GABRIEL NASCIMENTO SILVA

Interactions between unicellular eukaryotes and Porifera: physiological function and biotechnological applications of associated Labyrinthulomycetes

Interações entre eucariontes unicelulares e Porifera: função fisiológica e aplicações biotecnológicas de Labyrinthulomycetes associados

São Paulo

2022

GABRIEL NASCIMENTO SILVA

Interactions between unicellular eukaryotes and Porifera: physiological function and biotechnological applications of associated Labyrinthulomycetes

Interações entre eucariontes unicelulares e Porifera: função fisiológica e aplicações biotecnológicas de Labyrinthulomycetes associados

Dissertação apresentada ao Instituto de Biociências da Universidade de São Paulo, para a obtenção de Título de Mestre em Ciências, na Área de Fisiologia Geral.

Orientador(a):
Prof. Dr. Márcio Reis Custódio

São Paulo

2022

Ficha Catalográfica

Ficha catalográfica elaborada pelo Serviço de Biblioteca do Instituto de Biociências da USP, com os dados fornecidos pelo autor.

Nascimento-Silva, Gabriel.
Interações entre eucariontes unicelulares e Porifera: função fisiológica e aplicações biotecnológicas de Labyrinthulomycetes associados / Gabriel Nascimento Silva; orientador Márcio Reis Custódio -- São Paulo, 2022.

110 f.

Dissertação (Mestrado) – Instituto de Biociências da Universidade de São Paulo. Departamento de Fisiologia.

1. Simbiose. 2. Protistas. 3. Cultura-dependente. 4. Esponjas. 5. Potencial Biotecnológico. I. Custódio, Márcio Reis. II. Título.

Bibliotecária responsável pela catalogação: Elisabete da Cruz Neves. CRB - 8/6228.

Comissão Julgadora:

_____ _____

Prof(a). Dr(a). Prof(a). Dr(a).

_____ _____

Prof(a). Dr(a). Prof. Dr. Márcio Reis Custódio

Orientador(a)

“À minha mãe, Berenice.
eu sei que você estaria
orgulhosa de mim”

“Aos meus avós.
Ednalva, Benta e Nonato
que estão longe, mas sempre comigo”

Ya me cansé de esperar,

ahora me levanto,

ya no quiero escapar

ahora siento las manos,

ya no pienso dejarme llevar

El cansancio no es pretexto para dejar de caminar

si el destino está lejos, sólo hace falta querer llegar

Ahora luzco huesos y dientes aunque te moleste mirar,

lamo mis heridas para que no sangren más.

Ahora me agarro al timón, para ser yo mi capitán

y ya no voy a la deriva, si caigo me vuelvo a levantar.

Abro las alas cuando e acerque un vendaval,

voy a intentar cambiar las cosas que no me dejan volar

Ahora celebro la vida

que hay un mundo entero por conquistar.

Stella

DÛRGA (De Lira Ire)

Agradecimentos

Agradeço meu irmão Rafael, meu pai Gilson e minha tia Solange. Sem eles eu simplesmente não teria chegado até aqui. Lidar com todas as dificuldades inerentes da nossa vida é mais tolerável graças a vocês. Obrigado por todo apoio e por estarem comigo em todos os momentos, bons ou ruins. Nossa sorte ainda vai mudar.

Agradeço profundamente o meu orientador Márcio Custódio. Sua inteligência, humor e sagacidade são invejáveis. Obrigado por me motivar e me ensinar a pensar como um cientista. Obrigado por todas as ideias mirabolantes (...ideia #7989...) que funcionaram e pelas que não funcionaram também. Também agradeço ao Vagner pela disposição em me ajudar em absolutamente todas elas, pelas tardes de companhia, conversas e por todos os "ajustes técnicos".

Sou eternamente grato por meus amigos. Todos vocês têm um lugar especial no meu coração. Sem vocês os dias seriam intermináveis e entediantes. Agradeço a Liv, por ser essa pessoa maravilhosa, empática e especial que ela é. Mariana e Michelle por todas as nossas conversas, risadas, choros e alegrias. Vinicius, Lícia e Priscílla por todas as nossas milhares de aventuras incríveis. À Camila, Pedro, Ricardo e mais recentemente a Júlia, por todas nossas noites em claro passando nervoso em jogos falidos. Essas horas foram essenciais para minha saúde mental, /tts obrigado por me manterem contraproducente. Daniel, nossos cafés da tarde e conversas sobre a vida, o universo e tudo mais me deram fôlego nos dias difíceis, ainda vamos sair na nossa coleta, prometo. Guilherme seu apoio incondicional foi importantíssimo, espere o mesmo de mim sempre. Aos meus *consagrados*, David, Vinicius, Gabriel, Rafael, Henrique e Andressa: vocês tomaram 2d8 de dano psíquico ao lerem isso e têm desvantagem no próximo turno. Agradeço pela nossa amizade mágica, entretanto rolem suas iniciativas.

Agradeço à Profª. Dra. Cristiane Hardoim por todo o incentivo e confiança depositados em mim. Também agradeço à Profº. Dra. Renata Moreira-Whitton pelo auxílio na investigação de ácidos graxos e ao Professor Dr. Daniel Lahr pela colaboração no estudo geral desses protistas. Agradeço ao Instituto de Biociências (IB-USP) e ao Centro de Biologia Marinha da USP (CEBIMar) pela infraestrutura cedida para o desenvolvimento deste trabalho assim como os esforços durante os tempos difíceis que vivemos. Também agradeço enormemente à FAPESP que me concedeu e manteve minha Bolsa de Pesquisa durante tempos incertos através do processo nº 2019/04707-8, Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).

Sumário

Lista de Figuras e Tabelas

PREFÁCIO

I. Organização da Dissertação

A presente dissertação está organizada em quatro capítulos. O capítulo 1 consiste em uma introdução geral onde apresentamos ao leitor a problemática dessa investigação, a justificativa dessa pesquisa e as abordagens metodológicas gerais que foram desenvolvidas aqui. O capítulo 2 é uma revisão narrativa da literatura, tendo surgido a partir de reflexões sobre a lacuna no conhecimento científico referente ao microeucarioma de Porifera. Nessa revisão nós discutimos as associações entre Porifera e diversas linhagens de eucariontes unicelulares, problemas metodológicos dessas investigações e perspectivas futuras. O capítulo 3 aborda diferentes aspectos da associação entre as esponjas marinhas tropicais *Hymeniacidon heliophila* e *Haliclona melana* e os protistas pertencentes ao táxon Labyrinthulomycetes (Stramenopiles). Por fim, o Capítulo 4 apresenta as principais conclusões depreendidas a partir das evidências presentes neste trabalho. Os capítulos 2 e 3 estão sendo submetidos para publicação.

II. Contribuição para a Área de Estudo

O estudo do microbioma dos animais é uma área em franca expansão dada as (r)evoluções tecnológicas ocorridas nas últimas décadas. Enquanto a nossa compreensão do microbioma dos vertebrados cresce vertiginosamente a cada ano (*viz*. Human Microbiome Project: https://www.hmpdacc.org) comparativamente pouco se sabe sobre as diversas associações entre microrganismos e os invertebrados. As esponjas constituem um grupo de animais que possui inúmeras peculiaridades. Essas características únicas podem nos dar pistas de como ocorreu a evolução das associações entre os primeiros animais multicelulares e os diversos filos de microrganismos que habitam os mares. Frente a isso, enquanto a maioria dos estudos foca nas frações bacteriana e arqueana do microbioma das esponjas, o presente trabalho estuda a natureza da associação entre Porifera e um grupo de protistas pouco conhecido, mas muito relevante no contexto de culturas celulares, usando uma abordagem tradicional dependente de cultura. Além disso, nós ainda abordamos as possibilidades de aplicações biotecnológicas que esses protistas isolados de esponjas apresentam.

RESUMO GERAL

Ao longo de suas histórias evolutivas, os representantes do filo Porifera estabeleceram complexas associações com vários filos de microrganismos. Essa microbiota está diretamente relacionada à fisiologia desses animais e tem sido o foco de vários estudos nas últimas décadas. Historicamente, a maioria dessas investigações foca na fração do microbioma composta por bactérias, arqueias e fungos. Entretanto, nossa compreensão da natureza das complexas associações entre protistas e esponjas ainda é limitada e carece de investigações. Esses microrganismos possuem uma grande diversidade de formas e metabolismos e ocupam vários nichos ecológicos. Buscando entender como eles se relacionam com as esponjas, realizamos uma revisão detalhada da literatura que versa sobre as interações entre protistas e Porifera observando o estado do conhecimento e principais desafios da área. Identificamos que um grupo de protistas conhecido como Labyrinthulomycetes constitui um dos principais obstáculos para o estabelecimento de culturas celulares desses invertebrados marinhos. Investigamos a natureza da associação entre Labyrinthulomycetes e duas esponjas marinhas tropicais: *Hymeniacidon heliophila* e *Haliclona melana*. Através de uma abordagem dependente de cultura, caracterizamos a morfologia geral dos isolados e inferimos sua identificação. Em conjunto, fizemos testes de degradação de diferentes substratos e a identificação de seus ácidos graxos, a fim de obter um perfil metabólico e avaliar seu potencial biotecnológico. Neste trabalho nós discutimos o papel dos protistas como um importante componente do microeucarioma das esponjas assim como reportamos os primeiros isolamentos sistemáticos de Labyrinthulomycetes a partir de culturas primárias de células de esponjas marinhas. Obtivemos três isolados com características morfológicas e bioquímicas distintas pertencentes às famílias Thraustochytrida e Labyrinthulida. Observamos que um dos isolados (HAL1) possui grande potencial para futuras explorações biotecnológicas, porém os outros (HYM1 e HAL2) ainda necessitam ser melhor investigados. Em vista das evidências, concluímos que estes protistas podem estar associados a essas esponjas marinhas de forma comensal ou mutualista. O trabalho até então realizado constitui um importante passo no entendimento da complexa e pouco estudada associação entre protistas e Porifera.

Palavras-chave: protistas; microeucarioma; microbioma; cultura-dependente; traustoquitrídeos; labirintulídeos; esponjas marinhas

ABSTRACT

Throughout their evolutionary histories, the representatives of the phylum Porifera have established complex associations with various microorganisms. This microbiota is directly related to the physiology of these animals and has been the focus of several studies in recent decades. Historically, most of these investigations have focused on the fraction of the microbiome composed of bacteria, archaea, and fungi. However, our understanding of the nature of the complex associations between protists and sponges is still limited and needs further investigation. These microorganisms possess a great diversity of forms and metabolisms and occupy various ecological niches. Seeking to understand how they relate to sponges, we conducted a detailed review of the literature on the interactions between protists and Porifera, observing the state of knowledge and the main challenges in the area. We identified that a group of protists known as Labyrinthulomycetes constitutes one of the main obstacles for the establishment of cell cultures of these marine invertebrates. We investigated the nature of the association between Labyrinthulomycetes and two tropical marine sponges: *Hymeniacidon heliophila* and *Haliclona melana*. Using a culture-dependent approach, we characterized the general morphology of the isolates and inferred their identification. Also, we performed degradation tests of different substrates and the identification of their fatty acids in order to obtain a metabolic profile and evaluate their biotechnological potential. In this work we discuss the role of protists as an important component of the sponge microeukaryome as well as report the first systematic isolations of Labyrinthulomycetes from primary cultures of marine sponge cells. We obtained three isolates with distinct morphological and biochemical characteristics belonging to the families Thraustochytrida and Labyrinthulida. We observed that one of the isolates (HAL1) has the greatest potential for future biotechnological exploitation, but the others (HYM1 and HAL2) still need further research. In view of the evidence, we conclude that these protists are associated with these marine sponges in a commensal or mutualistic manner. The work done so far constitutes an important step in the understanding of the complex and little studied association of protists and Porifera.

