de oxigênio e nitrogênio levando a susceptibilidade à doença. Por outro lado, os isolados de *C. glabrata* não apresentaram tais respostas, mostrando susceptibilidade a ação dos neutrófilos. Neste contexto, observamos a habilidade da β-glucana, extraída de *Laminarina digitata*, em imunomodular a resposta de neutrófilos. β-glucana aumentou significativamente a produção de espécies oxidantes (EROs), e modulou a liberação de citocinas, sugerindo que este polissacarídeo pode ser um imunomodulador eficaz da resposta microbicida de neutrófilos para ambas as espécies isoladas de CVV. Conhecendo o comportamento diferenciado dos isolados clínicos de *C. albicans,* estudamos fatores relacionados à adesão as células cervicais humanas (*HeLa*) e a expressão de genes importantes neste processo, e.g. *ALS* (*agglutinin-like sequence*) e *SAP* (secreted aspartyl proteinas). Observamos que apesar do isolado ASS, ter maior capacidade de adesão ás células *HeLa*, houve menor expressão de genes relacionados à patogenicidade. Por outro lado, os isolados CVV e CVVR tiveram menor potencial de adesão, mas foram capazes de aumentar significativamente a regulação de genes importantes no processo de infecção. Com base nestes resultados podemos salientar a relação fungo-hospedeiro, principalmente a que ocorre nos casos de recorrência da CVV, além de sugerir β-glucana como um potencial imunomodulador para a resposta da CVVR.

ABSTRACT

Bonfim-Mendonça, P.S.; **Relevant aspects of the etiopathophysiology of vulvovaginal candidiasis and immunomodulatory action of β-glucan**. 2014, Tese (doutorado) – Universidade Estadual de Maringá.

The high incidence of vulvovaginal candidiasis (VVC), and the clinical relevance of this disease, have been driving research studies to explain the mechanisms of pathogenicity and identification of virulence factors of *Candida* spp. However, few studies have been targeted in the evaluation of the fungus-host interaction, protective mechanisms involved (modulation of the immune response) or factors associated with susceptibility to infection, particularly in patients with CVVR (recurrent vulvovaginal candidiasis). Therefore, the aim of this study was to evaluate relevant aspects about the etiopathophysiology of vulvovaginal candidiasis and immunomodulatory action of β-glucan. Experimental model used was clinical isolates of *C. albicans* and *C. glabrata* originating from women with different symptomatology for VVC: ASS (no symptoms), VVC (one episode) and RVVC (several episodes/year). The summary of our results showed that, the isolated RVVC of the *C. albicans* caused biochemical changes in the inflammatory response triggered by neutrophils, especially in the production of reactive oxygen and nitrogen species leading to disease susceptibility. On the other hand, isolates of *C. glabrata* did not show such responses, demonstrating susceptibility to the action of neutrophils. In this context, we note the ability of β-glucan extracted from laminarin digitata in immunomodulate the neutrophil response. β-glucan significantly increased the production of oxidant species, and modulated the release of cytokines, suggesting that this polysaccharide may be a immunomodulatory effective of microbicidal response of the neutrophils. Knowing the different behavior of the clinical isolates of *C. albicans*, we studied factors related to adherence to human cervical cells (HeLa) and the expression of important genes in this process, eg ALS (agglutinin-like sequence) and SAP (secreted aspartyl proteins). Observed that despite the isolated ASS, have greater adhesion capacity on *HeLa* cells, a down-regulation genes related with pathogenicity. On the other hand, the VVC and RVVC isolates had lowerest potential for adhesion, but were able to significantly up-regulation important genes in the infection process. Based on these results we can highlight the relationship fungal-host, which occurs mainly in cases of recurrent VVC and suggests β-glucan as an potential immunomodulatory for RVVC response.

CAPÍTULO I

Este capítulo tem por objetivo contextualizar os pontos que serão discutidos nos trabalhos que compõe a tese.

REVISÃO DA LITERATURA

1. Candidíase Vulvovaginal

Candidíase vulvovaginal (CVV) é uma infecção da vulva e da vagina, causada por várias espécies do gênero *Candida* (Sobel, 1988). Apesar disso, é amplamente conhecido que a espécie *Candida albicans* é o principal agente etiológico da CVV, representando 70-80% dos casos, enquanto que 10 a 30% deles são causadas por espécies *Candida* não-*Candida albicans* (CNCA), tais como: *C*. *glabrata*, *C*. *tropicalis* e *C*. *parapsilosis* (Dan et al., 2002; Grigoriou et al., 2006; Hamad et al., 2014; Kennedy and Sobel, 2010).

A CVV é considerada uma das formas mais comum de infecção fúngica e a transformação da condição assintomática para a sintomática indica uma transição da forma comensal para a forma patógena (vaginite sintomática), devido a determinadas condições que alteram o ambiente vaginal (Calderone and Clancy, 2002; Taguti Irie et al., 2006). Neste sentido, tanto a diminuição na resposta do sistema imune do hospedeiro quanto o aumento na virulência do fungo podem desencadear a doença (Jaeger et al., 2013).

A literatura mostra que as condições associadas ao hospedeiro está muitas vezes relacionadas à imunossupressão, diabetes mellitus, gravidez, uso de antibióticos, contraceptivos orais, entre outros (Foxman et al., 2013; Kennedy and Sobel, 2010; Rosa et al., 2013; Sobel, 1992; Spinillo et al., 1999). Na ausência destes fatores, observações clínicas mostram que CVV ocorre predominantemente durante a fase lútea do ciclo menstrual, quando os níveis de progesterona e estrogênio são elevados. Apesar dessas descrições sobre o desenvolvimento da CVV, ainda não há um consenso sobre a etiofisiopatologia desta doença.

Clinicamente a CVV se caracteriza por prurido intenso, dispaneuria, disúria, eliminação de corrimento vaginal em grumos, semelhante a nata de leite, eritema e edema vulvovaginal. E a ausência de sintomas pode estar relacionada com a presença de espécies CNCA (Fidel et al., 2004; Sobel, 1996; Sobel et al., 1990). Além disso, as

lesões podem se estender pelo períneo, região perianal e inguinal (Almeida Filho et al., 1995).

Episódios repetidos de infecção por *Candida* spp. são observados na candidíase vulvovaginal recorrente (CVVR) que é caracterizada pela ocorrência de, no mínimo, três a quatro episódios de vaginites no período de um ano (Patel et al., 2004). Dentre as mais de 180 espécies de *Candida* descritas, *C*. *albicans* é responsável por 90% das infecções em pacientes com CVVR (Calderone and Clancy, 2002; Sobel, 2002).

1.1 Fatores relacionados ao hospedeiro

A transição de *Candida* spp. de comensal para patógeno consiste de uma linha tênue e que é atribuível a um extenso repertório de determinantes de virulência seletivamente expressos em condições predisponentes adequadas.

A integridade da pele e das mucosas é considerada de grande relevância na defesa do hospedeiro. Todas as circunstâncias que alteram a superfície da pele ou de mucosas por traumatismo, maceração e oclusão, favorecem a aderência do fungo e sua invasão no tecido, propiciando assim a instalação da infecção (Urizar, 2002).

O papel da imunidade humoral na proteção contra infecções fúngicas em mucosas e sistêmicas permanece controverso, pois há pacientes com infecções fúngicas e níveis elevados de anticorpos anti-*Candida* no soro e mucosas, e por outro lado, pacientes com níveis normais desses anticorpos. Foi demonstrado ainda resposta acentuada de IgA, IgG1 e IgG4 anti-*C*. *albicans* no lavado vaginal de mulheres sintomáticas com cultura positiva, sugerindo importante papel desses anticorpos na resposta imune local estimulada pela presença do fungo (Fidel and Sobel, 1996).

Alguns autores afirmam que a imunidade mediada por células é considerada o mecanismo de defesa do hospedeiro predominante contra infecções causadas por essa espécies de *Candida*. Modelos experimentais tem mostrado a imunidade celular no controle da infecção causada por *C*. *albicans*, nos quais a dicotomia da resposta imune do tipo CD4+ Th1 e CD4+ Th2 é considerada um fator importante para a suscetibilidade ou resistência à infecção por *Candida* (Lilic et al., 1996; Puccetti et al., 1994). Além disso, estudos demonstram que a presença de substâncias alergênicas no lúmen vaginal e seu transporte pelos canais interepiteliais propiciam a produção de IgE

específica (Taylor et al., 2000; Witkin et al., 1989; Wormley et al., 2001). Por outro lado, algumas pesquisas sugerem que ocorra alguma forma de imunorregulação, que iniba a resposta mediada por células local ou sistêmica contra a candidíase vaginal, com destaque para os episódios de recorrência (Taylor et al., 2000; Wormley et al., 2001). Devido à alta incidência de CVVR em pacientes sem nenhuma condição predisponente acredita-se que esta infecção também esteja relacionada com um processo de depressão da resposta imune na mucosa local, o que causaria certa "tolerância" da mucosa ao fungo (Amouri et al., 2013; Mårdh et al., 2002). A patogênese da CVVR em mulheres que não apresentam fatores de risco, que são a grande maioria, está sob investigação. Acredita-se ainda que a ausência de uma completa erradicação por um tratamento inadequado ou pela existência de cepas resistentes, pode causar uma recidiva (Ziarrusta, 2002).

Assim, mecanismos mediados por imunidade celular e humoral constituem uma eficiente proteção contra infecções por fungos do gênero *Candida* (Richardson and Warnock, 1994). O papel da atividade de neutrófilos contra infecções sistêmicas pode ser evidenciado pelo aumento de infecções disseminadas em pacientes submetidos à quimioterapia (Merz, 1990). A defesa do hospedeiro com relação a neutrófilos e macrófagos é realizada através da ação microbicida destas células (Greenfield, 1992). A imunidade humoral é efetuada pela formação de anticorpos, que associados ao sistema complemento, atuam como opsonizadores para as células fagocitárias ou impedem a aderência do microrganismo às células IgA secretoras (Odds, 1988; Samaranayake and Macfarlane, 1990).

1.1.1 Atividade microbicida de neutrófilos.

A atividade fagocítica de neutrófilos e células mononucleares é considerada um mecanismo importante de proteção do hospedeiro contra candidíase. São células abundantes na circulação sanguínea e são as primeiras células a migrar para o sítio inflamatório. Estas células apresentam um papel central na defesa do hospedeiro durante o processo inflamatório porém, podem também contribuir para o dano celular sendo responsáveis por exemplo, pela destruição de tecidos (Fang, 2004; Fradin et al., 2005; Segal, 2005). A atuação bactericida ou fungicida de neutrófilos, dependerá da ação de enzimas proteolíticas liberadas de grânulos citoplasmáticos como mieloperoxidase

(MPO) e de espécies reativas de oxigênio (EROs) produzidas durante o *burst oxidativo*, além da liberação de citocinas. Apesar disso, *C*. *albicans* tem se mostrado capaz de inibir a explosão oxidativa em uma variedade de fagócitos murinos e humanos (Wellington et al., 2009), e tal inibição pode representar um mecanismo de evasão da resposta imune.

Os neutrófilos quando recrutados para o local da lesão apresentam a capacidade de englobar micro-organismos e/ou fragmentos do tecido danificado por um processo denominado fagocitose. Neste processo, é formada uma vesícula denominada fagossomo, que quando se une a outras vesículas contendo grânulos digestivos passa a ser chamada de fagolisossomo permitindo a ativação do sistema fosfato de dinucleotídeo nicotinamida e adenina (NADPH) oxidase (Luerman et al., 2010).

O sistema NADPH oxidase é parte do arsenal microbicida de células fagocíticas e é composto por frações proteicas distintas (Cross and Segal, 2004; Groemping and Rittinger, 2005; Nauseef, 2004; Robinson, 2009). Na vigência de um processo infeccioso e/ou inflamatório o sistema NADPH oxidase é ativado através da translocação das proteínas citosólicas para a membrana do fagossomo. Esta ativação é regulada por diversos fatores sinalizadores.

A produção de EROs pelo sistema NADPH oxidase foi por muito tempo considerada a primeira linha de defesa dos neutrófilos. No entanto, sabe-se hoje que esse sistema é responsável também pelo influxo de íons para o vacúolo fagocítico o que desencadeia a ativação de enzimas lisossomais. Esses dados colaboram com o fato de que a atividade microbicida de neutrófilos é um fenômeno complexo, não dependente apenas de EROs (Segal, 2005).

1.2 Fatores relacionados as espécies de *Candida* spp.

Embora o sistema imune do hospedeiro seja o principal fator responsável pelo equilíbrio entre a transição de comensalismo para a patogenicidade, vários atributos de virulência, como habilidade de transição morfológica (formação de pseudo-hifas), hidrofobicidade de superfície celular, capacidade em aderência à superfície bióticas e abióticcas, formação de biofilme, produção de enzimas extracelulares (Consolaro et al., 2005; Fang, 2004; Seneviratne et al., 2008; Taguti Irie et al., 2006) têm sido sugeridos

como importantes fatores de virulência e podem contribuir para a persistência da colonização e do desenvolvimento de infecções fúngicas.

1.2.1 Adesinas

A aderência das leveduras na mucosa vaginal é reconhecida como um passo essencial para colonização microbiana e um evento chave no início do processo patogênico. Já é bem descrito que as diferentes espécies de *Candida* possuem tal atributo e que este contribui para persistência da levedura no hospedeiro, sendo fator fundamental para desenvolvimento da CVV (Kamai et al., 2002; Liu and Filler, 2011).

Os mecanismos de aderência dessa levedura aos tecidos humanos são variados e ocorrem como resultado do reconhecimento celular de sistemas *Candida*-hospedeiro, que são extremamente complexos e envolvem uma variedade de fatores (Hoyer, 2001; Hoyer et al., 2008).

A interação primária fungo-hospedeiro ocorre através da parede celular. O processo de adesão ao hospedeiro, a evasão do sistema imune e a integridade estrutural das proteínas envolvidas constituem fatores relavantes para a infecção (Hiller et al., 2011). Entre eles, a família de genes *ALS* (agglutinin-like sequence) de *C*. *albicans* codifica glicoproteínas da parede celular, algumas das quais estão diretamente envolvidas na adesão a superfícies do hospedeiro e formação de biofilme (Chaffin et al., 1998; Hoyer et al., 2008; Kamai et al., 2002). As proteínas Als são ancoradas a proteínas glicosilfosfatidilinositol (GPI) e sua função é mediar a adesão entre células e substratos do hospedeiro.

Estas adesinas são codificadas por uma família de genes *ALS* composta de oito integrantes distintos no genoma de *C*. *albicans*. O papel dessas proteínas na adesão não parece ser o mesmo para todas, já que a expressão heteróloga e a deleção de genes *ALS*, resultam em padrões singulares de adesão a diferentes superfícies. Dentre todas as proteínas, Als1, Als3, Als5 têm sido extensivamente caracterizadas e os estudos sugerem que estas proteínas exercem um papel importante na aderência de *C*. *albicans* às células do hospedeiro. Als3 é uma das principais enzimas desta família, sendo normalmente detectada *in vivo* em e *in vitro,* em estudos com diferentes epitélios (Alves et al., 2014; Aoki et al., 2012; Liu and Filler, 2011). A expressão desta proteína parece

estar associada à processos de adesão e formação de tubo germinativo, sendo importante nos processos infecciosos (Coleman et al., 2010). Além disso, tem sido considerada uma invasina fúngica que imita caderinas de células hospedeiras, induzindo a endocitose ligando à N-caderina nas células endoteliais (Phan et al., 2007), e também um receptor de ferritina, possibilitando assim a exploração do ferro da ferritina através da ligação morfológica dependente de Als3, sugerindo então que essa proteína pode ter atributos de virulência múltiplos (Almeida et al., 2008). Outras proteínas também são conhecidas proteínas específicas relacionadas à adesão de *C. albicans* ao epitélio, o que demonstra a eficiência deste fungo em colonizar superfícies do hospedeiro (Hiller et al., 2011; Naglik et al., 2011).

O gene da adesina de *C. albicans HWP1 (hyphal wall protein 1),* também faz parte do importante quadro de proteínas aderentes, e tem destaque com o desenvolvimento de tubo germinativo, regulando fortes ligações aos diferentes epitélios (Nobile et al., 2006). Alguns estudos mostram que, esta proteína tem uma participação essencial para a formação de biofilme, inclusive *in vivo*. Além disso, já foi demostrado através do uso de mutantes que, a ausência desta proteína faz com que tenha-se uma fraça ligação à células epiteliais e apresentam uma redução significativa na virulência da candidíase. (Nobile et al., 2006; Staab et al., 1999).

1.2.2 Proteinases

Atualmente, as proteases secretadas por fungos do gênero *Candida* têm sido consideradas um importante fator de virulência. A correlação de virulência e atividade de proteásica pode ser demonstrada através do conhecimento que isolados de *Candida* fortemente proteolíticos mostram maior aderência às células do epitélio bucal e às células endoteliais quando comparadas às cepas menos proteolíticas (Ghannoum and Abu Elteen, 1986; Hoegl et al., 1996; Klotz et al., 1983; Kumar et al., 2006).

As aspartil proteinases secretadas (*SAP*) são enzimas hidrolíticas que contribuem diretamente para virulência de *C. albicans*. Possui pelo menos 10 membros da família de genes *SAP*, designados de *SAP-1-10*, todos já sequenciados e bem caracterizados. As proteinases de um modo geral estão envolvidas em vários processos (Davies 1990), que incluem formação de pseudohifas, aderência e fenômeno “switching”, que contribuem

ainda mais para a patogenicidade das leveduras (Bektić et al., 2001; Naglik et al., 2003b).

A atividade de proteinase no gênero *Candida* está envolvida entre outros fatores com as proteínas geradas pela expressão dos genes de *SAP*, as quais estão implicadas na patogênese dos fungos (Hube, 1996; Naglik et al., 2004). As proteinases são capazes de degradar proteínas como albumina, hemoglobina, queratina, colágeno, mucina, lactoferrina, lactoperoxidase e imunoglobulinas como as da classe IgA (da Costa et al., 2009; Naglik et al., 2003a; Naglik et al., 2003c).

A atividade de proteinase de *Candida* está associada a família de isoenzimas Sap com pesos moleculares entre 35 e 50 kDa, codificadas pelos genes *SAP1-10* que aumentam a capacidade do microrganismo de colonizar e penetrar nos tecidos do hospedeiro e evadir da resposta imune (Naglik et al., 2003a). As Saps 1-3 estão associadas às células na forma de leveduras, têm atividade ótima em pH 3-5. As Saps 4-6 estão associadas à formação de hifas, têm atividade em pH 5-7. Dessa maneira pode-se dizer que as Saps com atividade em intervalos de pH entre 3 e 7 são importantes para a sobrevivência e infecção de espécies de *Candida* dentro do hospedeiro (Monod et al., 1998; Ribeiro et al., 2004).

Estudos prévios criaram um modelo hipotético que correlaciona a expressão do gene *SAP* com a patogenicidade de espécies de *Candida* (Naglik et al., 2004). A aderência dessas leveduras nas células epiteliais da mucosa bucal assim como, a invasão e danos aos tecidos do hospedeiro os quais contribuem para o desenvolvimento de infecções sistêmicas, ocorrem pela secreção das Saps 1 e 3. A habilidade de formar lesões na pele está relacionada à secreção da Sap 1, já as interações com o sistema imune do hospedeiro, ocorrem quando a Sap 2 é secretada, pois esta proteinase é responsável pela hidrólise das proteínas do sistema imune. No entanto, para que esse microrganismo escape da fagocitose dos macrófagos e consiga sobreviver é necessário a expressão do gene *SAP4*, que também está ligada à infecções sistêmicas junto com a secreção da Sap 6. A expressão dos genes *SAP*s 4, 5 e 6 foi induzida em macrófagos de murino após fagocitose de células de *Candida* (Borg-von Zepelin et al., 1998). O dimorfismo apresentado por *C. albicans* é conferido pelas Saps 4, 5 e 6. Nos biofilmes formados por culturas de *Candida* verifica-se uma alta secreção das Saps 5, 6 e 9

(Naglik et al., 2003c). Pouco se sabe sobre a relação das Saps, 8 e 10 com a patogenicidade em *Candida*. Genes da família *SAP* podem ser expressos em vários estágios do processo de infecção e Saps específicas tem papel especial neste processo (Naglik et al., 2004). Os genes que codificam para proteinase mais comumente expressa na cavidade bucal de indivíduos carreadores de *Candida* são *SAP* 2 e *SAP*s 4-6, enquanto que na candidíase bucal são expressos os genes de *SAP* 1 e *SAP* 3 (Samaranayake et al., 2002).

2. β-glucana

Os polissacarídeos presentes na parede celular fúngica apresentam a capacidade de alterar a resposta imunológica, sendo assim denominados imunomoduladores. Entre os polissacarídeos mais estudados estão as β-glucanas, tanto particuladas quanto as solúveis em água, as quais podem ser obtidas de diversas espécies de plantas e micro-organismos (Wakshull et al., 1999).

Há mais de 50 anos estuda-se estes produtos com atividade imunomodulatória derivados da parede celular da levedura *Saccharomyces cerevisiae* denominado Zimosan (constituído de proteínas, lipídios e polissacarideos), sendo que na década de 60 foi relatada que a substância ativa deste produto era o polissacarídeo β-D-glucana (Brown and Gordon, 2003; Brown and Gordon, 2005).

Ainda não está completamente elucidada qual a estrutura básica necessária para o desencadeamento da atividade biológica das glucanas. Sabe-se que as características físico-químicas, entre elas solubilidade, peso molecular, conformação espacial e quantidade de ramificações β(1-6) presente na partícula utilizada, podem influenciar no tipo de estimulação (Brown and Gordon, 2003). Assim, as glucanas com altos pesos moleculares e insolúveis, estimulam de forma eficaz o sistema imune, porém podem causar diversos efeitos colaterais como formação de abcessos, asma e em casos extremos toxicidade letal quando associada a determinados fármacos anti-inflamatórios.

