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ATIVIDADE BIOLÓGICA DO EXTRATO DE MOLÉCULAS DE *EUTERPE OLERACEA* Mart. (AÇAÍ) SOBRE BIOFILME DE *Candida parapsilosis e tropicalis*

LARISSA LIRA BRITO

São Luís-MA.
2017

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Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Saúde do Adulto e da Criança da Universidade Federal do Maranhão para obtenção do Título de Mestre em Saúde do Adulto e da Criança.

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Orientadora: Profa. Dra. Maria do Desterro Soares Brandão Nascimento

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“A tarefa não é tanto ver aquilo que ninguém viu, mas pensar o que ninguém ainda pensou sobre aquilo que todo mundo vê”.

(**Arthur Schopenhauer**)

RESUMO

BRITO, Larissa Lira. Atividade biológica do extrato de moléculas de *Euterpe oleracea* Mart. (Açaí) sobre biofilme de *Candida parapsilosis e tropicalis*. 2017, 74 folhas. Tese (Mestrado) Universidade Federal do Maranhão.

A candidíase ocorre como consequência de um distúrbio imunológico do hospedeiro e dos fatores de virulência destas leveduras. A Candida albicans têm sido relatada como a mais prevalente, seguida de C. parapsilosis, C. glabrata, C. tropicalis e C. krusei. A resistência dos microrganismos vem aumentando em função do uso indiscriminado de antimicrobianos utilizados no tratamento de doenças infecciosas, impulsionando pesquisadores a estudarem novas substâncias antimicrobianas de várias fontes, incluindo as plantas medicinais. Esse estudo tem por função elucidar o potencial antifúngico do açaí sobre biofilmes de Candida. São designados biofilmes as formas de crescimento resistentes a medicamentos representando uma grave ameaça aos indivíduos imunocomprometidos. O objetivo deste trabalho foi avaliar a atividade antifúngica in vitro do extrato do açaí (Euterpe Oleracea) frente aos biofilmes formados por cepas de Candida. O extrato da casca e do caroço da Euterpe tem forte efeito na formação de biofilme por ambas as espécies C. tropicallis e C. parapsilosis, as quais inibiram a formação daquele em diferentes concentrações dos extratos, mostrando o efeito de produtos naturais, evidenciando fitoquímicos, atividades biológicas e potencial da Euterpe para futuras aplicações industriais.

Palavras-chave: Biofilme; Candida spp.; C. albicans; C.parapsilosis; C. tropicallis; *Euterpe oleracea* Mart.

ABSTRACT

BRITO, Larissa Lira. Biological activity of the *Euterpe Oleracea* molecules extract Mart. (Açaí) about biofilme de *Candida parapsilosis e tropicalis*. 2017, 74 sheets. Thesis (Master degree) Federal University of Maranhão.

Candidiasis occurs as a consequence of an immune disorder of the host and of the virulence factors of these yeasts. Candida albicans has been reported as the most prevalent, followed by C. parapsilosis, C. glabrata, C. tropicalis and C. krusei. The resistance of microorganisms is increasing due to the indiscriminate use of antimicrobials used in the treatment of infectious diseases. Prompted researchers to study new antimicrobial substances from various sources, including medicinal plants. This study aims to elucidate the antifungal potential of açaí on biofilms of Candida. Biofilms are designated as drug resistant growth forms representing a serious threat to immunocompromised individuals. The objective of this work was to evaluate the in vitro antifungal activity of açaí extract (Euterpe Oleracea) against biofilms formed by Candida strains. Euterpe peel and core extract has a strong effect on the formation of biofilms by both C. tropicallis and C. parapsilosis species, inhibiting its formation in different concentrations of the extracts, showing the effect of natural products, evidencing phytochemicals, Activities and potential of Euterpe for future industrial applications.

Key words: Biofilm; Candida spp.; C. albicans; C.parapsilosis; C. tropicallis; Euterpe oleracea Mart.

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SUMÁRIO

1. INTRODUÇÃO

Os fungos do gênero Candida naturalmente compõem a microbiota do corpo humano e animais, colonizando a pele e as mucosas dos tratos digestivo e urinário, bucal e vaginal. Aproximadamente 200 leveduras são encontradas incluídas no as do gênero Candida, sendo que destas, pouco mais de 20 espécies são responsáveis por infecções no homem, estas leveduras são consideradas o principal grupo de fungos patógenos oportunistas, representando cerca de 8-10% das causas de infecções sanguíneas nosocomias em Unidades de Terapia Intensiva (UTIs) (HOSSAIN *et al*., 2003; BORG-VON *et al*. 2007; KUMAR *et al*. 2008; KARKOWSKA-KULETA *et al*. 2009; NEGRI *et al*. 2010).

Durante muitos anos a Candida albicans foi relatada como a espécie predominante responsável pela maioria (60-80%) das infecções causadas pelo gênero Candida, no entanto, as espécies não-albicans, como a *C. glabrata*, a e a *C. parapsilosis* foram frequentemente isoladas principalmente devido ao uso indiscriminado de agentes antifúngicos. (KRCMERY; BARNES, 2002; ARENDRUP, 2013; GUINEA, 2014).

As infecções por espécies de Candida ocorrem em decorrência de distúrbio imunológico do hospedeiro e dos fatores de virulência expressos por estas leveduras, que contribuem para habilidade de colonizar, penetrar e invadir o tecido, na maioria das vezes de origem endógena (BROWN; ODDDS; GOW, 2007; HOLLENBACH, 2008) desenvolvendo, assim, a Candidíase. Espécies não-albicans envolvidas nesse tipo de infecção, C. parapsilosis, C. glabrata, C. tropicalis e C. krusei, têm sido relatadas como a mais prevalentes (LU; LEE; CHUEN, 2004; ODDS *et al*., 2006, PFALLER; DIEKEMA, 2007; PANIZO *et al*., 2009).

Além dos fatores endógenos, o uso indiscriminado de antifúngicos de amplo espectro e ao aumento de dispositivos médicos implantados influenciam o aumento das candidíases por espécies não-albicans que surgem como segunda e terceira causa principal de Candidíase sistêmica. Geralmente, as espécies de Candida, são responsáveis por infecções fúngicas superficiais em imunocompetentes e sistêmicas em imunodeprimidos. Nesse mecanismo de infecção, ocorre a expressão de fatores de virulência que medeiam a relação hospedeiro e microbiota autócto, ou seja, do comensalismo à doença sistêmica fatal. A variedade de apresentações da doença leva à necessidade de utilização de diferentes métodos diagnósticos e esquemas terapêuticos (PAULA *et al.*, 1998; SANDAI *et al.*, 2016).

A patogênese da Candidíase é comum a todas as espécies de Candida sendo facilitada por uma série de fatores virulentos, dentre os quais podemos destacar: a capacidade de aderir a dispositivos médicos ou células hospedeiras, desenvolvimento de biofilmes e transição para a forma filamentosa (SILVA *et al.*, 2012). Como consequência do rompimento do equilíbrio parasita-hospedeiro pode ocorrer a Candidíase, que é desencadeada por alterações na barreira tecidual e na microbiota autóctone e pelo comprometimento das defesas naturais do organismo como a imunológica. Nas enfermidades que requerem uma permanência prolongada hospitalar há uma ocorrência maior do rompimento deste equilíbrio (PLAYFORD *et al*., 2008).

Naqueles pacientes que sofrem traumatismos constantes devido a procedimentos médicos invasivos, como uso de cateteres intravenosos, nutrição parenteral, sondas e com extensas queimaduras, sofrem alterações na superfície epitelial ou de mucosas, possibilitando a proliferação ou mudança do sítio anatômico da levedura, contribuindo para a instalação e infecção por Candida no organismo do hospedeiro (PULCINI *et al*., 2006; CELEBI *et al*., 2008).

A resistência dos microrganismos vem aumentando em função do uso indiscriminado de antimicrobianos utilizados no tratamento de doenças infecciosas. Essa situação tem impulsionado pesquisadores a estudarem novas substâncias antimicrobianas de várias fontes, incluindo as plantas medicinais. Nesse sentido, considerando a ampla atividade biológica apresentada pela Euterpe Oleracea Mart., esse estudo tem por função elucidar o potencial antifúngico do açaí e viabilizar um novo fármaco ou alimento funcional no tratamento dos biofilmes de Candida.

2. FUNDAMENTAÇÃO TEÓRICA

2.1. Candidíase

As espécies pertencentes ao gênero Candida, fazem parte do filo Ascomycota, da classe Hemiascomycetes, da ordem Saccharomycetales, englobando as leveduras de interesse clínico e científico (DIEZMANN *et al*., 2004; CHAI; DENNING; WARN, 2010). Atualmente já foram descritas cerca de 300 espécies de Candida dentre as quais A C. albicans é a mais frequente nas infecções humanas, entretanto espécies não-albicans estão avançando cada vez mais nas infecções oportunistas (LACHANCE *et al*., 2011).

Em geral, as espécies do gênero Candida habitam o organismo humano e de outros animais, dessa forma são considerados comensais ou podem colonizar cerca de 50% dos indivíduos em um determinado momento de sua vida (LIONAKIS; NETEA, 2013). Neste sentido, essas espécies colonizam a pele, o trato gastrointestinal e o trato geniturinário, algumas vezes podem ser isolados no trato respiratório superior (EGGIMANN; GARBINO; PITTET, 2003). As condições normais de imunidade limitam o desenvolvimento de infecções por esse microrganismo, todavia, quando o indivíduo adquire uma baixa imunidade permite um ambiente favorável que permite conversão de espécies de Candida em patógeno oportunista, desenvolvendo a invasão nas mucosas e disseminação sanguínea (LIONAKIS; NETEA, 2013).

Nas últimas duas décadas houve um aumento na incidência de infecções por espécies de Candida, sendo esta a principal causa de infecções fúngicas em seres humanos (FOURNIER *et al*., 2011). Este aumento tem sido atribuído, em parte, ao crescente número de pacientes portadores de neoplasias ou doenças degenerativas, indivíduos transplantados e portadores do vírus HIV (CONDE-ROSA *et al*., 2010).

A invasão das células do hospedeiro inicia-se com a aderência dos blastósporos de Candida em células epiteliais, seguido da formação de hifa, penetrando na célula ativamente ou por endocitose, causando dano progressivo ao tecido (MODRZEWSKA; KURNATOWSKI, 2013). Em pacientes hospitalizados, Candida spp. pode acessar a corrente sanguínea via cateteres vasculares ou se disseminar a partir do intestino e provocar candidemia, doença associada com elevada mortalidade (FILLER, 2012).

O cenário atual das infecções pelo gênero Candida vêm ganhando destaque para as espécies não-albicans que avançam em diversos tipos de infecções, dentre as quais as mais frequentes são *C. glabrata, C. tropicalis, C. parapsilosis* e *C. krusei* (WILLIAMS; LEWIS, 2011; DE LUCA *et al*., 2012; SPAMPINATO; LEONARDI, 2013). Estudos apontam que a *C. glabrata* é frequentemente isolada de pacientes idosos, pacientes com câncer e aqueles expostos primariamente a fluconazol, piperacilina-tazobactam ou vancomicina. Relatam que a *C. parapsilosis* é predominante nas infecções de neonatos, de pacientes transplantados e nas infecções associadas ao uso de cateter venoso. Além disso, *C. parapsilosis* também pode ter relação com infecções adquiridas pelo uso de nutrição parenteral, pois tais fungos crescem em soluções extremamente ricas em glicose. Observa-se também um aumento nos casos de infecção por *C. tropicalis* associada a doenças hematológicas. E mostram ainda que *C. krusei* é a quinta espécie com resistência intrínseca ao fluconazol, tornando-se um fator de risco para candidemias nosocomiais (ALANGADEN, 2011).

O Programa ARTEMIS de Vigilância Antifúngica Global (ARTEMIS Global Antifungal Surveillance Program), nos EUA, mostrou *C. glabrata* (44%), C. *tropicalis* (6%). O impacto da doença fúngica na saúde humana foi, portanto, aumentando em especial devido ao número crescente de pacientes imunocomprometidos, resultado da epidemia de Aids, aumento do transplante de órgãos e quimioterapia para câncer e uso indiscriminado de antibióticos que causou um grande impacto sobre a microbiota humana. Espécies de Candida, representam um componente importante da carga de doenças causadas por fungos e são a quarta causa mais comum de infecções nosocomiais em hospitais norte-americanos (SELLAM; WHITEWAY, 2016).