Keywords: protists; microeukaryome; microbiome; culture-dependent; thraustochytrids; labyrinthulids; marine sponges

CAPÍTULO 1

Introdução e Métodos Gerais

1.1 Introdução Geral

1.1.1 O filo Porifera

As esponjas (filo Porifera) são organismos multicelulares, filtradores, sésseis, dotados de poucas especializações morfológicas, tendo representantes tanto marinhos quanto dulciaquícolas (Simpson, 1984). Dados moleculares e suas características únicas colocam esses animais evolutivamente na base de Metazoa (Müller, 2003; Redmond e Mclysaght, 2021). Há evidências de sua presença no Pré-Cambriano (Sperling et al., 2010) e recentemente análises de fósseis recifais apontam a existência desses animais já no Neoproterozoico (Turner, 2021). São organismos comuns na maioria dos ambientes aquáticos e desempenham funções vitais no funcionamento dos ecossistemas (Wörheide et al., 2005; Bell, 2008; Chang et al., 2019). Atualmente são reconhecidas 9489 espécies de esponjas (de Voogd et al., 2022), divididas em quatro classes: Demospongiae, Calcarea, Homoscleromorpha e Hexactinellida.

O *bauplan* ou a organização do corpo de Porifera é relativamente simples quando comparado com outros metazoários, contudo, podem assumir uma miríade de formas (Figura 1.1A-E). As esponjas não têm tecidos ou órgãos como os encontrados nos organismos mais derivados. Possuem uma fisiologia essencialmente celular, com células especializadas desempenhando funções únicas e vitais para a homeostase do animal (van Soest et al., 2012). A maioria das esponjas tem como principal característica um esqueleto composto por espículas de sílica ou carbonato de cálcio, embora várias espécies possuam apenas uma rede de fibras de colágeno (*e.g.* esponjas de banho naturais) ou não tenham nenhum elemento esquelético definido (Simpson, 1984). Estas estruturas dão suporte estrutural e fornecem algum grau de proteção contra a predação, e por serem extremamente diversas (Figura 1.1F) são um dos principais caracteres usados na taxonomia e sistemática desses animais (van Soest et al., 2012). Além disso, outros caracteres diagnósticos do grupo são a presença de um sistema aquífero e a ausência de órgãos e sistemas especializados.

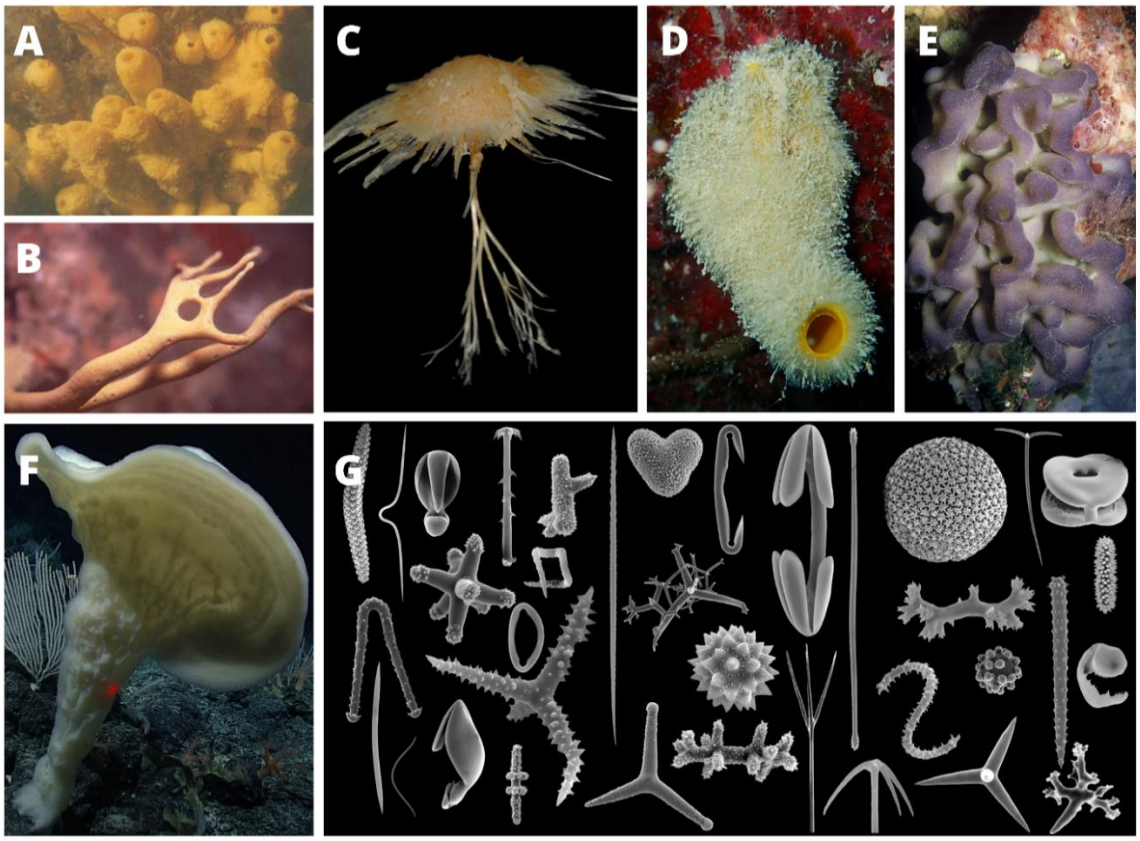

Figura 1.1. Representantes das quatro classes do filo Porifera: *Aplysina caissara* (A), *Aplysina cauliformis* e a esponja carnívora *Cladorhiza* sp. (C) pertencem a classe mais diversa, Demospongiae. Outras com esqueleto calcário como a *Sycettusa hirsutíssima* (C) pertence a classe Calcarea. Há também a classe Homoscleromorpha que tem representantes com características únicas como *Oscarella lobularis* (D). Por fim, há também as pouco estudadas esponjas de vidro da classe Hexactinellida, como essa da família Euretidae ainda não descrita (E). (F) Diversidade de espículas da classe Demospongiae, demonstrando algumas das distintas morfologias que as espículas desses animais podem assumir. As referências das imagens podem ser encontradas no Anexo 1.

O sistema aquífero de Porifera consiste em uma vasta rede de canais e câmaras de diferentes tamanhos que agrupam células flageladas, os coanócitos. O batimento destes flagelos cria uma corrente de água do exterior, trazendo microorganismos e matéria orgânica em suspensão, que são então capturados pelos coanócitos. O sistema aquífero desses animais pode assumir diferentes níveis de complexidade (Simpson, 1984; van Soest et al., 2012). No geral há pelo menos três níveis básicos de organização, sendo a mais simples a asconóide, seguida da siconóide e a mais complexa que é a leuconóide (Figura 1.2).

Dentro de Porifera a classe Demospongiae é a mais diversa, com 7861 spp. reconhecidas (de Voogd et al., 2022). Esse grupo compreende as esponjas que possuem um esqueleto composto por espículas de sílica, que podem ou não estar organizadas em

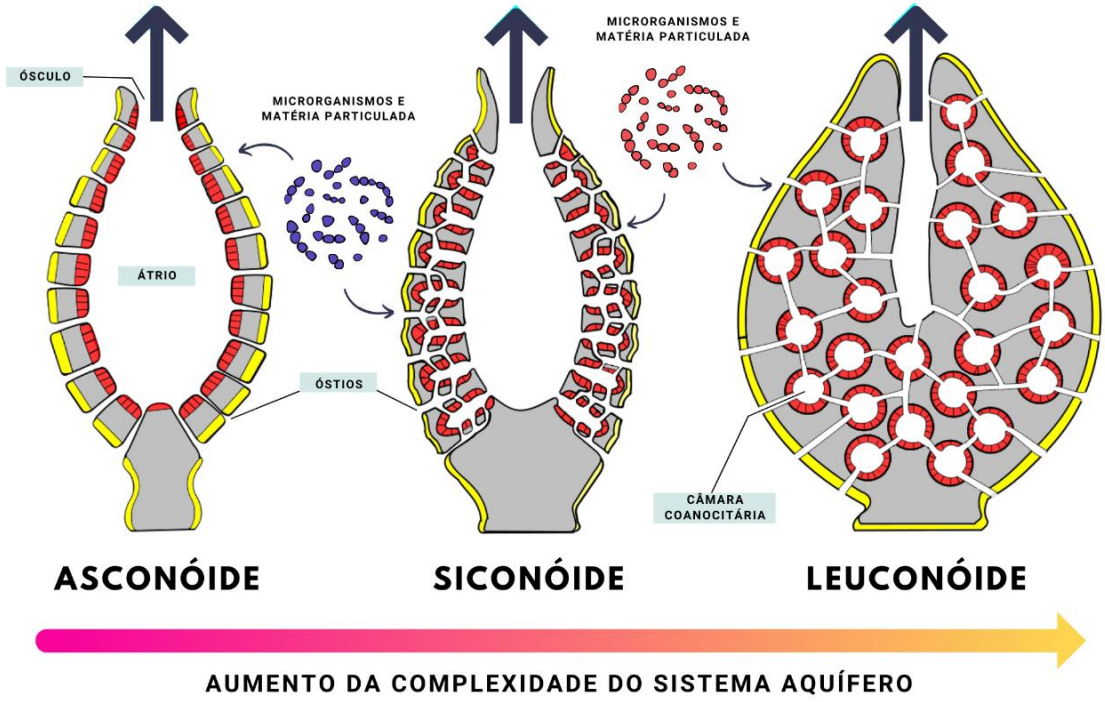

Figura 1.2. Esquema geral do sistema aquífero encontrado em Porifera, com três tipos básicos mais comuns que ilustram os diferentes graus de complexidade encontrados: Asconóide, Siconóide e Leuconóide. Nos três, a água ao redor da esponja contendo microrganismos e matéria orgânica particulada entra pelos óstios e é filtrada nas câmaras coanocitárias. O fluxo de água resultante é direcionado para fora do corpo da esponja pelo ósculo.

feixes embebidos por uma substância colágena, a espongina (van Soest et al., 2012). É a única classe conhecida que, além dos representantes marinhos, também inclui espécies de água doce (286 spp. são dulciaquícolas) e carnívoras (alguns representantes de Poecilosclerida). Além do potencial biotecnológico inerente dessa diversidade, as Demospongiae têm grande importância econômica, principalmente dentro de comunidades costeiras que usam a aquicultura sustentável desses animais como fonte de renda (Gaertner et al., 2020; Adler, 2020).

Por sua vez, Calcarea, possui 802 spp. reconhecidas (de Voogd et al., 2022). Nesse grupo estão as esponjas que secretam um esqueleto de carbonato de cálcio e produzem de espículas com esse mesmo mineral (van Soest et al., 2012). Assim como as Demospongiae, assumem várias formas, porém geralmente têm tamanhos menores, sendo medidas em milímetros ou poucos centímetros (van Soest et al., 2012). Interessante ressaltar que dada a natureza de seu esqueleto são espécies de interesse na investigação dos efeitos das mudanças climáticas nos oceanos, principalmente a

acidificação e o aumento das temperaturas médias (Ribeiro et al., 2020). A classe Hexactinellida, conhecidas como *esponjas de vidro*, têm 695 spp. reconhecidas (de Voogd et al., 2022). As diferentes espécies que compõem esse grupo têm morfologias e fisiologia únicas, e são geralmente encontradas em águas profundas, de 200 m até 6 km. Suas espículas têm simetria triaxônica (três eixos), possuem tecidos que são sinciciais e muitas espécies são vivíparas, com a produção de uma larva específica (revisão em Leys et al., 2007). Têm sido foco da Ciência de Materiais principalmente pelas características únicas de seu esqueleto, que permitem uma alta tramissibilidade de luz, semelhante às fibras ópticas (Brümmer et al. 2008). A classe mais recente e a menos diversa é Homoscleromorpha com 130 spp. aceitas (de Voogd et al., 2022). Historicamente esse táxon estava inserido dentro de Demospongiae, contudo análises moleculares demonstraram que esses animais constituem uma classe distinta (Gazave et al. 2012). Possuem espículas de sílica tetraxônicas (quatro eixos), porém outras morfologias também podem ser encontradas. Têm alguns caracteres diagnósticos como sua larva (cinctoblastula), porém a maior parte da identificação desse grupo é feita através de identificação molecular ou através do uso de métodos citológicos e histológicos (van Soest et al., 2012).