As glucanas são reconhecidas pelo sistema imune do hospedeiro através de receptores de superfície, que auxiliam no reconhecimento e controle de patógenos

fúngicos. Porém essa ativação do sistema imune é prejudicada pela camada externa da parede celular composta por mananas. Os receptores para D-manose, por sua vez, não são expressos em monócitos circulantes e a sua expressão em macrófagos é regulada por glicocorticóides que aumentam sua expressão e pela liberação de interferon gama (IFN-γ) que age de forma inversa diminuindo sua expressão. Várias células dos mamíferos possuem receptores capazes de reconhecer e interagir com a β-glucana principalmente leucócitos, incluindo macrófagos, monócitos, neutrófilos e Natural Killer (NK), assim como células endoteliais, e os fibroblastos (Brown, 2006; Brown and Gordon, 2005; Roeder et al., 2004). Os receptores de β-glucanas conhecidos são CR3, lactosilceramida e dectin-1 sendo que destes o último parece ter um papel essencial na ativação da defesa do hospedeiro. O receptor de complemento CR3 é expresso em células mielóides, células NK, e em alguns linfócitos, tem função de adesão celular e de estímulo a fagocitose em partículas de iC3b (molécula do sistema complemento) opsonizadas e não opsonizadas. Lactosilceramida é um glicoesfingolipideo encontrado em muitas células entre elas os linfócitos. O receptor dectin-1 é considerado o de maior importância na ativação das células imunes, possui dois ligantes um para polissacarídeos exógenos e outro co-estimulatório de células T. Estes receptores localizam-se nas células dendríticas, células de Langerhans da pele, monócitos/macrófagos e neutrófilos (Adachi et al., 2004; Brown and Gordon, 2005; Taylor et al., 2000).

Dentre os diferentes tipos de β-glucanas, destaca-se a Laminarina (LAM), polissacarídeo extraído de algas do gênero *Laminaria*. A estrutura molecular da LAM varia dependendo da espécie de *Laminaria* spp. a qual foi extraído. No entanto, é principalmente um polissacarídeo linear constituído por 25 a 50 unidades de glicose conectadas por ligações β-1,3-glicosídica e, em alguns casos tendo ligações β-1,6-glicosídicas e ramificações na posição O-6. Neste contexto, LAM derivada de *Laminaria digitata* são solúveis em água e contêm um menor número de conexões β-(1-6) nas cadeias laterais. Estes polissacarídeos apresenta-se como imunoestimulantes, afetando tanto a imunidade natural e adaptativa (Akramiene et al., 2006; Fuentes et al., 2011; Sweeney et al., 2012). Várias são as investigações sobre a bio-atividade de LAM, e estas incluem estudos *in vivo* e *in vitro* com a avaliação de citocinas pro e anti-inflamatórios, produção de vacinas, inibição da proliferação celular e atividade anti e

oxidante (Bonfim-Mendonça et al., 2014; Neyrinck et al., 2007; Peng et al., 2012; Ryan et al., 2012; Smith et al., 2011; Sweeney et al., 2012)

Justificativa

A CVV é uma patologia que acomete mulheres em idade reprodutiva, causada principalmente por *C. albicans* e *C. glabrata*, sendo um diagnóstico cada vez mais frequente na prática clínica. A alta incidência faz com que seja a segunda infecção genital mais frequente nos Estados Unidos e no Brasil. Na Europa, é a primeira causa de vulvovaginite (Foxman et al., 2000; Foxman et al., 2013; Ziarrusta, 2002). Representa em torno de 1/4 dos corrimentos vaginais de natureza infecciosa, precedida apenas pela vaginose bacteriana (Foxman et al., 2013).

O caráter recorrente da CVV pode estar ligado a aumento da virulência do fungo ou diminuição da resposta imune do hospedeiro, e ainda à terapêutica prolongada (Fidel and Sobel, 1996; Mårdh et al., 2002; Rosa et al., 2013). No entanto, existem dados mostrando que a CVVR também pode acometer mulheres sem nenhuma condição predisponente.

Assim, apesar da importância clínica da infecção causada por *Candida* spp., poucos estudos tem sido realizados para avaliação da interação hospedeiro-fungo, dos mecanismos protetores envolvidos ou fatores associados à susceptibilidade à infecção, principalmente nas pacientes com CVVR. E além disso, não se conhece estudos com a aplicação de β-glucana como modulador da resposta neste contexto.

Objetivo

Portanto, nossa proposta neste trabalho foi avaliar aspectos relevantes sobre a etiofisiopatologia da candidíase vulvovaginal e ação imunomoduladora de β-glucana.

Neste contexto, avaliamos alterações na relação celular levedura-hospedeiro, buscando fatores envolvidos nesta resposta. Como os atributos de virulência dos diferentes isolados clínicos e a resposta imune conferida pelas células de defesa

imunológica. E por fim, a participação da β-glucana como possível moduladora nesta resposta celular.

Objetivos específicos

1. Avaliar a capacidade microbicida dos neutrófilos frente a diferentes isolados de *C*. *albicans* e *C*. *glabrata* (episódios recorrentes, único episódio e assintomática) e cepas padrão de *C*. *albicans* ATCC 90028 e *C*. *glabrata* ATCC 90030, antes e após à exposição à β-glucana.
2. Avaliar a fagocitose dos neutrófilos ativados com diferentes isolados de *C*. *albicans* e *C*. *glabrata* (episódios recorrentes, único episódio e assintomática) e cepas padrão de *C*. *albicans* ATCC 90028 e *C*. *glabrata* ATCC 90030, antes e após à exposição à β-glucana.
3. Avaliar a produção de HOCl por neutrófilos ativados com diferentes isolados de *C*. *albicans* e *C*. *glabrata* (episódios recorrentes, único episódio e assintomática) e cepas padrão de *C*. *albicans* ATCC 90028 e *C*. *glabrata* ATCC 90030, antes e após à exposição à β-glucana.
4. Detectar a produção total de espécies oxidantes intracelulares de neutrófilos ativados com diferentes isolados de *C*. *albicans* e *C*. *glabrata* (episódios recorrentes, único episódio e assintomática) e cepas padrão de *C*. *albicans* ATCC 90028 e *C*. *glabrata* ATCC 90030, antes e após à exposição à β-glucana.
5. Avaliar a atividade de mieloperoxidase (MPO) de neutrófilos ativados com diferentes isolados de *C*. *albicans* e *C*. *glabrata* (episódios recorrentes, único episódio e assintomática) e cepas padrão de *C*. *albicans* ATCC 90028 e *C*. *glabrata* ATCC 90030, antes e após à exposição à β-glucana.
6. Avaliar a liberação de citocinas por neutrófilos ativados com diferentes isolados de *C*. *albicans* e *C*. *glabrata* (episódios recorrentes, único episódio e assintomática) e cepas padrão de *C*. *albicans* ATCC 90028 e *C*. *glabrata* ATCC 90030, antes e após à exposição à β-glucana.
7. Avaliar a taxa de adesão de diferentes isolados de *C*. *albicans* (episódios recorrentes, único episódio e assintomática) e cepa padrão *C*. *albicans* ATCC 90028,

em células humanas de carcinoma cervical (*HeLa)* durante diferentes tempos (2 e 6 horas).

8. Avaliar a expressão de genes da família *ALS (agglutinin-like sequence)* e *HWP1 (hyphal wall protein 1)*, após a adesão em células cervicais (2 e 6 horas) de diferentes isolados de *C*. *albicans* (episódios recorrentes, único episódio e assintomática) e cepa padrão *C*. *albicans* ATCC 90028.

9. Avaliar a expressão de genes da família *SAP* (secreted aspartyl proteinas), após adesão em células cervicais (2 e 6 horas) de diferentes isolados de *C*. *albicans* (episódios recorrentes, único episódio e assintomática) e cepa padrão *C*. *albicans* ATCC 90028.

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CAPÍTULO II

Este capítulo tem por objetivo apresentar os artigos publicados e aqueles a serem submetidos.

Artigo 1: Potential applications of β-glucans from *Laminaria* spp. involving immunologic and apoptosis modulation.
a ser submetido para a Revista Current Opinion in Immunology.

Potential applications of β-glucans from *Laminaria* spp involving immunologic and apoptosis modulation.

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ABSTRACT

Glucans are a heterogeneous group of glucose polymers, present in bacteria, algae, fungi and plants. The benefits of glucan are well known, however, there is considerable variation in the biochemical and solubility characteristics and applications of glucans obtained from different sources, and little is known about the potential of β-glucans extracted from the genus *Laminaria,* which is denominated Laminarin (LAM). Research on the bioactivity of LAM has been reported, including *in vivo* and *in vitro* studies on evaluation of pro and anti-inflammatory cytokines, vaccine production, inhibition of cell proliferation and anti and oxidant activity. So, the objective of this review is to revise the potential applications of β-glucans from *Laminaria* spp., highlighting immune modulating properties, vaccine development and activity anti-cancer.

INTRODUCTION

Glucans are a heterogeneous group of glucose polymers, distributed in bacteria, algae, fungi and plants, which are involved in cell wall structure and other biological functions. They are classified by their interchain linkage as being either α-or β-linked. The common structure comprising a main chain of β-(1,3)- and/or β-(1,4)-D-glucopyranosyl unit in non-repeating buth non-random order, along with side chains of varying lengths [1,2]. There is considerable variation in the biochemical and solubility characteristics of β-glucans from different sources [3].

The glucans extracted from seaweed of the genus *Laminaria* (denominated of the Laminarin or Laminaran) seem to be a potent immunostimulant, affecting both natural and adaptive immunity [4-7]. The molecular structure of Laminarin (LAM) varies depending on the species which *Laminaria* spp was extracted. However, it is mainly a linear polysaccharide constituted by 25 to 50 glucose units linked by β-1,3-glycosidic bonds and in some cases having β-1,6-glycosidic bonds and ramifications in o-6 position (**Figure 1**)**.** Thus, LAM derived from *Laminaria digitata* are water soluble and contain small numbers of β-(1 → 6)-linked side chains. In contrast, LAM derived from *Laminaria hyperborea* are water insoluble and only contain linear β-(1 → 3)-linked residues [1]. LAM have an average molecular weight of 5000 kDa (3400 to 7700 kDa) but they can differ in the terminal reducing end corresponding to a glucose residue in G-type laminarin and to a mannitol residue in M-type laminarin [2,8].

Research on the bioactivity of this group of glucans, has been reported both *in vivo* and *in vitro* studies, as evaluation of pro- and anti-inflammatory cytokines [9-14], anti oxidant activity [11,15], inhibition of cell proliferation [16], and vaccine production [17-19]. So, the objective of this review is to give an overview of the potential applications of β-glucans extracted from *Laminaria* spp., highlighting immune modulating properties, activity anti-cancer and the vaccine development.

IMMUNE MODULATING PROPERTIES

The use of immunomodulators for enhancing host defence responses has been considered the most promising alternatives. Recent researches have shown that LAM from *Laminaria* spp. has significant immunostimulatory activity, both *in vivo* and *in vitro*. *Laminaria digitata* followed by *L. japonica* and *L. hyperborea* are the species

most used in this context. Thus, *Laminarin* species that are most studied in this review and their main characteristics are listed in **Table 1**.

LAM vary in their structure and chemical composition which may modulate their effect on animal performance and gastrointestinal health [13]. Thereby, modulation of immune function by LAM has been promising in the field of nutrition [20].

Smith et al. (2011) [12] working with LAM from *L. digitata* in the diet of pigs, analyzed the cytokine expression in both unchallenged and bacterial lipopolysaccharide (LPS)-challenged ileal and colonic tissues. In the ileum no effect was observed, however, significant effect was observed for IL-6 and IL-8 gene expression in the colon of LPS challenged tissue. The potential benefit of this gene up-regulation of IL-6 and IL-8 cytokines for the host is to be pro-inflammatory cytokines, that plays an important role in acute inflammation in the early immune response [21]. In addition, IL-8 is responsible for neutrophil recruitment and activation to the initial site of infection [22]. These data are of biological interest, since they support the concept of collaborative signalling between TLR and non-TLR pattern recognition receptors in immune stimulation [23]. LAM bind to mammalian non-Toll-like receptor (TLR) pattern-recognition receptors such as dectin-1, complement receptor 3, lactosylceramide and scavenger receptors, thereby stimulating innate immunity through the activation of macrophages, dendritic cells, neutrophils, natural killer cells and helper T-cells [24]. The activation/proliferation of these cells results in enhanced phagocytosis and oxidative burst, cytokine production, activation of the alternative complement pathway and release of lysosomal enzymes [25].

Sweeney et al., (2012) [13] worked comparing the gene expression profiles of the markers of pro- and anti-inflammation in both unchallenged and lipopolysaccharide (LPS)- challenged ileal and colonic tissues, with supplementation diet with LAM derived from *L. digitata*, *L. hyperborea* and β-glucan of the *S. cerevisiae*. The results showed that the expression of a number of pro-inflammatory cytokine genes (IL-1a, IL-10, TNF-a and IL-17A) was down-regulated in the colon of pigs exposure to β-glucans from all the three sources. Seaweed-derived polysaccharides are resistant to hydrolysis by digestive enzymes in the upper gastrointestinal tract, which may explain the lower expression of the cytokine markers in the ileum compared with the colon of supplemented pigs. There was an increase in IL-8 gene expression in the gastrointestinal

tract from the animals exposed to *L. digitata* following an LPS ex vivo challenge (in accordande with Smith et al. (2011) [12]), that was not evident in the other two treatment groups. The authors suggest that inclusion of LAM from *L. digitata* in the diet could enhance the pro-inflammatory response to a microbial challenge as far as can be extrapolated from an LPS challenge.

The combined use of the extracts of different species of *Laminarin* also are being tested, as were recently reported use of the extracts containing both LAM and Fucoidan (FUC, sulfated polysaccharides isolated from *L. japonica*). These laminarins are water-soluble polysaccharides of brown algae, and have been explored as a novel source of bioactive compounds containing immunomodulatory and antimicrobial properties [26]. Walsh et al. (2013) [27] designed a factorial arrangement, involving the isolated use of LAM, FUC and the interaction between these in different concentrations (LAM between 0 and 300 parts per million (ppm) and FUC (0 and 240 ppm). The results showed the expression of a panel of pro- and anti-inflammatory cytokines (IL-6, IL-17A, IL-1β and IL-10) was down-regulated in the colon of pigs exposure to LAM isolated. The same results were found for FUC alone. However, there was a significant interaction between LAM and FUC supplementation on IL-10 mRNA expression. These results are important because anti-inflammatory cytokines such as IL-10 play an important role in maintaining the balance between pro- and anti-inflammatory mediators [28].

An unregulated Th17 inflammatory response has been highlighted as a major contributor to the underlying pathology of inflammatory bowel diseases (IBD) whereas regulatory T (T_{REG}) cells have been highlighted as pivotal in suppressing autoimmune and inflammatory responses in the gut. Depth study on dietary supplementation with LAM of the *L. hyperborea*, *L. digitata* and β-glucan of the *S. cerevisiae*, was realized by Ryan et al. (2012) [29], on gene expression of a range of cytokines, receptors, and signal transducing molecules relevant to the Th17 and T_{REG} pathways in the porcine colon. All sources of LAM significantly decreased the expression of Th17-related cytokines (IL-17a, IL-17F, and IL-22), receptor IL-23R, and IL-6. There was no alteration to the T_{REG} related target, Foxp3, or to TGF-β, although a significant reduction in IL-10 was observed in the *L. digitata* supplementated group. Differences were observed between all sources of β-glucans, regarding the magnitude of the

decrease, with the extract of the *S*. *cerevisiae* demonstrating the largest effects on these cytokines.

In vivo studies with rats and LAM also are being conducted, with similar objectives to studies in pigs. Neyrinck et al. (2007) [30] utilized LAM focused your study on gut-liver interactions to support the importance of interesting nutrients in the control of systemic infection by Gram-negative bacteria, more specifically if LAM can modulate the response to a systemic inflammation. The inflammatory mediators, anti-inflammatory prostaglandin (PGE_2), TNF-α and NO_2 concentration were analyzed. The authors observed that LAM treatment decreased serum monocytes number, NO_2 and TNF-α. Also observed modulated intra-hepatic immune cells: it lowered the occurrence of peroxidase-positive cells (corresponding to monocytes/neutrophils) and, in contrast, it increased the number of ED2-positive cells, corresponding to resident hepatic macrophages, i.e. Kupffer cells. Thus, these authors concluded that the hepatoprotective effect of LAM during endotoxic shock may be linked to its immunomodulatory properties.

Cheng et al. (2011) [31] investigated antioxidant activities of LAM in sepsis in rats, evaluating their effect on lung oxidative stress and lipid peroxidation. The results indicated that LAM polysaccharides significantly normalized catalase activity ($p<0.01$), increased glutathione peroxidase activity ($p<0.05$), superoxide dismutase activity and reduced malondialdehyde concentrations in animal. According authors, LAM polysaccharides appeared to be more effective in reducing sepsis-induced oxidative stress, lipid peroxidation in rats.

In an article recent, Guzmán-Villanueva et al. (2014) [32] evaluated the dietary effect of LAM (isolated from *L*. *digitata*) and Pdp 11 (*Shewanella putrefaciens*, probiotic isolated from gilthead seabream skin), single or combined, on growth, humoural (seric level of total IgM antibodies and peroxidase and antiprotease activities) and cellular innate immune response (peroxidase and phagocytic activities of head-kidney leucocytes), as well as the expression of immune-related genes in fish gilthead seabream (*Sparus aurata*). Immunoglobulin M (IgM), the primary antibody of fish, is a major component of the teleost humoural immune system, and it is used to identify and neutralise foreign antigens such as bacteria and viroses. IgM gene expression tended to be down-regulated being significant after one week of dietary in seabream specimens

fed with LAM or LAM plus Pdp 11. The seric antiprotease activity was increased in fish fed with both supplements.

Regarding the cellular immune activity, the phagocytic activity is a key aspect of innate immunity, and is part of the first line of cellular defence [33]. When β-glucan receptors are engaged by β-1,3/1,6-glucans all immune functions are improved, including phagocytosis, release of certain cytokines such as IL-1β, TNF-α, IL-6, interferons, and the processing of antigens [34]. These study LAM and combined diet increased phagocytic activity after 2 or 4 weeks. At gene level, IL-1β and INF-γ transcripts were always up-regulated in head-kidney (HK) but only the interleukin reached significance after 4 weeks in the group fed with LAM. These genes are mainly expressed by natural killer and phagocytic cells, respectively which are in turn the major responders to β-glucans [35]. Thus, the results of study suggested that LAM and Pdp 11 modulate the immune response and stimulates growth of the gilthead seabream. Other lines of research on *Laminarina* spp. are about *in vitro* involving microbicidal and immunomodulatory activity. These studies attempt to better explain the pathways of action of these polysaccharides, and are usually performed with different cell lines, **Table 1**.

Despite LAM modulate the immune response, some studies suggest that LAM has reduced potential to stimulate the immune system when compared to others β-glucan. Fuentes et al, 2011 [5], compared the phagocytosis of zymosan particles, by murine RAW 264.7 macrophages, before and after of the treatment with LAM, mannan (from *S. cerevisiae*), LPS and dexamethasone. The results showed that the LAM extracted of *L. digitata,* inhibited phagocytosis, the number of particles per cell (MNP) and the phagocytic index (PI). Additionally, this effect was concentration-dependent (3, 30 and 100ug/mL). LAM and dexamethasone suppressed phagocytosis rates, inverse results occurred with the treatment of LPS. To the authors, this observation suggests that native RAW macrophages recognize and ingest zymosan particles in a β-glucan-dependent manner.

Sonck et al, 2010 [11] wanted to know the response of different cells (monocytes, neutrophils and lymphocytes of pigs), in presence of different β-glucans, testing in vitro preparations. Lymphocyte proliferation, reactive oxygen species (ROS), production by neutrophils and monocytes and cytokine production, were tested. The

assays performed showed that LAM induced lower activity that other β-glucans with higher molecular weight. Similar results were found by Noss et al. 2013_[14], which tested 13 different β-glucans. LAM showed less potential to induce release of interleukins, compared with glucans from other sources . These results were expected, once other authors have already pointed that there appears a proportional order between the molecular weight of the β-glucans and biological effect [36,37] .

On the other hand, a large number of papers showed that LAM is potentially immunostimulating, furthermore is need highlights that, these works try to unravel the signaling pathway for the effects generated by the stimulus of LAM. Recently, Lee 2012, [38] investigated the immunostimulatory effects of LAM obtained from *L. digitata* with respect to inflammatory mediators such as calcium, hydrogen peroxide (H_2O_2), nitric oxide (NO), cytokines (monocyte chemotactic protein-1 (MCP-1), vascular endothelial growth factor (VEGF), leukemia inhibitory factor (LIF), granulocyte-colony stimulating factor (G-CSF), IL-6, MIP-1α, transcription factors (STAT1, STAT3, c-Jun, c-Fos, COX-2, and TLR2), in RAW 264.7 mouse macrophages. The tests were performed with different concentrations of laminarin: 100, 200, 300, 400, and 500 μg/mL. Overall, laminarin were effective in all assays performed. In addition, this response was present in most of the concentrations tested. Significantly increased intracellular calcium and H_2O_2 production in RAW 264.7 cells were observed in the research. The authors suggest that activation of macrophages exerted by LAM was even safer than traditional activation by LPS (bacterial lipopolysaccharide). Whereas the LPS cause cytotoxic effect on macrophages, unlike the results with laminarin. Furthermore, laminarin was able to increase tow-fold the concentration of NO.

This increase was attributed to calcium signaling, because when this pathway was inhibited there was a decrease in the production of the NO. In addition, LAM was able to increase the transcription of proinflammatory target genes, STAT3, c-Jun, c-Fos and cyclooxygenase (COX)-2. The literature states that the increase in cytosolic calcium, in turn, activates calcium-dependent transcription factors (TFs) including Signal Transducer and Activator of Transcription (STAT) 1, STAT3, and Activator protein-1 (AP-1; a heterodimeric protein composed of c-Fos and c-Jun), subsequently increasing the transcription of proinflammatory target genes [39].

LAM extracted of *L. japonica*, has been used suppress the invasion, infection and inflammation caused by *Listeria monocytogenes* (Lm), into human enterocyte-like Caco-2 cells and immune and/or inflammatory reactions of murine macrophage RAW 264.7 cells. For this effects, were used crude soluble polysaccharides (SPS) and ethanolic extract (EE) fractions of frond (kombu) and holdfast (ganiashi) parts of *L. japonica*. The sub-products of LAM, has been used successfully for suppression of Lm invasion and infection. The characterization the ethanolic extract showed higher phenolic content and antioxidant, than SPS. The characteristics of EE, allowed greater efficiency than SPS. EE suppressed the Lm invasion into the differentiated Caco-2 cells and the production of NO, suppressing the inflamation [40].

Our group recently also found promising results using LAM. The assays were conducted with neutrophils from human blood. The LAM, from *L. digitata*, was able to activate the pathway of reactive oxygen species in neutrophils. This carbohydrate increased microbicidal activity of neutrophils inducing significant oxygen consumption by the NADPH oxidase system, followed by high production of intracellular oxidant species, reflected by hypochlorous acid (HOCl) and Dihydrorhodamine 123 (DHR) assays and also myeloperoxidase (MPO) activity. These results were evaluated in the context of recurrent vulvovaginal candidiasis (RVVC). Clinical isolates of women with RVVC were resistant to the action of neutrophils, mainly by releasing detoxication enzyme. LAM was able to potentiate the action of neutrophils, and make them potentially able to kill resistant isolates [7].