A imunidade celular desempenha um papel importante na infecção causada pelas espécies Candida, determinando a susceptibilidade ou resistência dos indivíduos à infecção por este microrganismo. O sistema imune elabora mecanismos de defesa específicos e inespecíficos contra as leveduras com o intuito de impedir a proliferação e progressão de candidíases. Dentre os mecanismos envolvidos com esta defesa, as imunoglobulinas da classe IgA presentes nas secreções e saliva desempenham papel fundamental. Nas infecções por Candida das superfícies epiteliais, a IgA-s (secretora) age promovendo agregação dos fungos e inibe sua aderência às células epiteliais da mucosa, impedindo, consequentemente, sua proliferação. Pacientes com candidíase recorrente apresentam uma baixa resposta imune celular para antígenos de C. albicans. Este fato contribui para o entendimento acerca da patogênese da cadidíase recorrente, abrindo perspectivas para a utilização de agentes

imunomoduladores com a finalidade de restaurar a resposta imune destes pacientes. Clinicamente, a doença pode surgir como manifestações em mucosas até quadros sistêmicos, com a invasão de diversos órgãos. As mucosas oral, vaginal e esofágica são as mais acometidas em quadros de candidíases e de forma geral, a colonização é controlada por antagonismo competitivo da microbiota comensal, por competições nutritivas e pela produção de substâncias tóxicas que podem também interferir no mecanismo de aderência dessas leveduras às células epiteliais. A manutenção do pH salivar e a produção de ácido lático por essas células são fatores limitantes da colonização dessas leveduras. Com relação às infecções sistêmicas, pode-se observar a participação ativa do endotélio vascular, havendo interação entre receptores presentes nas células endoteliais e adesinas expressas pelas leveduras, podendo levar a disseminação hematogênica e causar microabscessos por todo corpo (CASTRO, 2010).

2.2. Aspectos gerais sobre *Candida tropicalis* e *Candida parapsilosis*

a) *Candida tropicalis*

Inicialmente a Candida tropicalis, era denominada de Oidium tropicale, a partir de 1910 foi diferenciada das demais espécies de Candida, pelo patologista e bacteriologista italiano Aldo Castellani. Desde então, recebeu outros como *Monilia tropicalis*, Candida vulgaris, Mycotorula dimorpha, Candida paratropicalis e outros 58 sinônimos. E só a partir de 1923, adquiriu o nome atual, o qual foi denominado por Christine Marie Berkhout (NEGRI *et al*., 2012a; OLIVEIRA, 2011).

A partir de 1960, C. *tropicalis* ganhou importância clínica desde então, é considerada a espécie causadora de candidíases invasivas graves. As infecções causadas por espécies podem ser adquiridas endogenamente, principal via de infecções, ou exogenamente, por meio do contato com pessoas ou fômites contaminados e estão relacionadas com fatores predisponentes como leucemia aguda, neutropenia e terapia anti-neoplásica, podendo ser infecções superficiais e localizadas de mucosa vaginal, do trato urinário, e infecções invasivas e disseminadas (CHAI; DENNING; WARN, 2010; NEGRI *et al*., 2012a).

Dessa forma, a C. *tropicalis* é considerada uma das espécies de Candida não-albicans mais prevalente nas infecções sanguíneas (candidemia) e do trato urinário (candidúria)

podendo ser a primeira ou segunda espécie mais isolada (COLOMBO et al., 2006; NUCCI *et al*., 2013; YISMAW *et al*., 2013), com frequência entre 3 a 66% das espécies de Candida isoladas de infecções de corrente sanguínea no mundo (CHAI; DENNING; WARN, 2010). Além disso, C. tropicalis demonstra ter mais sucesso na invasão da superfície de mucosas ou na colonização de cateteres intravasculares que as espécies C. albicans e C. glabrata (CHEN *et al*., 2012), apresentando também a habilidade de disseminação rápida após a colonização em hospedeiros imunocompetentes, causando alta mortalidade (CHAI; DENNING; WARN, 2010). Entre as espécies de Candida não-albicans, C. tropicalis vem sendo considerada a espécie mais frequente isolada de candidíases na região Ásia-Pacífico, Brasil e Europa (NEGRI *et al*., 2012a). Vários estudos mostram a frequência com que C. tropicalis é isolada de casos de candidíase no Brasil (COLOMBO et al., 2006; DA SILVA *et al*, 2014; DA COSTA *et al*, 2009; BRUDER-NASCIMENTO *et al*., 2010; NUCCI *et al*., 2013). Nos hospitais terciários brasileiros, C. tropicalis é apontada como a causa de 33-48% das infecções da corrente sanguínea por Candida (MORALEZ *et al*., 2013). Em 1998, 53% dos casos de candidúria no Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto-SP, no Brasil, foram causados por C. tropicalis (OLIVEIRA; MAFFEI; MARTINEZ, 2001). Um estudo prospectivo realizado por Colombo *et al*. (2006) em onze centros médicos brasileiros mostrou que espécies de Candida não-albicans foram mais frequentemente isoladas (59%), sendo que C. tropicalis (21%), C. parapsilosis (21%) e C. glabrata (5%) foram as mais isoladas.

b) *Candida parapsilosis*

Estudos sobre Candida parapsilosis surgiram a parti de 1928 quando Ashford em Porto Rico isolou das fezes diarreicas de um paciente, então nesta época foi pela primeira vez denominada por ele de *Monilia parapsilosis*, caracterizava-se por ser incapaz de fermentar a maltose. Esta nomenclatura permitiu distingui-la de *Monilia psilosis*, nomenclatura dada na época para Candida albicans (NOSEK *et al*., 2002; ALVAREZ-LERMA *et al*., 2003).

Morfologicamente esta espécie apresenta formas ovalares, redondas ou cilíndricas. Seu isolamento em Agar Sabouraud dextrose mostra colônias brancas e cremosas, brilhante e lisa ou rugosa. Podemos diferenciá-la de outras espécies do gênero Candida por não formar hifas verdadeiras e suas múltiplas formas fenotípicas que na fase leveduriforme forma fenótipos coloniais com textura lisa com forma neive, e na forma pseudohifal formam colônias concêntricas em forma crepe. Acredita-se que o desenvolvimento de pseudohifa nesta espécie está associado a um conjunto de aminoácidos como a citrulina, que causam

mudanças na sua morfologia celular e fenotípica da colônia (LAFFEY; BUTTER, 2005; KIM; BISSATI; BEM MAMOUN, 2006).

Inicialmente, esta espécie não foi considerada patogênica, mas estudos relatam o seu isolamento como agente etiológico de infecções desde 1940 (JOACHIM; POLAYES, 1940), e atualmente o aumento nos quadros infecciosos exógenos é favorecido pelo uso de instrumentos médico-hospitalares invasivos e soluções utilizadas em alimentação parenteral (KUHN, *et al*., 2004).

Os avanços nos estudos de C. parapsilosis permitiu a separação desta espécie em três grupos (I, II e III) até 2005. Atualmente mais estudos, embasados em características genéticas revelaram que há características genéticas distintas nesta espécie e permitiu outra classificação separando-a em espécies distintas intimamente relacionadas e foram denominadas em Candida parapsilosis, Candida orthopsilosis, Candida metapsilosis (TAVANTI, *et al*., 2005).

Entretanto, essa espécie possui um padrão de distribuição bem distinto e atualmente é considerado um patógeno em potencial, comumente isola-se de diferentes amostras clínicas de humanos e de outras fontes, como animais domésticos, água doce e salgada, solo, insetos. Um dos motivos para o avanço nas infecções pode ser o fato de esta espécie ser um comensal humano normal, este fator favorece a prevalência de infecções como infecção do trato urinário, fungemia, endocardites, meningites, peritonites, artrites, infecções oculares, otomicoses e onicomicoses (ÁLVARES; SVIDZINSKI; CONSOLARO, 2007; TROFA; GÁCSER; NOSANCHUK, 2008) e pode está associado ao fato de suas características genéticas (TOZZO; GRAZZIONE, 2012; BERTINI, *et al*., 2013). No Brasil, a C. Parapsilosis está entre primeira ou a segunda causa mais comum das lesões de onicomicose (FIGUEREDO, *et al*., 2007; MARTINS *et al*., 2007) e está cada vez mais frequente nos pacientes imunocomprometidos (JAYATILAKE, *et al*., 2009).

2.3. Fatores de virulência e Patogenicidade

Podemos entender como fatores de virulência as características requeridas pelo microrganismo para desenvolver a doença (YANG, 2003). Dessa forma, considera-se a propriedade de aderência como o primeiro passo para o desenvolvimento de biofilme, durante neste processo de aderência, várias proteínas, denominadas adesinas, medeiam o

reconhecimento e a ligação das células fúngicas às superfícies celulares e às superfícies inertes, entretanto (DA COSTA *et al*., 2009; LI *et al*., 2007).

As espécies de Candida podem expressar uma variedade de fatores de virulência que contribuem para sua patogenicidade (LIONAKIS; NETEA, 2013), entre esses fatores podemos citar a aderência nas células do hospedeiro por meio de adesinas, transição morfológica, hidrofobicidade da superfície celular e secreção de enzimas hidrolíticas como fosfolipases, lipases e proteases (NEGRI *et al*., 2010; DE LUCA *et al*., 2012; COSTA *et al*., 2012). Outro importante fator de virulência de espécies de Candida é a formação de biofilme, tanto em tecidos do hospedeiro como em dispositivos médicos intracorpóreos (NEGRI *et al*., 2012b; RAMAGE *et al*., 2012). Vários mecanismos de patogenicidade já foram associados a C. tropicalis, como a adesão a diferentes superfícies, formação de biofilme, capacidade de disseminação, secreção de fator hemolítico e a produção de enzimas hidrolíticas (SILVA *et al*., 2012; FAVERO *et al*., 2011; NEGRI *et al*, 2012a; GALÁN-LADERO *et al*., 2013).

Biofilmes são comunidades de microorganismos devidamente organizados e incorporados em uma matriz extracelular. Este modo de crescimento é um potente fator de virulência para todas as espécies de Candida. Além disso, os isolados C. albicans, C. parapsilosis, C. tropicalis e C. glabrata são bons formadores de biofilmes. Durante a infecção a presença de biofilmes tem sido relacionada as maiores taxas morbidade e de mortalidade em comparação com isolados incapazes de formar biofilmes. A formação de biofilme é um fenômeno sequencial que envolve a aderência, a maturação e desprendimento como ilustrado na Figura 1. A formação de biofilme é um fenômeno sequencial que envolve a aderência, a maturação e desprendimento, como ilustrado na Figura 1. Aderência e colonização de células de Candida a um superfície abiótica e / ou biótica é a primeiro passo para o desenvolvimento do biofilme (Figura 1A). Após aderência inicial das células de Candida segue a divisão celular, essa proliferação leva à formação basal de uma camada de microcolônias de ancoragem (Figura 1B), e depois maturação subsequente do biofilme (Figura 1C). A maturação do biofilme é, geralmente, caracterizada pela presença de estrutura filamentosa.

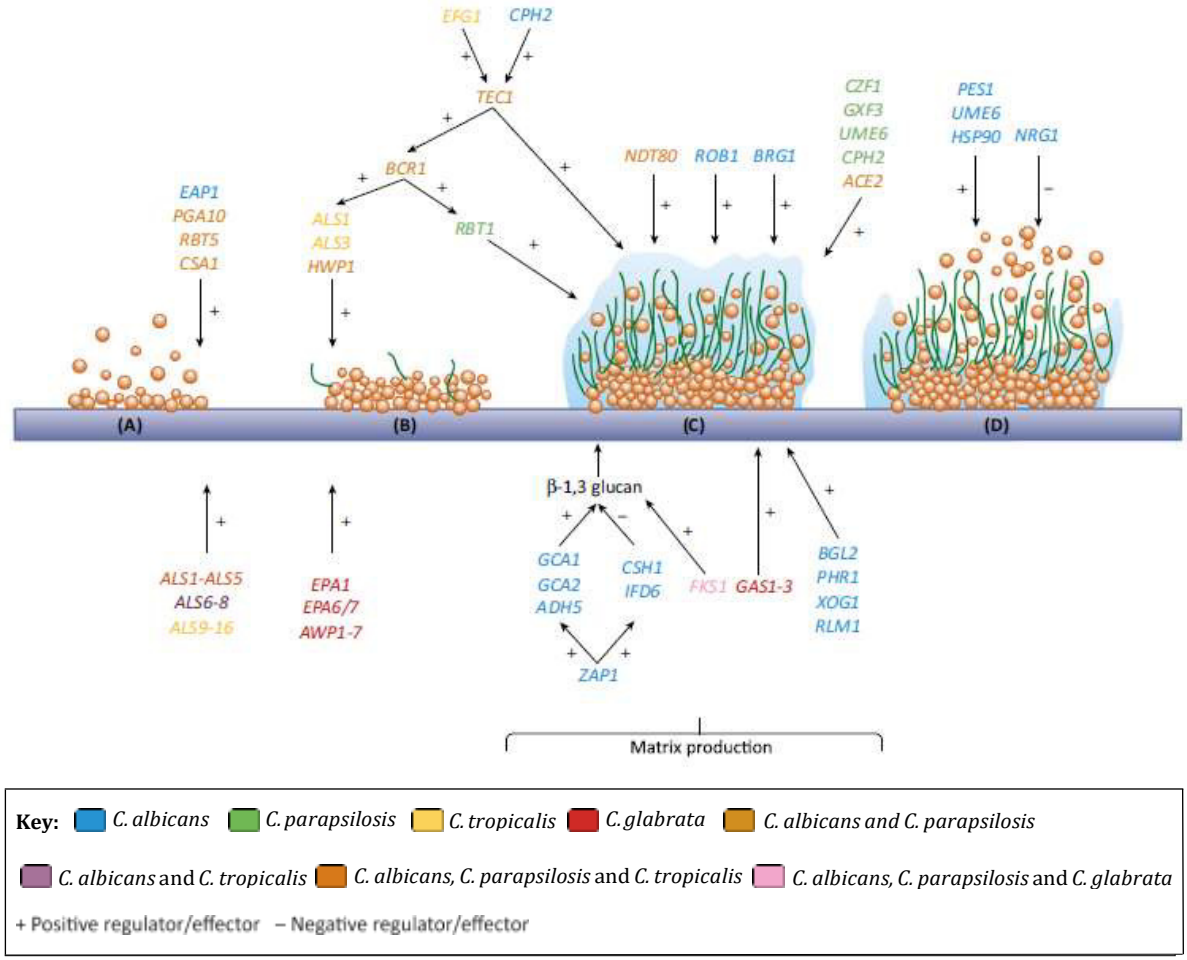


Figura 1. Genes de rede regulatória para os diferentes estádios de biofilme de Candida albicans, Candida parapsilosis, Candida tropicalis e Candidaglabrata. (A) Aderência inicial. (B) Formação de camadas de microcolonia basal. (C) Biodefólio maduro constituído por células com diversas morfologias e matriz extracelular. (D) Desprendimento e dispersão de biofilmes. Adaptado de ARAÚJO; HENRIQUES; SILVA, 2017.