1.1.2 Microbioma de Porifera

As esponjas são reconhecidas como animais com ampla capacidade de associação com microrganismos. Ao longo de suas histórias evolutivas, os representantes de Porifera estabeleceram complexas associações com diversos filos de bactérias, arqueias, fungos, algas, vírus e protistas (Taylor et al., 2007; Webster e Taylor, 2012; Webster e Thomas, 2016). Essas associações permitiram uma grande diversidade de soluções para desafios ambientais aos quais esses animais estão submetidos (van Soest et al., 2012). Os microrganismos associados podem desempenhar funções essenciais no metabolismo energético ou de compostos como nitrogênio e fósforo (Fiore et al., 2015; Zhang et al; 2015; revisão em Webster e Thomas, 2016), no desenvolvimento (Ereskovsky e Boury-Esnault, 2002; Fieth et al., 2016), na ecologia e evolução (Usher, 2007; Thomas et al., 2016) e até mesmo na modulação do sistema imune e na resiliência desses animais frente às futuras condições climáticas (Pita et al., 2018; Schmittmann et al., 2021; Posadas et al., 2021).

Existe um grande número de trabalhos que abordam a composição, estrutura e função das comunidades de bactérias e arqueias que habitam os tecidos de Porifera, sendo boa parte do conhecimento do microbioma desses animais restritos a esses grupos. De maneira geral, esses e outros microrganismos tais quais vírus, fungos e eucariontes unicelulares habitam a matriz extracelular das esponjas, ou mesohilo (Webster e Thomas, 2016). No entanto, vale ressaltar que algumas espécies de Porifera possuem também células chamadas bacteriócitos, que mantém os microrganismos endosimbiontes em vacúolos ou cápsulas dentro do seu citoplasma (Taylor et al., 2007). Embora todas as esponjas possuam microorganismos associados, nem todas os possuem grandes quantidades, e esta relação é algo associado com a história evolutiva das espécies. Enquanto alguns taxa podem ter até 10^9 células bacterianas por cm^3 (conhecidas como *High Microbial Abundance* - *HMA*) há outros que possuem 10^5 a 10^6 células bacterianas por cm^3 (*Low Microbial Abundance* - *LMA*), números semelhantes à água do mar no habitat do animal (Gloeckner et al., 2014). No geral essas classificações HMA/LMA são feitas com base em microscopia de transmissão (Gloeckner et al., 2014; Moitinho-Silva et al., 2017), onde é possível observar a presença de células bacterianas em diferentes densidades nos tecidos do animal estudado (Figura 1.3A-B).

1.1.3 O potencial biotecnológico dos microrganismos associados às esponjas

Em um cenário onde os recursos terrestres estão sendo depletados, os oceanos e seus habitantes se tornaram uma fonte de interesse para exploração biotecnológica, um movimento chamado *Blue Biotechnology* (Steinert et al., 2017). Neste sentido, as esponjas são historicamente muito promissoras. O metabolismo secundário, isto é, a produção de moléculas que não são utilizadas para o crescimento e reprodução, é bastante pronunciado estes organismos. Já foi verificado que aproximadamente 32% de todos os produtos naturais marinhos descobertos nos últimos 50 anos têm como origem extratos obtidos destes organismos (Blunt et al., 2015). Dentre estes, já foram identificados centenas de compostos bioativos com propriedades anticancerígenas, antimicrobianas ou anti-inflamatórias (Radwan et al., 2012; Brinkmann et al., 2017).

Parte destes compostos tem como origem os seus microrganismos simbiontes, os quais têm se revelado uma das principais fontes de novos metabólitos e processos industriais. Bactérias, arqueias e, mais recentemente, fungos têm sido amplamente

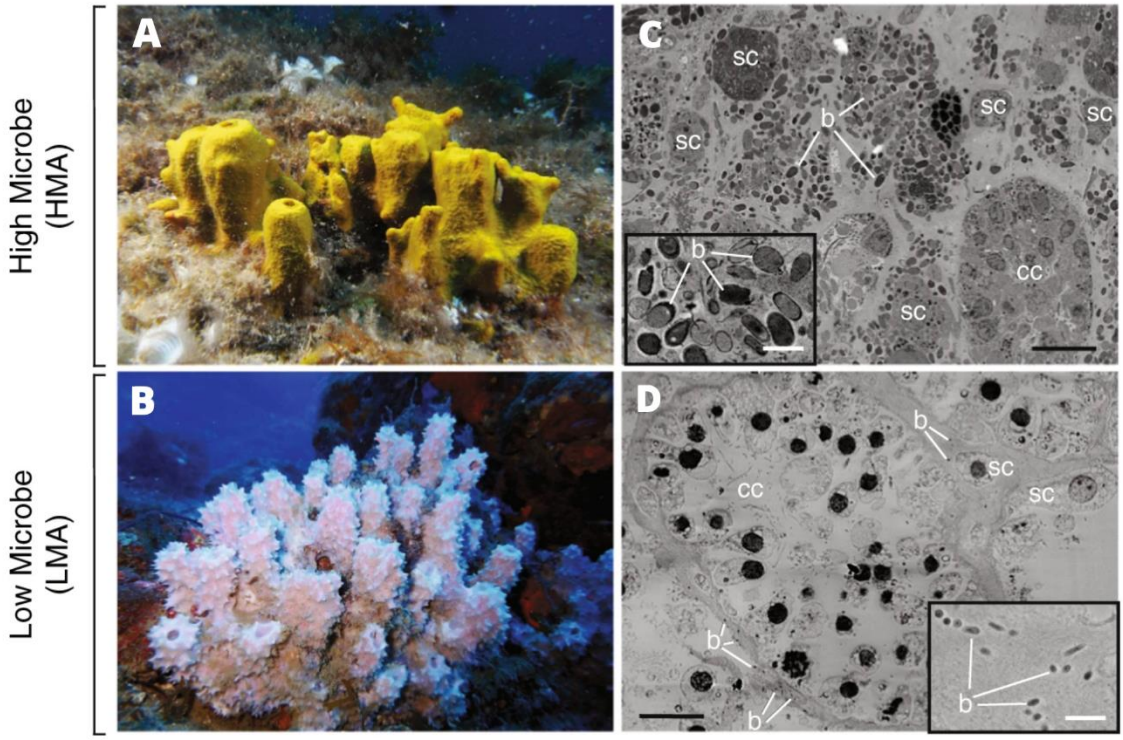

Figura 1.3. Morfologia geral de (A) *Aplysina aerophoba* classificada como *High Microbial Abundance* (HMA) e (B) *Dysidea avara* classificada como *Low Microbial Abundance* (LMA). Em (C) observa-se imagens de microscopia de transmissão de *A. aerophoba* onde há uma grande quantidade de bactérias associadas aos tecidos dessa esponja. Em contrapartida, nos tecidos de *D. avara* (D) a abundância relativa de microrganismos associados é bem menor. Legenda: cc – câmara coanocitária; sc – célula de esponja, b – células bacterianas. Barras de escala: figuras 10 µm, insets 1 µm. Adaptado de Rix et al. (2020).

explorados nesse sentido (Thakur e Müller, 2004, Suryanarayanan et al., 2012; Brinkmann et al., 2017). Microrganismos isolados de esponjas têm sido aplicados com sucesso no desenvolvimento de tratamentos de rejeitos eletrônicos, como é o caso das bactérias lixiviadoras de cobre (Rozas et al., 2017). Bactérias também mostraram uma significante capacidade de biorremediação ao degradar poluentes orgânicos persistentes, como o pesticida organoclorado Lindano (Aresta et al., 2015). Ainda nessa linha investigativa, com a pandemia causada pelo SARS-CoV2, a busca por produtos naturais se intensificou. Compostos como bromotirosinas e polifosfatos inorgânicos (polyP) produzidos pelas esponjas e/ou seus microrganismos associados apresentam o potencial de inibir a entrada de SARS-CoV2 em células humanas (Geahchan et al., 2021). Leveduras tolerantes a baixas temperaturas associadas às esponjas antárticas possuem um grande repertório enzimático ainda pouco explorado (Vaca et al., 2013). Apesar do interesse, muito ainda pode ser investigado. Fungos dos gêneros *Aspergillus* sp. e *Penicillium* sp. bem conhecidos por produzirem compostos variados com atividades que vão desde ação antimicrobiana e antitumoral à neuroprotetora, foram identificados

como contribuintes do perfil de metabólitos secundários de diversas esponjas (revisão em Suryanarayanan et al., 2012). Apesar dessa enorme diversidade de compostos bioativos, poucas dessas moléculas chegam às etapas de testes clínicos (Steinert et al., 2017). Isso se dá em boa parte pelas baixíssimas concentrações dos metabólitos de interesse que são encontrados nos animais, mas também pelas grandes dificuldades de isolamento e escalabilidade de cultivo dos microrganismos associados (Steinert et al., 2017).

1.1.4 O contexto das culturas de células primárias e os microrganismos associados

Para compreender o funcionamento desses animais primordiais assim como permitir uma exploração sustentável de moléculas bioativas de interesse humano, houveram diversas tentativas de estabelecimento de culturas de células de esponjas. Apesar de um recente avanço nessa área (Conkling et al., 2019), de uma forma geral, a cultura de células de esponja caminha à passos lentos, uma tendência que se estende virtualmente para todos os filos de invertebrados marinhos (Domart-Coulon e Blanchoud, 2022). Já foram publicados diversos trabalhos que tentam encontrar formas de manter a viabilidade, replicação e a condição axênica das culturas de células derivadas de todos os grandes filos (Porifera, Cnidaria, Mollusca, Platyhelminthes, Annelida, Crustacea, Echinodermata e Urochordata). Contudo, até hoje há pouco sucesso, já que as células desses organismos normalmente não se replicam quando *in vitro*, ou quando se replicam geralmente o processo ocorre de maneira limitada (Grasela et al., 2012; Conkling et al., 2019).

Para Porifera, o método mais usado ainda hoje consiste na formação e manutenção das células como estruturas tridimensionais, denominadas *primorfos* (Custodio et al., 1998), utilizadas até hoje em diversos experimentos (*e.g.* Müller et al., 1999; Zhang et al., 2003; Müller et al., 2004; de Caralt et al., 2007; Grasela et al., 2012). No entanto, embora o método permita que as células permaneçam viáveis por períodos que podem chegar a 12 meses (Chernogor et al., 2013), não há proliferação. Não há fatores de crescimento ou mesmo um meio de cultura nutriente definido. Assim como para outros invertebrados marinhos, tentativas de adicionar estes componentes em geral levam à proliferação rápida de microrganismos associados. Estes geralmente acabam por ultrapassar o crescimento das células animais e tem sido um grande

obstáculo nas diversas tentativas de avanço dessa área da ciência (Rinkevich, 1999; 2005; Grasela et al., 2012). Enquanto as contaminações por bactérias e fungos podem ser relativamente controladas por técnicas estéreis rigorosas e a utilização de antibióticos e antifúngicos, a proliferação de protistas não pode ser contida de maneira equivalente (Schippers et al., 2012). A variedade de metabolismos, hábitos e estratégias evolutivas deste grupo (Worden et al., 2015; Leray e Knowlton, 2016), associado à falta de conhecimento sobre as interações protistas-esponjas torna o processo de cultura celular de Porifera uma tarefa complexa.