VACCINES

As described above, β-glucan itself can elicit broad anti-infective effects. Furthermore, LAM has been established a protective effect against different microorganisms such as oral microbial species [41], *L.monocytogenes* [40] and *Candida albicans* [4,7]. On the other hand, there was observed that β-glucan, is recognized by neutrophils or polymorphonuclear leukocytes (PMNs) in response to *C. albicans* infection [42]. Basic molecular structure of β-glucan polymers is similar and highly conserved in different pathogenic fungal species, mainly consisting of β-(1,3)- and β-(1,6)-linked repeating units of D-glucose cross-linked together, and variously complexed with chitin and other glycoproteins [43]. So this mechanism, to recognize

and respond to their conserved structural components, particularly β-glucans, has evolved in mammals as defense against fungal pathogen [4].

According to LAM caracteristics with a β-glucan of non-fungal source, recent researches have shown the use of LAM as a possible vaccine against different fungi (**Table 2**). In particular, a number of β-glucan protein conjugates vaccines have shown efficacy in experimental models of candidiasis, aspergillosis and cryptococcosis, therefore, a research with LAM from brown alga *L.digitata* conjugated with the genetically-inactivated diphtheria toxin CRM197 has been found an interesting alternative to induce the production of anti-β-glucan antibodies capable of conferring protection against all three the above infections [18,19,44].

LAM, like most free polysaccharides, is a poor immunogen, so Torosantucci et al., (2005) [18] used LAM conjugate with the diphtheria toxoid CRM197, a carrier protein used in some glyco-conjugate bacterial vaccines. After, Bromuro et al. (2010) [17] formulated LAM-CRM197 conjugate plus MF59, a human-acceptable adjuvant. This LAM-CRM197/MF59 conjugate proved to be immunogenic and protective as immunoprophylactic vaccine, against both systemic and mucosal infections by *Candida albicans* and LAM-CRM 197 -vaccinated mice also were protected from a lethal challenge with conidia of *Aspergillus fumigatus*, showing this novel conjugate vaccine, can efficiently immunize and protect against two major fungal pathogens by mechanisms that may include direct antifungal properties of anti-beta-glucan antibodies [17-19]

However the vaccine format used in that previous investigation had some limitations as the relative complexity of the β-glucan antigen used in the conjugate. It was difficult to discern, in LAM-CRM197 vaccine, the most protective β-glucan epitopes, it should be considered that only selected anti-β-glucan antibodies appear to possess a protective value, as previously indicated by the observation that a monoclonal antibody recognizing exclusively a β-(1,3)-glucan epitope, but not one recognizing both β-(1,3)- and β-(1,6)-glucan, was protective in passive vaccination experiments [17-19]

Therefore, it was speculated whether presence or arrangement of this β-(1,6) branching on LAM molecule impacted on the immune response induced by the glycoconjugate in terms of antibody specificity and/or protective effect. After, Bromuro et al. (2010) [17] conducted a study with a series of different β-glucan-based,

conjugated vaccines, replacing LAM with β-glucan molecules with absent or defined number and positions of β-(1,6) branching, using a natural β-glucan curdlan (Curd) from *Alcaligenes faecalis* (Curd-CRM197); a synthetic β-glucan linear of 15 repeats (15mer-CRM197) conjugates; and synthetic b-glucan β-(1,6)-branched β-(1,3)-oligosaccharides with a branching point every 5 repeats (17mer-CRM197) conjugates. They observed that the protective β-glucan epitope is conformationally defined and this protective conformation is fulfilled by LAM-CRM197, Curd-CRM197 and 15mer-CRM197 but not by the 17mer-CRM197, indicatting the possibility of developing a synthetic vaccine against fungal diseases, replacing the highly heterogeneous LAM polysaccharide by a synthetic molecule. Despite this progress observed using β-glucan as a potential vaccine against human pathogenic fungi further studies are needed to completely define the structural requirements of β-(1,3)-glucan oligosaccharides–protein conjugates as candidate vaccine.

CYTOTOXICITY

Another feature elucidated the LAM is its antitumor activity and low side-effects, listed in the **Table 3**. In recent studies Park et al., 2013 [45] examined the mechanisms through which LAM from *L. digitata* induces apoptosis in HT-29 colon cancer cells, as well as the involvement of the ErbB signaling pathway and than cell viability assay revealed that LAM induced cell death in a dose-dependent manner, causing apoptosis in cancer cells highly proliferative. They confirmed that LAM inhibits the proliferation and survival of colon cancer cells by regulating the ErbB receptor signaling pathway. The Erb2 gene is an important regulator of aberrant growth in colon cancer.

Other authors reported the potential effect of the purification of LAM polysaccharides. Zhai et al, 2014 [46] investigated antitumor effects of a sulfated polysaccharide fraction from *L. japonica* (LJSP) on cervical carcinoma. In the results *in vitro*, they observed that LJSP showed inhibitory effects on five tumor cell lines (HeLa (cervical carcinoma), U14 (cervical carcinoma), A549 (lung carcinoma), Bel-7402 (hepatoma), and HCT-8 (colon carcinoma). The cervical carcinoma cell lines were more sensitive to LJSP exposure than other cell lines. However, *in vivo* tests showed that LJSP can not only significantly inhibit the growth of U14 implanted tumor but also

markedly induce apoptosis of tumor tissue cells by modulating the Bax/Bcl-2 ratio, which play a crucial role in apoptosis [47].

Others polysaccharides purified from aqueous extracts of *L. japonica* denominated novel polysaccharide WPS-2-1, was evaluated by Peng et al, 2012 [48]. Cytotoxicity assay showed that WPS-2-1 exhibited significant antitumor activities against BGC823 and A375 carcinoma cells *in vitro*. No anti- proliferative effects of WPS-2-1 on VSMCs (aortic vascular smooth muscle cells) were observed, implying that these polysaccharides had no direct cytotoxicity to non-cancer cells. These same authors in 2013 [50] investigated the mechanism of antitumor activity of *L. japonica* polysaccharide WPS-2-1 on A375 cells *in vitro*. They observed that WPS-2-1 induced A375 cells apoptosis. Moreover, WPS-2-1-induced apoptosis was associated with the alteration in expressions of Bcl-2 family proteins. Mitochondrial apoptotic pathway was involved in WPS-2-1-induced apoptosis, which included the loss of mitochondrial membrane and activation of caspase-3/9 [49]. Bcl-2 family proteins play central roles in regulating cell apoptosis.

On the other hand, Kim et al, 2006 [51] decribed the use of laminarin polysaccharides (LP1) from *L. japonica* and LP1-derived oligosaccharides (LO) in the immunomodulatory activity inhibiting death by apoptosis in rat thymocytes. More than 50% of the mouse thymocytes cultured in the unsupplemented medium died within 3 days of culture. However, most of the thymocytes in the medium treated with LO or LP1 remained alive in culture for 3 days, and more than 85% of the cells were alive when cultured for 2 weeks. Supplementation of the thymocyte culture with LO or LP1 remarkably increased survival of the cells in a dose-dependent manner, indicating that LO or LP1 indeed interacted with thymocytes and suppressed apoptotic cell death.

CONCLUSIONS

In summary, studies *in vitro* and *in vivo* showed that LAM from seaweed of the genus *Laminaria* and their derived can induce an initial oxidative burst and activation of numerous interleukins resulting in antifungal and antibacterial activities. In addition, LAM can activate signaling pathways, pre-activating dormant immune cells, acting as a priming agent. Discrepancies in the literature regarding the effects of LAM, may be due to differences in experimental conditions, including animal models, cell types and

phagocytic targets employed. In addition to these variables, we note that many studies have not adequately specify which the species laminarin used, making it difficult to classify the biological effects.

Another feature of LAM is related with inducing cancer cells to death. These research are very promising, showing that LAM acts a in a dose-dependent manner and causing apoptosis of these cells with no direct cytotoxicity to non-cancer cells.

Furthermore, due to LAM characteristics with branched polysaccharide containing both β-(1,3)- and β-(1,6)-linked D-glucose sequences and a β-glucan of non-fungal source, researches have shown the use of LAM conjugate with adjuvant as a possible vaccine inducing to production of anti-β-glucan antibodies capable of conferring protection against different pathogenic fungi. Nevertheless, recent studies suggest replacing LAM by a synthetic molecule of β-glucan with less heterogeneous LAM polysaccharide. Despite this fact observed, further studies are needed using β-glucan (LAM or a synthetic molecule of β-glucan) as a potential vaccine against human pathogenic fungi.

Finally, the biological effects of LAM on different signaling pathways, invites us to believe that this carbohydrate goes beyond the classic effects of a single immunomodulator, with good prospects for use in anti-cancer activity, vaccines and we believe that the combined use of LAM to microbial agents may to promote the host response.

Conflicts of interest

The authors have declared that no competing interests exist.

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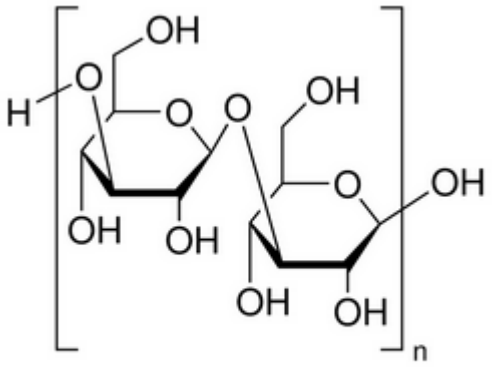

Fig 1. Glucose standard units of *Laminarin* spp.

Table 1: Applicability of β-glucans from seaweed of the genus *Laminaria* spp. in immune modulating properties.

Source	Product	Significant effects	Design	Ref
Laminaria digitata and L. hyperborea	Purified β-glucans	-not stimulate any pro- or anti-inflammatory cytokine in the ileal epithelial cells; -expression of a panel of pro- and anti-inflammatory cytokines (IL-1a, IL-10, TNF-a and IL-17A) was down-regulated in the colon; -reduce pro-inflammatory markers in the colon**;**	*In vivo* (piglet performance)	[13]
Laminaria digitata	Laminarin	- enhanced IL-6 and IL-8 cytokine expression	*In vivo* (pigs (mean body weight 17·9 kg)	[12]
Laminaria spp.	-	- lower IL-6 (P,0·05), IL-17A (P,0·01) and IL-1 b (P,0·01) mRNA expression in the colon	*In vivo* (pigs)	[27]
Laminara digitata and Laminara hyperborea	β-glucan	- IL-6 ($p < 0.05$) and TLR-4 ($P < 0.05$) were less expressed in the *L. digitata* supplementation group; - IL-10 ($p = 0.06$) and IL-1α ($p = 0.02$) were more expressed in the *L. hyperborea* supplementation group; - ~3-fold increase in both IL-10 and IL-1α in the liver samples of *L. hyperborea* relative to the *L. digitata* supplementation groups ($p < 0.01$)	*In vivo* (pigs mean body weight of 15.3 kg)	[29]
-	Laminarin	- decreased serum monocytes number, NO2 and TNF-α; - modulated intra-hepatic immune cells;	*In vivo* (Male Wistar rats weighing 136±1 g)	[30]
Laminarin (purchased from a local market in Shenyang city (Shenyang, China))	Laminarin polysaccharides	- normalized CAT activity ($p<0.01$), increased GPx activity ($p<0.05$), SOD activity; - reduced MDA concentrations in animal; - reducing sepsis-induced oxidative stress, lipid peroxidation in rats;	*In vivo* (SD rats of either sex (210–280g;)	[31]

Laminarina digitata	β-1,3/1,6-glucan	- β-1,3/1,6-glucan and combined diet increased phagocytic activity after 2 or 4 weeks. - IL-1β and INFγ transcripts were always up-regulated in HK but only the interleukin reached significance after 4 weeks in the group fed with β-glucan - IgM gene expression tended to be down-regulated being significant after 1 week in seabream specimens fed with β-glucan or β-glucan plus Pdp 11	*In vivo* (14.0+/- 1.5 g body weight and 9.6 +/- 0.4 cm body-length) of the hermaphroditic protandrous seawater teleost gilthead seabream (S. aurata L.)	[32]
Laminaria digitata	Laminarin	- inhibited ingestion of zymosan particles by the murine macrophage cell line RAW 264.7	*In vitro* (murine RAW 264.7 macrophages)	[5]
Laminaria digitata	Laminarin	- not activate ROS-production of monocytes and neutrophils;	*In vitro* (peripheral blood)	[11]
Laminaria digitata	Laminarin	- very low cytokine production IL-1β, IL-6, IL-8 and TNF-α	*In vitro* (whole blood cultures)	[14]
Laminaria digitata	Laminarin	- not reduce the cell proliferation of RAW 264.7 mouse macrophages; - increased the release of hydrogen peroxide, calcium, nitric oxide, monocyte chemotactic protein-1, vascular endothelial growth factor, leukemia inhibitory factor, and granulocyte-colony stimulating fator; - enhancing expression of Signal Transducer and Activator of Transcription 1 (STAT1), STAT3, c-Jun, c-Fos, and cyclooxygenase-2 mRNA;	*In vitro* (RAW 264.7 mouse macrophages)	[38]
Laminaria japonica	Crude soluble polysaccharides (SPS) and ethanolic extract (EE) fractions of frond (kombu) and holdfast	- EE of ganiashi suppressed the *Listeria monocytogenes* invasion into the differentiated Caco-2 cells; - Ganiashi SPS increased the NO production of intact RAW 264.7 cells; - suggest that *L. japonica*, particularly ganiashi, might	*In vitro* (Invasion into human enterocyte-like Caco-2 cells and immune and/or inflammatory reactions of murine macrophage RAW 264.7 cells)	[40]

	(ganiashi) parts	suppress the invasion and infection of *Listeria monocytogenes* and also the inflammation;		
Laminarina digitata	Laminarin	- increased oxidant species production - reduction of cytokines	*In vitro* (neutrophils and total leukocytes)	[7]
Laminaria spp.	Laminarin	- lower IL-6 ($p<0.05$), IL-17A ($p<0.01$) and IL-1β ($p<0.01$) mRNA expression in the colon;	*In vivo* (pigs, 24d of age, weight of 6·9 (SD 0.50) kg)	[27]
Laminaria japonica	Ethanol extracts	- antimicrobial agent	*In vitro (Streptococcus* spp, *Actinomyces* spp, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, and *P. gingivalis)*	[41]
-	Laminarin	- antioxidant effects	*In vitro* (murine macrophage cell line, J774a.1)	[15]

Table 2: Applicability of β-glucans from seaweed of the genus *Laminaria* spp. in vacinne against different pathogenic fungi.

Source	Product	Significant effects	Design	Ref
Laminaria digitata	Laminarin	vacinne against *C. albicans* e *A. fumigatus*	*In vivo* (Female 4-wk- old CD2F1 mice (Harlan))	[18]
Laminaria digitata	Laminarin	vacinne against *C. albicans*	*In vivo* (Female 4-week-old CD2F1 mice (Harlan-Nossan))	[17]
Laminaria digitata	Laminarin	vacinne against *C. albicans*	*In vivo* (Female BALB/c and BALB/cnu/nu(nude) mice between 6 and 8 weeks of age)	[52]
Laminaria digitata	Laminarin	- increased levels of TGF-β e IL-6 - high titers of Ab recognizing *C. albicans* b-mannan Ag	*In vitro* (mouse macrophage cell line RAW264.7 cells; Bone marrow–derived DCs (BMDCs)	[6]

Table 3: Applicability of β-glucans from seaweed of the genus *Laminaria* spp. in antitumor activity.

Source	Product	Significant effects	Design	Ref
Laminaria digitata	Laminarin	- induced cell death in a dose-dependent manner; - increased the percentage of cells in the sub-G1 and G2-M phase; - inhibited the heregulin-stimulated phosphorylation of ErbB2; - decrease in cellular proliferation;	*In vitro* (HT-29 colon cancer cells)	[45]
Laminaria japonica	Sulfated polysaccharide fraction (LJSP)	- highest cell growth inhibitory effect on cervical carcinoma U14 cells among five tumor cell lines; - *In vivo*, LJSP could not only inhibit the growth of the tumor but also enhance the spleen and thymus indexes, as well as the bodyweight of U14 tumor-bearingmice; - prominent antitumor activities and low toxic effects;	*In vitro* (HeLa (cervical carcinoma), U14 (cervical carcinoma), A549 (lung carcinoma), Bel-7402 (hepatoma), and HCT-8 (colon carcinoma) cell) *In vivo* (Female Kunming mice (6–8 weeks old), weighing 18–22 g)	[46]
Laminaria japonica	Novel polysaccharide WPS-2-1	- antitumor activities against A375 and BGC823 cells with a dose-dependent manner - lower cytotoxicity to vascular smooth muscle cells.	*In vitro* (human gastric carcinoma cell line BGC823, human melanoma cell line A375 and aortic vascular smooth muscle cells)	[48]
Laminaria japonica	Novel polysaccharide WPS-2-1	- induced apoptosis associated with the alteration in expressions of Bcl-2 family proteins; - mitochonadrial apoptotic pathway was involved in WPS-2-1-induced apoptosis, which included the loss of mitochondrial membrane and activation of caspase-3/9. - WPS-2-1 could effectively inhibit proliferation of A375 cells in vitro and induce apoptosis via mitochondrial apoptotic pathway.	*In vitro* (human melanoma cell line A375)	[49]
Laminaria japonica	Laminarin polysaccharides (LP1)	- suppressed apoptotic death around 3- or 2-fold - extended cell survival in culture at a rate of about 30 or 20%	*In vitro* (mouse thymocytes)	[51]

Artigo 2: β-glucan induces reactive oxygen species production in human neutrophils to improve the killing of *Candida albicans* and *Candida glabrata* isolates from vulvovaginal candidiasis

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β-Glucan Induces Reactive Oxygen Species Production in Human Neutrophils to Improve the Killing of *Candida albicans* and *Candida glabrata* Isolates from Vulvovaginal Candidiasis

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Abstract

Vulvovaginal candidiasis (VVC) is among the most prevalent vaginal diseases. *Candida albicans* is still the most prevalent species associated with this pathology, however, the prevalence of other *Candida* species, such as *C. glabrata*, is increasing. The pathogenesis of these infections has been intensely studied, nevertheless, no consensus has been reached on the pathogenicity of VVC. In addition, inappropriate treatment or the presence of resistant strains can lead to RVVC (vulvovaginal candidiasis recurrent). Immunomodulation therapy studies have become increasingly promising, including with the β-glucans. Thus, in the present study, we evaluated microbicidal activity, phagocytosis, intracellular oxidant species production, oxygen consumption, myeloperoxidase (MPO) activity, and the release of tumor necrosis factor α (TNF-α), interleukin-8 (IL-8), IL-1β, and IL-1Ra in neutrophils previously treated or not with β-glucan. In all of the assays, human neutrophils were challenged with *C. albicans* and *C. glabrata* isolated from vulvovaginal candidiasis. β-glucan significantly increased oxidant species production, suggesting that β-glucan may be an efficient immunomodulator that triggers an increase in the microbicidal response of neutrophils for both of the species isolated from vulvovaginal candidiasis. The effects of β-glucan appeared to be mainly related to the activation of reactive oxygen species and modulation of cytokine release.

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Introduction

Vulvovaginal candidiasis (VVC) is an important public health problem that affects a large number of healthy women of child-bearing age, resulting in an estimated cost of USD$1 billion per year in the United States [1]. In Europe, *Candida* species are listed as the primary cause of vaginal infections. In the United States and Brazil, although VVC is also the most common, it is preceded some times by bacterial vaginosis [2,3]. Approximately 75% of adult women will experience at least one episode of VVC during their lifetime, among which approximately 40–50% will experience further episodes, and 5% will develop recurrent VVC (RVVC) [4]. According to the literature, *Candida albicans* is still the species that is most responsible for symptomatic episodes of VVC [5,6]. However, a significant trend toward the emergence of non-*Candida albicans Candida* (NCAC) species, such as *C. glabrata*, has been observed in recent years [4,7].

The pathogenesis of these infections has been intensely studied over the past two decades. Recent research has presented new hypotheses to better understand this infection [4,5,8]. Nevertheless, no consensus has been reached on the pathogenicity of VVC or RVVC [9,10]. Host factors, such as cellular immune deficiency, combined with pathogen virulence and its ability to effectively evade host immune defenses appear to be important. Additionally, inappropriate treatment or the presence of resistant strains can lead to RVVC [2,4,10]. For infections caused by NCAC species, RVVC might be closely related to inappropriate treatment. Relatively little information is available on the immune system and susceptibility to recurrent episodes [10].

Chemotherapies that seek to improve the host immune response are an alternative to control fungal infections. β-glucans are

polymeric carbohydrates that have been reported to modulate inflammatory responses *in vitro* and *in vivo* [11–13]. The immunomodulatory effects of β-glucans are influenced by their degree of branching, polymer length, and tertiary structure, but no agreement has been reached on the basic structural requirements for biological activity, and different types of glucans have different biological effects [14,15]. The beneficial effects of β-glucan treatment have been attributed to modulation of the immune response, such as the stimulation of phagocytosis and activation of oxidative burst, which contribute to microbicidal activity [16]. Moreover, these carbohydrates can stimulate or suppress the secretion of cytokines [11,17]. Among the β-glucans, laminarin has been highlighted recently because its immunomodulatory properties [13,18].

Recent research in our laboratory demonstrated that RVVC isolates of *C. albicans* induced alterations in immunological defense mechanisms, especially those related to the microbicidal activity of neutrophils (Ratti, *et al.*, unpublished data). The present study sought to determine whether host susceptibility to RVVC caused by *C. albicans* might be reduced in β-glucan-treated neutrophils. Furthermore, better understand whether the lower incidence of RVVC caused by *C. glabrata* might be related to the immune response triggered by neutrophils. Our results suggesting that β-glucan induces biochemical alterations in the microbicidal activity of neutrophils mainly through reactive oxygen species production. We also suggest that RVVC isolate caused by *C. glabrata* does not involve impairment of the microbicidal activity of neutrophils.