Portanto, a formação de biofilme é um processo complexo, que envolve múltiplos tipos de células e fases. Recentemente, uma rede de regulação transcricional foi identificada na gênese da formação de biofilme, que inclui vários fatores de transcrição e circuitos interligados de controle (NOBILE *et al.*, 2012; NOBILE; JONHSON, 2015).

Experimentos in vitro demonstraram que o desenvolvimento de biofilme ocorre em uma série de passos sequenciais ao longo de um período de 24-48 horas. O passo inicial consiste na aderência de células leveduriformes de fungos individuais para formar uma camada basal de células. Em seguida vem a fase de proliferação celular em toda superfície e filamentos, que formam projeções alongadas que continuam a crescer em forma de hifas filamentosas. A produção de hifas é um marcador do início da formação de biofilme, seguido por um acúmulo da matriz extracelular de polissacarídeo culminando na maturação do

biofilme. O último passo consiste na liberação de células de levedura não aderentes a partir do biofilme em um ambiente onde podem colonizar outras superfícies (TSUI *et al*., 2016).

Investigações quantitativas e qualitativas das propriedades de biofilmes demonstraram que a maioria das células dispersadas são filamentosas, sugerindo que a transição de filamentosa para hifa pode ser revertida para dispersão. Esse achado indica que em tese, as células liberadas durante a etapa de dispersão são designadas exclusivamente para semear novos biofilmes e novos sítios de infecção. A análise genética indica que ambas as células, leveduras e hifas, são cruciais para formação de biopelícula, sugerindo que cada tipo de célula tem um papel exclusivo no processo (TSUI *et al*., 2016).

As investigações sobre a regulação temporal da formação de biofilme expandiu o circuito inicial, mostrando que não só são os complexos de controle transcricional, mas também a modificação ao longo do tempo de como o biofilme progride do estágio inicial até a maturidade. No entanto, a regulação da transcrição não é o único processo que contribui para regulação e formação de biofilme. O circuito de transcrição pode ser ligado à rede de Hsp90, já implicado em vários processos celulares (DIEZMANN; LEACH; COWEN, 2015). Além disso, os processos pós-transcrição que controlam a estabilidade do RNA através da proteína Puf3 e a Cccr4 desidrogenase foram encontrados desempenhando um papel na regulação da produção de matriz e na ligação da formação de biofilme para a função mitocondrial (SELLAM; WHITEWAY, 2016). A maior parte da matriz extracelular do biofilme é constituída de α-mannosidase e β-1 3-glucanase, essa última envolvida nos mecanismos de resistência dos antifúngicos (TSUI *et al*., 2016).

A produção extracelular de enzimas, como fosfolipases e proteinases, contribui para a virulência, já que são capazes de promover destruição dos tecidos do hospedeiro. Os mecanismos moleculares relacionados com tal virulência estão envolvidos com a ativação da via de transdução de sinal MAP (mitogen-activated protein) Kinase, onde respostas celulares envolvidas com formação de parede celular, crescimento invasivo, reprodução e adaptação ao estresse osmótico ocorrem mediante vias de sinalização intracelular como MKc1, Cek1/2 e HOG1 MAP Kinase. A ativação da via MAPK também proporciona a ativação do fator de transcrição Cph1, responsável pela forma filamentosa, considerada fator de virulência para ocorrência de infecções sistêmicas, e do CLA4, responsável pela formação do tubo germinativo e hifas. A via de ativação PKA proporciona a formação de AMPc, que regula o fator Efg1, responsável pela formação de hifas. Ressalta-se que outras vias de sinalização intracelular, como a p38 MAPK, também estão envolvidas com a patogenicidade da C. albicans. Uma vez instalada a infecção, mediadores pró-

inflamatórios, como TNF-α, IL-1α e IL-2α, são sintetizados e, consequentemente, induzem a resposta inflamatória. As vias de sinalização intracelular podem sofrer interferências, proporcionando às células de Candida maior complexidade na expressão dos seus fatores de virulência.

O conhecimento acerca desses mecanismos pode contribuir para a descoberta de novos agentes anti-Candida (MONGE *et al*., 2006; CASTRO, 2010). Progressos recentes têm sido feitos na elucidação de circuitos diretamente implicados na virulência da C. albicans, tais como a formação de biofilme, resposta ao stress e a adaptação metabólica. Resposta ao estresse é uma função crítica para um patógeno oportunista como C. albicans, uma vez que é necessário ter a capacidade de superar as defesas do hospedeiro e ter um potencial de virulência satisfatório para invadir o organismo (SELLAM; WHITEWAY, 2016).

Evidências recentes salientam o papel da resposta de choque de calor ubíquo nos processos celulares ligados à virulência (O'MEARA; COWEN, 2014). Isto inclui HSF1 e Hsp90, o fator de transcrição de choque térmico e uma chaperonina envolvida no choque de calor, respectivamente, coordenando arquitetura da cromatina e a expressão do gene de resposta ao stress para permitir adaptação à resposta do hospedeiro, potencialmente, feita por meio da febre (LEACH *et al*., 2016). Além disso, a rede reguladora de choque térmico liga a vias metabólicas principais que estão intimamente relacionadas com a resposta celular ao stress. Estas observações fornecem a visão de quão complexo e interrelacionados são os processos ligados à resposta ao stress e o papel da virulência da nas infecções fúngicas (O'MEARA *et al*., 2016). Recentemente, foram encontradas várias vesículas de C. Albicans transportando fatores de virulência estimulando respostas C. albicans imunes em macrófagos e células dendríticas (VARGAS *et al*., 2015).

A forma de hifa é ainda mais implicada como mecanismo de virulência por expressar vários fatores de virulência, como adesinas e proteases. É interessante ressaltar que vários genes expressos durante a transição da forma de levedura para hifa não são importantes para morfogênese, mas são importantes nos mecanismos de virulência (KADOSH; JOHNSON, 2005; KUMAMOTO; VINCES, 2005). Como exemplo, há a expressão de proteases aspárticas (FELK *et al*., 2002; NAGLIK *et al*., 2008), assim como as adesinas HWP1 (Hyphal wall protein 1) e ALS3 (Aglutinin-like sequence) (FU *et al*., 2002; SUNDSTROM, 2002).

2.4. Atividade biológica de produtos naturais sobre Biofilme

Em vista as ameaças crescentes apresentadas por leveduras resistentes a medicamentos incentivaram os pesquisadores na procura vigorosa de antifúngicos alternativos vindos de produtos naturais, que não só podem ser mais efetivos como também possuírem menos efeitos colaterais (ALVES *et al.*, 2014; SARDI *et al.*, 2011; GOEL *et al.*, 2016). Várias substâncias que não são antibióticos, como por exemplo, os óleos essenciais, azeite e óleo de canela, foram identificados como eficazes sobre leveduras e biofilmes (UPADHYAY, 2010).

O interesse sobre produtos naturais aumentou independente de estarem associados a outras terapias e estratégias promissoras, como o uso de nanopartículas, anticorpos e mais recentemente a inativação fotodinâmica como tratamentos antifúngicos (SARDI *et al.*, 2013).

O uso de plantas medicinais como terapia alternativa pela população tem sido uma prática comum desde antes de Cristo. Como exemplo, tem-se o uso de papoula (Papaver somnniferum) e maconha (Cannabis sativa) ao longo de 4.000 anos.

No entanto, a procura pelos componentes presentes em plantas medicinais só começou no século XIX, levando à concepção da primeira droga com as características que conhecemos hoje. Friedrich Serturner, em 1806, foi pioneiro quando isolou a morfina alcalóide de Papoula: um evento que levou a uma busca contínua de outros medicamentos derivados de plantas. Em 1824, Pierre-Jean Robiquet isolou codeína, um agente antitussígeno também da papoula, em 1848, George Merck Fraz isolou o anti- espasmódico alcalóide papaverina desta mesma planta. Outros exemplos importantes de componentes ativos isolados a partir de plantas compreendem atropina (antagonista muscarínico) isolada de Atropa belladonna por Mein em 1831; Cafeína obtida por Runge em 1820 da Coffea arabica; Digoxina (digitálicos) isolado por Claude-Adolphe Nativelle em 1869 da Digitalis lanata; E curare (músculo Relaxante) isolado por Winstersteiner e Dutcher em 1943 da Chondrodendron Tomentosum, entre muitos outros exemplos.

Estudos recentes buscam novos modelos de plantas capazes de inibir a formação de biofilme, como exemplo a Bixa orellana L. comumente conhecida como annatto nativo da América Central e América do Sul. Tem sido usado há séculos em muitas partes do mundo para a prevenção e tratamento de uma série de distúrbios de saúde. Nas últimas décadas foram isolados várias classes diferentes de fitoconstituintes, incluindo carotenóides, apocarotenóides, esteróis, compostos alifáticos, monoterpenos e sesquiterpenos, triterpenoides, óleos voláteis e outros compostos diversos de todas as partes desta planta.

Esses fitoquímicos exibem uma ampla gama de atividades farmacológicas que incluem antibacterianos, antifúngico, antioxidante, antiinflamatório, anticancerígeno, motilidade gastrointestinal aumentada, neurofarmacológica, atividades anticonvulsivantes, analgésicas e antidiarreicas. As investigações modernas desta planta revelaram a presença de corante avermelhado natural em sementes de B. orellana (ALVES *et al.*, 2009)

O gênero Euterpe possui cerca de 28 espécies localizadas na América Central e do Sul tendo uma distribuição por toda a bacia amazônica. As três espécies que ocorrem mais freqüentemente são E. oleracea, E. precatoria e E. edulis. Apesar dessa distribuição, apenas as duas primeiras usam os seus frutos comercialmente. A E. oleracea, popularmente conhecida é encontrada principalmente em terras baixas e em florestas inundadas pelo estuário do rio Amazonas, nos estados brasileiros do Pará, Maranhão, Tocantins, Amapá, e também na Guiana Francesa e Venezuela. Apesar da maior quantidade de espécies de Euterpe concentrados no lado oriental da floresta amazônica, também é observada uma quantidade considerável na região setentrional da América do Sul (YAMAGUCHI *et al*., 2015).

As raízes de *E. oleracea* também são utilizadas na Guiana como agente anti-malárico, mas sempre em combinação e de preferência com outras plantas medicinais, Caricapapaya, Citrus sp. (Limão) e Quassia amara, mostrando baixa atividade quando comparada com outras espécies. Além dessa atividade, há relatos do uso da E. oleracea para o tratamento de Leishmaniose tegumentar pela população da Guiana Francesa. Na Colômbia e no Suriname, é usado no tratamento da diarreia (YAMAGUCHI *et al*., 2015).

A composição fitoquímica do fruto "açaí" tem sido bem caracterizada e inclui: ácidos fenólicos, antocianinas - especialmente cianidina-3-orutinosida, Cianidina-O-glucosido - proantocianidinas, lignanas - tais como ariltetrahidronaftaleno, dihidrobenzofurano, furofurano, 8-O-4 '-neolignano, Tetrahidrofurano - e constituintes polifenólicos - tais como a epicatequina, a catequina Homoorientina, orientina, isovitexina, taxifolino desoxihexose.