1.1.5 Os eucariontes unicelulares que habitam as esponjas

Apesar da compreensão sobre a microbiologia das esponjas ter crescido nos últimos anos, a maioria das investigações ainda são focadas nas bactérias e arqueias associadas, um viés já observado no estudo do microbioma de outros animais (del Campo et al., 2020). Ao longo de nossas observações e experimentos nós constatamos a presença de diferentes microrganismos eucariontes. Os fungos geralmente são organismos bem conspícuos e em vários de nossos experimentos, leveduras marinhas e fungos filamentosos cresceram em abundância quando não controlados. Embora a origem de algumas cepas possa ser de contaminações, há dados que indicam que sejam verdadeiramente associados. Isto foi observado por Rozas et al. (2011) ao isolar diversas cepas fúngicas a partir de culturas de células estabelecidas das esponjas *Haliclona melana* e *Hymeniacidon heliophila*. Em outros experimentos também foi observada a presença de uma levedura marinha *Rhodorutula* spp. nas espécies estudadas neste trabalho, também a partir de culturas *in vitro*. Esses microrganismos já foram descritos como simbiontes de *Chondrilla* sp. e outras esponjas (Maldonado et al., 2005; Rosado-Rodríguez et al., 2019) inclusive existindo evidências de sua transmissão vertical. Contudo, para além de Fungi, são observados também diversos grupos de microrganismos eucariontes comumente conhecidos como *protistas*. Estes também aparentam estar associados de alguma forma com os tecidos das esponjas estudadas, visto a ubiquidade desses microrganismos nas diversas culturas de células.

O termo *protista* é uma denominação informal utilizada para diversos eucariotos unicelulares, que formam um grupo parafilético composto de múltiplas linhagens evolutivas (Pawlowski, 2014; Adl et al., 2019). A diversidade dos protistas associados

às esponjas não é insignificante, apesar da pouca atenção que recebem dentro da espongologia. As esponjas marinhas e dulcíaquícolas mantêm íntimas associações com diversas linhagens de protistas, como dinoflagelados, diatomáceas, foraminíferos, ciliados dentre outros (vide Capítulo 2), adicionando uma nova dimensão ao já complexo problema de criação de uma linhagem celular de Porifera. Para ilustrar a complexidade dessas associações, como efeito positivo da interação esponja-protista um bom exemplo é simbiose entre a esponja de água doce *Lubomirskia baicalensis* e a microalga verde *Mychonastes*. Essa associação alga-hospedeiro é a principal responsável pela sobrevivência dos primorfos obtidos dessas esponjas, que podem ser mantidos em condições experimentais por até 10 meses sem um meio de cultura nutriente (Chernogor et al., 2011). Por outro lado, não faltam observações e registros dos impactos negativos causados pela proliferação de protistas nas culturas de Porifera (Schippers et al., 2012). Dentre as diversas linhagens mais comumente encontradas, podemos destacar um pequeno grupo de estramenópilos basais conhecidos como Labyrinthulomycetes. Os representantes desse táxon são conhecidos inviabilizarem a cultura celular de diversos invertebrados, sendo consistentemente observados nas culturas de células de esponjas (Rinkevich, 1999; Schippers et al., 2012). Tal ubiquidade torna esses microrganismos um alvo válido no estudo de suas interações com Porifera.

1.1.6 Labyrinthulomycetes: taxonomia, morfologia, ubiquidade e potencial inexplorado

A taxonomia e filogenia deste táxon são bastante complexas. Observa-se por muitas vezes na literatura o intercambiamento do termo “Labyrinthulomycetes” usado no *International Code of Nomenclature* (ICN, para fungos, algas e plantas) com “Labyrinthulea” ou “Labyrinthulomycota” usados pelo *International Code of Zoological Nomenclature* (ICZN, para animais) (revisão em Bennet et al., 2017 e Marchan et al., 2018). Aqui priorizamos o uso de “Labyrinthulomycetes”. Estes são microrganismos marinhos, semelhantes a fungos, que adquirem matéria particulada por absorção osmótica (osmotrofia). O posicionamento filogenético e taxonomia desse grupo ainda é foco de discussão, porém há evidências que suportam esse táxon como um grupo basal dentro de Stramenopila (Figura 1.4) (Bennet et al., 2017; Marchan et al., 2018). Esses microeucariotos têm uma morfologia aparentemente simples, contudo,

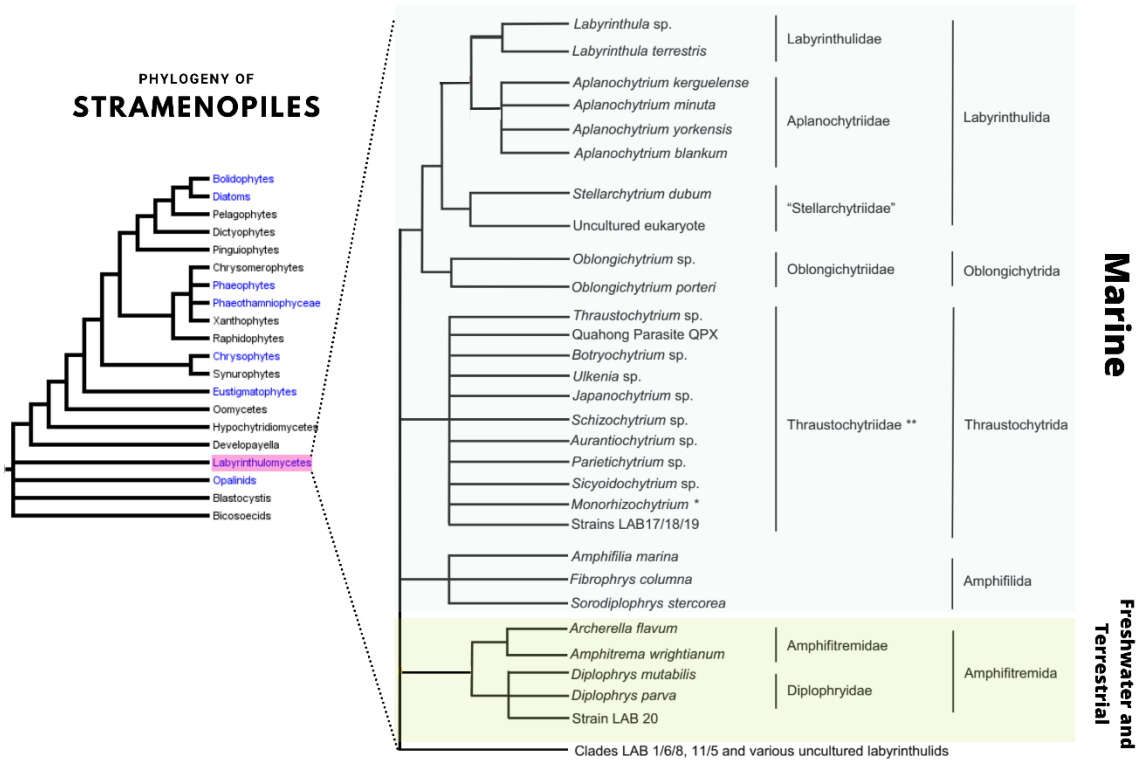

Figura 1.4. Filogenia simplificada de Stramenopila e Labyrinthulomycetes. Nota-se que grande parte dos protistas dentro de Labyrinthulomycetes são marinhos, porém há grupos terrestres e/ou de água doce. Adaptado de Bennet et al. (2017) e Bailey (2010).

uma inspeção mais aprofundada revela uma série de detalhes e diversidade formas (Figura 1.5A-J). Os Labyrinthulomycetes são recobertos de escamas ovóides ou hexagonais, não-celulósicas, geralmente compostas por polissacarídeos fosfatados (Figura 1.5J) (Bennet et al., 2017). Detalhes ultraestruturais mostram que possuem mitocôndrias tubulares dotadas de uma crista e sua parede celular tem escamas circulares organizadas em uma ou mais camadas (Moss, 1985; Dick, 2001). Outra característica importante desse grupo é que as formas vegetativas produzem redes ectoplasmáticas ramificadas a partir de uma organela denominada botrossomo ou sagenogenotossomo (Figura 1.5C-D) (Iwata et al., 2017). Podem viver associados a algas, invertebrados e vertebrados como parasitas, comensais ou mutualistas, mas podem ser encontrados também em vida livre, compondo uma parte do fenômeno de "neve marinha" ou "*marine snow"* (Figura 1.5E-I) (Raghukumar, 2002; Marchan et al., 2018).

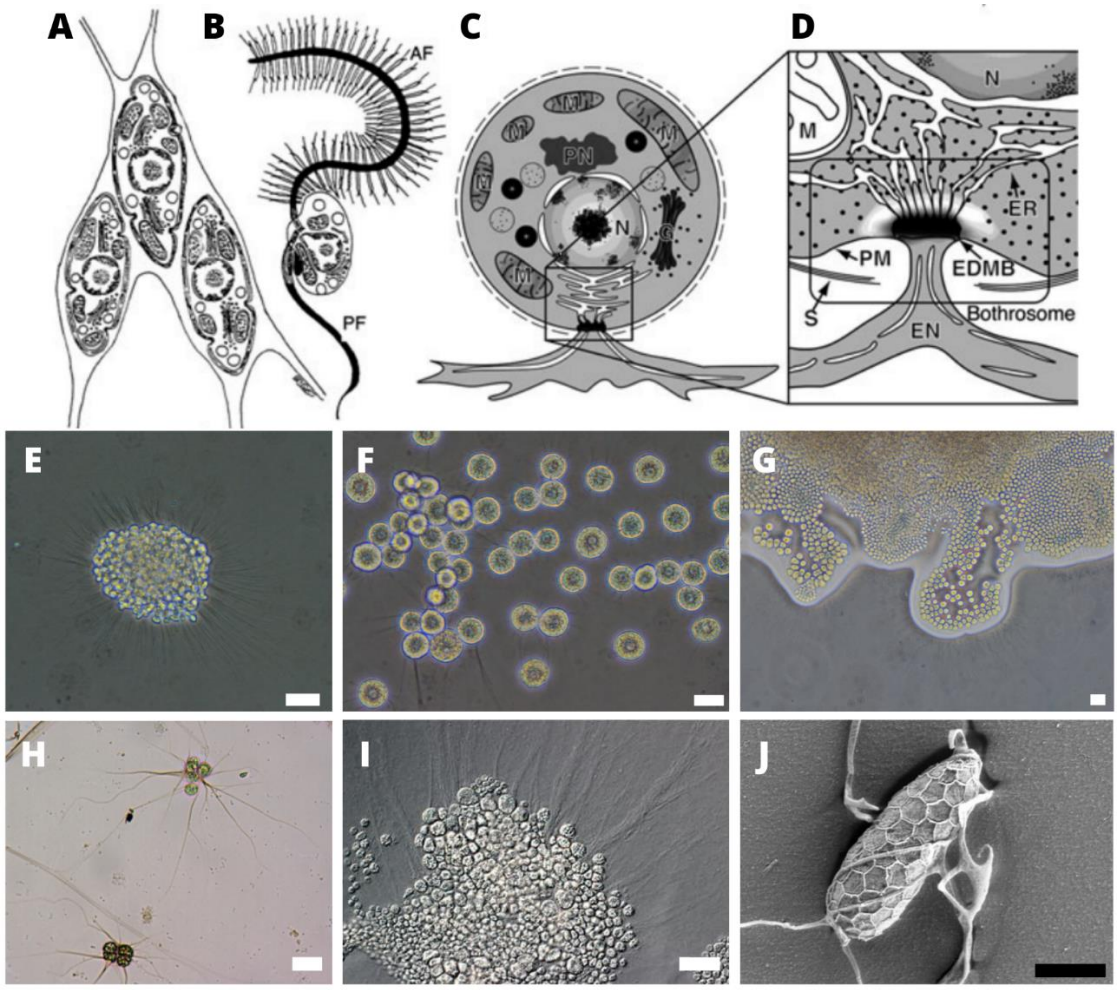

Figura 1.5. Características morfológicas de representantes de Labyrinthulomycetes. (A) Esquema geral de *Labyrinthula* demonstrando a organização das células dentro da rede ectoplasmática. (B) Zoósporo biflagelado, característico dos Stramenopila. (C) Esquema geral de *Schizochytrium aggregatum* mostrando uma célula vegetativa uninucleada e (D) detalhes da organela botrossomo. Legenda: N – Núcleo; G - Complexo de Golgi; M – mitocôndria; EDMB – material eletrodenso; EN – rede ectoplasmática; ER - retículo endoplasmático; PM – membrana plasmática; S – escamas que formam a parede celular. (E - G) Labyrinthulomycetes isolados de esponjas marinhas no presente trabalho. (H) *Aurantiochytrium* sp isolado de manguezais no litoral de São Paulo. (I) *Stellarchytrium dubum* isolado a partir de tecidos da estrela do mar *Pisaster ochraceus.* Microscopia de transmissão de uma célula vegetativa *Aplanochytrium* sp. onde é possível observar as escamas que formam a parede celular do protista. Barras brancas = 20 µm, barra preta = 2 µm. As referências das imagens podem ser encontradas no Anexo 1.