Materials and Methods

Chemicals

Dextran, Histopaque, taurine, 3,3′,5,5′-tetramethylbenzidine (TMB), phorbol 12-myristate 13-acetate (PMA), N-formyl-Met-Leu-Phe (fMLP), fluorescein isothiocyanate (FITC), dihydrorhodamine 123, catalase, RPMI-1640 medium, lipopolyssacharide (LPS) and 5–5′-dithio-bis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sabouraud Dextrose Agar (SDA) was obtained from Difco, (Difco, Detroit, MI, USA). Hypochlorous acid (HOCl) was prepared by diluting a concentrated commercial chlorine solution and calculating its concentration using its absorption at 292 nm. PMA was dissolved in 10 mg/ml dimethylsulfoxide (DMSO). TMB solution was prepared by dissolving 10 mM TMB and 100 mM potassium iodide in 50% dimethylformamide and 50% acetic acid (400 mM). FITC was prepared by dilution with 10% DMSO in 1 ml phosphate-buffered saline (PBS). Dihydrorhodamine 123 was dissolved in 10 mg/ml DMSO. All of the experimental groups, including the controls, were tested with final DMSO concentrations of less than 1%, a concentration that was found to not affect neutrophil viability (data not shown).

β-glucan

β-glucan derived from *Laminaria digitata* was purchased from Sigma Chemical Co. (St. Louis, MO, USA; L-9634). This glucan has the molecular form β-(1,3;1,6)-D-glucan with a molecular weight of 5.85 kDa, and it is soluble in neutral water [12]. Stock solutions of 6 mg/ml β-glucan were aseptically prepared. The concentration of β-glucan that was used in the assays was 3 mg/ml, a concentration that does not affect the viability of neutrophils and induces a neutrophil response. The test of cell viability was determined by means of trypan blue, with 95%±10 viability. This concentration was based on a dose-response curve that used 0.5, 1.0, 3.0, 5.0, and 10 mg/ml (data not shown).

Candida albicans and *Candida glabrata*

A total of six clinical isolates (5 V, 7 V, 9 V, 11 V, 55 V, and 125 V) from female vaginal secretions that belonged to the archive collection of the Laboratory of Medical Mycology, Universidade Estadual of Maringa, Brazil, and two reference strains from the American Type Culture Collection (*C. albicans*, ATCC 90028; *C. glabrata*, ATCC 90030) were used. The clinical isolates were separated into groups according to symptoms presented by the patients: asymptomatic (ASS), vulvovaginal candidiasis (VVC) and recurrent vulvovaginal candidiasis (RVVC) [7]. The identity of all of the isolates was confirmed using CHROMagar *Candida* (CHROMagar, BioMerieux, Paris, France) and matrix-assisted laser-desorption/ionization time-of-flight mass spectroscopy (MALDI TOF-MS). For the MALDI TOF-MS method, the yeasts were prepared according to a previous report [19]. Measurements were performed according to a previous study [20] with a Microflex LT mass spectrometer (Bruker Daltonics) using FlexControl software (version 3.0, Bruker Daltonics).

Growth conditions and opsonization

For each experiment, the isolates were subcultured on Sabouraund Dextrose Agar (SDA) overnight at 37°C. The cellular density was adjusted to 2×10^7 yeasts/ml in phosphate-buffered saline (PBS) using a Neubauer chamber. Opsonization using 2.5% serum (v/v) was achieved by incubating the samples for 30 min at room temperature. Opsonized yeast was used for all of the assays.

Neutrophils and Total Leukocytes

Neutrophils and total leukocytes were isolated from peripheral venous blood obtained from healthy volunteers by centrifugation over a Ficoll-Hypaque gradient according to [21]. The volunteers signed a consent form, this study was approved by the Committee for Ethics in Research Involving Humans at the State University of Maringa (UEM)/Paraná, Brazil (No. 293/2006). Cell concentration and viability, determined by trypan blue exclusion, were determined in a Neubauer chamber. The purity was estimated to be higher than 98%. Neutrophils (2.0×10^6 cells/ml) and total leukocytes (2.0×10^6 cells/ml) were suspended in RPMI1640.

Microbicidal activity

Killing activity was monitored in the presence and absence of neutrophils, yeast, and β-glucan according to the method described by [22] with modifications. Neutrophils (2.0×10^6 cells/ml) that were previously treated or not with 3 mg/ml β-glucan for 30 min at 37°C were activated or not with different isolates of *C. albicans* or *C. glabrata* (RVVC, VVC, and ASS) and the reference strain (2.0×10^7 CFU/ml). These cells were maintained at 37°C with moderate shaking for 0, 30, 60, 90, and 120 min. The samples were diluted in sterile cold distilled water and mixed vigorously on a whirlmixer for 5 min to lyse the neutrophils and then diluted in sterile saline. The number of viable yeast was then calculated by spread-plating suitable diluted samples onto SDA, followed by incubation at 37°C for 24 h. The quantity of viable yeast was calculated by colony formation unit (CFU) enumeration.

Phagocytosis assay

The phagocytosis assay for *C. albicans* or *C. glabrata* was performed using microorganisms labeled with FITC according to the method described previously [23] with some modifications. Different isolates of *C. albicans* or *C. glabrata* (RVVC, VVC, and ASS) and the reference strain (2.0×10^7 CFU/ml) were incubated in 10 mM PBS with 5.0 mg/ml FITC for 30 min at 37°C. The

neutrophils (2.0×10^6 cells/ml) that were previously treated or not with 3 mg/ml β-glucan for 30 min at 37°C were then placed in contact with yeast for 1 h at 37°C. The negative control consisted of neutrophils alone. The fluorescence of gated neutrophils was detected using a FACSCalibur flow cytometer (Becton-Dickinson, Rutherford, NJ, USA) equipped with CellQuest software (Joseph Trotter, The Scripps Research Institute, La Jolla, CA, USA). A total of 10,000 events were acquired in the region previously established as the one that corresponded to the neutrophils. The results were recorded as the fluorescence intensity of positive cells in the sample.

Dihydrorhodamine 123 assay

Dihydrorhodamine 123 (DHR) is normally used to detect intracellular oxidant species production by cellular systems. The non-fluorescent probe DHR is oxidized to the fluorescent product rhodamine 123 (Rh123) when it is in contact with intracellular oxidant species as described previously [24]. Total leukocytes (2.0×10^6 cells/ml) that were previously treated or not with 3 mg/ml β-glucan for 30 min at 37°C were activated or not with different isolates of *C. albicans* or *C. glabrata* (RVVC, VVC, and ASS) and the reference strain (2.0×10^7 CFU/ml) or 400 nM PMA for 30 min at 37°C. After stimulation, the cells were incubated with 50 μM DHR for 30 min, washed once, and suspended in PBS. The fluorescence of gated neutrophils was detected at FL1, with 10,000 events/gate, using a FACSCalibur flow cytometer (Becton-Dickinson, Rutherford, NJ, USA). The data were analyzed using CellQuest software (Joseph Trotter, The Scripps Research Institute, La Jolla, CA, USA), and the results were recorded as fluorescence intensity and the percentage of positive cells in the sample.

Determination of hypochlorous acid

The formation of hypochlorous acid (HOCl) was based on the formation of taurine-chloramine that results from the reaction of HOCl with taurine as described previously [25]. Neutrophils (2×10^6 cells/ml) that were previously treated or not with 3 mg/ml β-glucan for 30 min at 37°C were activated or not with different isolates of *C. albicans* or *C. glabrata* (RVVC, VVC, and ASS) and the reference strain (2.0×10^7 CFU/ml) in 10 mM phosphate buffer (pH 7.4) that contained 140 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 1 mg/ml glucose and incubated with 15 mM taurine at 37°C under gentle agitation. The reaction was stopped after 30 min by the addition of 20 mg/ml catalase, followed by centrifugation for 10 min at $700\times g$ at 24°C. The concentration of taurine-chloramine present in the supernatant was estimated by adding 50 μl of TMB solution. After 5 min, the resulting blue product was spectrophotometrically detected at 655 nm using a plate reader (WaveX5 power-Biotech USA) and related to the standard curve (constructed by adding known concentrations of HOCl) to determine the concentration of HOCl produced.

Luminol-enhanced chemiluminescence assay for measurement of MPO release from neutrophils

Neutrophils (2×10^6 cells/ml) that were previously treated or not with 3 mg/ml β-glucan for 30 min at 37°C were activated or not with different isolates of *C. albicans* or *C. glabrata* (RVVC, VVC, and ASS) and the reference strain (2.0×10^7 CFU/ml) or the presence of 400 nM PMA (positive control) for 30 min at 37°C. After incubation, the cells were immersed in ice and centrifuged at $500\times g$ for 10 min at 4°C to separate the supernatant from the cells. The supernatant was used to measure MPO activity. The reaction was run in PBS, 0.1 mM H_2O_2, and 1 mM luminol at 37°C in a final volume of 0.3 ml [26]. Luminol (5-amino-2,3-dihydro-1,4-phthalazindione) is a chemical light amplifier. Myeloperoxidase-derived metabolites are responsible for the excitation of luminol [27]. The chemiluminescent response was monitored for 20 min at 37°C in a microplate luminometer (EG&G Berthold LB96V).

Determination of oxygen consumption by neutrophils

Oxygen consumption by neutrophils was polarographically measured at 37°C using a Clark-type electrode positioned in a closed Plexiglas chamber. Neutrophils were placed in the oxygen electrode vessel with 2 ml of PBS solution. To estimate oxygen consumption, neutrophils (2×10^6 cells/ml) that were previously treated or not with 3 mg/ml β-glucan for 30 min at 37°C were activated or not with different isolates of *C. albicans* or *C. glabrata* (RVVC, VVC, and ASS) and the reference strain (2.0×10^7 CFU/ml) and 400 nM PMA (positive control) for 30 min. Oxygen uptake was monitored for 5–10 min. Oxygen uptake rates were calculated from the polarographic records using an initial concentration of dissolved oxygen of 190 μM at 37°C [28].

Cytokine assay

Neutrophils (2×10^6 cells/ml) that were previously treated or not with 3 mg/ml β-glucan for 30 min at 37°C and activated or not with different isolates of *C. albicans* or *C. glabrata* (RVVC, VVC, and ASS) and the reference strain (2.0×10^7 CFU/ml) or 1 μg/ml lipopolysaccharide (LPS; positive control) were cultured for 18 h at 37°C and 5% CO_2. The supernatants obtained from the cell cultures were collected and frozen at −40°C until tumor necrosis factor α (TNF-α), interleukin-8 (IL-8), IL-1Ra, and IL-1β were determined using an enzyme-linked immunosorbent assay (ELISA; Quantikine, R&D Systems, Minneapolis, MN, USA). As expected, the amount of cytokines found in the supernatant of the cultures displayed a broad interval because of considerable individual variability in the basal release of cytokines.

Estimation of reduced thiol level

In the thioredoxin system, thioredoxin (Trx) is reduced to a dithiol $T(SH)_2$ by thioredoxin reductase (TR). The inhibition of TR decreases total reduced thiol [29]. Free thiol levels were determined using DTNB. Different isolates of *C. albicans* or *C. glabrata* (RVVC, VVC, and ASS) and the reference strain (2.0×10^7 CFU/ml) were centrifuged for 5 min at 8,000 g and lysed by adding 0.5 ml of lysis buffer (50 mM Tris-Cl, 150 mM NaCl, 50 mM ethylenediamine tetraacetic acid [EDTA], pH 7.2), and approximately 0.5 g of glass beads (diameter, 425–600 μm; Sigma Chemical Co.). Lysis was performed by vortexing for 3 mixing cycles of 3 min with 1 min intervals on ice. Cellular debris was removed by centrifugation, and 100 μl of the supernatant and 100 μl of 500 mM phosphate buffer (pH 7.5) were taken in each microtiter well, followed by the addition of 20 μl of 1 mM DTNB to each well. Absorbance was measured at 412 nm using a plate reader (WaveX5 power-Biotech, USA).

Statistical analysis

The data distribution was verified using the Kolmogorov-Smirnov and Lilliefors tests. Data with a non-normal distribution are expressed as the mean ± standard deviation (SD) of at least three independent experiments. Significant differences among means were identified using analysis of variance (ANOVA) followed by the Kruskal-Wallis test and Mann-Whitney U-test. The data were analyzed using Prism 6.0 software (GraphPad, San

Diego, CA, USA). Values of $p \leq 0.05$ were considered statistically significant.

Results

β-glucan increases microbicidal activity by neutrophils activated by different isolates of *C. albicans* and *C. glabrata* (RVVC, VVC, and ASS) and the reference strain

Our previous work demonstrated that the resistance of RVVC isolates caused by *C. albicans* involves the detoxification of oxidant species produced by neutrophils through TR activity, an antioxidant enzyme in fungi. Thus, the killing of the RVVC isolates was markedly impaired by the microbicidal response of neutrophils. Our first step was to evaluate the microbicidal activity of neutrophils that were previously treated with β-glucan and activated by the RVVC isolate compared with VVC and ASS isolates and the reference strain of *C. albicans* and *C. glabrata*. Fig. 1A shows that the microbicidal activity of β-glucan-treated neutrophils activated with all of the isolates of *C. albicans* increased significantly (>30%) after 60 min incubation compared with the activated and untreated neutrophil group. Importantly, this increase was time-dependent, and β-glucan-treated neutrophils killed 70% of the RVVC isolate after 120 min incubation (Fig. 1Ad). For *C. glabrata*, all of the isolates were able to induce the microbicidal activity of untreated neutrophils after 60 min incubation (Fig. 1B). This microbicidal activity was significantly higher after 60 min for the reference strain and ASS isolates (approximately 57% yeast killing) followed by the VVC and RVVC isolates (approximately 45% yeast killing) compared with the yeast alone (Fig. 1Ba'-d'). After treating the neutrophils with β-glucan, microbicidal activity significantly increased for all of the isolates, with the exception of the reference strain. Interestingly, β-glucan-treated neutrophils induced the death of more than 60% of the VVC and RVVC isolates compared with the activated and untreated neutrophil group.

β-glucan increases the phagocytosis activity by neutrophils activated by different isolates of *C. albicans* and *C. glabrata* (RVVC, VVC, and ASS) and the reference strain

Based on the effect of β-glucan on the microbicidal activity of neutrophils, we evaluated whether this carbohydrate modulates the phagocytosis of neutrophils that were activated by the RVVC isolate compared with the VVC and ASS isolates and reference strain of *C. albicans* and *C. glabrata*. All of the isolates of *C. albicans* induced neutrophil phagocytosis, with a marked rate of phagocytosis for the RVVC isolate (76%; Fig. 2A). After treating the neutrophils with β-glucan, the rate of phagocytosis increased for all of the isolates, with a significant increase for the ASS and VVC isolates (35%) compared with the activated and untreated neutrophil group (Fig. 2A). Additionally, all of the *C. glabrata* isolates induced high neutrophil phagocytosis (>50%; Fig. 2B). After treating the neutrophils with β-glucan, the rate of phagocytosis for all of the *C. glabrata* isolates increased by approximately 20% compared with the activated and untreated neutrophil group.

β-glucan increases intracellular oxidant species production by neutrophils stimulated with different isolates of *C. albicans* and *C. glabrata* (RVVC, VVC, and ASS) and the reference strain

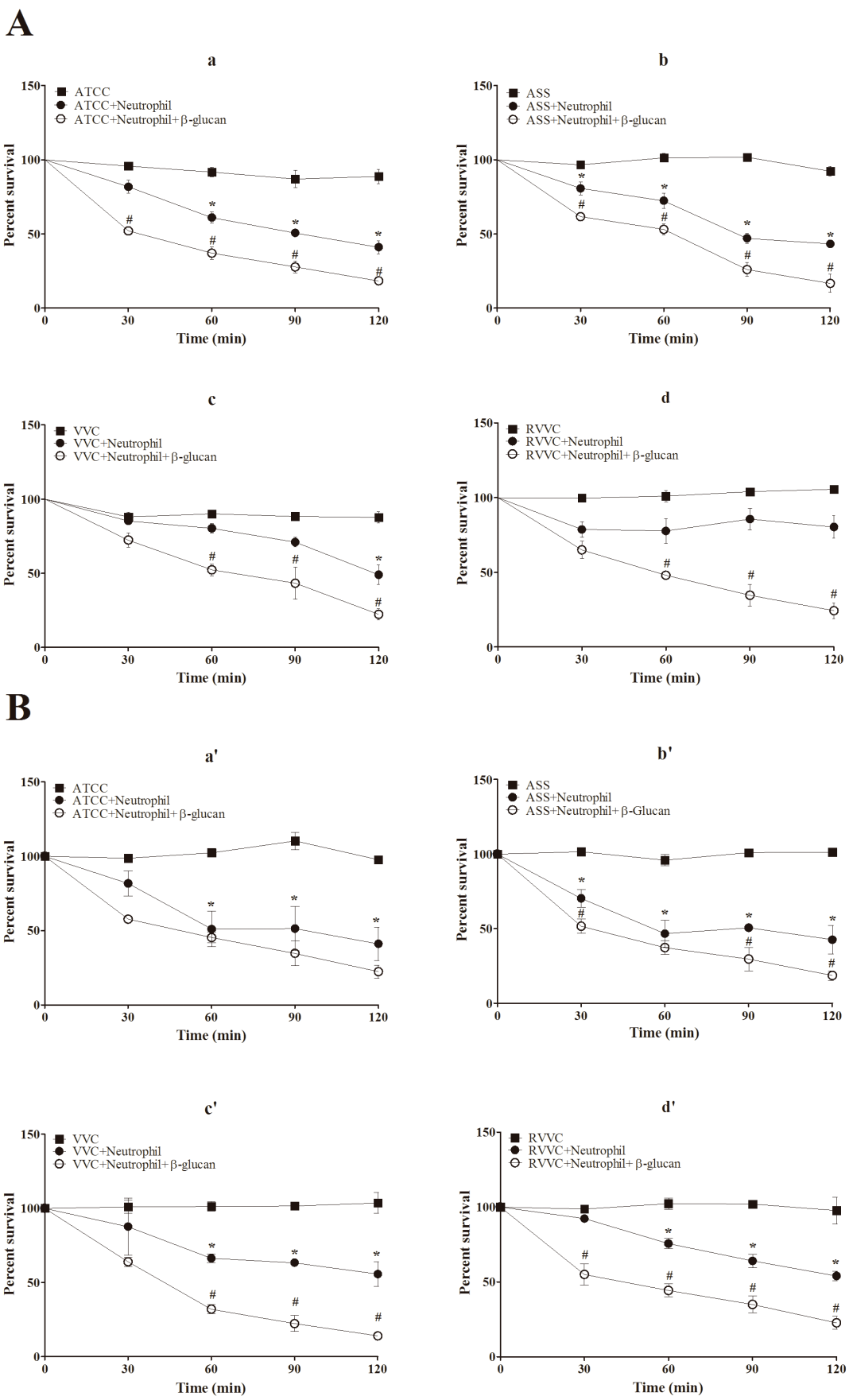
After phagocytosis, neutrophils undergo numerous signaling events that lead to the production of oxidative compounds in a complex mechanism known as oxidative burst [30]. Our early work demonstrated that the VVC and RVVC isolates of *C. albicans* did not induce significant intracellular oxidant species formation by neutrophils. Thus, we evaluated the production of total intracellular oxidant species in β-glucan-treated neutrophils that were activated by the RVVC isolate compared with the VVC and ASS isolates and reference strain of *C. albicans* and *C. glabrata*. β-glucan induced an increase in Rh123 fluorescence for all of the isolates of *C. albicans* compared with the activated and untreated neutrophil group (Fig. 3A), indicating intracellular oxidant species production by β-glucan-treated neutrophils. Interestingly, this increase in Rh123 fluorescence was significantly higher for the VVC and RVVC isolates (126% and 196%, respectively) compared with the activated and untreated neutrophil group. For *C. glabrata*, all of the isolates induced significant intracellular oxidant species production (>222%) by untreated neutrophils compared with neutrophils alone (Fig. 3B). After treating the neutrophils with β-glucan, intracellular oxidant species production significantly increased when the neutrophils were activated by the ASS and RVVC isolates (approximately 37% and 58%, respectively) compared with the activated and untreated neutrophil group. Untreated neutrophils that were activated with PMA also exhibited significant intracellular oxidant species production (418%) compared with neutrophils alone. Notably, after treating the neutrophils with β-glucan, intracellular oxidant species production increased even in inactivated neutrophils.

β-glucan increases HOCl production by neutrophils stimulated by different isolates of *C. albicans* and *C. glabrata* (RVVC, VVC, and ASS) and the reference strain

HOCl plays an important role in the microbicidal activity of neutrophils [31]. We studied HOCl production by β-glucan-treated neutrophils that were activated by the RVVC isolate compared with the VVC and ASS isolates and reference strain of *C. albicans* and *C. glabrata*. Untreated neutrophils that were activated by all of the isolates of *C. albicans* exhibited an increase in HOCl production. However, this increase was significant only for the ASS isolate and reference strain (at least two-fold; Fig. 4A). In neutrophils activated by all of the *C. albicans* isolates, β-glucan increased the production of HOCl after 30 min compared with the activated and untreated neutrophil group. A significant effect was observed for the ASS, VVC, and RVVC isolates, with more than a two-fold increase in HOCl production (Fig. 4A). In contrast to *C. albicans*, all of the isolates of *C. glabrata* induced a significant increase (approximately three-fold) in HOCl production by untreated neutrophils compared with neutrophils alone (Fig. 4B). After treating the neutrophils with β-glucan, HOCl production significantly increased (approximately two-fold) with all of the isolates compared with the activated and untreated neutrophil group. After treating the neutrophils with β-glucan, HOCl production increased even in inactivated neutrophils.

β-glucan increases MPO activity by neutrophils stimulated by different isolates of *C. albicans* and *C. glabrata* (RVVC, VVC, and ASS) and the reference strain

The route of HOCl generation is known to rely on MPO, a heme peroxidase enzyme that catalyzes the reaction between H_2O_2 and chloride [32]. Our data suggest that β-glucan activates neutrophil signaling pathways of oxidant species production, such as HOCl, leading to the removal of *C. albicans* and *C. glabrata* isolates. We evaluated the activity of MPO using luminol-enhanced chemiluminescence in both untreated neutrophils and β-glucan-treated neutrophils that were activated by the RVVC


A
a
ATCC
ATCC+Neutrophil
ATCC+Neutrophil+β-glucan
Percent survival
Time (min)
b
ASS
ASS+Neutrophil
ASS+Neutrophil+β-glucan
Percent survival
Time (min)
c
VVC
VVC+Neutrophil
VVC+Neutrophil+β-glucan
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Percent survival
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RVVC+Neutrophil
RVVC+Neutrophil+β-glucan
Percent survival
Time (min)

Figure 1. Microbicidal activity of β-glucan-treated neutrophils activated by different isolates of *C. albicans* and *C. glabrata*. Neutrophils (2.0×10^6 cells/ml) were previously treated or not with 3 mg/ml β-glucan and incubated with the reference strain and different isolates of (A) *C. albicans* and (B) *C. glabrata* (RVVC, VVC, and ASS; 2.0×10^7 CFU/ml) at 37°C for different times (0, 30, 60, 90, and 120 min). The quantity of viable yeast was estimated by plating the samples in Sabouraund Dextrose Agar (SDA) at 37°C for 24 h. The data are expressed as the mean ± SD of three separate experiments. *$p\leq0.05$, significant difference compared with the control group (yeast alone); #$p\leq0.05$, significant difference compared with untreated and activated neutrophils.
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isolate compared with the VVC and ASS isolates and reference strain of *C. albicans* and *C. glabrata*. The kinetics experiment showed an increase in light emission in the first 6 min, indicating MPO activity, followed by a slight decrease in all of the groups tested (Fig. 5, inset). Fig. 5A shows that all of the *C. albicans* isolates induced an increase in MPO activity compared with neutrophils alone. Interestingly, this increase was lower for the RVVC isolate, which induced a 24% increase in MPO activity (Fig. 5Ad). Myeloperoxidase activity in β-glucan-treated neutrophils was higher for the RVVC and VVC isolates (approximately 120%) followed by the reference strain and ASS isolate (approximately 100%; Fig. 5Aa–d). For *C. glabrata*, all of the isolates induced a similar increase (>90%) in MPO activity compared with neutrophils alone (Fig. 5B). After treating the neutrophils with β-glucan, MPO activity also increased in all of the isolates compared with the activated and untreated neutrophil group (≥90%). β-glucan induced an increase in MPO activity in both inactivated neutrophils (124%) compared with neutrophils alone and neutrophils activated by all of the isolates compared with the activated and untreated neutrophil group.