Vários estudos abordaram os efeitos farmacológicos do açaí, incluindo: atividade antitumoral na linha celular MCF-7 - uma linha celular do câncer de mama (SILVA *et al*., 2014); inibição de disfunção cardíaca em ratos submetidos a infarto do miocárdio; efeitos analgésicos durante ensaios de dor aguda e neuropática (SUDO *et al*., 2015) e propriedades anticonvulsivantes em camundongos (SOUZA-MONTEIRO *et al*., 2015). Um achado clínico recente mostrou que o consumo de "açaí" reduz Stress e melhora a tolerância ao esforço em atletas profissionais (CARVALHO-PEIXOTO *et al*., 2015).

A resistência antimicrobiana aos fármacos é um obstáculo no tratamento de numerosas doenças infecciosas (MAH, 2012). Um dos tipos mais comumente reconhecidos de resistência aos fármacos é a implantação de biofilmes nos dispositivos médicos. Esses biofilmes exibem uma resistência inata a múltiplas classes de fármacos, e são capazes de suportar concentrações antifúngicas 1000 vezes mais altas do que aquelas necessárias para inibir a Candida em sua forma livre (RAMAGE *et al*., 2005). Quando a terapia farmacológica não erradica os biofilmes, a remoção do dispositivo infectado é quase sempre necessária para resolver o quadro de infecção. O tratamento é difícil, uma vez que os dispositivos médicos são frequentemente críticos para a sobrevivência do paciente e as terapêuticas antifúngicas atualmente disponíveis são praticamente ineficazes. As infecções por biofilme de Candida, se não forem tratadas com sucesso, podem ter consequências devastadoras, evoluindo para difusão hematogênica e infecções fúngicas invasivas com altos riscos de mortalidade (TAFF *et al*., 2013).

3. OBJETIVOS

3.1. Objetivo Geral

Analisar atividade biológica *in vitro* do extrato da *Euterpe oleracea* sobre biofilme de *Candida parapsilopisis e Candida tropicalis*.

3.2. Objetivos Específicos

- Preparar extrato bruto hidroalcoólico liofilizado de *Euterpe oleracea*;
- Determinar o potencial de formação de biofilmes de *Candida parapisiolosis* e *Candida tropicalis* em placas microtiter de poliestireno;
- Comparar o potencial antibiofilme do extrato bruto hidroalcoólico liofilizado da casca e do caroço de *Euterpe oleracea* Mart na formação de biofilme por *Candida parapisiolosis* e *Candida tropicalis*.

4. METODOLOGIA

4.1. Obtenção do extrato hidroalcoólico liofilizado do fruto total, casca e caroço de *Euterpe oleracea* Mart.

Os frutos de juçara (*Euterpe oleracea* Mart) utilizados neste estudo foram oriundos do Parque da Juçara (São Luís, Maranhão, Brasil). Uma amostra do exemplar foi armazenada sob exsicata número 30 expedida pelo Herbário Rosa Mochel do Núcleo de Estudos Biológicos da Universidade Estadual do Maranhão (UEMA) e depositado no World International Property Organization sob o registro nº PI0418614-1. Os frutos foram previamente acondicionados sob refrigeração a -20ºC no Laboratório de Farmacologia e Psicobiologia da Universidade Estadual do Rio de Janeiro (UERJ). Após descongelamento em temperatura ambiente, a amostra foi separada em três porções: casca, caroço e fruto total (casca + caroço). O processo de extração seguiu de acordo com a metodologia desenvolvida por de Moura *et al*., (2011). Aproximadamente 360 g de juçara foram lavadas em água corrente e fervidas em água destilada por 5 a 10 minutos. Posteriormente, as porções foram trituradas e em seguida, homogeneizadas com 400 ml de etanol sob agitação por 2 h. Os extratos resultantes foram armazenados a 4ºC protegidos da luz por 10 dias. Após esse período de maturação, os extratos hidroalcoólicos foram filtrados em papel de filtro #1 Whatman e a fase líquida concentrada em um evaporador rotatório de baixa pressão (Fisatom Equipamentos Científicos Ltda. São Paulo, São Paulo, Brasil) a aproximadamente 40ºC e então liofilizados (LIOTOP modelo 202, Fisatom Equipamentos Científicos Ltda. São Paulo, São Paulo, Brasil) em temperatura de −30 a −40 ºC e vácuo de 200 mm Hg. Os extratos foram mantidos a -20 ºC até o dia de uso. Posteriormente, foi analisada a quantidade total de polifenóis da juçara através do método de Folin Ciocaulteau segundo Oliveira *et al*. (2009).

4.2. Obtenção dos isolados de *Candida*

Foram utilizadas espécies de *Candida parapsilosis* ATCC 1369 e *Candida tropicalis* ATCC 1369 oriundas da Plast-Labor Microbiologia© figura 2. Ambas foram mantidas sob

refrigeração no laboratório de Micologia do Núcleo de Imunologia Básica e Aplicada (NIBA, DEPAT/CCBS/UFMA).

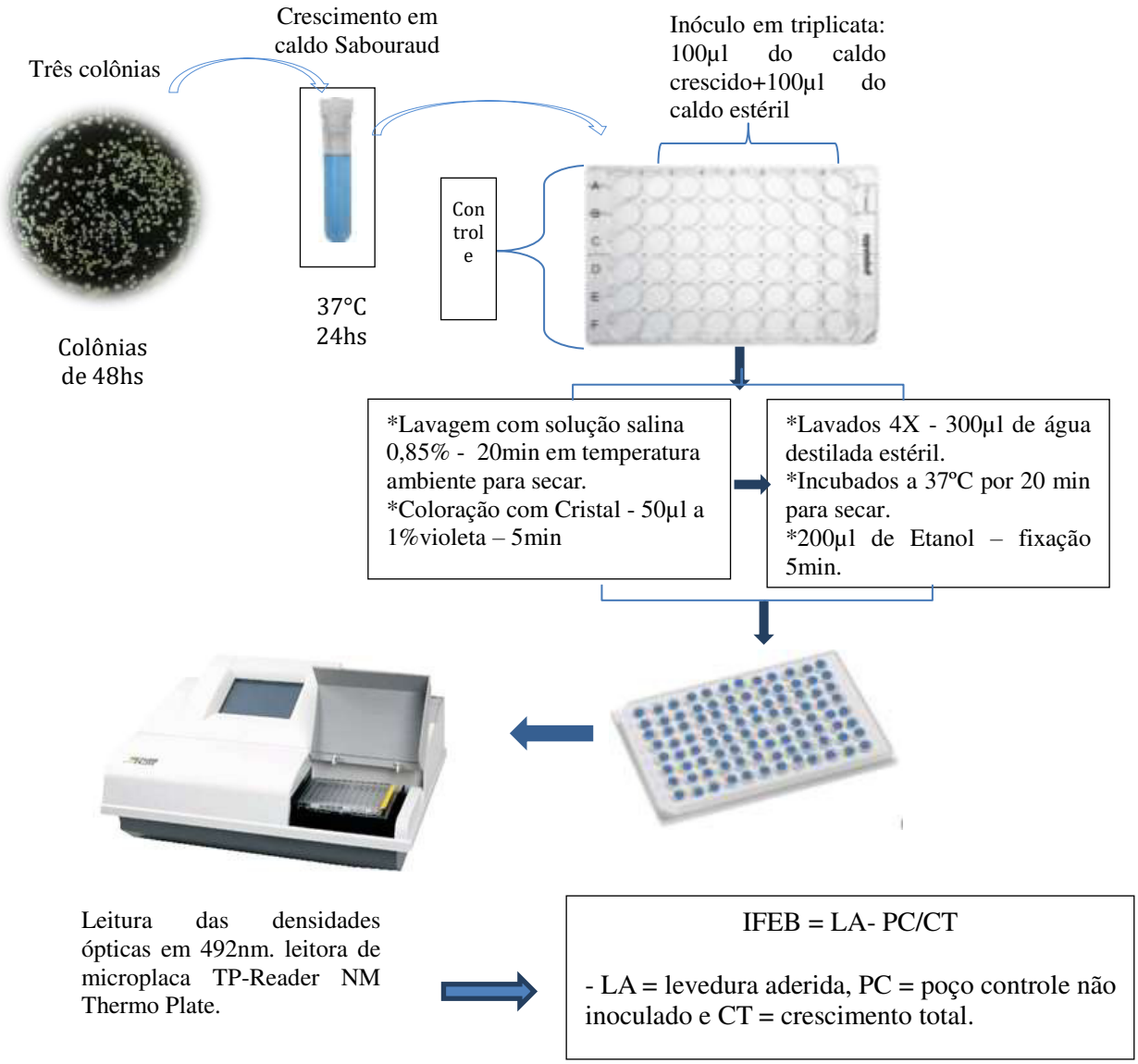
Figura 2. Cepas de *Candida parapsilosis* ATCC 1369 e *Candida tropicalis* ATCC 1369 oriundas da Plast-Labor Microbiologia©

4.3. Formação de Biofilme

Os isolados de *C. parapsilosis* e *Candida tropicalis* foram reativadas em ágar Sabouraud dextrose a 37°C por 48 horas, em seguida foram transferidas três colônias de cada isolado para tubos de ensaio com 5mL de caldo Sabouraud dextrose os quais foram incubadas a 37°C por 24 horas. Posteriormente alíquotas de 100µL do caldo crescido mais 100µL de caldo estéril, com inoculo variando entre $3x10^7$ a $1,8x10^8$ UFC/mL, foram transferidas para os poços de placas microtiter de poliestireno estérieis que foram incubadas por 24 horas a 27ºC (JAIN *et al*., 2007) (Figura 5).

As placas foram posteriormente tratadas segundo metodologia descrita por Neves *et al*., (2008), com a determinação do crescimento total (CT) pela medida da densidade óptica a 630nm, em leitora de microplaca TP-Reader NM Thermo Plate, seguida da remoção do caldo crescido, lavagem dos poços com solução fisiológica e secagem em temperatura ambiente por

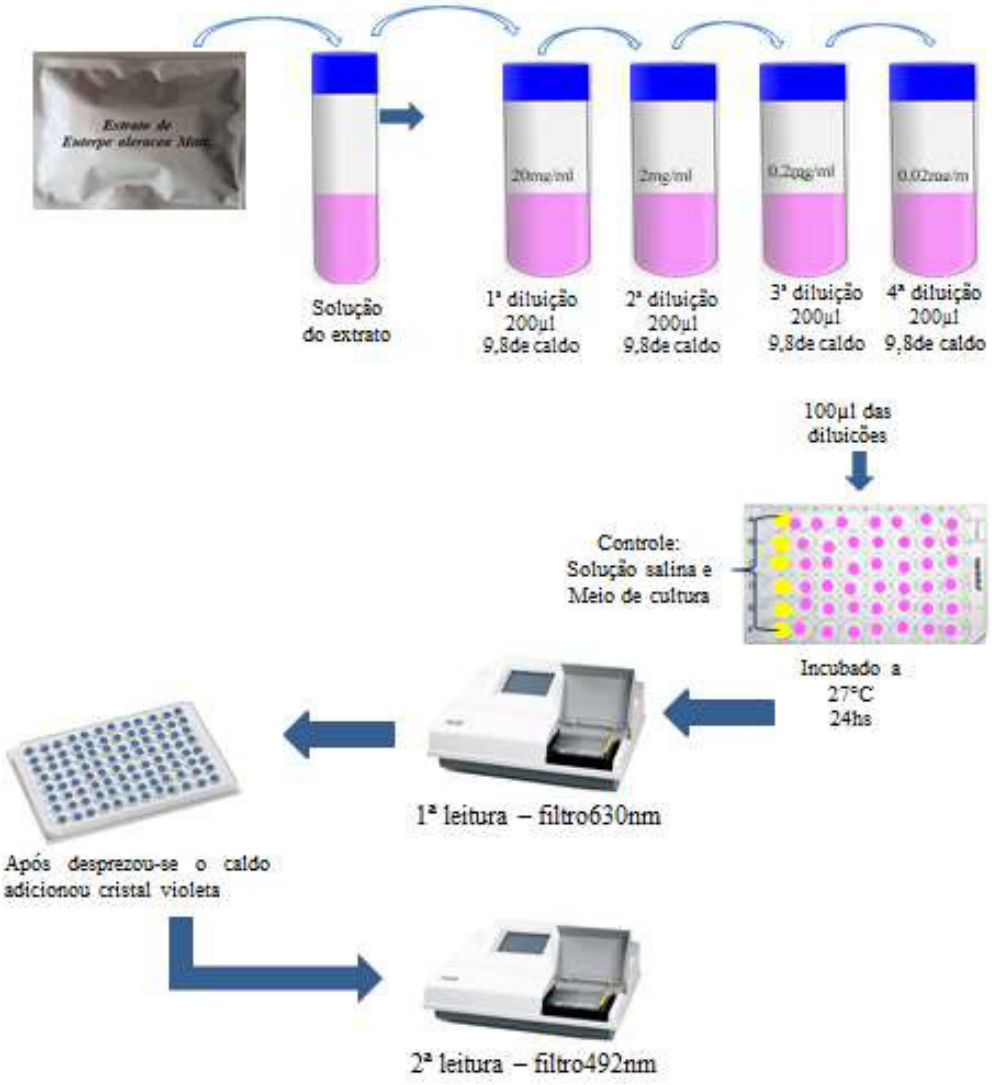
20 minutos. Subsequentemente os poços foram corados com 50µL de cristal violeta a 1% por cinco minutos, após esse período o corante foi desprezado e os poços lavados quatro vezes com 300µL de água destilada. As placas foram incubadas a 37ºC por 20 minutos para secagem, posteriormente adicionou-se 200µL de etanol absoluto em cada poço para fixação e a placa microtiter foi incubada durante 5 minutos. Após o processamento de coloração das placas as densidades ópticas foram lidas a 492 nm em leitora de microplaca TP-Reader NM Thermo Plate. As leituras de densidade óptica permitiram o cálculo do Índice de Formação Específica de Biofilme (IFEB) por meio da fórmula IFEB = LA- PC/CT, aonde LA = levedura aderida, PC = poço controle não inoculado e CT = crescimento total. Todos os ensaios foram realizados em triplicata para cada espécie de *Cândida* em experimentos independentes (Algoritmo 1).