Como parasitas esses protistas causam impactos econômicos significativos, pois afetam invertebrados de consumo humano, como moluscos (Figura 1.6A-B) (Shinn et al., 2015). Por estarem associados com muitos invertebrados, são um obstáculo no processo de culturas celulares (Rinkevich, 1999, 2005; Rabinowitz et al., 2006; Han et al., 2013, Nowotny et al., 2021) e já foram publicados trabalhos onde formas vegetativas desses protistas foram erroneamente identificadas como as células dos animais

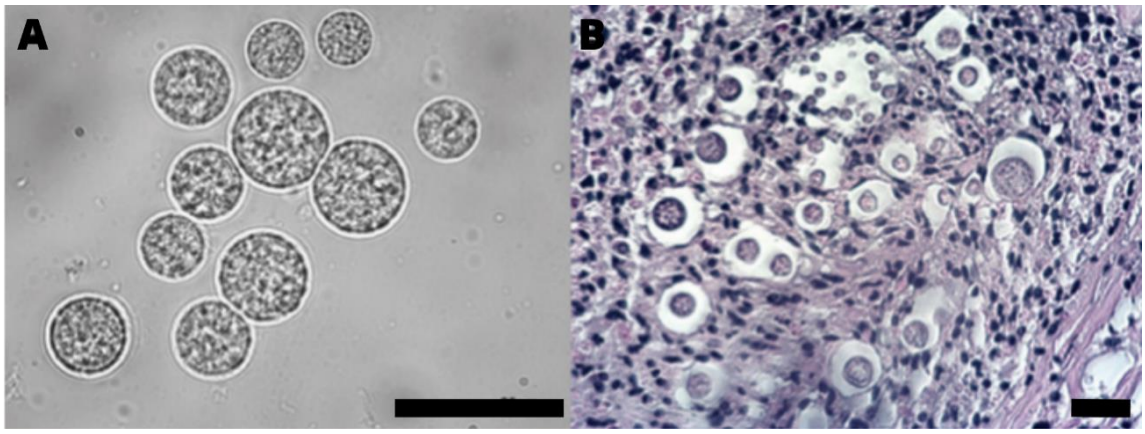

Figura 1.6. (A) Cultura de células vegetativas de um protista parasita pertencente ao grupo dos Labyrinthulomycetes (Thraustochytrida) denominado Quahog Parasite Unknown (QPX). (B) Histologia de tecidos do manto de um bivalve da espécie *Mercenaria mercenaria* infectado por QPX Corado com Hematoxilina/Eosina. Barra de escala = 20 µm. Adaptado de Collier et al. (2017).

(Rinkevich e Rabinowitz, 1993; Richelle-Maurer et al., 2003). Em relação a Porifera, a natureza da interação entre esses protistas e esponjas é muito pouco conhecida. A maioria dos registros refere-se à presença desses microrganismos em culturas de células primárias ou estudos de *high-throughput sequencing*. Além disso, até o momento existem pouquíssimos estudos aprofundados sobre esses protistas isolados a partir de tecidos de esponjas (Raghukumar, 2002).

Entretanto, embora seja um problema em alguns contextos, os Labyrinthulomycetes também possuem um grande repertório metabólico, o que abre a possibilidade do seu uso para uma ampla variedade de aplicações biotecnológicas (Raghukumar, 2008; Marchan et al., 2018). Esses protistas são reconhecidos pela síntese de ácidos graxos poliinsaturados (PUFAS), como DHA (ácido docosahexaenóico) e EPA (ácido eicosapentaenóico). Tais compostos, principalmente óleos ricos em DHA oriundos de traustoquitrídeos têm sido aplicados em uma miríade de processos. A produção significativa dessas substâncias torna esses protistas alvo de exploração pela indústria nutracêutica, onde são usados como suplemento tanto para humanos quanto na pecuária e aquicultura (Marchan et al., 2018). O uso dos óleos derivados desses Labyrinthulomycetes, como é o caso das espécies *Schizochytrium* sp. and *Ulkenia* sp., tem sido um substituto válido para componentes de origem animal (Marchan et al., 2018).

Esses microeucariotos também possuem em seu repertório metabólico a capacidade de produção de carotenóides, esqualeno e exsudatos de polissacarídeos. Os

carotenóides são pigmentos naturais que podem ser usados como ingredientes em alimentos, cosméticos e produtos farmacêuticos, além de terem propriedades antioxidantes e serem precursores de outras moléculas bioativas como vitaminas (Park et al., 2018). Por sua vez o esqualeno é um hidrocarboneto essencial que atua como precursor de esteróides, ácidos biliares e vitaminas, além de também possuir atividades antioxidantes (Spanova e Daum, 2011). Esse composto também é bastante utilizado como adjuvante de vacinas, sendo um ingrediente usado em certos imunizantes com o objetivo de promover uma resposta imune mais robusta (Mendes et al., 2022). Sendo uma das principais fontes de esqualeno o fígado de elasmobrânquios como tubarões de profundidade (Spanova e Daum, 2011), as populações desses animais tornam ameaçadas sendo uma prática insustentável a longo prazo. Nesse contexto, os Labyrinthulomycetes têm sido estudados como possíveis alternativas para esse problema (Aasen et al., 2016; Patel et al., 2020; Mendes et al., 2022). Já os exsudados de polissacarídeos, também produzidos por esses protistas têm diversas aplicações. Recentemente esses compostos têm sido usados pelas suas propriedades antitumorais e anticoagulantes, mas também pelas suas aplicações na indústria de cosméticos (Nham Tran et al., 2020). Além disso, há também uma diversidade ainda pouco explorada nas atividades metabólicas de diversas espécies de Labyrinthulomycetes. Representantes desse grupo possuem várias enzimas capazes de degradar substratos como celulose, lignina, amido, colágeno, compostos aromáticos, proteínas complexas e lipídeos (Raghukumar, 2008; Liu et al 2014; Li et al., 2021). Dada essas diversas características, os Labyrinthulomycetes constituem um táxon de interesse biotecnológico único, mas que ainda é muito pouco explorado, ainda mais no Brasil.

Sendo assim, neste trabalho foram efetuados o isolamento, o cultivo e a identificação morfológica de cepas Labyrinthulomycetes obtidas a partir de culturas de células i*n vitro* de duas espécies de esponjas marinhas do litoral brasileiro. Um perfil preliminar de atividades metabólicas foi efetuado para tentar identificar possíveis vias de interação com os hospedeiros. Dada a dispersão das poucas informações a respeito destes organismos e de outros microeucariotos associados à Porifera, foi feita também uma revisão da literatura disponível a respeito do tópico submetida para a revista *Microbiological Research* (https://www.sciencedirect.com/journal/microbiological-research). Nas próximas seções são apresentados os objetivos gerais do trabalho e detalhes da metodologia que não são abordados nos capítulos posteriores.

1.2 Objetivos Gerais e Específicos

O objetivo geral deste trabalho foi investigar diferentes aspectos da associação entre Labyrinthulomycetes e esponjas marinhas. Foram feitos o isolamento e cultivo desses microrganismos a partir de culturas primárias de células de duas esponjas marinhas comuns no Canal de São Sebastião, SP: *Hymeniacidon heliophila* e *Haliclona melana*. Foi realizada a identificação taxonômica dos protistas obtidos nas culturas, seguida da caracterização lipídica, metabólica e avaliação do potencial biotecnológico dos isolados. Também foram avaliadas as possíveis interações fisiológicas entre o protista e a esponja. Mais detalhadamente, os objetivos específicos desse trabalho foram:

a) Isolar e identificar os Labyrinthulomycetes associados a células de esponjas modelo e caracterizar seus perfis lipídicos e metabólicos.
b) Investigar a natureza das interações na associação entre esses protistas e as esponjas.
c) Avaliar o potencial biotecnológico dos isolados.

1.3 Materiais e Métodos Gerais

Nesta seção serão apresentadas as abordagens metodológicas gerais que foram desenvolvidas neste trabalho. Aqui elas serão detalhadas em maior profundidade do que nos manuscritos subsequentes.

1.3.1 *Coletas dos organismos-modelo*

Foram utilizados como animais modelos as esponjas marinhas *Hymeniacidon heliophila* (Wilson, 1911) (Demospongiae: Halichondriidae) e *Haliclona melana* Muricy e Ribeiro, 1999 (Demospongiae: Chalinidae) (Figura 1.7A-B). Ambas são comuns no litoral em locais de fácil acesso e já foram utilizadas em trabalhos anteriores do laboratório. Os espécimes foram coletados na região entremarés da Praia Grande (23° 49'22,5 "S, 45° 24'57,4" W) São Sebastião, São Paulo - Brasil, por meio de mergulho livre ou diretamente do costão rochoso exposto na maré baixa. As esponjas coletadas foram mantidas em tanques do Centro de Biologia Marinha (CEBIMar/USP) para aclimatação e triagem. O transporte foi feito em recipientes de plástico contendo aproximadamente 100 mL de volume de esponjas em 800 mL de água do mar local. Os recipientes contendo os organismos foram transportados em isopores a uma temperatura de 18ºC ± 2ºC até o Laboratório de Biologia Celular de Invertebrados Marinhos (LabCel/USP) em São Paulo - SP. Na impossibilidade de acesso ao CEBIMar durante a pandemia os organismos coletados foram mantidos em aquários de 30L com compressores de ar (BOYU Sc-7500 e BOYU ACQ-001) e transportadas no dia seguinte conforme já descrito. No laboratório, os animais foram acondicionados em aquários previamente montados, com os principais parâmetros ambientais (*i.e.* pH, oxigênio dissolvido, temperatura e salinidade) ajustados de acordo com aqueles observados no local de coleta. Os envolvidos na execução do projeto possuem experiência prévia com o trabalho de campo e laboratório, assim como licença para coletas no SISBIO (28917-1).