β-glucan increases oxygen consumption by neutrophils stimulated by different isolates of *C. albicans* and *C. glabrata* (RVVC, VVC, and ASS) and the reference strain

Based on the HOCl and intracellular oxidant species production results, we evaluated whether β-glucan induces oxygen consumption by neutrophils when the neutrophils were activated by the RVVC isolate compared with the VVC and ASS isolates and reference strain of *C. albicans* and *C. glabrata*. All of the *C. albicans* isolates, with the exception of the VVC isolate, significantly increased oxygen consumption by neutrophils. We showed that after treating the neutrophils with β-glucan, oxygen consumption increased with all of the *C. albicans* isolates, including the VVC isolate (Fig. 6A). This increase was significant for the ASS, VVC, and RVVC isolates (1.4-, 2.9-, and 1.4-fold, respectively). For *C. glabrata*, oxygen consumption by neutrophils was significantly higher for all of the isolates compared with neutrophils alone (Fig. 6B). This increase was more pronounced for the ASS isolate (approximately 5.2-fold), followed by the reference strain and VVC and RVVC isolates (2.8-, 2.7-, and 2.6-fold, respectively). After treating the neutrophils with β-glucan, they consumed more oxygen compared with the activated and untreated neutrophil group. This oxygen consumption was significantly higher for the ASS, VVC, and RVVC isolates (1.3- to 1.9-fold). The positive control, PMA, induced 4.2-fold higher oxygen consumption by neutrophils compared with neutrophils alone. After treating the neutrophils with β-glucan, oxygen consumption increased, even in inactivated neutrophils.

β-glucan decreases the release of IL-8, IL-1B and TNF-a by neutrophils stimulated by different isolates of *C. albicans* and *C. glabrata* (RVVC, VVC, and ASS) and the reference strain

The production of intracellular oxidant species by neutrophils is important for both microbicidal activity that leads to the oxidative damage of essential pathogenic molecules and biological signaling events that act as second messengers that regulate the production of biomolecules. We further evaluated IL-8, IL-1β, IL-1Ra, and TNF-α release in untreated neutrophils and β-glucan-treated neutrophils activated by the RVVC isolate compared with the VVC and ASS isolates and reference strain of *C. albicans* and *C. glabrata*. Fig. 7 shows that all of the isolates of *C. albicans* were able to induce the release of IL-8, IL-1β, IL-1Ra, and TNF-α

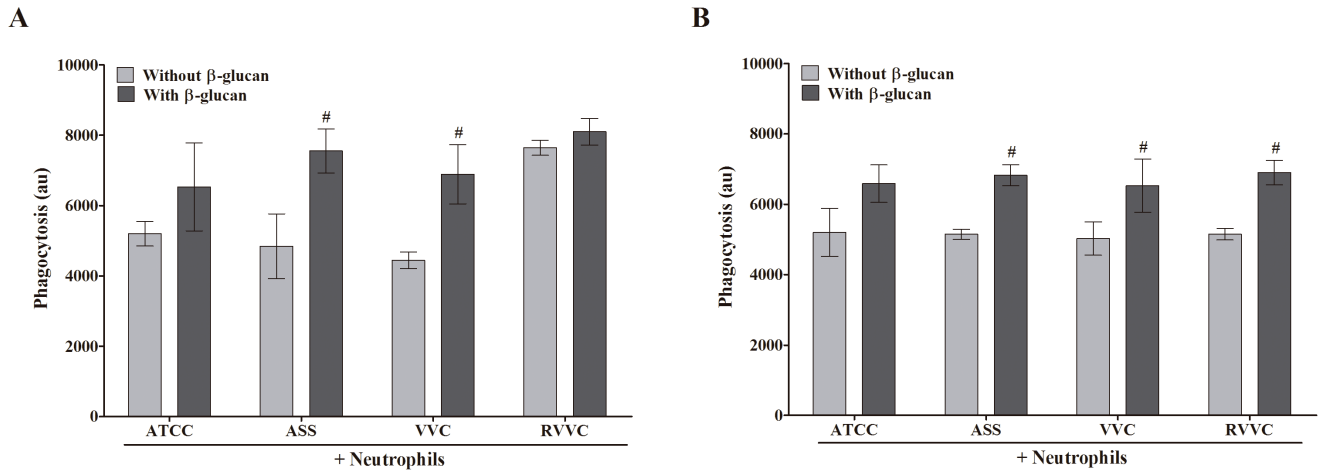

Figure 2. Phagocytosis activity of β-glucan-treated neutrophils activated by different isolates of *C. albicans* and *C. glabrata*. Neutrophils (2.0×10^6 cells/ml) were previously treated or not with 3 mg/ml β-glucan and incubated for 1 h at 37°C with the reference strain and different isolates of (A) *C. albicans* and (B) *C. glabrata* (RVVC, VVC, and ASS; 2.0×10^7 CFU/ml) labeled with FITC. Phagocytosis was determined by flow cytometry, and the results are expressed as the mean fluorescence (in arbitrary units [au]) ± SD of three independent experiments. #$p\leq0.05$, significant difference compared with untreated and activated neutrophils.
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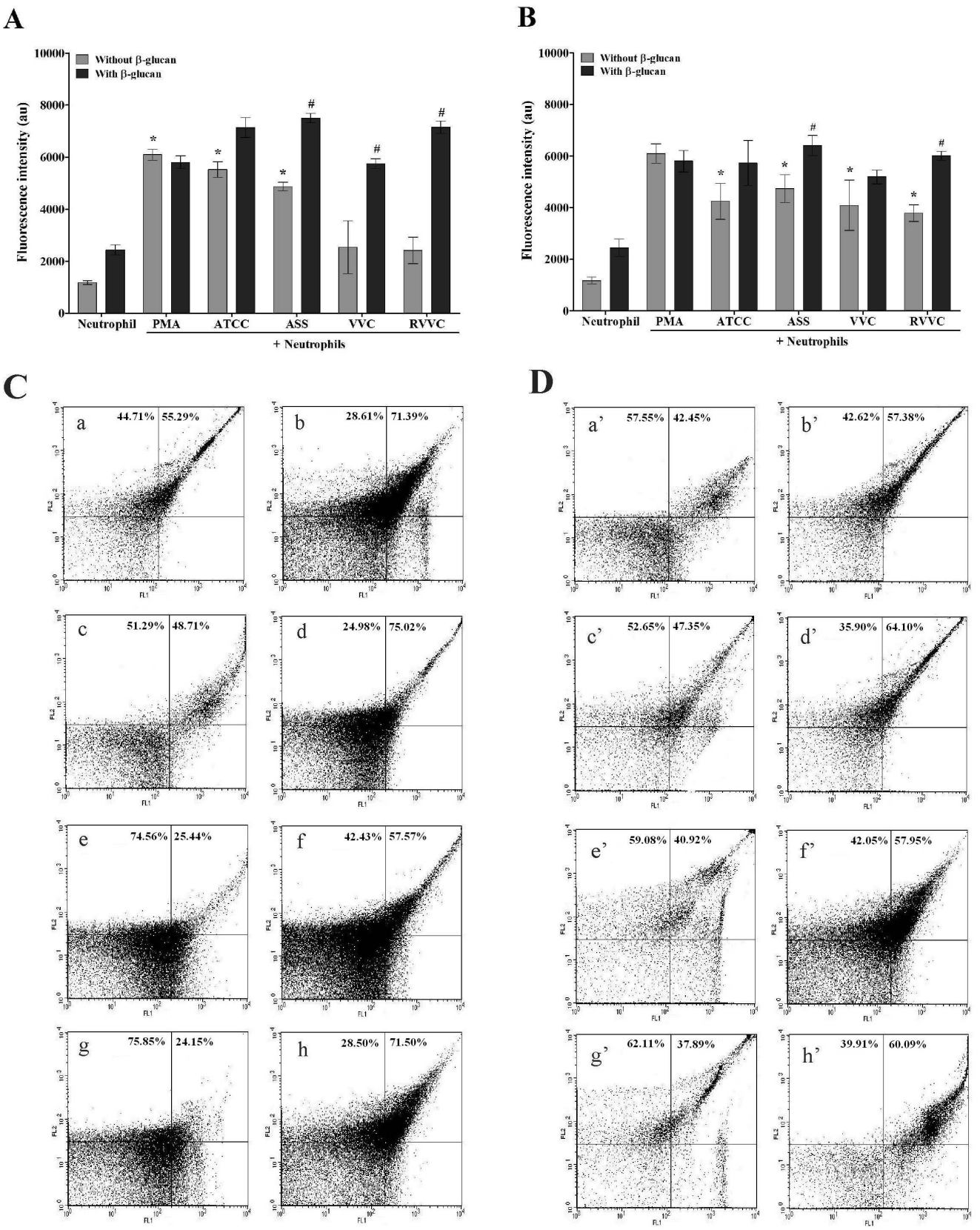

Figure 3. Intracellular oxidant species production by β-glucan-treated neutrophils activated by different isolates of *C. albicans* and *C. glabrata* determined by flow cytometry. Neutrophils (2.0×10^6 cells/ml) were previously treated or not with 3 mg/ml β-glucan and incubated for 1 h with the reference strain and different isolates of (A) *C. albicans* and (B) *C. glabrata* (RVVC, VVC, and ASS; 2.0×10^7 CFU/ml), followed by 30 min incubation with DHR. The data are expressed as the mean ± SD of at least three independent experiments. *$p\leq0.05$, significant difference compared

with the control group (neutrophils alone); $^{\#}p \leq 0.05$, significant difference compared with untreated and activated neutrophils. (C and D) Representative dot plot display of FL1 (green fluorescence) *vs.* FL2 on a logarithmic scale. (C – (a) ATCC, (c) ASS, (e) VVC,(g) RVVC) *C. albicans* with untreated neutrophils. (C – (b) ATCC,(d) ASS,(f) VVC,(h) RVVC) *C. albicans* with neutrophils previously treated with 3 mg/ml β-glucan. (D – (a') ATCC,(c') ASS,(e') VVC,(g') RVVC) *C. glabrata* with untreated neutrophils. (D – (b') ATCC,(d') ASS,(f') VVC,(h') RVVC) *C. glabrata* with neutrophils previously treated with 3 mg/ml β-glucan.
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compared with neutrophils alone. After treating the neutrophils with β-glucan, they exhibited a decrease in the release of TNF-α, IL-8, and IL-1β (Fig. 7Aa–c) in both inactivated and activated neutrophils. This decrease was significant for IL-8 for all of the *C. albicans* isolates (approximately 74%) and significant for IL-1β for the ASS and VVC isolates (75% and 70%, respectively). Fig. 7Ad shows that β-glucan-treated neutrophils exhibited an increase in the release of IL-1Ra in both inactivated and activated neutrophils. This increase was similar among all of the isolates (approximately 4.1-fold). For *C. glabrata*, the release of TNF-α, IL-8, and IL-1β by untreated neutrophils followed the same pattern as the one observed for *C. albicans* (Fig. 7B). However, neutrophils that were activated by the isolates of *C. glabrata* exhibited a higher level of cytokine release compared with isolates of *C. albicans*. After treating the neutrophils with β-glucan, they exhibited a significant decrease in TNF-α, IL-8, and IL-1β release for all of the isolates, with the exception of the reference strain (Fig. 7Ba–c). This decrease was similar among all of the isolates and more marked for TNF-α (approximately 8.5-fold), followed by IL-8 (4.3-fold) and IL-1β (3.5-fold). For IL-1Ra, β-glucan induced an increase in release when the isolates were activated by all of isolates of *C. glabrata*. This increase was significant for the reference strain (2.7-fold). After treating the neutrophils with β-glucan, IL-8, IL-1β, and TNF-α release decreased in inactivated neutrophils, and IL-1Ra release increased.

Estimation of reduced thiol levels in different isolates of *C. albicans* and *C. glabrata* (RVVC, VVC, and ASS)

The thioredoxin system, including thioredoxin (Trx) and TR, is used for oxidative stress defenses in fungi [33]. The conditions of oxidative imbalance depend on both increased oxidant species and decreased antioxidant effectiveness [34]. Our previous work (e.g. Ratti, *et al.*, unpublished data) suggested that the susceptibility of the host to the RVVC isolate of *C. albicans* is related to the activity of TR. The present data showed that *C. glabrata,* including the RVVC isolate, is sensitive to host defenses. We evaluated thiol levels in the ASS, VVC, and RVVC isolates of *C. glabrata* and reference strain. Similar to our previous work, Fig. 8A shows that thiol levels were significantly higher (approximately 39% and 55%) for the VVC and RVVC isolates of *C. albicans* compared with the reference strain. Thiol levels for *C. glabrata* were similar among the clinical isolates, with no statistically significant difference (Fig. 8B).

Discussion

The pathogenesis of VVC caused by *Candida* species is complex and involves factors related to both fungi and the host. Depending on these factors, recurrent episodes of candidiasis might increase, making the treatment of candidiasis more challenging [2,6]. The incidence of recurrent episodes of candidiasis is closely related to *Candida* species. To address these issues, research has sought to describe new virulence factors and fitness attributes of *Candida* species. In recent years, increased information about the mechanisms of fungal recognition by the host immune system has been reported [34,35]. In addition to understanding how host defenses are activated, fungal pathogens are known to possess strategies to evade recognition and inhibit antifungal defenses [4,36,37].

In VVC episodes, neutrophils are known to be markedly recruited to the site of infection, and reactive oxygen species play a central role in host defense by killing microbes in phagocytic cells [9]. In the present study, we investigated differences in yeast-neutrophils interactions with *C. albicans* and *C. glabrata* isolates from VVC in the presence of β-glucan as an immunomodulator of the VVC response.

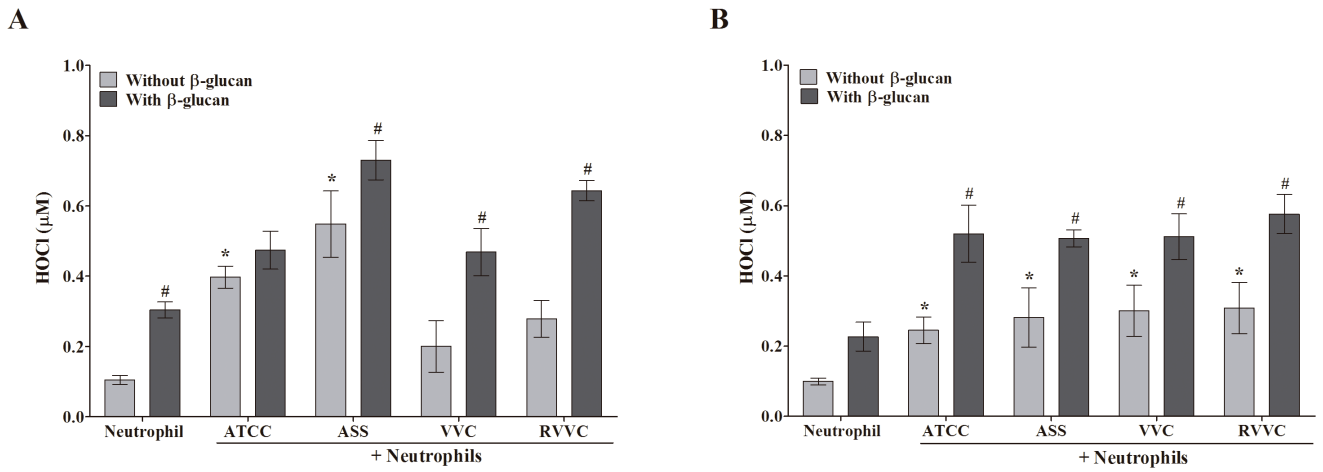
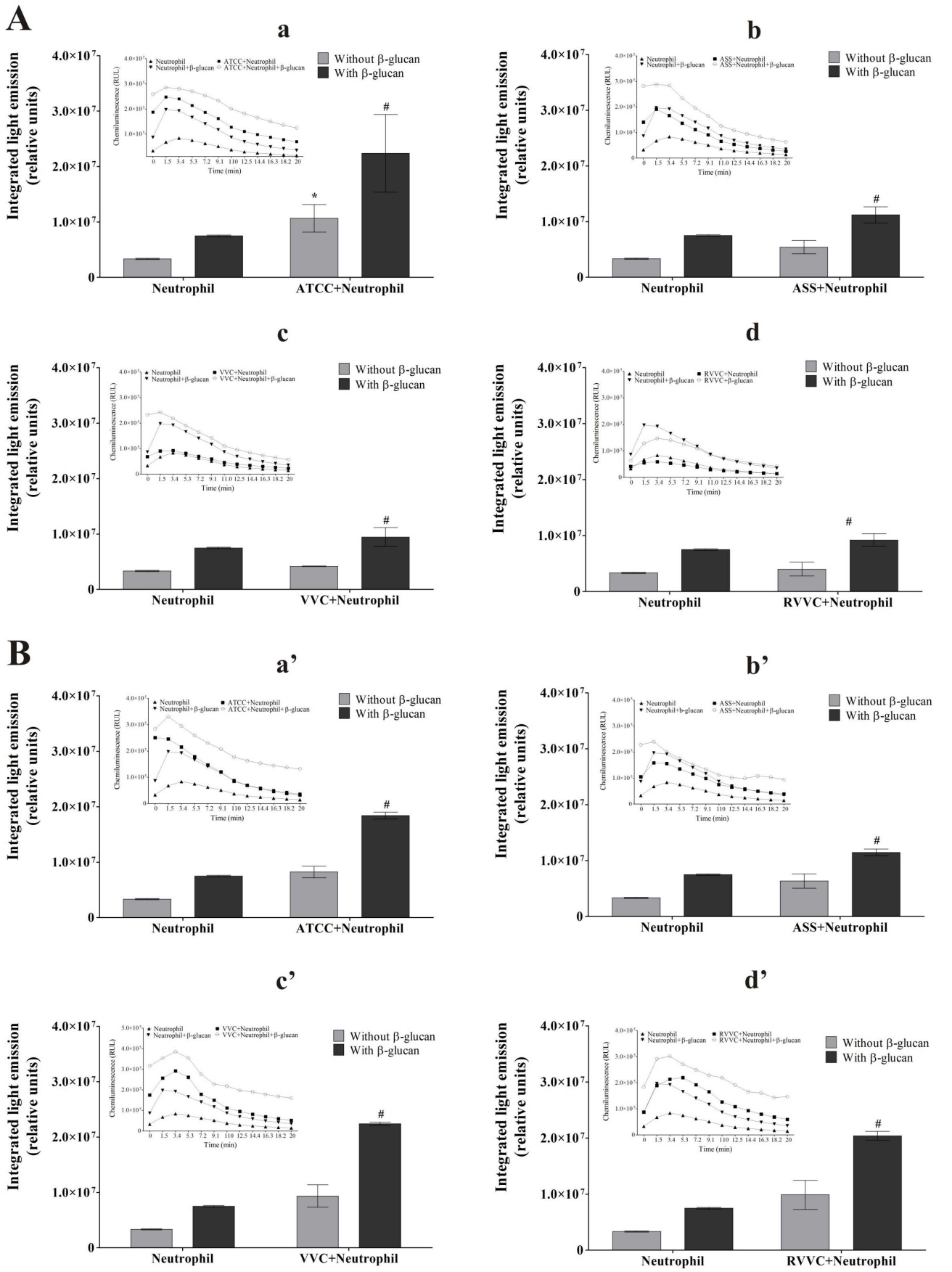


Figure 4. HOCl production by β-glucan-treated neutrophils activated by different isolates of *C. albicans* and *C. glabrata* determined by spectrophotometry. Neutrophils (2.0×10^6 cells/ml) were previously treated or not with 3 mg/ml β-glucan and activated or not by the reference strain and different isolates of (A) *C. albicans* and (B) *C. glabrata* (RVVC, VVC, and ASS; 2.0×10^7 CFU/ml) and read at 655 nm. The data are expressed as the mean ± SD of three independent experiments. $^{*}p \leq 0.05$, significant difference compared with the control group (neutrophils alone); $^{\#}p \leq 0.05$, significant difference compared with untreated and activated neutrophils.
doi:10.1371/journal.pone.0107805.g004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Integrated light emission (relative units)
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ASS+Neutrophil
VVC+Neutrophil
RVVC+Neutrophil
Chemiluminescence (RUL)
Time (min)
Neutrophil+β-glucan
ATCC+Neutrophil+β-glucan
ASS+Neutrophil+β-glucan
VVC+Neutrophil+β-glucan
RVVC+Neutrophil+β-glucan

Figure 5. Myeloperoxidase activity of β-glucan-treated neutrophils activated by different isolates of *C. albicans* and *C. glabrata* (integrated light emission). The inset represents kinetic study of MPO activity of β-glucan-treated neutrophils after 20 minutes of incubation. Neutrophils (2.0×10^6 cells/ml) were previously treated or not with 3 mg/ml β-glucan and incubated with the reference strain and different isolates of (A) *C. albicans* and (B) *C. glabrata* (2×10^7 CFU/ml) for 30 min. (a,a') ATCC. (b,b') ASS. (c,c') VVC. (d,d') RVVC. After incubation, chemiluminescence was monitored for 20 min at 37°C in a microplate luminometer using luminol as a chemical light amplifier. The data are expressed as the mean ± SD of three independent experiments. *$p\leq0.05$, significant difference compared with the control group (neutrophils alone); $^{\#}p\leq0.05$, significant difference compared with untreated and activated neutrophils.
doi:10.1371/journal.pone.0107805.g005

The incidence of *C. glabrata* ranks second in the epidemiology of VVC [4,7], but little is known about its interactions with the host. Our results provide clear evidence that RVVC infection caused by *C. glabrata* does not appear to involve impairment of the microbicidal activity of neutrophils, in which all of the isolates were susceptible to the killing action of neutrophils. The microbicidal activity of neutrophils was activated by all of the isolates inducing significant oxygen consumption by the NADPH oxidase system, followed by high production of intracellular oxidant species, reflected by HOCl and DHR assays and also MPO activity. Interestingly, all of *C. glabrata* isolates had similarly low levels of thiol groups, reflecting TR activity. The efficient oxidative activity of neutrophils combined with the low antioxidant activity of *C. glabrata* leads to the marked removal of these isolates, revealing the susceptibility of *C. glabrata* to the action of neutrophils. Other authors reported that the lower incidence of VVC caused by *C. glabrata* might be related to the ability of this species to induce an adequate immune response [4,38,39]. More evidence of this was found when immunocompromised hosts developed the infection with recurrent episodes [39]. After treating the neutrophils with β-glucan, the neutrophil oxidative parameters were markedly improved, with the exception of cytokines, resulting in the greater killing of *C. glabrata* isolates by neutrophils.