4.4. Determinação de Susceptibilidade *in vitro* de *Euterpe oleracea* Mart.

Após a formação do Biofilme dos isolados de *Candida* de acordo com a metodologia e as espectrofotométricas supracitada, as microplacas foram submetidas ao ensaio de susceptibilidade de acordo com a metodologia proposta por Messier *et al*., (2011) com modificações.

Para esse ensaio foi utilizado diluições seriadas do extrato de *Euterpe oleracea* Mart. Primeiramente foi preparado a solução mãe dissolvendo 1g do extrato liofilizado em 1mL em solução salina, dessa solução preparou-se diluições seriadas e transferidos 0,2mL da solução inicial para outro tubo com 9,8 mL de caldo Sabouraud, para obtenção da primeira concentração, com auxílio da pipeta multicanal foram feitas sucessivas diluições (0,02; 0,2; 2 e 20 mg/mL) na microplaca. A coluna vertical representa o controle Positivo (três primeiros poços) e o controle negativo (três últimos). Foi colocado 100 ul do caldo crescido de 24 horas mias 100ul do extrato em todos os pocinhos. Incubado a 27° C por 24 hs. Após esta incubação foi realizada a primeira leitura em filtro de 630 nm. Em seguida desprezou-se a suspensão, para realizar a coloração com cristal violeta que determinaria a presença ou ausência do biofilme. Após esta coloração realizou-se a segunda leitura em filtro de 492 nm para confirmar o efeito do extrato de *Euterpe oleracea* Mart na destruição ou não do biofilme, conforme algorítimo 2.



Algoritmo 2 – Ensaio da atividade de *Euterpe oleracea* Mart sobre biofilme de *Candida parapisilosis* e *Candida tropicalis*

4.5. Análise estatística

Para a avalição o grau de associação entre o Extrato e a formação de biofilme utilizou-se a correlação de Spearman. Afim de avaliar se as diferenças foram estatisticamente significativas foi realizado o Kruskal Wallis, com $p > 0,05$. As análises dos dados obtidos foram realizadas com o auxílio do programa STATA® (versão 14).

5. RESULTADOS

As espécies testadas para o ensaio de formação de biofilme foram *Candida parapsiolosis* e *Candida tropicalis*. Na Tabela 1 podemos observar que segundo o teste aplicado as *C. tropicalis* e *C. parapsilosis* formaram biofilme.

Tabela 1: Associação entre os extratos de *Euterpe oleracea* e a formação de biofilme por *C. tropicalis* e *C. parapsilosis*

Extrato	**Espécie**	
	C. tropicalis	***C. parapsilosis***
Casca	-0,727	-0,859
Caroço	-0,908	-0,783

Segundo a correlação de Spearman (Tabela 2) tanto o extrato da casca quanto do caroço tem forte influência negativa na formação de biofilme por ambas espécies do gênero Candida. Desta forma demonstra-se que os extratos inibem significativamente a formação de biofilme. Nota-se, também, que a o extrato da casca possui melhor inibição sobre a formação de biofilme por *C. parapsilosis* que em *C. tropicalis*; todavia o extrato do caroço tem melhor efeito em *C. tropicalis* que em *C. parapsiolosis*.

Tabela 2: Efeito do extrato da casca de *Euterpe oleracea* na aderência e formação de biofilme em *C. tropicalis* e *C. parasislosis* de acordo com a absorbância.

Concentração	**Espécie**	
	C. tropicalis	***C. parapsilosis***
0,02 mg/ml	0,438 ±0,133[a]	0,243 ±0,133[a]
0,2 mg/ml	0,375 ±0,187[a]	0,184 ±0,110[a b]
2 mg/ml	0,397 ±0,420[a]	0,044 ±0,063[b c]
20 mg/ml	-0,031 ±0,059[b]	-0,060 ±0,059[c]

Considerou-se significativamente diferente resultados com valor de $p<0,05$

O extrato da casca inibiu totalmente ou parcialmente a formação de biofilme por ambas as espécies (Tabela 3). A formação de biofilme por *C. tropicalis* tendeu a ser reduzida nas concentrações de 0,02 a 2 mg/ml e inibida na concentração de 20 mg/ml do extrato da

casca. A *C. parapsilosis* teve a formação do biofilme significativamente inibidas parcialmente nas concentrações de 0,2 e 2 mg/ml; e sendo completamente inibida na concentração de 20 mg/ml. O aumento da concentração do extrato foi proporcionalmente inverso a quantidade de biofilme indicando a dose dependência.

Tabela 3: Efeito do extrato do caroço de *Euterpe oleracea* na formação de biofilme por *C. tropicalis* e *C. parasislosis* de acordo com a absorbância.

Concentração	**Espécie**	
	C. tropicalis	***C. parapsilosis***
0,02 mg/ml	0,275 ±0,073[a]	0,123 ±0,074[a]
0,2 mg/ml	0,222 ±0,074[a b]	0,111 ±0,063[a]
2 mg/ml	0,104 ±0,035[b c]	0,018 ±0,035[b]
20 mg/ml	-0,018 ±0,043[c]	-0,059 ±0,062[b]

Considerou-se significativamente diferente resultados com valor de $p<0,05$

Na tabela 3 observou-se que o extrato do caroço inibiu a formação de biofilme em diferentes concentrações, sendo dose dependente também. Nas concentrações de 0,2 e 2 mg houveram redução parcial de biofilme, e na concentração de 20 mg/ml inibiu completamente a formação do biofilme em ambas as espécies.

6. DISCUSSÃO

No presente estudo as espécies submetidas à formação de biofilme por *C. parapsiolosis* e *C. tropicalis* foram satisfatórias para formação de biofilme em placa de Elisa com 96 poços. Os resultados encontrados na literatura relatam que isolados de *C. parapisilosis*, *C. pseudotropicalis* e *C.grabrata* possuem menor desenvolvimento de biofilme comparado a *C. albicans*. Porém, no presente trabalho observou-se que as espécies *C. tropicallis* e *C. parapsilosis* podem produzir quantidade significante de biofilme.

Grande parte dos experimentos relacionados as informações sobre a formação de biofilmes por *Candida* provém de experimentos com uma variedade de substratos (plásticos, acrílico, poliestireno, etc) (RAMAGE *et al*, 2007).

Como existem dados escassos sobre o impacto da mídia de crescimento no fenômeno do desenvolvimento do biofilme por *Candida*, foi realizado um estudo para avaliar a eficácia de três meios de cultura dextrose sabouraud (SDB), base nitrogenada de fermento (YNB) e RPMI 1640 sobre crescimento, adesão e formação de biofilmes de duas leveduras patogênicas *C. albicans* e *C. parapsilosis* em que concluíram *que C. albicans* e *C. tropicalis* apresentaram crescimento variável, heterogêneo, adesão, bem como potencial de formação de biofilmes e arquitetura em diferentes meios de crescimento (WEERASEKERA, 2016). O nosso meio utilizado foi o dextrose saboround.

Em relação a forte influência inibitórtia do extrato de *Euterpe oleracea* Mart na formação de biofilme para ambas espécies estudadas *C. tropicallis e C. parapsilosis*. Muitos extratos e óleos essenciais isolados de plantas demonstraram exercer atividade biológica que justifica a investigação de sua potencial atividade antimicrobiana (FURLETTI *et al*, 2011; PIETRELLA *et al*, 2011). De acordo com estudos bioquímicos que já foram realizados para revelar a composição do açaí existem vários tipos de fitoquímicos, dentre eles antocianinas, proantocianidinas e outros flavonóide (SCHAUSS *et al*., 2006). A atividade bioativa tem sido objeto de pesquisas frente ao seu poder de regeneração do tecido epitelial por sua ação antioxidante, hidratante, reguladora de lipídeos (UDANI *et al.*, 2011) e estimulante do processo de cicatrização (MACHADO, 2010). As antocianinas presente neste fruto são conhecidas cientificamente por suas propriedades antimicrobianas, antiinflamatória e anticarcinogênicas (ALASALVAR, 2005). Diversos trabalhos realizados na Amazônia (ABREU *et al.,* 2014) comprovam o efeito antimicrobiano de palmeiras comumente

cultivadas em solos como o açaí (*Euterpe oleraceae* Mart.) e a pupunheira (Bactris gasipaes e Bactris dahlgreniana), que em trabalho realizado por Araújo, Henriques e Silva (2017) foi observado efeito bactericida dos óleos provenientes de seus frutos.

Em relação a dose dependência em que foi observada, quanto maior a concentração do extrato de *Euterpe Oleracea* Mart maior foi o efeito inibitório na formação do biofilme em ambas as espécies estudadas neste trabalho, com inibição na concentração de 20mg/ml. Entretanto, esta maior concentração vai de encontro aos achados de Abreu *et al* (2014) que avaliou o efeito das doses dos óleos de murmuru (*Astrocaryum ulei* Mart.) e açaí (*Euterpe oleracea* Mart). Apesar de serem fungos de espécies diferentes da estudada neste trabalho e por ter sido óleo e o nosso terem sido extratos da casca e do caroço da *Euterpe Oleracea* Mart possuem resultados semelhantes ao achado no presente trabalho.

Pode-se observar ainda neste estudo que houve uma diferença na tendência de inibição do biofilme das *Candidas* estudadas, relacionadas aos extratos da casca e do caroço. O extrato do caroço inibiu completamente a formação de biofilme.

As antocianinas são flavanóides com ação antioxidante (OLIVEIRA, 2013). No caroço há uma presença de compostos fenólicos: ácidos fenólicos, flavonas, flavonóis, antocianinas, ácido protocatecuico, catequinas, epicatequina e procianidinas oligoméricas (diméricas a pentaméricas) (RODRIGUES *et al*., 2015). Estes protegem contra danos causados pela radiação eletromagnética e também podem exibir propriedades defensivas, tais como antibacteriana e antifúngica (MONTONARI; BOLZANI, 2001), sendo necessários estudos específicos que indiquem as frações responsáveis pelo efeito antifúngico. Vários estudos sobre extratos e óleos extraídos de substâncias naturais já foram testados sobre ação do biofilme de *Candidas* mostrando seus diferentes efeitos. Em um estudo (JABEUR *et al.*, 2016) sobre propriedades bioativas e constituintes funcionais de Hypericum Androsaeum L. com foco no perfil fenólico, o extrato foi eficaz na inibição da produção de óxido nítrico como indicador do potencial anti-inflamatório. Os efeitos anti-*Candida* variaram entre as espécies *C. grabratta* e *c.tropicallis* sendo esta mais sensível estando diretamente relacionado as concentrações testadas. Foi observado um potencial significativo de formação de biofilmes com redução maior que 90%. Os compostos observados, a maioria fenólicos, podem explicar as ações: antioxidantes, citotóxicas, anti-inflamatórias e atividades anti-*Candidas*.

O extrato de própolis também foi testado para combater infecções de espécies de *Candida* tanto em células plactônicas quanto em biofilmes mostrou-se como potente antifúngico em ambos os casos (TOBALDINI-VALERIO *et al*., 2016), em que os flavanóides constituem uma classe muito importante de polifenóis amplamente presente no própolis,

responsável por sua atividade biológica. Vários extratos brutos de plantas do cerrado brasilerio foram testados contra espécies de *Candida*, os seis extratos apresentaram atividade antifúngica (CORREIA *et al.*, 2016), sendo que *Eugenia dysenterica, Pouteria ramiflora* mostraram maior efeito contra as espécies não-albicans testadas. A análise química dos extratos destas revelou presença de polifenóis (flavanóide e catequinas), uma classe química importante como atividade antifúngica. O que nos leva a crê que baseado nesses estudos com outras substâncias naturais a possível atividade antifúngica e antibiofilme do extrato do açaí esteja nos compostos fenólicos, sendo necessário ainda estudos *in vitro* para fracionar quais compostos são responsáveis por essa ação requerida em nosso estudo.