1.3.2 Cultura in vitro das células de esponjas

As esponjas coletadas foram dissociadas em água do mar artificial sem cálcio e magnésio, suplementada com EDTA (CMFSW+E: 460 mM NaCl, 7 mM Na_2SO_4, 10 mM KCl, 10 mM HEPES (ácido 4-(2-hidroxietil)-1-piperazin-etanossulfônico), 2.5 mM

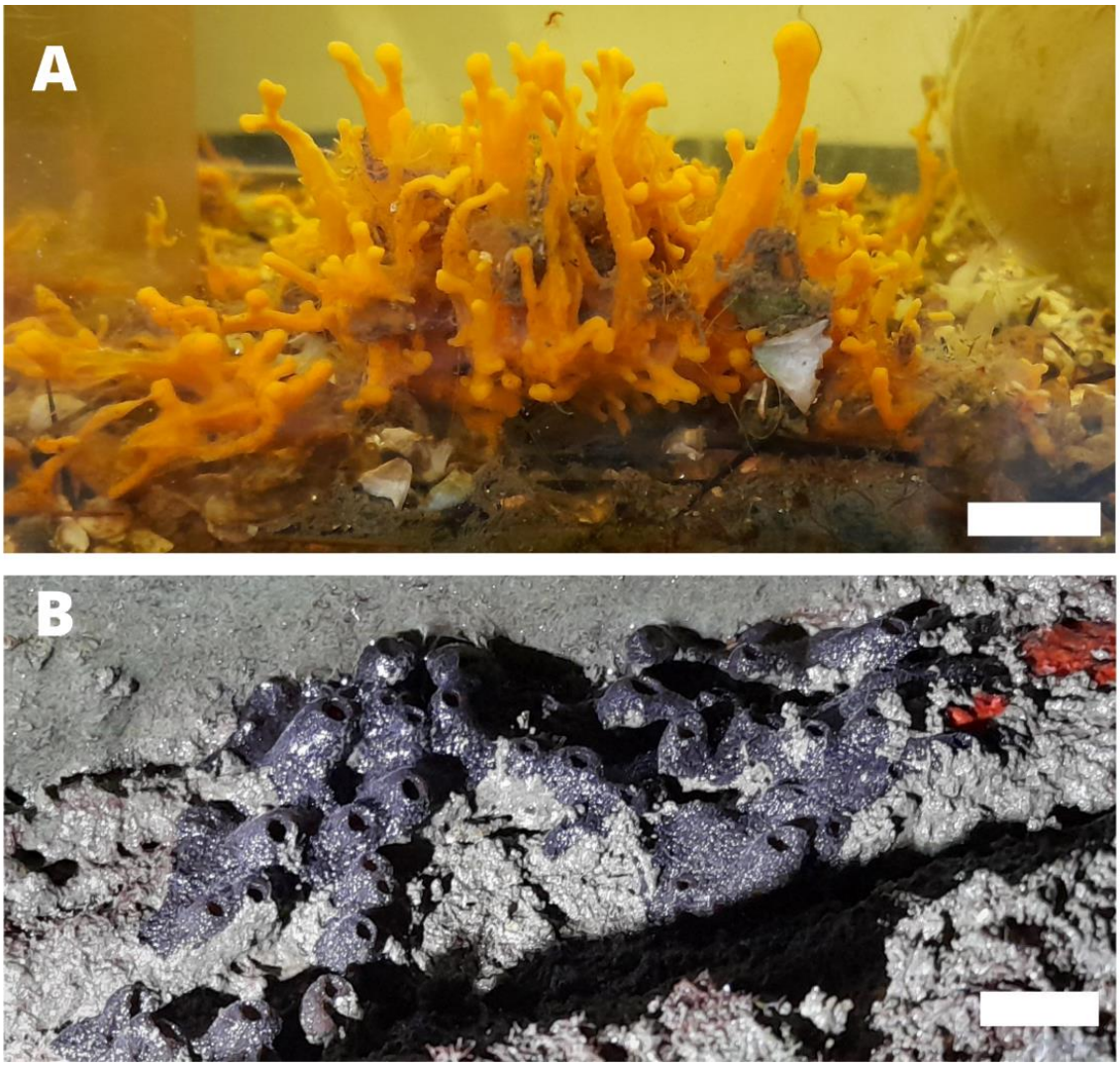

Figura 1.7. Morfologia geral das esponjas (A) *Hymeniacidon heliophila* (aquário); e (B) *Haliclona melana* (campo). A esponja *H. heliophila* pode apresentar diferentes morfologias de papilas. Estas podem ser bem pronunciadas, reduzidas e até mesmo inexistentes, tendo hábito similar às esponjas incrustantes. Já *H. melana é* mais presente na zona infralitoral, ficando exposta apenas em marés muito baixas, e geralmente têm papilas conspícuas dotadas de ósculos bem pronunciados no topo das projeções. Barra de escala = 1 cm.

EDTA (ácido etilenodiaminotetracético), pH 8.2) de acordo com Custodio et al. (1998). Os indivíduos foram limpos de outros organismos ou detritos e recortados em fragmentos de aproximadamente 2 mm. Esses fragmentos foram colocados em tubos de 50 mL contendo CMFSW+E (5:1 v/v) por 30 minutos em agitação leve. Após este tempo, os tubos foram decantados por 2 minutos e o sobrenadante descartado. Em seguida adicionou-se novo CMFSW+E aos tubos e estes foram mantidos em agitação leve por mais 1 hora. Ao final, o sobrenadante foi filtrado em uma malha de nylon (100 µm) e centrifugado a 250 x g por 10 minutos. O pellet foi ressuspendido em água do mar natural esterilizada por filtração (0,22 µm) e suplementada com antibióticos

(penicilina e estreptomicina - PS, 0,2 mg/mL) para remoção de bactérias transientes. A suspensão celular derivada desse processo foi colocada em placas de cultura (1 a 2 x 10^6 células/mL). Quando dissociadas e recolocadas em meio contendo cálcio, como a água do mar, as células de esponja se organizam primeiro em agregados (Figura 1.8A) e depois em estruturas tridimensionais denominadas *primorfos* (Custodio et al., 1998), que possuem uma pinacoderme contínua recobrindo a massa celular do seu interior (Figura 1.8B). Com intuito de se obter estas estruturas, o meio das culturas celulares foi trocado diariamente durante a primeira semana para diminuir possíveis contaminações de fungos e protistas transientes, extremamente comuns na cultura de invertebrados marinhos e um dos principais contaminantes (Custodio et al., 1998; Rinkevich, 1999).

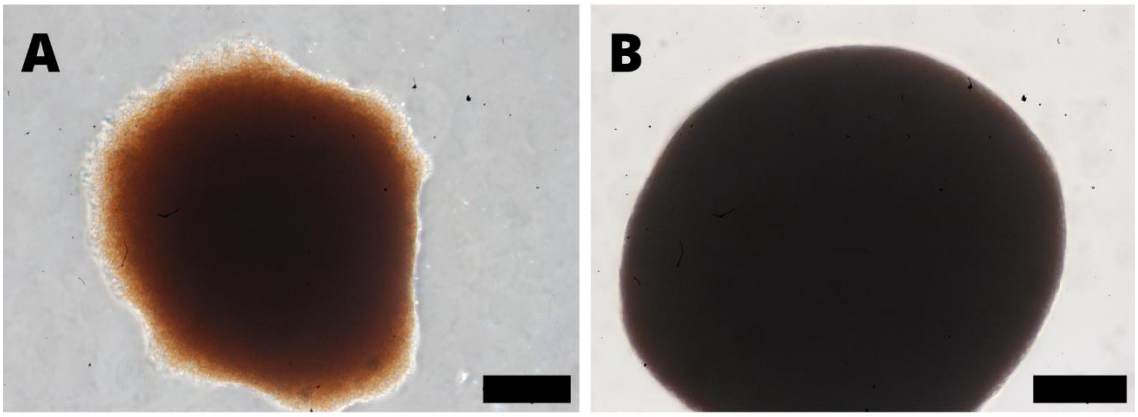

Figura 1.8. Agregados de *Hymeniacidon heliophila* em diferentes estágios de desenvolvimento. (A) Agregado recém-formado, com uma semana de cultura, mostrando as bordas irregulares. (B) Primorfo depois de formado, com alta densidade celular e uma pinacoderme completa, conferindo o aspecto liso à superfície. Barra de escala = 100 µm. Contraste de fase. Nikon TE300.

1.3.3 Isolamento dos Labyrinthulomycetes associados

O isolamento desses organismos foi feito através de inoculação de amostras de cultura de células de esponjas em meios já descritos na literatura para este grupo, além de outros meios que são utilizados para crescimento de outros organismos isolados de esponjas marinhas. Neste trabalho foram usados os meios *Dextrose-Yeast Nitrogen Base* (DYNB - ROZAS et al., 2011), *Brain-Heart Infusion agar* (BHI - ROZAS et al., 2017), YTD-H e YTD (adaptado de Harel et al., 2008) em diferentes condições. O meio DYNB é composto por 1% de dextrose e 1% de Yeast Nitrogen Base (Fluka) em água do mar natural. Após a esterilização por filtração em 0,22 µm, foi adicionada ao meio uma solução de antibióticos (PS) na concentração de 0,2 mg/mL. Já o BHI-ágar, um

meio mais rico em nutrientes do que o DYNB, foi preparado segundo as instruções da fabricante: 26g de BHI-ágar (Acumedia) foram diluídos em 500 mL de água do mar natural e em seguida esterilizado em autoclave por 15 minutos. Após isso foi adicionado PS na mesma concentração acima. O meio YTD-H é composto de 0.1% triptona, 0.1% extrato de leveduras e 0.1% dextrose diluídos em água do mar artificial (Red Salt, pH 8, salinidade 33 ppt), suplementada com PS na concentração de 0,2 mg/mL. Já o meio YTD é composto a partir da diluição do meio YTD-H dez vezes atingindo uma concentração de 0,01%. Nos experimentos de isolamento foram utilizados tanto o meio líquido quanto meio sólido (meio + 1,5% ~ 2% ágar bacteriológico - Merck), com exceção do meio BHI-ágar. Em seguida foram inoculadas amostras de 2 a 5 primorfos de *Hymeniacidon heliophila* ou *Haliclona melana* nos meios de cultura descritos. As placas e garrafas de cultura celular 25 cm² (Kasvi) foram colocadas em uma estufa a 24ºC ± 1ºC por duas semanas e observadas diariamente em microscopia de luz em busca de microrganismos que se encaixassem nas descrições de Labyrinthulomycetes presentes na literatura.

Somado a utilização do meio YTD foi aplicada a técnica de *baiting*, já utilizada na literatura para isolamento de Labyrinthulomycetes (Raghukumar, 2002; Boro et al., 2018). Esta se baseia na capacidade desses microrganismos saprófitos em utilizar fontes recalcitrantes de nutrientes, como celulose, esporopolenina e quitina (Raghukumar, 2002). Os protistas de interesse aderem a esses materiais e então é possível seu isolamento e replicação até a obtenção de culturas axênicas. Para isso, usamos cistos do crustáceo *Artemia* sp. que foram autoclavados e distribuídos em placas de 12 poços (50 mg de cistos por poço) contendo água do mar natural estéril suplementada com PS na concentração usual. Também usamos outro material recalcitrante como isca, consistindo de fragmentos do esqueleto de espongina oriundos da esponja *Aplysina fulva*. Em seguida, amostras com dois a cinco primorfos oriundos de novas culturas de *Hymeniacidon heliophila* ou *Haliclona melana* foram adicionadas em cada poço. As placas foram deixadas em uma câmara escura, à temperatura ambiente (24ºC ± 2ºC) e observadas diariamente. Após a colonização, os materiais foram transferidos para placas de cultura com YTD-sólido (ágar 1,5%) para o desenvolvimento e em seguida foi feita a replicação desses isolados em YTD-líquido. Um esquema simplificado do processo de isolamento é visto na Figura 1.9. Todas as manipulações foram realizadas em fluxo laminar com materiais estéreis de fábrica ou autoclavados por 15 minutos à 121ºC.

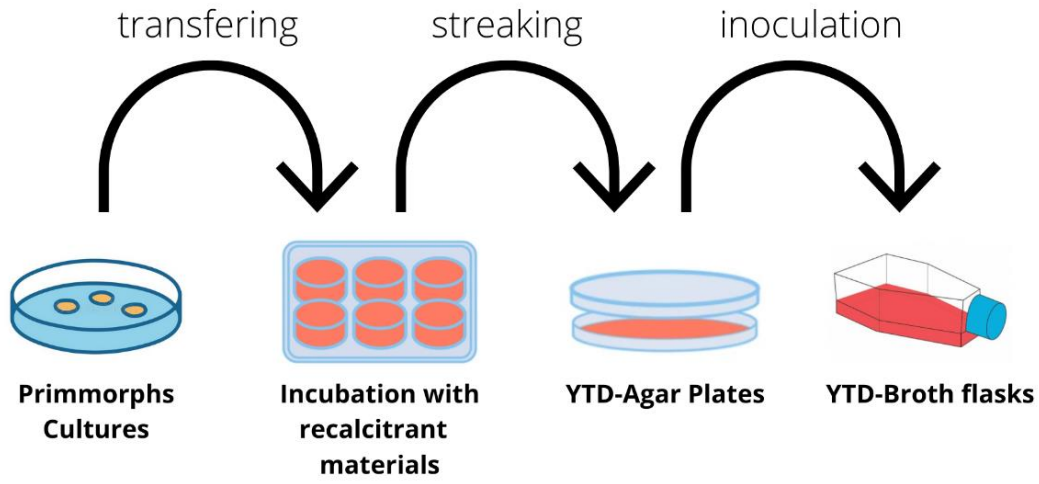

Figura 1.9. Etapas envolvidas no isolamento de Labyrinthulomycetes associados a partir de culturas de células de esponjas marinhas. Os primorfos são incubados com materiais recalcitrantes como cistos de *Artemia* sp. ou fibras de espongina. Após a colonização destes os cistos são espalhados em placas de YTD-ágar onde se proliferam. Após isso são feitas subculturas em garrafas de cultura celular que permitem melhor manutenção dos isolados.