In contrast to isolates of *C. glabrata*, which were equally susceptible to the action of neutrophils, *C. albicans* showed heterogeneous behavior among the clinical isolates, demonstrated mainly for VVC and RVVC isolates. For *C. albicans*, the NADPH oxidase was activated, reflected by high oxygen consumption, but MPO and HOCl production was impaired. These results suggest that high concentrations of TR in RVVC isolates caused by *C. albicans* enable them to detoxify ROS/RNS and hinder the microbicidal action of neutrophils. The VVC isolates, in contrast, had moderate TR activity, which would also be able to detoxify oxidative products. However, the low oxygen consumption in these isolates indicated possibly no activation of the NADPH oxidase system. For *C. albicans* isolates, the treatment of neutrophils with β-glucan resulted in significant phagocytosis of the ASS and VVC isolates, but the phagocytosis of the RVVC isolate remained the same as before treatment. The RVVC isolate has high virulence factors that direct the pathogenesis of recurrent episodes through the activation of an exacerbated immune response [40]. Thus, we believe that RVVC isolates boost the highest neutrophil phagocytosis response, which cannot be improved by β-glucan. β-glucan also improved the microbicidal activity of neutrophils, killing all of the isolates tested, including the RVVC isolate, which is considered resistant to the microbicidal action of neutrophils (Ratti, *et al.*, unpublished data). Based on the literature, this effect of other types of β-glucan might be related to the induction of ROS production by leukocytes [16]. Our results show that β-glucan-treated neutrophils activated by all of the isolates exhibited high ROS production, including HOCl, an important fungicidal component of oxidative metabolism in neutrophils. Thus, we believe that β-glucan induces signaling pathways that are strongly related to the activation of neutrophil oxidative burst through the NADPH oxidase system. We confirmed this action, showing that this carbohydrate induced high amounts of oxygen consumption, even when the neutrophils were activated by VVC isolates. β-glucans has also been described as an efficient immunomodulator of leukocytes that

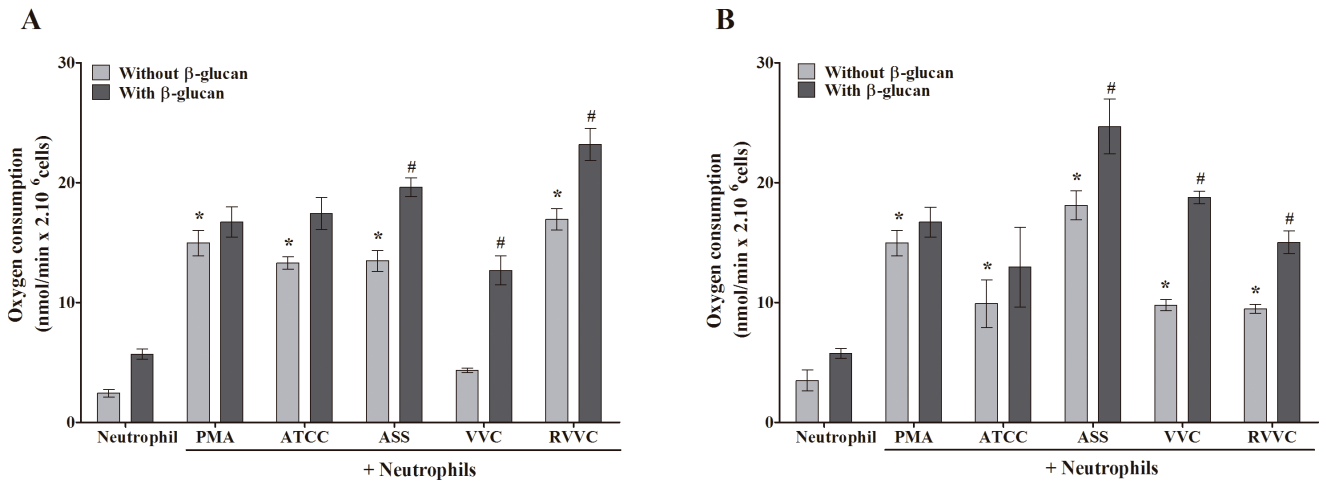

Figure 6. Oxygen consumption by β-glucan-treated neutrophils activated by different isolates of *C. albicans* and *C. glabrata*. Neutrophils (2.0×10^6 cells/ml) were previously treated or not with 3 mg/ml β-glucan and activated or not by the reference strain and different isolates of (A) *C. albicans* and (B) *C. glabrata* (RVVC, VVC, and ASS; 2.0×10^7 CFU/ml). Oxygen consumption was monitored for 5–10 min and calculated from the polarographic recordings using an initial concentration of dissolved oxygen of 190 μM at 37°C. The data are expressed as the mean ± SD of three independent experiments. *$p\leq0.05$, significant difference compared with the control group (neutrophils alone); $^{\#}p\leq0.05$, significant difference compared with untreated and activated neutrophils.
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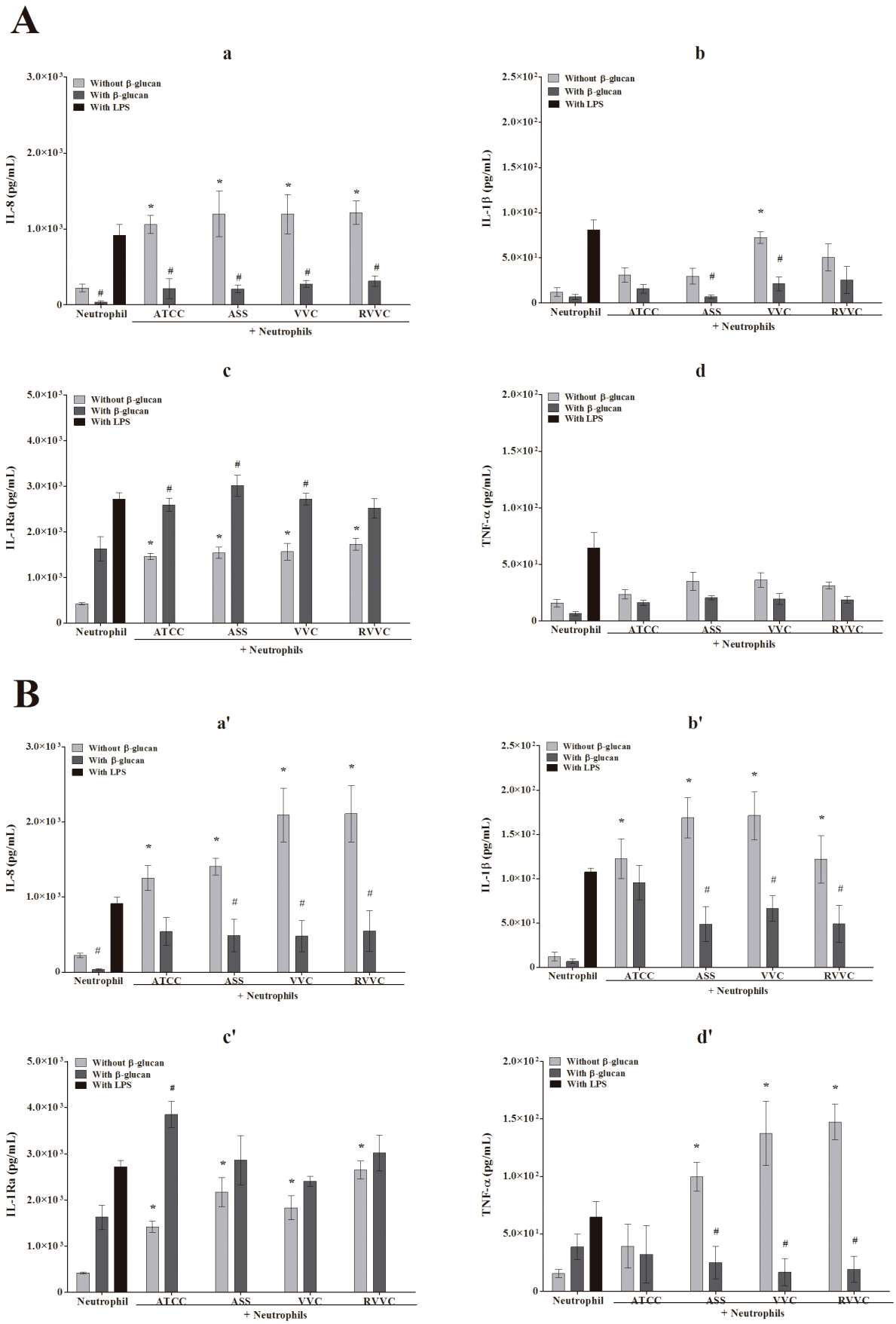
A
a
Without β-glucan
With β-glucan
With LPS
IL-8 (pg/mL)
Neutrophil
ATCC
ASS
VVC
RVVC
+ Neutrophils
b
IL-1β (pg/mL)
c
IL-1Ra (pg/mL)
d
TNF-α (pg/mL)
B
a'
b'
c'
d'

Figure 7. Cytokine release by β-glucan-treated neutrophils activated by different isolates of *C. albicans* and *C. glabrata*. Neutrophils (2.0×10^6 cells/ml) were previously treated or not with 3 mg/ml β-glucan and activated or not by the reference strain and different isolates of (A) *C. albicans* and (B) *C. glabrata* (RVVC, VVC, and ASS; 2.0×10^7 CFU/ml) and 1 μg/ml LPS and cultured for 18 h. (a, a′) IL-8. (b, b′) IL-1β. (c, c′) IL-1Ra. (d, d′) TNF-α. The data are expressed as the mean ± SD of three independent experiments. *$p\leq0.05$, significant difference compared with the control group (neutrophils alone); $^{\#}p\leq0.05$, significant difference compared with untreated and activated neutrophils.
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increases different biochemical processes [16]. We found that this carbohydrate might also induce the activity of reactive oxygen species-forming enzymes, reflected by an increase in MPO activity in all of the isolates tested. Interestingly, these isolates induced very low MPO activity in untreated neutrophils.

The isolates from both species were able to stimulate the production of the cytokines tested, mainly IL-8 and IL-1β. Proinflammatory cytokine release is important in the recruitment of neutrophil activation (e.g., IL-8) and stimulation of the proliferation and production of inflammatory molecules (e.g., IL-1β) [40]. The literature shows that epithelial cells release cytokines and chemokines, confirming that epithelial cells that are in contact with *Candida* spp. initiate an inflammatory response to cause tissue irritation, a common feature of CVV [40,41]. Our results showed that *C. glabrata* was able to induce higher levels of cytokine release by neutrophils compared with *C. albicans*. β-glucan was shown to be strongly related to the oxidative metabolism of neutrophils, which induces a decrease in cytokine production compared with untreated neutrophils. These results are supported by the observation of an increase in the release of IL1-Ra by neutrophils. This interleukin is a member of the IL-1 family that binds to IL-1 receptors but does not induce any intracellular response and is characterized as a receptor antagonist [42]. The balance between IL-1β and IL-1Ra in local tissue plays an important role in the susceptibility to and severity of many diseases [40,42]. Our results show clear evidence that β-glucan was able to maintain this balance, decreasing the release of IL-1β, likely by increasing its antagonist. A defined and balanced immunomodulatory response is crucial for protecting mucosal surfaces from coming into contact with pathogenic microorganisms [35,41,42]. Therefore, β-glucan serves as a modulator of the immune response, increasing ROS/RNS independently of the type of prior neutrophil activation (e.g., *C. albicans* or *C. glabrata*), improving the microbicidal activity of the host, and reducing the inflammatory response. For VVC, this response is apparently important because it enables the reduction of the classic symptoms of this pathology [40,41].

Our results also showed that β-glucan, beyond activating signaling pathways, pre-activated dormant neutrophils, acting as a priming agent. This effect has been described for other types of β-glucan, and its action might be related to interactions with neutrophil cell surface receptors [16,43].

Altogether, we hypothesize that β-glucan induces reactive oxygen species production at high concentrations. Reactive oxygen species are part of the microbicidal repertoire of neutrophils. More than that, ROS at very low concentration are involved in signaling events as second messenger leading to the production of essential molecules, such as cytokines. All of these functions of reactive oxygen species are very thin regulated, and the specific concentrations of ROS play an important role in this process [43].

Our analysis of untreated neutrophils indicated that the microbicidal response was activated in both *C. glabrata* and *C. albicans*. However, all of the parameters analyzed for *C. glabrata* were significantly lower compared with *C. albicans*, with the exception of cytokines. *C. glabrata* has lower virulence factors than *C. albicans*, mainly related to the ability of *C. albicans* to undergo a morphological transition from blastoconidia to hypha formation [44,45]. In fact, the relatively low pathogenicity of *C. glabrata* compared with *C. albicans* in animal models suggests that infections caused by *C. glabrata* may not require the stringent host immune response mechanisms that are required for infections caused by *C. albicans* [46]. Additionally, the increased prevalence of *C. glabrata* infections in immunocompromised patients indicates the importance of at least a moderate host immune response. Furthermore, one of the reasons for the proliferative success of *C. glabrata* is its relatively high drug resistance, particularly toward different azole antifungals. In the last decade, *C. glabrata* emerged as a cause of mucosal and invasive fungal infections, in part because of its intrinsic or acquired resistance to azole antifungals [47].

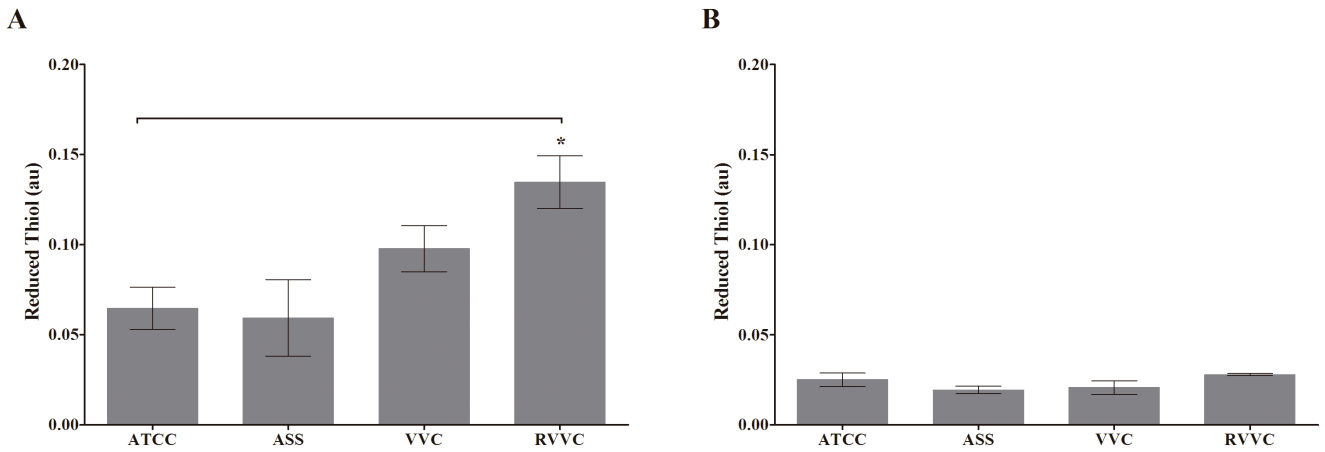

Figure 8. (A) Thiol levels in different isolates of *C. albicans* (ASS, VVC, and RVVC) and (B) thiol levels in different isolates of *C. glabrata* (ASS, VVC, and RVVC) incubated with DTNB. The data are expressed as the mean ± SD of three independent experiments. *$p\leq0.05$, compared with ASS or control group (neutrophils).
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In conclusion, as more is understood about the pathogenesis of fungal diseases from immunological, infectious disease, and microbiological perspectives, more we hope about thedirect development of future therapeutics for VVC and RVVC. Our results suggest that β-glucan is an efficient immunomodulator that triggers an increase in the microbicidal response of neutrophils that is mainly related to reactive oxygen species production for both species studied. For *C. albicans*, β-glucan decreased host susceptibility to the RVVC isolate, which has an efficient detoxification system. Additionally, β-glucan markedly increased ROS production by neutrophils that were activated by the VVC isolate, which has a low ability to induce neutrophil oxidative bursts. This effect of β-glucan is consistent with previous studies that reported that β-glucan is an immunomodulator that improves human response to infections process [12,16]. Considering that these species, mainly *C. glabrata*, are becoming more resistant to conventional treatments, β-glucan might be used in combination with classical fungicidal drugs to induce a better host response.

Author Contributions

Conceived and designed the experiments: PSBM SOS TIES AF. Performed the experiments: PSBM BAR JSRG NCAL. Analyzed the data: PSBM BAR JSRG MN EH. Contributed reagents/materials/analysis tools: EH MELC SOS TIES. Contributed to the writing of the manuscript: PSBM SOS TIES BAR MN EH MELC. Revised the manuscript and helped to provide information and suggestions: SOS TIES.

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Artigo 3: Transcriptional response showing more virulence of *Candida albicans* from recurrent candidiasis vulvovaginal in human cervical cells.

a ser submetido para a Journal of Medical Microbiology.

Transcriptional response showing more virulence of *Candida albicans* from recurrent candidiasis vulvovaginal in human cervical cells

Running title: Transcriptional response of candidiasis vulvovaginal

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ABSTRACT

Vulvovaginal candidiasis (VVC) is one of the main genital tract. However several literatures describe risk factors associated with this disease, there is still no consensus, and in addition, little is known about the interaction yeast-host for women with different symptomatology. In this context, we observed the difference in adhesion among clinical isolates of *C. albicans* from women with VVC; recurrent VVC (RVVC) and; asymptomatic (ASS). Second, we also determined the transcriptional response of these isolates during adhesion to the cervical cells. Our results show a significant difference in adhesion ability between the clinical isolates tested. ASS isolate had greater ability to adhere compared to VVC and RVVC isolates. On the other hand, VVC and RVVC up-regulation of a large number of *ALS* and *SAP* gene family, besides *HWP1*, important genes for yeast-host interaction. Than, this results suggest that RVVC isolate may have a significant participation in the pathology of VVC. In this research we suggest that this isolate may be more related to invasion than colonization.

INTRODUCTION

Vulvovaginal candidiasis (VVC) is a frequent consequence of *Candida* infection and the incidence of VVC has markedly increased over the last decades (Hamad et al., 2014). Most women have at least one event of VVC in their lifetime, others are asymptomatic candida carriers. However, it is important to highlighter that approximately 5-8% women suffer with four or more recurrences per year leading to the diagnosis of recurrent vulvovaginal candidiasis (RVVC) (Nyirjesy, 2001; Sobel, 2007). The specie *Candida albicans* is the more frequently responsible for VVC. The complexity of interactions between *C*. *albicans* and its human host suggests that this fungus has numerous mechanisms to adapt to a diversity of host sites. Although host factors are certainly involved in these processes, the versatility of interactions between *C*. *albicans* and its host is also undoubtedly controlled by differential expression of genes encoding key factors the fungus requires to adapt to this wide variety of environments (Hoyer et al., 2001).

Considering *C*. *albicans* is a commensal of human mucosa, become interesting to know about possible differences on host-yeast in colonization or infection process. Recently, we observe that there is a distinct behavior among clinical isolates from asymptomatic (ASS), with one episode (VVC) and recurrent VVC (RVVC) women (Bonfim-Mendonça et al., 2014). However that early study was based on human neutrophils, and there is no known about relation of this isolates group regarding epithelial cells.

The genes of secreted aspartic proteinases (*SAP*) and the adhesins, such as agglutinin like sequence (*ALS*) family and hyphal wall protein 1 (*Hwp1*), are some these factors and are closely related to the pathogenicity of *C*. *albicans*.

The Sap are encoded by 10 *SAP* genes (Naglik et al., 2003a) which are thought to have different roles during an infection, e.g. the degradation of tissue barriers during invasion, destruction of host defence molecules, or nutrient supply (Naglik et al., 2003b). The Als family and Hwp1 of *C*. *albicans* encodes large cell-surface glycoproteins that are implicated in the process of adhesion to host surfaces and can be divided into subfamilies based on the sequence of the central tandem repeat domain (Hoyer et al., 2001). Thus, our goal in this work was to investigate the potential

interaction of these isolates with a human vaginal epithelium and the relative expression these virulence factors (*SAP* and *ALS* genes) in same condition.

METHODS

1. *Candida albicans* and growth conditions

Four strains of *C*. *albicans* were used in this study, one reference strain from the American Type Culture Collection (*C*. *albicans* ATCC 90028) and three clinical isolates (5V, 7V and 9V) obtained from female vaginal secretions that belonged to the archive collection of the Laboratory of Medical Mycology, Universidade Estadual of Maringa, Brazil. The clinical isolates were separated according to symptoms presented by the patients: asymptomatic (ASS), vulvovaginal candidiasis (VVC) and recurrent vulvovaginal candidiasis (RVVC) (Lopes Consolaro et al., 2004). The identity of all of the isolates was confirmed using CHROMagar *Candida* (CHROMagar, BioMerieux, Paris, France) and matrix-assisted laser-desorption/ionization time-of-flight mass spectroscopy (MALDI TOF-MS). For the MALDI TOF-MS method, the yeasts were prepared according to a previous report (Spanu et al., 2012). Measurements were performed according to a previous study (Pascon et al., 2011) with a Microflex LT mass spectrometer (Bruker Daltonics) using FlexControl software (version 3.0, Bruker Daltonics). For each experiment, strains were subcultured on Sabouraud dextrose agar (SDA; Difco, Detroit, MI, USA) for 48 h at 37ºC.