7. CONCLUSÃO

Pode-se concluir com este trabalho que o extrato da *Euterpe oleracea* Mart inibiu a formação do biofilme de *Candida tropicallis* e *Candida parapsilosis*, sendo que os melhores resultados de inibição foram do extrato do caroço. Estudos posteriores serão necessário para comprovar a eficácia do extrato como inibidor dos biofilmes de *Candida*e para identificar qual substância é responsável por essa inibição.

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ANEXOS

ANEXO A: Comprovante da submissão do artigo "Use of phytochemicals as a new promise for Candidiasis therapy", na revista Frontiers in Micobiology.

Use of phytochemicals as a new promise for Candidiasis therapy

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MSBN:idealizador
LLB,KRB,GXS,IVPR,WEMF,LAP,LOC,JPPS:participated in the search of the articles and assembly of the article
GFBB,RMTF:reviewed the article

Keywords

Biofilm, C. albicans, C. parapsilosis, phytochemicals, Fungi

Abstract

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Non-albicans Candida infections such as C. glabrata, C. krusei, C. tropicallis and C. parapsilosis are increasingly frequent, and the resistance of such microorganisms is increasing due to the indiscriminate use of antifungal agents. Biofilms are one of the major factors involved in the success of candidiasis therapy, prompting researchers to study new antimicrobial substances from various sources, including medicinal plants, in the search for phytochemicals capable of inhibiting, interfering or undoing this structure. The aim of this study is to compile relevant publications published since 2011 focusing on Candida biofilms and phytochemicals. The research was conducted by searching in the PubMed platform using the keywords "Factors of virulence", "biofilm", "Candida albicans and non-albicans", and "Phytochemicals". 2546 articles were found, and among them 28 were selected according to the study proposal guidelines. Among the articles found, 2519 were excluded because they did not present the necessary requisites of this study. Based on the results, we conclude that candidiasis by Candida non-albicans is increasingly emerging and is associated with the expression of its factors of virulence, especially biofilm. This is a mechanism capable of interfering with the action of available antifungal agents. In addition, the search for new phytochemicals with properties capable of inhibiting, interfering or undoing this fungal matrix is extremely important due to an increasing resistance that these microorganisms have been developing over the last decades.

Keywords: Biofilm, C. albicans, C. parapsilosis, Phytochemicals

ANEXO B: Artigo científico - Use of phytochemicals as a new promise for Candidiasis therapy

Use of phytochemicals as a new promise for Candidiasis therapy.

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ABSTRACT

Non-albicans *Candida* infections such as *C. glabrata*, *C. krusei*, *C. tropicallis* and *C. parapsilosis* are increasingly frequent, and the resistance of such microorganisms is increasing due to the indiscriminate use of antifungal agents. Biofilms are one of the major factors involved in the success of candidiasis therapy, prompting researchers to study new antimicrobial substances from various sources, including medicinal plants, in the search for phytochemicals capable of inhibiting, interfering or undoing this structure. The aim of this study is to compile relevant publications published since 2011 focusing on *Candida* biofilms and phytochemicals. The research was conducted by searching in the PubMed platform using the keywords "Factors of virulence", "biofilm", "*Candida albicans* and non-*albicans*", and "Phytochemicals". 2546 articles were found, and among them 28 were selected according to the study proposal guidelines. Among the articles found, 2519 were excluded because they did not present the necessary requisites of this study. Based on the results, we conclude that candidiasis by *Candida* non-albicans is increasingly emerging and is associated with the expression of its factors of virulence, especially biofilm. This is a mechanism capable of interfering with the action of available antifungal agents. In addition, the search for new phytochemicals with properties capable of inhibiting, interfering or undoing this fungal matrix is extremely important due to an increasing resistance that these microorganisms have been developing over the last decades.

Keywords: Biofilm, *C. albicans*, *C. parapsilosis*, Phytochemicals.

INTRODUCTION

Infections by *Candida* non-*albicans*, such as *C. glabrata*, *C. krusei*, *C. tropicallis* and *parapsilosis*, are increasingly common (1, 2, 3). Such infections are a result of host immune disorders and virulence factors expressed by these yeasts, which contribute to the ability to colonize, penetrate and invade tissues (4, 5), thus leading to candidiasis. *C. tropicallis* and *C. parapsilosis* have the ability of forming biofilms, a mechanism by which treatments using most antifungal agents are hindered (6, 7, 8, 9).

The pathogenesis of candidiasis is common to all species of *Candida*, being facilitated by a number of virulent factors, among which we may highlight the ability to adhere to medical devices or host cells, development of biofilms and transition to filamentous forms (10). As a

consequence of the disruption of the parasite-host balance, candidiasis may occur triggered by changes in the tissue barrier and the autochthonous microbiota and by the compromise of the body's natural defenses. In diseases requiring a long hospital stay, there is a greater occurrence of disruption of this balance (2).

In addition to these virulence mechanisms expressed by *Candida* species, biofilm is one of the main factors for the development of fungal resistance and the difficulty in responding to treatment since it has the capacity to form a community of planktonic cells over cell and abiotic surfaces, on which substances with antifungal properties generally fail to overcome and succeed in therapy (11).

According to Araújo, Henrique and Silva (12), mucosal infections may be associated with the formation of biofilms by microorganisms to the extent that the pathogen is able to adhere to a surface and produce an extracellular matrix or biofilm. Several genes are involved in this relationship, including several common genetic mechanisms for the formation and development of biofilms on abiotic and mucosal surfaces. Thus, the different stages of biofilm development (adhesion/colonization, maturation and dispersion) are mediated by complex molecular events. However, biofilm formation is strongly dependent on environmental conditions, which makes it difficult to compare regulatory genetic changes among *Candida* species.

Thus, the biofilm is a mechanism of great interest in the medical field. Many authors (13, 14, 15) emphasize its importance as part of the defense strategies of the pathogen forming the biofilm, and also because it is a matrix or a community of microorganisms that can be formed over biotic and abiotic surfaces, including medical devices such as catheters and bladder probes. In this sense, the search for natural products or phytochemicals capable of inhibiting, interfering or undoing this structure is crucial in the exploration of new antifungals (16, 17, 18, 19). Therefore, this study aims to compile articles on approaches to *Candida* biofilms and phytochemicals with antifungal activity and antibiofilms that may stimulate new research in the area.

METHODOLOGY

We conducted a review of articles on the PubMed platform emphasizing *Candida* biofilms, phytochemicals with antifungal activity and *Candida* antibiofilm proprieties. The articles were organized according to author, year, title and objectives. The search was carried out using the following keywords: Factors of virulence, biofilm, *Candida* non-*albicans* and Phytochemicals. All selected articles are listed in Table 1 and Table 2. Subsequently, the results were compared and described below.

RESULTS

In this study, we found 2,546 articles related to biofilm-*Candida*-Phytochemicals presenting approaches on the virulence factors of *Candida*. Of these, 483 articles discussing biofilm and phytochemicals were separated. After analysis, 28 articles that were in agreement with the proposed objective were selected. Publications from 2011 were reviewed and among the articles found, 2519 were excluded because they did not present the characteristics chosen for this study, according to the flowchart below.

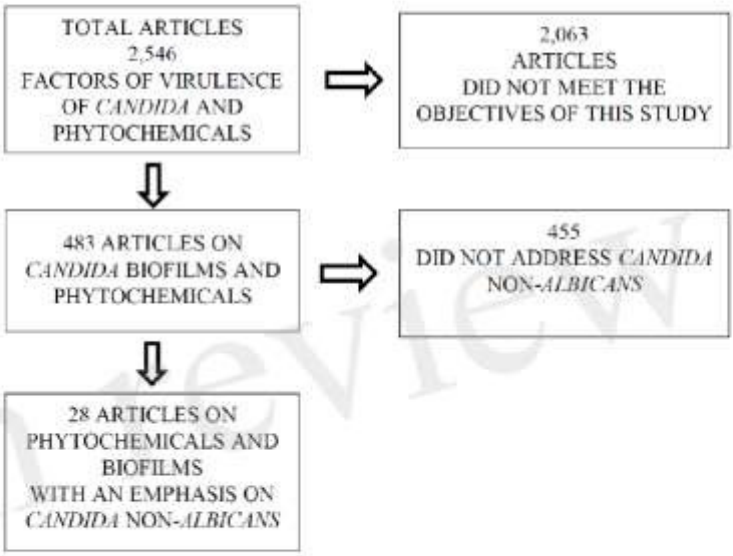

Figure 1. Methodology flowchart.

Tables 1 and 2 present, in order of publication, articles that address biofilm formation, antifungal and antibiofilm activity, and Phytochemicals, respectively.

Authors	Title	Objectives
Zárójelentés, 2011.	Investigation of virulence factors of *Candida parapsilosis*	Investigate the virulence of *Candida parapsilosis* and analyze the pathogen-host relationship.
Seabra, 2011.	Study on virulence factors of mixed cultures of *Candida albicans* and *Candida parapsilosis* after adhesion to an abiotic surface	Evaluate and compare the expression of different virulence factors, namely adhesion capacity, formation of oral clinical biofilms of *C. albicans* and *C. parapsilosis*.
Revino-Rangel, 2015.	Biofilm formation and genetic variability of BCR1 gene in the *Candida parapsilosis* complex	Quantify biofilm formation of a subset of 65 clinical isolates of the complex *C. parapsilosis* by two different methods and analyze the nucleotide sequence of a fragment of the BCR1 gene.
Deorukhkar et al., 2015.	Virulence Factors Attributed to Pathogenicity of non-albicans *Candida* Species Isolated from Human Immunodeficiency Virus Infected Patients with Oropharyngeal Candidiasis	Determine the expression of virulence factors of NAC spp. isolated from HIV-infected patients with OPC.
Brandi et al., 2016.	Demineralizing potential of dental biofilm added with *Candida albicans* and *Candida parapsilosis* isolated from	Investigate the demineralization potential of dental biofilms added with *Candida albicans* (CA) and *Candida parapsilosis* (CP) isolated

	preschool children with and without caries	from the saliva of preschoolers with and without cavities.
Goel; Mittal; Chaudhary, 2016.	Role of non-albicans *Candida* spp. and Biofilm in Neonatal ICU. Infectious disorders drug targets.	Understand the prevalence of different *Candida* species that cause blood infections, their ability to form biofilms and evaluate the relationship between biofilms and resistance to antifungal drugs.
Islam; Rather; Mohammad, 2016.	Phytochemistry, biological activities and potential of annatto in natural colorant production for industrial applications - A review.	Provide updated systematic and organized information on traditional use, phytochemistry and pharmacology of annatto. Highlight their non-food industrial applications to stir more interest in this plant, identify the existing gaps and make suggestions for future studies.

Table 1. Published articles referring to the formation of biofilm by species of *Candida*

Author	Title	Objectives
Fonseca; Botelho, 2011.	Antifungal Activity of Leaf Extract of *Psidium guajava* on Yeasts of the Genus *Candida*	Test antifungal activity of *Psidium guajava* on *Candida albicans*, *C. krusei* and *C. tropicalis*.
Raga; Espiritu; Shen; Ragasa, 2011.	A bioactive sesquiterpene from *Bixa or ellana*.	Verify antifungal (against *C. albicans*) and antibacterial activities of dichloromethane extract from dried leaves of *Bixa orellana*
Santana; Naves, 2012.	Action of chalcones on biofilm formation of *Candida albicans* isolated from the oral cavity	Evaluate biofilm formation by *Candida albicans* isolated from the oral cavity and the impact of chalcone derivatives on the inhibition of this microbial structure by susceptibility assays of planktonic and sessile forms.
Freires et al., 2014.	*Coriandrum sativum* L. (Coriander) essential oil: antifungal activity and mode of action on *Candida* spp., and molecular targets affected in human whole-genome expression.	Investigate the antifungal activity and the mode of action of essential oil of *Coriandrum sativum* L. leaves on different species of *Candida* and detect the affected molecular targets in the expression of the total genome of human cells.
Scarsini, et al, 2015.	Antifungal activity of cathelicidin peptides against planktonic and biofilm cultures of *Candida* species isolated from vaginal infections.	Investigate the antifungal activities of the cathelicidin peptides LL-37 and BMAP-28 against *Candida* spp., also including *Candida albicans*, isolated from vaginal infections, and against *C. albicans* SC5314 as a reference strain.
Jovito; Castro, 2016.	Anti-*Candida* activities and cytotoxicity analysis of the leaf extract of *Schinopsisi brasiliensis* Engl.	Evaluate the antifungal, anti-biofilm and cytotoxic potential of the rota-evaporated extract of leaves of *Schinopsis brasiliensis* Engl. on 6 strains of *Candida* spp.
Islam; Rather; Mohammad, 2016.	Phytochemistry, biological activities and potential of annatto in natural colorant production for industrial applications - A review.	Provide updated systematic and organized information on traditional use, phytochemistry and pharmacology of annatto. Highlight their non-food industrial applications to stir more interest in this plant, identify the existing gaps and make suggestions for future studies.
Assaf et al., 2016.	Antimicrobial and anti-inflammatory potential therapy for opportunistic microorganisms.	Evaluate antimicrobial and anti-inflammatory activities of some medicinal plants known to reduce the risk of opportunistic infections of the oral cavity of humans caused by *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*.
Raut; Karuppayil, 2016.	Phytochemicals as inhibitors of *Candida* biofilms.	Explore and review the potential of phytochemicals as a new strategy against *Candida* biofilms. In addition, describe the efficacy of some phytochemicals taking into account their inhibitory concentrations of biofilm.
Karygianni, et al 2016	Natural antimicrobials and oral microorganisms: a systematic Review on herbal interventions for the eradication of multispecies oral biofilms.	Critically present antimicrobial effects of various medicinal herbs against multispecies oral biofilms *in vitro*, *ex vivo* and *in situ*.
Pinheiro; Carreira; Rollo; Fernandes; Ferreira; Monteiro, 2016.	Blad-containing Oligomer Fungicidal Activity on Human Pathogenic Yeasts. From the Outside to the Inside of the	Demonstrate the antifungal activity of a polypeptide of *Lupinus albus* against *C. albicans* var. *albicans* (CBS 562). *C.*