1.3.4 Testes de frascos de cultura para cultivo dos protistas isolados

Buscando aprimorar o cultivo dos Labyrinthulomycetes testamos qualitativamente diferentes frascos de cultura. Usamos frascos de cultivo feitos de plástico, como garrafas de cultura celular 25 cm² (Kasvi), tubos Falcon® (50 mL) e tubos Nalgene® (40 mL). Também foram testados frascos de vidro, como tubos simples (10 mL), Erlermeyers (125 mL) e garrafas graduadas (500mL - Schott). Os isolados de interesse foram cultivados nesses diferentes frascos sob agitação horizontal (60-100 RPM) e temperatura média de 25ºC ± 1ºC.

1.3.5 Extração do DNA genômico dos protistas isolados

A morfologia dos eucariontes unicelulares por muitas vezes não é suficiente para distinguir com clareza os táxons a que pertencem os microrganismos, sendo necessária uma abordagem molecular. Para isso extraímos o DNA genômico dos protistas de interesse para eventual sequenciamento do gene 18S rRNA. Utilizamos nessa extração de gDNA o PureLink DNA Extraction Minikit (Invitrogen). Na ausência do protocolo adequado para essa extração, foi necessário adaptar um novo, partindo das premissas

explicitadas no manual desse kit. As culturas cresceram por sete dias e em seguida foram centrifugadas em tubos de 50 mL por 10 minutos a 750 x g para obtenção do pellet de células. Estes foram transferidos para tubos Eppendorf (2 mL) estéreis. Os pellets de cada cultura (com volumes variando entre 100-150 µL) foram ressuspendidos em 180 µL de *Purelink Genomic Digestion Buffer*, e em seguida adicionamos 20 µL de proteinase K e os tubos foram deixados em um banho-maria a 55 ºC por 18 horas. Para eliminar qualquer vestígio de RNA adicionamos também 20 µL de RNAse A em cada amostra e incubamos por 2 minutos em temperatura ambiente. Os lisados resultantes foram centrifugados a 12.000 x g (Eppendorf MiniSpin) por 5 minutos em temperatura ambiente para remover qualquer particulado. Transferimos os sobrenadantes de cada amostra para novos tubos Eppendorf estéreis. Adicionamos em cada tubo 200 µL do PureLink Binding Buffer. Em seguida colocamos em cada amostra 200 µL de EtOH 100% e agitamos brevemente até a formação de uma solução homogênea.

1.3.6 Processamento do DNA genômico obtido

Para a purificação foram utilizadas colunas de centrifugação do *PureLink DNA Extraction Minikit* (ThermoFisher), de acordo com as instruções do fabricante. Para isso, colunas contendo os lisados obtidos na etapa anterior foram centrifugadas a 10.000 x g (Eppendorf MiniSpin) por um minuto em temperatura ambiente. Os tubos de coleta contendo impurezas foram descartados e novos tubos foram colocados em cada coluna. A cada amostra foram adicionados 500 µL de *Wash Buffer 1* e as colunas centrifugadas a 10.000 x g por um minuto em temperatura ambiente. Os tubos de coleta foram descartados e novos foram postos como substitutos. O mesmo procedimento foi repetido com 500 µL de *Wash Buffer 2*, porém as colunas foram centrifugadas a 12.000 x g por três minutos em temperatura ambiente. Os tubos coletores foram descartados.

O próximo passo consistiu em colocar as colunas em tubos Eppendorf 2 mL estéreis. Em seguida foi adicionado a cada uma 25 µL de *PureLink Genomic Elution Buffer* e as reações foram deixadas incubando por 1-2 minutos. Ao fim desse tempo as colunas foram centrifugadas a 12.000 x g por um minuto em temperatura ambiente. O resultado desse procedimento foi uma solução no tubo Eppendorf coletor contendo o DNA genômico extraído e purificado de cada uma das culturas dos protistas de interesse. Para recuperar mais DNA o mesmo procedimento foi repetido com um novo

tubo Eppendorf coletor, adicionando-se mais 25 µL de *Genomic Elution Buffer* nas mesmas colunas e centrifugando novamente. Obteve-se no total dois tubos Eppendorf contendo 25 µL de DNA genômico para cada uma das culturas de protistas de interesse. Após a devida rotulagem, as amostras de DNA foram armazenadas em um freezer -20ºC até o sequenciamento.

1.3.7 *Escolha de Primers*

Para o sequenciamento do gene 18S rRNA escolhemos os pares de primers NS1/NS4 e NS5/NS8 já utilizados na identificação de Labyrinthulomycetes (Hassett e Gradinger, 2018). As sequências no sentido 5'-3' são: NS1 – GTAGTCATATGCTTGTCTC; NS4 – CTTCCGTCAATTCCTTTAAG; NS5 – AACTTAAAGGAATTGACGGAAG; NS8 – TCCGCAGGTTCACCTACGGA.

1.3.8 Hibridização in situ dos Labyrinthulomycetes nos tecidos de Porifera

Com a intenção de localizar as células dos protistas de interesse nos tecidos de esponjas fixamos em metanol os tecidos e primorfos segundo o protocolo de Fierro-Constaín et al. (2021). Nós adquirimos uma sonda de DNA (Synbio) marcada com digoxina na porção terminal de 5'. Essa sonda é específica para o táxon Labyrinthulomycetes, já tendo sido utilizada na investigação do parasita QPX em bivalves (LABY1336 - Stokes et al., 2002), sendo essa a primeira aplicação dela em organismos do filo Porifera.

1.3.9 Extração dos polissacarídeos extracelulares

Visando investigar o potencial dos polissacarídeos extracelulares (PEs) produzidos pelos Labyrinthulomycetes foram feitos extratos brutos desses compostos a partir de adaptações feitas de Bajpai et al. (2016). As culturas utilizadas cresceram por 25 dias em garrafas de cultura com 20 mL de meio YTD. De cada cultura foi transferido 15 mL para tubos de 50 mL e adicionou-se 2,1 mL de uma solução 100% de ácido tricloroacético (TCA) com o objetivo de se obter uma concentração final de TCA de 14%. Os tubos foram deixados sob agitação de 60 RPM em temperatura ambiente

(24ºC) por uma hora. Essa solução foi centrifugada a 8.000 x g por 20 minutos para formar pellets de células e proteínas. Os sobrenadantes de cada cultura foram colocados em tubos de 50 mL e adicionou-se duas vezes o volume (2 x 15 mL) de etanol absoluto gelado, totalizando uma solução de 45 mL em cada tubo. A reação foi incubada por 48 horas a 4ºC para a precipitação do PEs. Passado esse período observou-se uma massa esbranquiçada no fundo de cada tubo. Estes foram centrifugados novamente para concentrar o precipitado e o sobrenadante foi descartado. Após a total evaporação do etanol, os tubos contendo os extratos brutos de polissacarídeos extracelulares derivados de culturas dos protistas de interesse, foram rotulados e guardados à 4º C para análises posteriores.

REFERÊNCIAS

Aasen, I.M., Ertesvåg, H., Heggeset, T.M.B., Liu, B., Brautaset, T., Vadstein, O., Ellingsen, T.E., 2016. Thraustochytrids as production organisms for docosahexaenoic acid (DHA), squalene, and carotenoids. Appl. Microbiol. Biotechnol. 100, 4309–4321. https://doi.org/10.1007/s00253-016-7498-4

Adl, S.M., Bass, D., Lane, C.E., Lukeš, J., Schoch, C.L., Smirnov, A., Agatha, S., Berney, C., Brown, M.W., Burki, F., Cárdenas, P., Čepička, I., Chistyakova, L., Campo, J., Dunthorn, M., Edvardsen, B., Eglit, Y., Guillou, L., Hampl, V., Heiss, A.A., Hoppenrath, M., James, T.Y., Karnkowska, A., Karpov, S., Kim, E., Kolisko, M., Kudryavtsev, A., Lahr, D.J.G., Lara, E., Le Gall, L., Lynn, D.H., Mann, D.G., Massana, R., Mitchell, E.A.D., Morrow, C., Park, J.S., Pawlowski, J.W., Powell, M.J., Richter, D.J., Rueckert, S., Shadwick, L., Shimano, S., Spiegel, F.W., Torruella, G., Youssef, N., Zlatogursky, V., Zhang, Q., 2019. Revisions to the Classification, Nomenclature, and Diversity of Eukaryotes. J. Eukaryot. Microbiol. 66, 4–119. https://doi.org/10.1111/jeu.12691

Adler, J., 2020. Meet The Underwater Sea Sponge Farmers of Zanzibar [WWW Document]. HuffPost. URL https://www.huffpost.com/entry/sea-sponge-farmers-zanzibar_n_5f2c2112c5b6e96a22af4194?guccounter=1 (accessed 2.20.22).

Aresta, A., Marzano, C.N., Lopane, C., Corriero, G., Longo, C., Zambonin, C., Stabili, L., 2015. Analytical investigations on the lindane bioremediation capability of the demosponge *Hymeniacidon perlevis*. Mar. Pollut. Bull. 90, 143–149. https://doi.org/10.1016/j.marpolbul.2014.11.003

Bell, J.J., 2008. The functional roles of marine sponges. Estuar. Coast. Shelf Sci. 79, 341–353. https://doi.org/10.1016/j.ecss.2008.05.002

Bennett, R.M., Honda, D., Beakes, G.W., Thines, M., 2017. Labyrinthulomycota, in: Handbook of the Protists. Springer International Publishing, Cham, pp. 507–542. https://doi.org/10.1007/978-3-319-28149-0_25

Blunt, J.W., Copp, B.R., Keyzers, R.A., Munro, M.H.G., Prinsep, M.R., 2015. Marine natural products. Nat. Prod. Rep. 32, 116–211. https://doi.org/10.1039/c4np00144c

Boro, M.C., Harakava, R., Pires-Zottarelli, C.L.A., 2018. Labyrinthulomycota from Brazilian mangrove swamps and coastal waters. Bot. Mar. 61, 65–74. https://doi.org/10.1515/bot-2017-0052

Brinkmann, C.M., Marker, A., Kurtböke, D.I., 2017. An overview on marine sponge-symbiotic bacteria as unexhausted sources for natural product discovery. Diversity 9. https://doi.org/10.3390/d9040040

Brümmer, F., Pfannkuchen, M., Baltz, A., Hauser, T., Thiel, V., 2008. Light inside sponges. J. Exp. Mar. Bio. Ecol. 367, 61–64. https://doi.org/10.1016/j.jembe.2008.06.036

Chang, S., Zhang, L., Clausen, S., Bottjer, D.J., Feng, Q., 2019. The Ediacaran-Cambrian rise of siliceous sponges and development of modern oceanic ecosystems. Precambrian Res. 333, 105438. https://doi.org/10.1016/j.precamres.2019.105438

Chernogor, L.I., Denikina, N.N., Belikov, S.I., Ereskovsky, A. V., 2011. Long-Term Cultivation of Primmorphs from Freshwater Baikal Sponges *Lubomirskia baikalensis*. Mar. Biotechnol. 13, 782–792. https://doi.org/10.1007/s10126-010-9340-9

Chernogor, L., Denikina, N., Kondratov, I., Solovarov, I., Khanaev, I., Belikov, S., Ehrlich, H., 2013. Isolation and identification of the microalgal symbiont from primmorphs of the endemic freshwater sponge *Lubomirskia baicalensis* (Lubomirskiidae, Porifera). Eur. J. Phycol. 48, 497–508. https://doi.org/10.1080/09670262.2013.862306