2. Human cells line

To assess the adhesion ability of *C*. *albicans* to human epithelial cells, was used as model *HeLa* cells derived from a human cervical carcinoma, donated by "Dr. Luísa Lina Villa, ICESP-USP, São Paulo, Brazil". Cells were cultured at 37ºC under 5% CO_2 in Dulbecco's modified Eagle's medium (D-MEM; Sigma) containing 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (P/S;Gibco). After achieving 80% confluence, cells were detached using 25% trypsin-EDTA (Gibco) solution; the cell concentration was adjusted to $2x10^5$cells/ml with fresh D-MEM without P/S and 0.5 ml of the suspension was added to the wells of a 24-well plate and incubated at 37ºC under CO_2 5% for 48h. Prior to the adhesion assays, the wells were washed twice with PBS.

3. Adhesion Assays

Yeast cells were inoculated in YEPD (yeast extract peptone dextrose) and incubated for 18h at 37ºC under agitation in shaker (60 rpm). After incubation, yeast were harvested by centrifugation at 3000 rpm for 5 min and washed twice with Phosphate Buffer Solution (PBS; pH7.0; 10mM). The remaining pellets were suspended D-MEM and the cellular density adjusted to $2x10^7$ yeasts mL^{-1}, using a Neubauer chamber. Then, 500 ul of the suspension was added to each well of a 24-well plate containing a confluent layer of cervical cells. After 2 and 6h of incubation (60rpm, at 37ºC), the wells were washed twice with PBS to remove unattached yeasts. The yeast were removed using 25% trypsin-EDTA (Gibco) solution, resuspended in ice-cold PBS and shaken for 30seconds. The suspesion was plated on SDA and incubated at 37°C/24h. After this time, the number of viable yest cells was evaluated by colonic forming unit (c.f.u. mL^{-1}). All procedures were performed in triplicate and repeated in three separate assays.

4. Analysis of *SAP* gene expression

4.1 RNA extraction and cDNA synthesis

After adhesion assays, adherent *Candida* cells were harvested by detaching the cervical cells monolayer for isolation of total RNA to perform transcriptional analyses. The total RNA was extracted using Trizol® reagent (Invitrogen), adding to solution acid-washed glass beads (Sigma) and vortexing for 10minutes with 30second on ice. RNeasy® Mini kit (Qiagen, Germany) was used for purification. The concentration and purity of RNA was evaluated with NanoDrop (Thermo Scientific 200c). The cDNA was obtained using Superscript III RT® Supermix kit (Invitrogen), according to the kit manufacturer.

4.2 *ALS* and *SAP* family and *HWP1* gene PCR primers

For detection of *SAP*, *ALS* family and *HWP1* transcripts, specific pairs primers were designed. These primers used in real-time PCR (q-PCR) are listed in Table 1. The complete gene sequences of *SAP*, *ALS*, *HWP1*, *ACT1* (actin) and *CEF3* (translation elongation factor 3) genes for *C. albicans* were obtained from the GenBank database. The primers were designed using software available on the website IDT Integrated

DNA Technologies (http://www.idtdna.com/scitools/Applications/RealTimePCR), using criteria such as size of the oligonucleotides (20-25 bp), size of the resulting fragment (70-110 bp), annealing temperature equal for all primers (60°C), percentage of base G (guanine) and C (cytosine) between 40 and 60%, and low percentage of dimers. The sequences were compared with the data base of *C. albicans* using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), to determine their specificity. Initiators with nonspecific signs were excluded. To validate the specificity of each primer pair for its corresponding *ALS*, *SAP* and *HWP1* gene, PCR products were amplified from *C. albicans* ATCC 90028 genomic DNA. PCR reactions contained 0.4uM of each primer, 200uM $MgCl_2$ (Invitrogen), 0.8x Taq polymerase buffer (Invitrogen), 2.5 units Taq polymerase (Invitrogen) and 50 ng genomic DNA. PCR products were resolved on 2% agarose gel and visualized by staining with ethidium bromide. The derived DNA sequences for each gene-specific primer pair matched exactly with the predicted sequence and validated the specificity of the primers against the *C. albicans* genome. Each primer pair only amplified its specific gene and no cross-reactivity was observed, further demonstrating the specificity of primer pairs across the *ALS*, *SAP* and *HWP1* genes.

4.3 Efficiency of the designed primers

The efficiency of the primers was performed with titration ranging from 100-600nM to evaluate the optimal concentration of the primer which generates a lower Ct, the higher ΔRn and absence of dimers. After evaluating the optimal concentration of primers, serial dilutions of cDNA from different samples were performed to verify the efficiency of experiment, is indicated by the slope of the standard curve. The ideal efficiency is 100% (90-110%) and standard curve with slope of -3.32.

4.4 *ALS*, *SAP* and *HWP1* genes expression by real-time PCR

Real-time PCR (CF X 48 Real-Time PCR System; Applied Biossistems) was used to determine the relative levels of S*AP1-10, ALS1-7* and *ALS9*, *HWP1* mRNA transcripts, with *ACT1* and *CEF3* as a reference housekeeping gene for normalization. Each reaction consisted of 10μL of SYBR®Green Real-Time PCR Master Mix, 300 nM forward and reverse primers, 3μL of water and 1μL of cDNA in a final reaction volume

of 20µl. Negative controls were included in each run. The relative quantification of *SAP*, *ALS* and *HWP1* genes expression was performed by the DDCt method. Each reaction was performed in triplicate and mean values of relative expression were determined for each gene.

5. Scanning electron microscopy (SEM)

For SEM study, cells were initially adhered on glass coverslips which were in 24-well microtitration plates (TPP, Trasadingen, Switzerland). After, was performed the kinetics of adhesion, as described above. After that, the wells were gently washed three times with sterile saline solution in order to remove the loosely adherent yeast cells. Then, the glass coverslips were fixed with glutaraldehyde 2.5% diluted in cacodylate 0.1M buffer (Sigma, St. Louis, Missouri, USA) pH 7,2, and dehydrated in ascending series of alcoholic solution. Samples were dried at 42°C at 30minutes, and submitted to the CO_2 critical point apparatus Balzers CPD-030 (Balzers Instruments, Balzers, Liechtenstein). Afterwards, samples were coated with gold by metallizer Shimadzu IC-50 Ion Coater and materials were viewed and photographed in a scanning electron microscope (FEI Quanta 200), State University of Londrina (UEL), Londrina - Paraná.

6. Statistical analysis

The data distribution was verified using the Kolmogorov-Smirnov and Lilliefors tests. Data with a non-normal distribution are expressed as the mean±standard deviation (SD) of at least three independent experiments. Significant differences among means were identified using analysis of variance (ANOVA) followed by the Kruskal-Wallis test. The data were analyzed using Prism 6.0 software (GraphPad, San Diego, CA, USA). Values of $p \leq 0.05$ were considered statistically significant.

RESULTS

Quality control of real-time PCR assays

Basic Local Alignment Search Tool (BLAST) analysis indicated that each primer pair was specific for a particular *C. albicans* gene, and would not cross-react

with sequences from other organisms (data not shown). PCR efficiencies ranged between 90% and 110% for each of the primer pairs (data not shown), indicating that all realtime assays had similar good efficiencies. Gelelectrophoresis and melting curve analysis confirmed the presence of the expected PCR products only, and the absence of unwanted non-specific products (data not shown). Non-inoculated cervical cells failed to show evidence of gene expression (data not shown), confirming that each primer pair was specific for its corresponding *C. albicans* gene. Using the optimized real-time PCR assays, we found that all *ALS* and *SAP* genes were expressed at all time points during adhesion growth in both times tested (and also in the start cultures), as evidenced from a detectable Ct value (Ct < 35; data not shown).

The adhesion efficiency of *C. albicans* into cervical cells

The results of the kinetics adhesion assay are shown in Fig. 1. In general, ASS, VVC and RVVC isolates of *C. albicans* were able to adhere in cervical cells at different times. After 2h of contact with cervical cells, the number of adherent yeast was $2.78x10^6$, $1.63x10^6$ and $1.68x10^5$ c.f.u. mL^{-1} to ASS, VVC and RVVC isolates, respectively. Curiously, ASS isolate had significantly higher potential adherence at 2h compared to VVC and RVVC isolates (1.7- and 1.6-fold, respectively, *p=0.401*). After 6h, the profile of all isolates adhesion was similar than happened at 2 h. The number of adherent yeast was $2.45x10^6$, $1.95x10^6$, $1.35x10^5$ c.f.u. mL^{-1} and to ASS, VVC and RVVC isolates, respectively. Therefore in 6h of contact with cervical cells RVVC isolate was significantly less able to adhere (more than 90%) than the ASS isolate (*p=0.026*).

Morphology of ASS, VVC and RVVC isolates in adhesion

SEM analysis was used to examine the adhesion of ASS, VVC and RVVC isolates of *C. albicans* in 2h and 6h, Fig. 2B, 3B and 4B, respectively. The results show that during the initial process of adhesion (2h), all clinical isolates analyzed were in yeast form. However, in 6h of adhesion there was different behavior among isolates from the initial time. ASS and VVC isolates, showed predominant morphology of yeast. Differently, RVVC isolate showed yeasts and predominantly pseudohyphae form, Fig. 4B. VVC and RVVC isolates seems to have tighter interaction with cervical cells (Fig. 3B and 4B) than the ASS isolate (Fig. 2B).

Quantitative expression of *ALS1-7*, *ALS9* and *HWP1* genes in experimental cervical epithelial infections

After knowing the different behavior in the adhesion process of *C*. *albicans* on cervical cells, we consider whether there was a differential expression among adhesins and isolates.

ALS1-7, *ALS9* and *HWP1* expression by ASS, VVC and RVVC isolates in contact with cervical cells are shown in Fig. 2C, 3C and 4C, respectively. All *ALS* genes were expressed, however, the amount of *ALS* and *HWP1* genes expressed were strain-dependent.

The Fig. 2C, show that ASS isolate expressed the highest number of *ALS* genes and the expression was homogeneous among 2 and 6h of adherence to cervical cells. For this strain, only *ALS2* and *ALS3* expression differed significantly between 2 and 6h, with a decrease of 90 and 60%, respectively ($p<0.05$). VVC isolate was also able to express a large number of adhesins, however, most of the genes were expressed in low amounts (Fig. 3C). Attention should be given for *ALS3* and *ALS6*, that were expressed in both times, in equivalent amounts. Among these genes, *ALS3* was significantly expressed compared to *ALS6* (six-fold, $p<0.05$). In general, RVVC isolate expressed a lowest number of adhesins (Fig. 4C). Although, the expression levels of *ALS2*, *ALS3* and *HWP1* at 6h were significantly higher to RVVC than ASS and VVC isolates, $p<0.05$. Furthermore, it was possible to observe that *ALS3* and *HWP1* increase expression over time (2h to 6h), significantly.

Quantitative expression of *SAP1–10* genes in experimental cervical epithelial infections

In general, we find that ASS, VVC and RVVC isolates of *C*. *albicans* were able to express *SAP* during colonization of the cervical cells in both times (Fig. 2D, 3D and 4D).

Regarding expression of *SAP* genes after interaction with cervical cells and yeast show that in the general form RVVC isolate showed the highest expression of *SAP* genes and there was an adhesion reduced *SAP* expression in ASS and VVC isolates.

In the first time of the adhesion (2h), ASS isolate expressed significantly higher levels of *SAP1* gene ($p<0.05$) than others genes of this family (Fig. 2D). For VVC

isolate, only the *SAP8* was expressed., while RVVC isolate expressed *SAP4-8* with significance for *SAP4*, *7* and *8* ($p<0.05$).

In the second period (6h) in cervical cells adhesion, there was an increase in the number and quantity of the *SAP* expressed by all isolates tested. For ASS isolate, *SAP1-3* and *SAP8* were significantly expressed ($p<0.05$), Fig. 2D. Considerable increase in the expression of *SAP* was observed for VVC isolate, highlighting *SAP3*, *SAP5*, *SAP7* e *SAP10* ($p<0.05$), Fig. 3D. On the other hand, it should be emphasized that similar to what happened to *ALS* family of gene expression, isolated RVVC expressed *SAP* gene family in higher levels than ASS and VVC isolates. In RVVC isolated, remained the expression of genes *SAP5*, *SAP7* and *SAP8,* besides significantly increased the expression of *SAP2-4*, *SAP6*, *SAP7* and *SAP10*, Fig. 4D.

DISCUSSION

Vulvovaginal candidiasis continues to be a health problem to women worldwide and *C. albicans* remains the major etiologic agent of VVC as it is commonly encountered in 60–80% of cases. Several studies related to this pathology, although the pathogenesis of VVC is still unclear, mainly the interaction between the host and fungus, which contributes to the development of this disease (Alves et al., 2014b; Cassone, 2014; Zhang et al., 2014). There is no consensus regarding the factors leading the development of VVC, some authors attribute to host factors, on the other hand, it is known virulence attributes of yeasts that are able to cause infection (Sârbu et al., 2013).

Adhesion of *C. albicans* to epithelia is fundamental, as a prerequisite, to yeast penetrate tissues in order to mediate pathogenesis in the host (Sohn et al., 2006). In this research, we study the early events of host–pathogen interaction by through an adhesion *in vitro* assay to human cervical cells. As vaginal cells model, we used human cervical carcinoma cells line (*HeLa*). These cells line are very often used to study, *in vitro*, mechanisms of interaction among different species of *Candida* and genital tract (Negri et al., 2011; Sudjana et al., 2012; Sârbu et al., 2013).

Adhesion potential of *C. albicans* to different epithelia has been studied by different authors (Jordan et al., 2014; Sohn et al., 2006). In the initial adhesion time, we found that despite all the isolates tested were able to adhere on cervical cells,

surprisingly, ASS isolate had greater potential of the adhesion compared to VVC and RVVC isolate ($p<0.05$), which showed similar adhesion pattern. Adherence of *C. albicans* to host epithelial cells is a critical first step in the infection process, however, others factors are involved in the development of the disease. Furthermore *C. albicans* intra-species do not adhere to the same extent to mucosal cells or epithelium and this fact can be related with isolate features (Alves et al., 2014a; Jordan et al., 2014; Sohn et al., 2006).

Moreover, this adhesion profile remained until the second time analysis (6h), Fig. 1. SEM revealed that ASS and VVC isolates had predominantly yeast forms in both times of adhesion kinetics. On the other hand, RVVC isolate showed markedly increased filamentation in time of 6h adhesion. Morphological transition from yeast- to hypha-form, is considered an important factor that can be a facilitate in the adhesion phenomenon, and contribute to virulence, as invasion and secretion of hydrolytic enzymes (Calderone and Clancy, 2002; Calderone and Fonzi, 2001; Romeo et al., 2011).

In this context, is known that during the adhesion process, there may be changes in the transcriptional profile of yeast, resulting in important alterations in the adhesion and virulence profile. Genes belonging to the *ALS* gene family and *HWP1* encode cell-surface associated glycosylphosphatidylinositol (GPI) anchored glycoproteins that mediate adhesion of *C. albicans* to mucosal surfaces, as the genital tract (Hoyer et al., 2008; Hoyer et al., 2001). In current study different levels of expression of the *ALS* genes and *HWP1* were detected in ASS, VVC and RVVC isolates, during the time course in our *in vitro* experiments. The ASS isolate was able to express most *ALS* gene family, and had higher adhesion ability. Other studies have already shown that these genes contibuem to the adhesion process (Zhao et al., 2004; Zhao et al., 2005). However, we must emphasize that, the VVC and RVVC isolates express fewer ALS gene family, but with the levels greater than the ASS isolated.

VVC isolate showed high level of expression for *ALS3* and *ALS6* in both time, and *ALS1* and *ALS9* in 6h. Hoyer, 2008 describes hat these *ALS* gene family, can build to the pathogenesis of *C. albicans*, primarily involved in host-pathogen interactions (Hoyer et al., 2008). Studies show that Als1 and Als3 may be involved in tissue destruction, and Als6 and Als9 with the ability to interact yeast-host (Coleman et al.,

2012; Coleman et al., 2010; Liu and Filler, 2011; Zhao et al., 2004). The RVVC isolate show express significant for *ALS2*, *ALS3* and *HWP1* genes, in both time. These results are consistent with findings from SEM assay (Figure 4Bd), which show the morphology changes (transition of the yeast for hypha form) of this isolate.

It is known that *SAP* gene products are known to contribute to virulence processes such as adhesion, invasion and immune evasion. Differential regulation of certain *SAP* genes has been reported (Schaller et al., 2001). Thus, they might play individual roles at certain stages of the infection (Aoki et al., 2011). So, in our study, we find that although of the ASS isolate has greater ability to adhere, it did not refecte in true virulence factor, since the ASS isolate showed expression of *SAP1-3* and *SAP9-10* genes, which are not involved aggressively in infectious process. Biu, 2013, described that *SAP1-3* are involved in yeast form, this results are reflected in the SEM analysis (Buu and Chen, 2014). In addition, Aoki, 2011 showed that *SAP9-10* provide low pathogenicity (Aoki et al., 2011). The VVC isolated had low expression of the *SAP* gene family, similar to isolated ASS.

On the other hand, the isolated RVVC showed expression of a high expression of *SAP* gene family, in relation to the VVC and ASS isolates. Furthermore, there was the expression of *SAP* genes involved in the process filamentation and pathogenicity (*SAP4-6*) (Buu and Chen, 2013; Felk et al., 2002; Naglik et al., 2003a). These results also reflected in the SEM assay (Figure 4Bb and 4Bd), that showed that during the adhesion kinetics was this change in morphology, with the presence of yeast filamentation in 6h, following *SAP4-6*, *ALS3* and *HWP1* up-regulation. Some authors suggest that *C*. *albicans*, increases the adhesion ability in the presence of pseudohyphae when compared with yeast form (Naglik et al., 2003a; Nikawa et al., 2003). However, in our study, the adhesion process has remained constant, despite to observer filamentation in 6h, according to Henriques, 2007 (Henriques et al., 2007). Hyphal formation did not seem to play an essential role in the adhesion of *Candida*, but may be tightly related with invasion mechanism. Hence, other factors, rather than physico-chemical properties seem to be ruling the process of adhesion (Alves et al., 2014b; Henriques et al., 2007; Negri et al., 2011). Among these factors the peripheral proteins are that promote adhesion, exemplified by the expression of *ALS3*, *HWP1*, *SAP3-6* genes. It is believed that after 2h yeast-cells contact, there is the start of biofilm

formation, which is considered an important virulence factor and has been recognized as a step for establishment of infection (Ramage et al., 2012). We observed that at 6h of the adhesion, RVVC isolated up-regulation genes important for biofilm formation, eg. *ALS3* and *HWP1*. Reserachs using mutant strains, it was demonstrated that *ALS1*, *ALS2*, *ALS3* and *HWP1* are important for biofilm growth *in vitro* and *in vivo* (Hoyer et al., 2008; Nobile et al., 2006; Zhao et al., 2004; Zhao et al., 2005) and that *ALS1*/*ALS3* and *HWP1* have complementary roles in biofilm formation, inclusive in mucosa surfaces (Naglik et al., 2008; Ricicova et al., 2010).

In summary, we observed that despite the ASS isolate, have greater adhesion capacity, showed down-expression of pathogenicity-related genes. And yet, that despite VVC and RVVC having less potential adhesion, which were able to markedly up-regulation genes involved in the pathogenicity, e.g. *ALS1*, *ALS3*, *HWP1* and *SAP3-6*. In this paper once again we show that the yeasts have an important potential virulence that contribute for the development of VVC. We believe that with the results of previous work, that the RVVC isolate may have a significant participation in the pathology of VVC due to its ability to detoxify reactive oxygen species generated by neutrophils, and consequently survive the action of these immune cells. Furthermore, in this research we suggest that RVVC isolate may be more related to invasion than colonization, since this isolated was able to produce virulence attributes providing greater potential for invasion of epithelial host. As a preliminary study, this work only explored a short period of contact among yeasts isolated from VVC and human cervical cells; more studies, incluing longer contact times, would be of interest, as would the inclusion of different clinical isolates. Thus, the knowledgement about the behavior of VVC isolates in process of colonization-infection of human cervical cells, could help in the development of new therapeutic strategies.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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Table 1. Primers used for real-time PCR analysis of *SAP*, *ALS* and *HWP1* gene expression

	Target	Foward (5'→3')	Reverse (5'→3')
Secreted Aspartyl Proteinase gene family	*SAP 1*	TTTCATCGCTCTTGCTATTGCTT	TGACATCAAAGTCTAAAGTGACAAAACC
	SAP 2	GGATACCGTTGGATTTGGTGG	GTGACAGGGACATTATCATAACTACCAC
	SAP 3	CCCAAGGTGAAATCAATACCAAC	GGAACCAACAGTAATATCAGAAGCATAA
	SAP 4	AGCGGCTCTTTAGTTGATTTGC	AGAATCTAAGAGGACACCAGCGTT
	SAP 5	CCAGCATCTTCCCGCACTT	TTTAGCGTAAGAACCGTCACCAT
	SAP 6	TGGTATTGGTGGTGCTTCTGTT	CGTAAGGAGTTCTGGTAGCTTCG
	SAP 7	GTGCTGTCTCACAAGTCGAACAA	CTGATGGTTGCGAAGAGGAAG
	SAP 8	CTGTTATTGTTGACACAGGTTCTTCTG	GTGATTGACCAGGATAAGTGACTTGA
	SAP 9	TTCGGGTTCAGGAACAACATCT	GCTGAATGACGTGTGCTGGTAC
	SAP 10	TCCTGACACCATCCAAATACACA	TCGAACCGATCTCCAATTCTG
Agglutinin-Like Sequence genes family	*ALS1*	TTACCTCATCCTCACCTTCAACC	CAAACCGTACAACCAAGTAGAATGTT
	ALS2	ACCAGGTGGCACTGACTCAGT	AGGTGGCTCTCTTATGATAACAGTAGC
	ALS3	GGTGGCACTGATTCGGTTATC	TGGTAGTGGTAGTTGCGTAAGATTG
	ALS4	CCCAGTCTTTCACAAGCAGTAAGTC	TCCCGACATGAACTGTGGAAC
	ALS5	ACTCCATTAGCACAAGTCAGGAGA	CACTTGACACTAAAATATCGGTGACTG
	ALS6	TACTGTTGAAATCCCTCCTGTGACT	CTCCAAGTGTTGCCGTAGAAGC
	ALS7	TCTCTTACCGTGGGGGAACTTT	TCCGATGGACGTTTCAGACA
	ALS9	TTCCCACGACCACAATTACAACT	TGACTGTCGCAGTACCACCAAT
Hyphal Wall Protein	*HWP1*	GTTCCACTCATGCAACCATC	GCACCGAAAGTCAATCTCATG
Housekeeping Gene	*ACT1*	AAGTGTGACATGGATGTTAGAAAAGAAT	ATGGAGCCAAAGCAGTAATTTCC
	CEF3	CAACCCAAGACGAATGTAAAACC	GTCAAACCAACTTCACCATCTTCA