	Target Cell.	*dubliniensis*, *C. glabrata*, *C. lusitaneae* and *C. parapsilosis* and provide some insights on its mode of action.
Jabeur et al., 2016.	Bioactive properties and functional constituents of *Hypericum androsaemum* L.: a focus on the phenolic profile.	Verify anti-oxidant, anti-tumor and antifungal activities against *C. albicans*, *C. glabrata*, *C. parapsilosis* and *C. tropicalis* of the ethanol:water extract of *Hypericum androsaemum* L.
Neji, et al 2017	Virulence factors, antifungal susceptibility and molecular mechanisms of azole resistance among Candida parapsilosis complex isolates recovered from clinical specimens.	Determine biofilm formation and extracellular enzymatic activities of 182 clinical isolates of the *Candida parapsilosis* complex.
Sony; Kalyani; Jeyakumari; Kanna; Sukumar, 2017.	In vitro antifungal activity of Cassia *fistula* extracts against fluconazole resistant strains of *Candida* species from HIV patients.	Evaluate the anti-candidiasis activity of leaves, bark and seeds of *Cassia fistula* against *Candida* species resistant to fluconazole: *C. glabrata*, *C. krusei*, *C. tropicalis*, *C. kefyr* and *C. parapsiolosis* isolated from HIV patients. The predominant phytochemical component responsible for the fungicidal activity was evaluated.
Quatrin et al., 2017	Antimicrobial and antibiofilm activities of nano-emulsions containing *Eucalyptus globulus* oil against *Pseudomonas aeruginosa* and *Candida* spp.	Prepare and characterize nano-emulsions containing *Eucalyptus globulus* oil and verify its antimicrobial and antibiotic activities against *P. aeruginosa* and *Candida* spp.
Sardi., et al 2017.	Unexplored endemic fruit species from Brazil: antibiofilm 1 properties, 2 insights into mode of action, and systemic toxicity of four *Eugenia* spp.	Describe the antifungal activity of four species of *Eugenia* spp. against *C. albicans* biofilms and demonstrate its mode of action and toxicity *in vitro* and *in vivo*.
Peixoto et al., 2017.	Antifungal activity, mode of action and anti-biofilm effects of *Laurus nobilis* Linnaeus essential oil against *Candida* spp.	Demonstrate the antifungal potential of the chemically characterized essential oil of *Laurus nobilis* L. against adhesion and formation of *Candida* spp. biofilms and establish its mode of action on *C. albicans*.
Vieira; Nascimento, 2017	Resistance to Antifungal Drugs by *Candida* and therapeutic approach.	Describe the mechanisms of resistance of *Candida* spp. to antifungal agents and propose susceptibility tests for existing antifungal agents to formulate a targeted therapy aiming a decrease in the development of resistant species.
Quinós; Villar-Vidal; Erasmo, 2017.	Activity of micafungin against *Candida* biofilms.	Describe the antifungal activity of micafungin against fungal biofilms based on a review of medical and scientific literature in recent years.
Scorzoni et al., 2017.	Antifungal Therapy: New Advances in the Understanding and Treatment of Mycosis.	Different approaches to prevent and treat fungal diseases are discussed in this review, focusing on mechanisms of resistance of fungi, aiming to develop efficient strategies to overcome and prevent resistance, as well as new advances in antifungal therapy.
Fernández-Rivero et al., 2017.	Activity of amphotericin B and anidulaf ungin, alone and combined, against *Can dida tropicalis* biofilms developed on Teflon® and titanium.	Evaluate the activity of amphotericin B (AMB) and anidulafungin (AND), isolated and in combination, against biofilms of *C. tropicalis* developed on polytetrafluoroethylene (teflon - PTFE) and titanium surfaces using time-kill assays.

Table 2. Articles published in 2011-2017 on Phytochemicals or other substances with antibiofilm activity on *Candida* spp.

DISCUSSION

Regarding virulence factors, such as enzyme production, adhesion genes and biofilm formation, *C. albicans* is the most studied species. In view of this, Santana et al. (20) studied the virulence factors of 32 samples of *C. albicans* isolated from oral cavity through morpho-typing, tube-typing, enzyme-typing and typing by killer toxins, besides biofilm formation. As a result, the authors verified that the isolates of *C. albicans* variably expressed virulence factors, all yeasts formed biofilm and there was no correlation between this property and the expression of other virulence factors studied.

In the study by Seabra (13), the authors found that when isolated species of *C. albicans* and *C. parapsilosis* in co-infection were separated, *C. parapsilosis* AM2 is influenced by the absence of *C. albicans* AM after 2 hours of adhesion, requiring more time to adapt to the new environment. However, *C. albicans* AM, after 2 hours, had a better ability to adapt to new environments in the absence of *C. parapsilosis* AM2, both in mono-species and two-species systems containing the strain *C. parapsilosis* AD.

It was further observed that, in single biofilms, the strains of *C. parapsilosis* expressed a greater amount of virulence factors than the strains of *C. albicans*. In a mixed biofilm, the expression of virulence factors was lower than when the expressions of each species in simple biofilm were summed. Thus, it was concluded from this study that the expression of virulence factors depends on the conditions under which the species are isolated (mono-infection or co-infection), strain and type of system (mono-species or two-species) (13).

In another study conducted by Treviño-Rangel et al (14), *C. parapsilosis stricto sensu* was associated with a low biofilm production phenotype, whereas *C. orthopsilosis* was associated with both phenotypes: low and high production of biofilm. In addition, no association was found between the biofilm formation phenotype and a particular genetic variant of the BCR1 gene fragment analyzed.

In their studies, Deorukhkar and Saini (21) observed a greater adhesion to oral epithelial cells (ABEC) by *C. dubliniensis*. However, when compared to other non-*albicans* Candidae, *C. glabrata* showed a low ABEC. A high activity of phospholipase was noted in *C. tropicalis*, followed by *C. kefyr*. A high proteinase activity was found in *C. dubliniensis*, followed by *C. tropicalis*, and a high production of hemolysin was found in *C. tropicalis*, followed by isolates of *C. kefyr*. This study evidenced that the different species of *Candida* have different profiles of virulence factors, both regarding the ability of adherence to oral epithelial cells, the activity of phospholipase and proteinase, and hemolysin production.

Several studies have now been conducted focusing on compounds present in natural products, such as phytochemicals, which are capable of interfering with the virulence factors of human pathogenic fungi, such as *Candida* species. In this sense, Shahid-Ul-Islam, Rather

and Mohammad (22) found in their experiments that the ethanol extract from the leaves and seeds of *Bixa orellana*, a plant belonging to the Bixaceae family and commonly known as annatto, has antifungal properties against *C. albicans*, with a zone of inhibition of the leaf extract of 22 cm and 20 cm for the seed extract. This result serves as a subsidy for the use of *B. orellana* in traditional medicine as gargle for sore throats and oral hygiene, since esophageal candidiasis affects this region of the human body.

In contrast, Raga et al. (23) found that sesquiterpenes with Ishwarano skeleton, a substance present in this plant and isolated by the dichloromethane extraction method, had an activity index of 0.3 against *C. albicans*, that is, this compound showed a moderate activity against this yeast. However, the author points out that it is not correct to state that such a compound is inactive for this property. The way the compound was administered has to be taken into consideration.

Regarding the use of phytochemicals, Fonseca and Botelho (24) carried out a study in which they verified an antifungal activity of the leaf extract of *Psidium guajava* on yeasts of the genus *Candida*. Likewise, Freires et al. (25) evaluated antifungal activity against some *Candida* species, as well as the mode of action of *Coriandrum sativum* L. essential oil and molecular targets that are affected in the expression of the complete human genome. Asaf et al. (26) evaluated anti-fungal (against *C. albicans*) and anti-bacterial activities (against *Staphylococcus aureus* and *Pseudomonas aeruginosa*) of the methanol extracts of six plants: *Arbutus andrachne*, *Chrysanthemum coronarium*, *Inula viscosa*, *Origanum syriacum*, *Punica granatum* and *Rosmarinus officinalis*. All extracts had antimicrobial activity, standing out *O. syriacum*, which showed the highest antimicrobial activity for the 3 microorganisms tested: the MIC of *C. albicans* was 1 mg/mL.

Pinheiro et al. (27) demonstrated the antifungal activity of a polypeptide of *Lupinus albus* against *C. albicans* var. *albicans* (CBS 562), *C. dubliniensis*, *C. glabrata*, *C. lusitaneae* and *C. parapsilosis* and provided some insights on its mode of action. They found that the polypeptide behaved similarly to Amphotericin B in relation to cell inhibition and cell death of the yeasts studied. In addition, its predictable multisite mode of action suggests a low risk of inducing resistance mechanisms, which constitute a major problem in face of currently available antifungal agents.

Jabeur et al. (28) evaluated anti-oxidant, antitumor and antifungal activities of the hydroalcoholic extract of *Hypericum androsaemum* L. against *C. albicans*, *C. glabrata*, *C. parapsilosis* and *C. tropicalis* and found that anti-*Candida* effects varied among the different strains of the same species. *C. glabrata* and *C. tropicalis* were the most sensitive species to the substance, whose effects were directly related to the tested concentrations of the extract.

In contrast, Sony et al. (29) evaluated the anti-candidiasis activity of leaves, bark and seeds of *Cassia fistula* against *Candida* species resistant to fluconazole (*C. glabrata*, *C. krusei*, *C. tropicalis*, *C. kefyr* and *C. parapsiolosis* isolated from HIV patients) and found that all extracts of *C. fistula* showed an excellent anti-*Candida* activity. The ethanol extract from the seed showed the greatest inhibitory effects and *C. krusei* and *C. parapsilosis* were the most inhibited yeasts and *C. kefyr* was the least inhibited.

CONCLUSION

With this study, which gathers updated information, it was possible to conclude that the emergence of *Candida* infections is associated with the expression of its virulence factors, especially biofilms, which are a mechanism capable of interfering with the action of available antifungals. Thus, it deserves special attention. In addition, the search for new phytochemicals with properties capable of inhibiting, interfering or undoing this fungal matrix becomes urgent as these yeasts are progressively developing mechanisms of resistance against the arsenal of antifungal agents currently available.

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ANEXO C: Normas da revista Frontiers in Micobiology

Author Guidelines

1. Summary Table

- Please view the table below for a summary on currently accepted article types and general manuscript style guidelines. Article types may vary depending on journal.