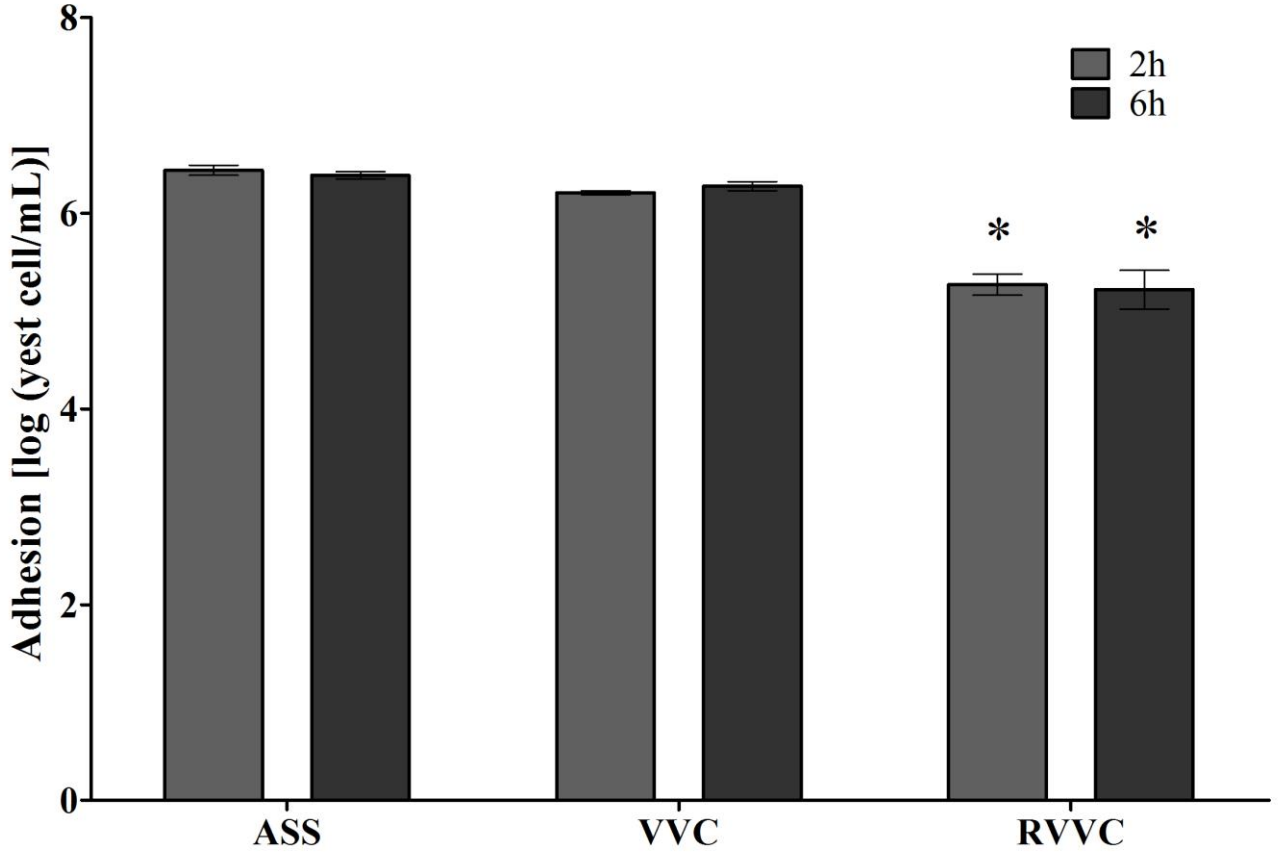

Fig. 1: Average log of CFUs of isolates of the *Candida albicans* (ASS, VVC and RVVC) obtained in adherence assays *in vitro* on cervical cells (*HeLa*). * $p \leq 0.05$, significant difference among RVVC isolate and the otheres samples tested in 2h and 6h of the adherence assay.

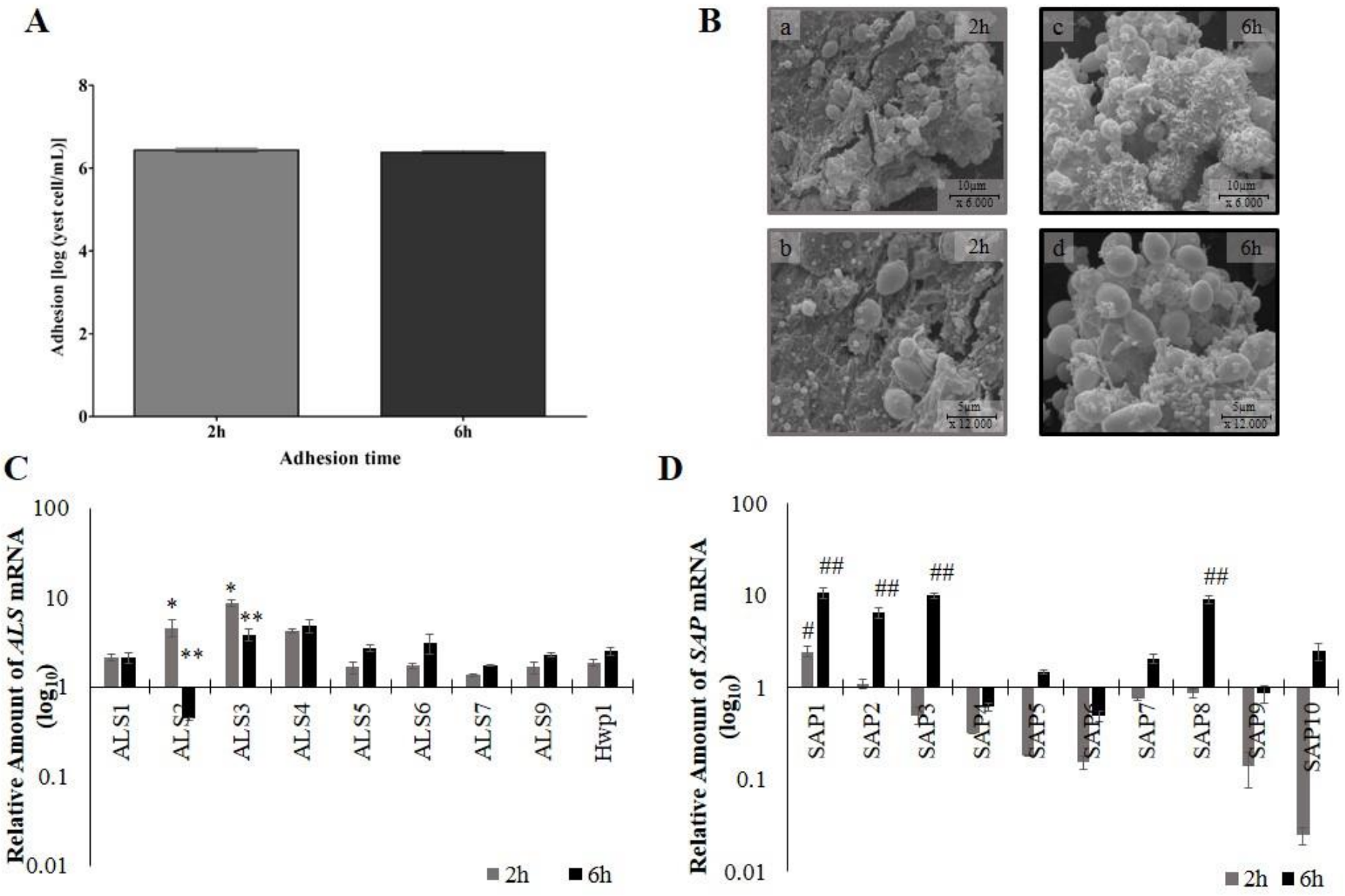
A
Adhesion [log (yest cell/mL)]
8
6
4
2
0
2h
6h
Adhesion time
B
a
2h
10μm
x 6.000
c
6h
10μm
x 6.000
b
2h
5μm
x 12.000
d
6h
5μm
x 12.000
C
Relative Amount of ALS mRNA (log10)
100
10
1
0.1
0.01
ALS1
ALS2
ALS3
ALS4
ALS5
ALS6
ALS7
ALS9
Hwp1
2h
6h
D
Relative Amount of SAP mRNA (log10)
100
10
1
0.1
0.01
SAP1
SAP2
SAP3
SAP4
SAP5
SAP6
SAP7
SAP8
SAP9
SAP10
2h
6h

Figure 2: Overview of the interaction process ASS isolated on cervical cells

A. Adhesion kinetics for 2 to 6 hours.

B. Monitoring by SEM, 1b and 2b representing adhesion process for 2 hours. 4b and 5b represent adhesion process for 6 hours.

C. Gene expression fold-change values derived from qPCR data. The changes in expression of nine genes: *ALS1-7*, *ALS9* and *HWP1*, of the ASS isolate in Hela-affected cells. The results were normalised to *ACT1* and *CEF3*, and plotted with respect to equivalent yeast from control.

D. Gene expression fold-change values derived from qPCR data. The changes in expression of nine genes: *SAP1-10* of the ASS isolate in *Hela*-affected cells. The results were normalised to *ACT1* and *CEF3*, and plotted with respect to equivalent yeast from control.

The data are expressed as the mean ± SD of three separate experiments. *,# $p \leq 0.05$, significant difference among all genes tested **, ## $p \leq 0.05$, significant difference among two and six hours.

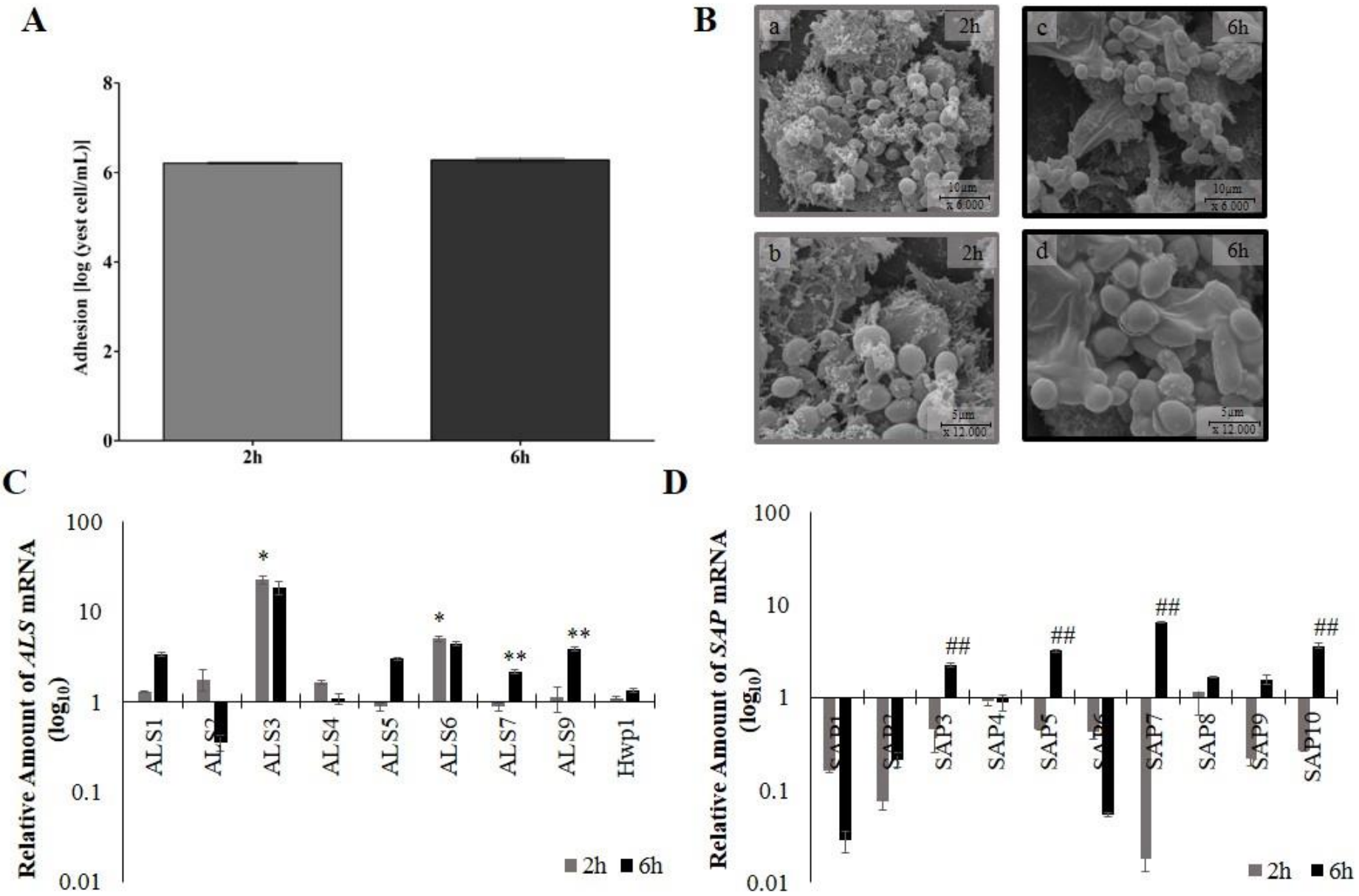
A
Adhesion [log (yest cell/mL)]
8
6
4
2
0
2h
6h
B
a
2h
10µm
x 6.000
c
6h
10µm
x 6.000
b
2h
5µm
x 12.000
d
6h
5µm
x 12.000
C
Relative Amount of ALS mRNA (log10)
100
10
1
0.1
0.01
*
*
**
**
ALS1
ALS2
ALS3
ALS4
ALS5
ALS6
ALS7
ALS9
Hwp1
2h
6h
D
Relative Amount of SAP mRNA (log10)
100
10
1
0.1
0.01
##
##
##
##
SAP1
SAP2
SAP3
SAP4
SAP5
SAP6
SAP7
SAP8
SAP9
SAP10
2h
6h

Figure 3: Overview of the interaction process VVC isolated on cervical cells.

A. Adhesion kinetics for 2 to 6 hours.

B. Monitoring by SEM, 1b and 2b representing adhesion process for 2 hours. 4b and 5b represent adhesion process for 6 hours. The White arrows indicate the interaction VVC-*HeLa* cells

C. Gene expression fold-change values derived from qPCR data. The changes in expression of nine genes: *ALS1*-7, *ALS9* and *HWP1*, of the ASS isolate in *Hela*-affected cells. The results were normalised to *ACT1* and *CEF3*, and plotted with respect to equivalent yeast from control.

D. Gene expression fold-change values derived from qPCR data. The changes in expression of nine genes: *SAP1-10* of the ASS isolate in *Hela*-affected cells. The results were normalised to *ACT1* and *CEF3*, and plotted with respect to equivalent yeast from control.

The data are expressed as the mean ± SD of three separate experiments. *,# $p \leq 0.05$, significant difference among all genes tested **, ## $p \leq 0.05$, significant difference among two and six hours.

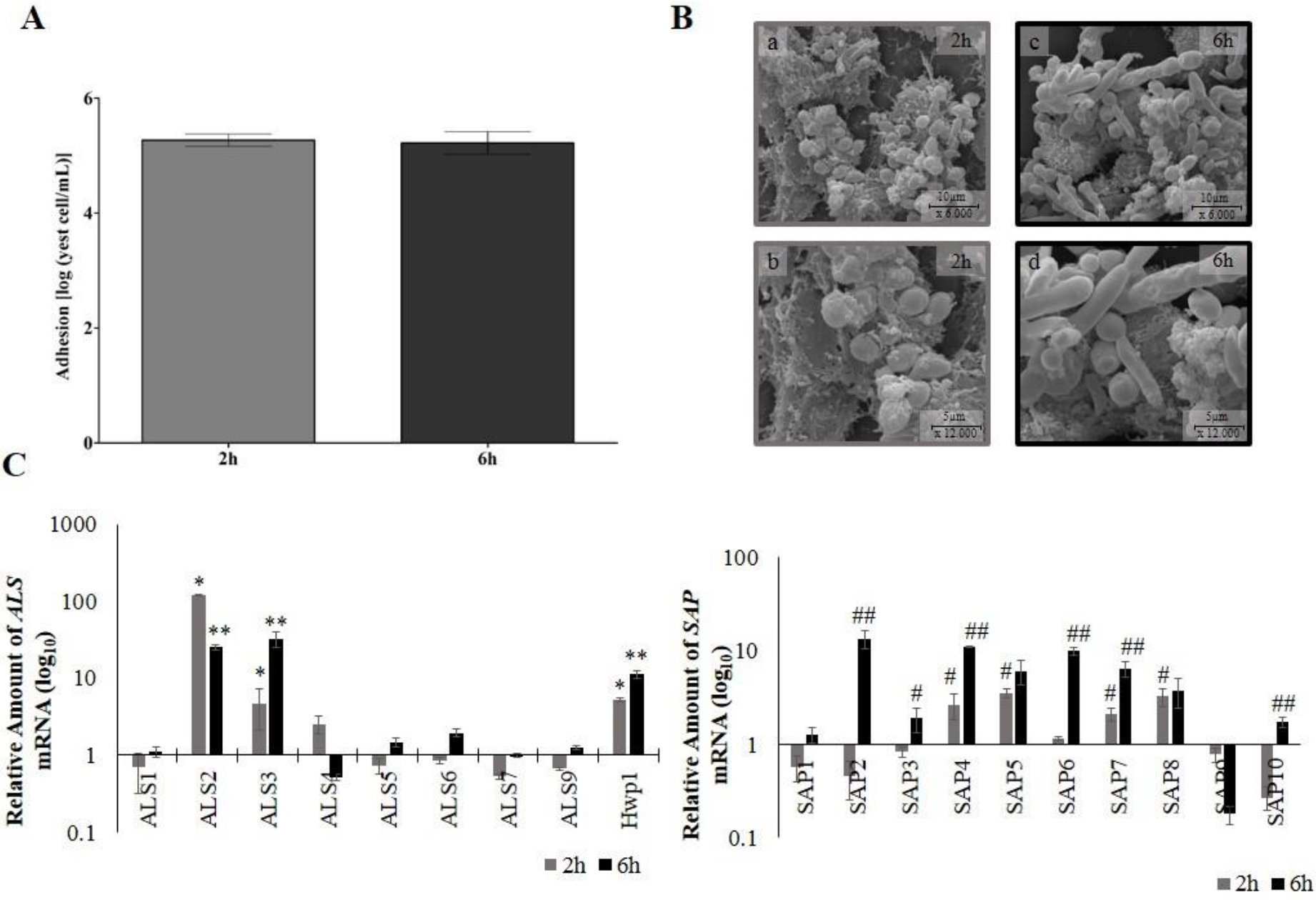
A
Adhesion [log (yest cell/mL)]
6
4
2
0
2h
6h
B
a
2h
10µm
x 6.000
c
6h
10µm
x 6.000
b
2h
5µm
x 12.000
d
6h
5µm
x 12.000
C
Relative Amount of ALS mRNA (log10)
1000
100
10
1
0.1
ALS1
ALS2
ALS3
ALS4
ALS5
ALS6
ALS7
ALS9
Hwp1
2h
6h
Relative Amount of SAP mRNA (log10)
100
10
1
0.1
SAP1
SAP2
SAP3
SAP4
SAP5
SAP6
SAP7
SAP8
SAP9
SAP10
2h
6h

Figure 4: Overview of the interaction process RVVC isolated on cervical cells.

A. Adhesion kinetics for 2 to 6 hours.

B. Monitoring by SEM, 1b and 2b representing adhesion process for 2 hours. 4b and 5b represent adhesion process for 6 hours. The white arrows indicate the presence of pseudohyphae and interaction RVVC-*HeLa* cells

C. Gene expression fold-change values derived from qPCR data. The changes in expression of nine genes: *ALS1-7*, *ALS9* and *HWP1*, of the ASS isolate in *Hela*-affected cells. The results were normalised to *ACT1* and *CEF3*, and plotted with respect to equivalent yeast from control.

D. Gene expression fold-change values derived from qPCR data. The changes in expression of nine genes: *SAP1-10* of the ASS isolate in *Hela*-affected cells. The results were normalised to *ACT1* and *CEF3*, and plotted with respect to equivalent yeast from control.

The data are expressed as the mean ± SD of three separate experiments. *,# $p \leq 0.05$, significant difference among all genes tested **, ## $p \leq 0.05$, significant difference among two and six hours.

CAPÍTULO III

Este capítulo tem por objetivo apresentar as conclusões e perspectivas futuras.

CONCLUSÕES

Nossos principais achados mostram que os isolados clínicos de candidíase vulvovaginal (CVV) pode desencadear diferentes respostas no hospedeiro. *C*. *albicans* foi capaz de detoxificar espécies de reativas de oxigênio geradas por neutrófilos, e, por conseguinte, sobreviver a ação destas células imunitárias. Por outro lado, nenhum isolado de *C*. *glabrata* foi capaz de alterar a resposta dos neutrófilos. Acreditamos então, que *C*. *glabrata* está emergindo na epidemiologia da CVV, muito provavelmente à sua resistência intrínsica aos antifúngicos azólicos, usados rotineiramente na prática clínica. A ação imunomoduladora de β-glucana foi eficiente, capacitando os neutrófilos a serem efetivos para todos isolados clínicos testados, inclusive para aqueles inicialmente resistentes. Além disso, notamos que esse polissacarídeo é capaz de estimular os neutrófilos mesmo sem um contato prévio com as leveduras.

Os ensaios de adesão *in vitro*, reforçam os dados sobre o isolado CVVR. Este por sua vez, parece estar mais relacionado à invasão do que colonização, uma vez que este foi capaz de produzir atributos de virulência proporcionando maior potencial de invasão epitelial.

PERSPECTIVAS FUTURAS

Os resultados obtidos neste trabalho, nos impulsiona a continuar pesquisas com diferentes espécies de *Candida*, com os seguintes objetivos:

- avaliar rotas bioquímicas induzidas nas células do sistema imunológico, durante a infecção por *Candida* spp.,

- avaliar o uso de β-glucana em estudos *in vivo*,

- aprofundar os ensaios de adesão *in vitro*, avaliando outras proteínas envolvidas no processo de adesão/invasão,

- avaliar possíveis danos celulares que podem ocorrer durante o processo de adesão dos isolados clínicos,

- dosar citocinas liberadas no processo de adesão à linhagem celular.