	Abstract (max. length)	Running title (5 words)	Figures and/or tables (combined)	Manuscript max. length	Peer review	Author fees	Submitted to PubMed Central or other indexing databases
Book Review	✘	✘	1	1'000 words	✔	✘	✔
Classification	250 words	✔	10	2'000 words	✔	✔	✔
Case Report	350 words	✔	4	3'000 words	✔	✔	✔
Clinical Trial	350 words	✔	15	12'000 words	✔	✔	✔
Code	250 words	✔	3	3'000 words	✔	✔	✔
Community Case Study	350 words	✔	5	5'000 words	✔	✔	✔
Conceptual Analysis	350 words	✔	10	8'000 words	✔	✔	✔
CPC	250 words	✔	6	2'500 words	✔	✔	✔
Curriculum, Instruction, and Pedagogy	350 words	✔	5	5'000 words	✔	✔	✔
Data Report	✘	✔	2	3'000 words	✔	✔	✔
Editorial	✘	✘	0	1'000 words*	✔	✘	✔
Empirical Study	350 words	✔	10	8'000 words	✔	✔	✔
Evaluation	350 words	✔	5	6'000 words	✔	✔	✔
Field Grand Challenge	✘	✔	1	2'000 words	✔	✘	✔
Focused Review [(1)]	350 words	✔	5	5'000 words	✔	✘	✔
Frontiers Commentary [(1)]	✘	✘	1	1'000 words	✔	✘	✔
General Commentary	✘	✘	1	1'000 words	✔	✘	✔
Hypothesis and Theory	350 words	✔	15	12'000 words	✔	✔	✔
Methods	350 words	✔	15	12'000 words	✔	✔	✔
Mini Review	250 words	✔	2	3'000 words	✔	✔	✔
Opinion	✘	✔	1	2'000 words	✔	✔	✔
Original Research	350 words	✔	15	12'000 words	✔	✔	✔
Policy Briefs	125 words	✔	5	3'000 words	✔	✔	✔
Protocols	350 words	✔	15	12'000 words	✔	✔	✔
Perspective	250 words	✔	2	3'000 words	✔	✔	✔
Policy Brief	125 words	✔	5	3'000 words	✔	✔	✔
Review	350 words	✔	15	12'000 words	✔	✔	✔
Specialty Grand Challenge	✘	✔	1	2'000 words	✔	✘	✔

Systematic Review	350 words	✔	15	12'000 words	✔	✔	✔
Technology Report	350 words	✔	15	12'000 words	✔	✔	✔

- (1) Tier 2 article - field level article reserved to authors of selected Tier 1 articles.
- * Editorials for Research Topics with 5 to 10 published articles have a maximum of 1'000 words, for Research Topics with more than 10 published articles the following applies: 1'100 words for 11 articles, 1'200 for 12 articles, 1'300 for 13 articles etc. up to maximum 5'000 words, for 50 or more papers.
- Appendices and footnotes will be considered in the total length and word count of the article.

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Please see Additional Requirements for specific article types including Focused Reviews, General Commentaries, Protocols and Data Reports.

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The title is written in title case, centered, and in 16 point bold Times New Roman font at the top of page.
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Witty or creative titles are welcome, but only if relevant and within measure. Consider if a title meant to be thought-provoking might be misinterpreted as offensive or alarming. In extreme cases, the editorial office may veto a title and propose an alternative.
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All names are listed together and separated by commas. Provide exact and correct author names as these will be indexed in official archives. Affiliations should be keyed to the author's name with superscript numbers and be listed as follows: Laboratory, Institute, Department, Organization, City, State abbreviation (USA, Canada, Australia), and Country (without detailed address information such as city zip codes or street names).
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This is a short text to acknowledge the contributions of specific colleagues, institutions, or agencies that aided the efforts of the authors.

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Sondheimer, N., and Lindquist, S. (2000). Rnq1: an epigenetic modifier of protein function in yeast. *Mol. Cell.* 5, 163-172.

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Sorenson, P. W., and Caprio, J. C. (1998). "Chemoreception," in *The Physiology of Fishes*, ed. D. H. Evans (Boca Raton, FL: CRC Press), 375-405.

Book:
Cowan, W. M., Jessell, T. M., and Zipursky, S. L. (1997). *Molecular and Cellular Approaches to Neural Development.*New York: Oxford University Press.

Abstract:
Hendricks, J., Applebaum, R., and Kunkel, S. (2010). A world apart? Bridging the gap between theory and applied social gerontology. *Gerontologist* 50, 284-293. Abstract retrieved from Abstracts in Social Gerontology database. (Accession No. 50360869)

Patent:
Marshall, S. P. (2000). *Method and apparatus for eye tracking and monitoring pupil dilation to evaluate cognitive activity.* U.S. Patent No 6,090,051. Washington, DC: U.S. Patent and Trademark Office.

Data:
[Dataset] Perdiguero P, Venturas M, Cervera MT, Gil L, Collada C. (2015) Data from: Massive sequencing of Ulms minor's transcriptome provides new molecular tools for a genus under the constant threat of Dutch elm disease. Dryad Digital Repository.http://dx.doi.org/10.5061/dryad.ps837

Theses and Dissertations:
Smith, J. (2008) Post-structuralist discourse relative to phenomological pursuits in the deconstructivist arena. [dissertation/master's thesis]. [Chicago (IL)]: University of Chicago

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Sondheimer N, Lindquist S. Rnq1: an epigenetic modifier of protein function in yeast. *Mol Cell* (2000) **5**:163-72.

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Sorenson PW, Caprio JC. "Chemoreception,". In: Evans DH, editor. *The Physiology of Fishes*. Boca Raton, FL: CRC Press (1998). p. 375-405.

Book:
Cowan WM, Jessell TM, Zipursky SL. *Molecular and Cellular Approaches to Neural Development.* New York: Oxford University Press (1997). 345 p.
Abstract:
Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, editor. *Genetic Programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002* Apr 3–5; Kinsdale, Ireland. Berlin: Springer (2002). p. 182–91.
Patent:
Pagedas AC, inventor; Ancel Surgical R&D Inc., assignee. *Flexible Endoscopic Grasping and Cutting Device and Positioning Tool Assembly.* United States patent US 20020103498 (2002).
Data:
[Dataset] Perdiguero P, Venturas M, Cervera MT, Gil L, Collada C. Data from: Massive sequencing of Ulms minor's transcriptome provides new molecular tools for a genus under the constant threat of Dutch elm disease. Dryad Digital Repository. (2015) http://dx.doi.org/10.5061/dryad.ps837
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- Abstract

  - Background
  - Methods
  - Results
  - Conclusions

- Introduction

  - Rationale
  - Objectives
  - Research question

- Methods

  - Study design

- Participants, interventions, comparators
- Systematic review protocol
- Search strategy
- Data sources, studies sections and data extraction
- Data analysis
  - Results

- Provide a flow diagram of the studies retrieved for the review
- Study selection and characteristics
- Synthesized findings
- Risk of bias
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- Summary of main findings
- Limitations
- Conclusions

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- example of use
- known issues
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Data-type	Recommended Repositories	Metadata Standard
Genetic and genomic sequence (DNA/ RNA)^	GenBank DNA Data Bank of Japan (DDBJ) European Nucleotide Archive (ENA)	MiXS
Metagenomic sequence	EBI Metagenomics	MiXS
DNA and RNA trace or short-read sequencing data	NCBI Trace Archive NCBI Sequence Read Archive	MiXS
Genetic polymorphism data, including SNP and CNV data	dbSNP dbVar European Variation Archive DGVa	MiXS
Gene expression data; chromatin immunoprecipitation data (deep-sequencing or microarray)	ArrayExpress Gene Expression Omnibus (GEO)	MIAME / MINSEQE
Data linking genotype to phenotype	dbGaP	
Protein sequence data	UniProt	
Proteome profiling data	PRIDE PeptideAtlas ProteomeXchange	MIAPE
Small molecule, protein, protein complex data structural data	Crystallography Open Database Cambridge Structural Database wwPDB (Protein DataBank)	CIF

	Electron Microscopy Databank
Taxonomy data	Zoobank

^ Genetic sequence variants should be annotated according to the guidelines established by the Human Variome Project.

Authors are encouraged to consider deposition in public, community-supported repositories of the data-types listed below:

Data-type	Recommended Repositories	Metadata Standard
Protein-protein interaction data	Database of Interacting Proteins (DIP)	MIMIx
Metabolite and metabolome profiling data	MetaboLights Human Metabolome Database	MSI
Small-molecule screening data, chemical compound data	PubChem	CIF
Flow cytometry data	Flow Repository	
Brain Imaging data / Neuroimaging data	OpenfMRI INDI NITRC NeuroVault [Statistical maps]	
Trait data	TRY database	
Phenology data	National Phenology Network	
Any data	FigShare Dryad Digital Repository	None

Inclusion of Zoological Nomenclature

The International Code of Zoological Nomenclature, in a recent 2012 amendment to the 1999 Zoological Code, allows all electronic-only papers, such as those published by the Frontiers journals, to have valid new taxon names and nomenclatural acts. However, these new names or nomenclatural acts must be registered in ZOOBANK and have associated Life Science Identifiers (LSIDs). Registration must be done by the authors before publication. Should your manuscript include any zoological new taxon names and/or nomenclatural acts, please ensure that they are registered prior to final publication.

>Inclusion of RNAseq Data

Studies employing RNASeq for comparative transcriptomic analyses must contain at least 3 biological replicates (unless otherwise justified). Each biological replicate should be represented in an independent library, each with a unique barcode if libraries are multiplexed for sequencing. Validation on a number of key transcripts highlighted in the study is also highly recommended.

Full data accompanying these experiments must be made available to reviewers at the time of submission in a freely accessible resource e.g the sequence read archive (SRA) or European Nucleotide Archive (ENA).

Depending on the question addressed in a manuscript, de novo assemblies of transcriptomes may also require multiple replicates and assembled sequences together with sequence annotation must be made freely available e.g figshare or dryad.

Inclusion of Proteomics Data

Authors should provide relevant information relating to how peptide/protein matches were undertaken, including methods used to process and analyze data, false discovery rates (FDR) for large-scale studies, and threshold or cut-off rates for peptide and protein matches. Further information should include software used, mass spectrometer type, sequence database and version, number of sequences in database, processing methods, mass tolerances used for matching, variable/fixed modifications, allowable missed cleavages, etc.

Authors should provide as supplementary material information used to identify proteins and/or peptides. This should include information such as accession numbers, observed mass (m/z), charge, delta mass, matched mass, peptide/protein scores, peptide modification, miscleavages, peptide sequence, match rank, matched species (for cross-species matching), number of peptide matches, etc. Ambiguous protein/peptide matches should be indicated.

For quantitative proteomics analyses, authors should provide information to justify the statistical significance, including biological replicates, statistical methods, estimates of uncertainty, and the methods used for calculating error.

For peptide matches with biologically relevant post-translational modifications (PTMs) and for any protein match that has occurred using a single mass spectrum, authors should include this information as raw data or annotated spectra, or submit data to an online repository (recommended option; see table below).

Raw or matched data and 2-DE images should be submitted to public proteomics repositories such as those participating in ProteomeXchange. Submission codes and/or links to data should be provided within the manuscript.

4. Figure and Table Guidelines

- General Style Guidelines for Figures

The maximum number of figures and tables for all article types are shown in the Summary Table. Frontiers requires figures to be submitted individually, in the same order as they are referred to in the manuscript, the figures will then be automatically embedded at the end of the submitted manuscript. Kindly ensure that each table and figure is mentioned in the text and in numerical order.
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- General Style Guidelines for Tables

Tables should be inserted at the end of the manuscript. If you use a word processor, build your table in word. If you use a LaTeX processor, build your table in LaTeX. An empty line should be left before and after the table. Please note that large tables covering several pages cannot be included in the final PDF for formatting reasons. These tables will be published as supplementary material on the online article abstract page at the time of acceptance. The author will notified during the typesetting of the final article if this is the case. A link in the final PDF will direct to the online material.

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Figure and table legends are required to have the same font as the main text (12 point normal Times New Roman, single spaced). Legends should be preceded by the appropriate label, for example "Figure 1" or "Table 4". Figure legends should be placed at the end of the manuscript (for supplementary images you must include the caption with the figure, uploaded as a separate file). Table legends must be placed immediately before the table. Please use only a single paragraph for the legend. Figure panels are referred to by bold capital letters in brackets: (A), (B), (C), (D), etc.

- Image Size

Figure images should be prepared with the PDF layout in mind, individual figures should not be longer than one page and with a width that corresponds to 1 column or 2 columns.

- **All articles are prepared using the 2 column layout:** 2 column articles can contain images 85 mm or 180 mm wide.

- Format

The following formats are accepted:
TIFF (.tif) TIFF files should be saved using LZW compression or any other non-lossy compression method.
JPEG (.jpg)
EPS (.eps) EPS files can be uploaded upon acceptance

- Color Image Mode

Images must be submitted in the color mode RGB.

- Resolution Requirements

All images must be uploaded separately in the submission procedure and have a resolution of **300 dpi at final size**. Check the resolution of your figure by enlarging it to 150%. If the resolution is too low, the image will appear blurry, jagged or have a stair-stepped effect.

Please note saving a figure directly as an image file (JPEG, TIF) can greatly affect the resolution of your image. To avoid this, one option is to export the file as PDF, then convert into TIFF or EPS using a graphics software. EPS files can be uploaded upon acceptance.

- Chemical Structures

Chemical structures should be prepared using ChemDraw or a similar program according to the guidelines given below:
Drawing settings: chain angle, 120° bond spacing, 18% of width; fixed length, 14.4 pt; bold width, 2.0 pt; line width, 0.6 pt; margin width 1.6 pt; hash spacing 2.5 pt. Scale 100%Atom Label settings: font, Arial; size, 8 pt. Assign all chemical compounds a bold, Arabic numeral in the order in which the compounds are presented in the manuscript text. Figures containing chemical structures should be submitted in a size appropriate for incorporation into the manuscript.

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Figures must be legible. Check the following:

- The smallest visible text is no less than 8 points in height, when viewed at actual size.
- Solid lines are not broken up.
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