Collier, J.L., Geraci-Yee, S., Lilje, O., Gleason, F.H., 2017. Possible impacts of zoosporic parasites in diseases of commercially important marine mollusc species: Part II. Labyrinthulomycota. Bot. Mar. 60, 409–417. https://doi.org/10.1515/bot-2016-0133

Conkling, M., Hesp, K., Munroe, S., Sandoval, K., Martens, D.E., Sipkema, D., Wijffels, R.H., Pomponi, S.A., 2019. Breakthrough in Marine Invertebrate Cell Culture: Sponge Cells Divide Rapidly in Improved Nutrient Medium. Sci. Rep. 9, 1–10. https://doi.org/10.1038/s41598-019-53643-y

Custodio, M.R., Prokic, I., Steffen, R., Koziol, C., Borojevic, R., Brümmer, F., Nickel, M., Müller, W.E.G., 1998. Primmorphs generated from dissociated cells of the sponge *Suberites domuncula*: a model system for studies of cell proliferation and cell death. Mech. Ageing Dev. 105, 45–59. https://doi.org/https://doi.org/10.1016/S0047-6374(98)00078-5

de Caralt, S., Uriz, M.J., Wijffels, R.H., 2007. Cell culture from sponges: pluripotency and immortality. Trends Biotechnol. 25, 467–471. https://doi.org/10.1016/j.tibtech.2007.08.006

del Campo, J., Bass, D., Keeling, P.J., 2020. The eukaryome: Diversity and role of microeukaryotic organisms associated with animal hosts. Funct. Ecol. 34, 2045–2054. https://doi.org/10.1111/1365-2435.13490

de Voogd, N.J., Alvarez, B., Boury-Esnault, N., Carballo, J.L., Cárdenas, P., Díaz, M.-C., Dohrmann, M., Downey, R., Hajdu, E., Hooper, J.N.A., Kelly, M., Klautau, M., Manconi, R., Morrow, C.C. Pisera, A.B., Ríos, P., Rützler, K., Schönberg, C., Vacelet, J., van Soest, R.W.M., 2022. World Porifera Database [WWW Document]. https://doi.org/https://doi.org/10.14284/359

Domart-Coulon, I., Blanchoud, S., 2022. From Primary Cell and Tissue Cultures to Aquatic Invertebrate Cell Lines: An Updated Overview, in: Ballarin, L., Rinkevich, B., Hobmayer, B. (Eds.), Advances in Aquatic Invertebrate Stem Cell Research. MDPI, Basel, Switzerland, pp. 1–64. https://doi.org/10.3390/books978-3-0365-1635-6-1

Dick, M.W., 2001. Straminipilous Fungi, Kluwer Academic Publishers. Springer Netherlands, Dordrecht. https://doi.org/10.1007/978-94-015-9733-3

Ereskovsky, A. V., Boury-Esnault, N., 2002. Cleavage pattern in *Oscarella* species (Porifera, Demospongiae, Homoscleromorpha): Transmission of maternal cells and symbiotic bacteria. J. Nat. Hist. 36, 1761–1775. https://doi.org/10.1080/00222930110069050

Fierro-Constaín, L., Rocher, C., Marschal, F., Schenkelaars, Q., Séjourné, N., Borchiellini, C., Renard, E., 2021. In Situ Hybridization Techniques in the Homoscleromorph Sponge *Oscarella lobularis*, in: Methods in Molecular Biology. United States, pp. 181–194. https://doi.org/10.1007/978-1-0716-0974-3_11

Fieth, R.A., Gauthier, M.E.A., Bayes, J., Green, K.M., Degnan, S.M., 2016. Ontogenetic changes in the bacterial symbiont community of the tropical demosponge *Amphimedon queenslandica*: Metamorphosis is a new beginning. Front. Mar. Sci. 3, 1–20. https://doi.org/10.3389/fmars.2016.00228

Fiore, C.L., Labrie, M., Jarett, J.K., Lesser, M.P., 2015. Transcriptional activity of the giant barrel sponge, *Xestospongia muta* Holobiont: molecular evidence for metabolic interchange. Front. Microbiol. 6. https://doi.org/10.3389/fmicb.2015.00364

Gaertner, H., Hamad, A.A., Walz, J.R., 2016. The Community Influence of Sponge and Coral Aquaculture in Zanzibar. DU Undergrad. Res. J. Arch. 1, 1–19.

Gazave, E., Lapébie, P., Ereskovsky, A. V., Vacelet, J., Renard, E., Cárdenas, P., Borchiellini, C., 2012. No longer Demospongiae: Homoscleromorpha formal nomination as a fourth class of Porifera. Hydrobiologia 687, 3–10. https://doi.org/10.1007/s10750-011-0842-x

Geahchan, S., Ehrlich, H., Rahman, M.A., 2021. The anti-viral applications of marine resources for COVID-19 treatment: An overview. Mar. Drugs 19. https://doi.org/10.3390/md19080409

Gloeckner, V., Wehrl, M., Moitinho-Silva, L., Gernert, C., Schupp, P., Pawlik, J.R., Lindquist, N.L., Erpenbeck, D., Wörheide, G., Hentschel, U., 2014. The HMA-LMA Dichotomy Revisited: an Electron Microscopical Survey of 56 Sponge Species. Biol. Bull. 227, 78–88. https://doi.org/10.1086/BBLv227n1p78

Grasela, J.J., Pomponi, S.A., Rinkevich, B., Grima, J., 2012. Efforts to develop a cultured sponge cell line: Revisiting an intractable problem. Vitr. Cell. Dev. Biol. - Anim. 48, 12–20. https://doi.org/10.1007/s11626-011-9469-5

Han, Q., Li, P., Lu, X., Guo, Z., Guo, H., 2013. Improved primary cell culture and subculture of lymphoid organs of the greasyback shrimp *Metapenaeus ensis*. Aquaculture 410–411, 101–113. https://doi.org/10.1016/j.aquaculture.2013.06.024

Harel, M., Ben-Dov, E., Rasoulouniriana, D., Siboni, N., Kramarsky-Winter, E., Loya, Y., Barak, Z., Wiesman, Z., Kushmaro, A., 2008. A new Thraustochytrid, strain Fng1, isolated from the surface mucus of the hermatypic coral *Fungia granulosa*. FEMS Microbiol. Ecol. 64, 378–387. https://doi.org/10.1111/j.1574-6941.2008.00464.x

Hassett, B.T., Gradinger, R., 2018. New Species of Saprobic Labyrinthulea (=Labyrinthulomycota) and the Erection of a gen. nov. to Resolve Molecular Polyphyly within the Aplanochytrids. J. Eukaryot. Microbiol. 65, 475–483. https://doi.org/10.1111/jeu.12494

Iwata, I., Honda, D., 2018. Nutritional Intake by Ectoplasmic Nets of *Schizochytrium aggregatum* (Labyrinthulomycetes, Stramenopiles). Protist 169, 727–743. https://doi.org/10.1016/j.protis.2018.06.002

Leray, M., Knowlton, N., 2016. Censusing marine eukaryotic diversity in the twenty-first century. Philos. Trans. R. Soc. B Biol. Sci. 371, 20150331. https://doi.org/10.1098/rstb.2015.0331

Leys, S.P., Mackie, G.O., Reiswig, H.M., 2007. The Biology of Glass Sponges. Adv. Mar. Biol. 52, 1–145. https://doi.org/10.1016/S0065-2881(06)52001-2

Li, X., Li, M., Pu, Y., Ragauskas, A.J., Tharayil, N., Huang, J., Zheng, Y., 2021. Degradation of aromatic compounds and lignin by marine protist *Thraustochytrium striatum*. Process Biochem. 107, 13–17. https://doi.org/10.1016/j.procbio.2021.05.006

Liu, Y., Singh, P., Sun, Y., Luan, S., Wang, G., 2014. Culturable diversity and biochemical features of thraustochytrids from coastal waters of Southern China. Appl. Microbiol. Biotechnol. 98, 3241–3255. https://doi.org/10.1007/s00253-013-5391-y

Maldonado, M., Cortadellas, N., Trillas, M.I., Rützler, K., 2005. Endosymbiotic yeast maternally transmitted in a marine sponge. Biol. Bull. 209, 94–106. https://doi.org/10.2307/3593127

Marchan, L.F., Lee Chang, K.J., Nichols, P.D., Mitchell, W.J., Polglase, J.L., Gutierrez, T., 2018. Taxonomy, ecology and biotechnological applications of thraustochytrids: A review. Biotechnol. Adv. 36, 26–46. https://doi.org/10.1016/j.biotechadv.2017.09.003

Mendes, A., Azevedo-Silva, J., Fernandes, J.C., 2022. From Sharks to Yeasts: Squalene in the Development Vaccine Adjuvants. Pharmaceuticals 15, 265. https://doi.org/10.3390/ph15030265

Moitinho-Silva, L., Steinert, G., Nielsen, S., Hardoim, C.C.P., Wu, Y.C., McCormack, G.P., López-Legentil, S., Marchant, R., Webster, N., Thomas, T., Hentschel, U., 2017. Predicting the HMA-LMA status in marine sponges by machine learning. Front. Microbiol. 8, 1–14. https://doi.org/10.3389/fmicb.2017.00752

Moss, S.T., 1985. An ultrastructural study of taxonomically significant characters of the Thraustochytriales and the Labyrinthulales. Bot. J. Linn. Soc. 91, 329–357. https://doi.org/10.1111/j.1095-8339.1985.tb01154.x

Müller, W.E.G., Wiens, M., Batel, R., Steffen, R., Schröder, H.C., Borojevic, R., Custodio, M.R., 1999. Establishment of a primary cell culture from a sponge: Primmorphs from Suberites domuncula. Mar. Ecol. Prog. Ser. 178, 205–219. https://doi.org/10.3354/meps178205

Müller, W.E.G., Grebenjuk, V.A., Le Pennec, G., Schröder, H.-C., Brümmer, F., Hentschel, U., Müller, I.M., Breter, H.-J., 2004. Sustainable Production of bioactive compounds by sponges-cell culture and gene cluster approach: A review. Mar. Biotechnol. 6, 105–117.

Müller, W.E.G., 2003. The origin of metazoan complexity: Porifera as integrated animals. Integr. Comp. Biol. 43, 3–10. https://doi.org/10.1093/icb/43.1.3

Nham Tran, T.L., Miranda, A.F., Gupta, A., Puri, M., Ball, A.S., Adhikari, B., Mouradov, A., 2020. The Nutritional and Pharmacological Potential of New Australian Thraustochytrids Isolated from Mangrove Sediments. Mar. Drugs 18, 151. https://doi.org/10.3390/md18030151

Nowotny, J.D., Connelly, M.T., Traylor-Knowles, N., 2021. Novel methods to establish whole-body primary cell cultures for the cnidarians *Nematostella vectensis* and *Pocillopora damicornis*. Sci. Rep. 11, 1–9. https://doi.org/10.1038/s41598-021-83549-7

Park, H., Kwak, M., Seo, J.W., Ju, J.H., Heo, S.Y., Park, S.M., Hong, W.K., 2018. Enhanced production of carotenoids using a Thraustochytrid microalgal strain containing high levels of docosahexaenoic acid-rich oil. Bioprocess Biosyst. Eng. 41, 1355–1370. https://doi.org/10.1007/s00449-018-1963-7

Patel, A., Liefeldt, S., Rova, U., Christakopoulos, P., Matsakas, L., 2020. Co-production of DHA and squalene by thraustochytrid from forest biomass. Sci. Rep. 10, 1–12. https://doi.org/10.1038/s41598-020-58728-7

Pawlowski, J., 2014. Protist Evolution and Phylogeny, in:eLS. Wiley, Chichester, UK. https://doi.org/10.1002/9780470015902.a0001935.pub2

Pinheiro UDS, Hajdu E, Custódio MR (2007) *Aplysina* Nardo (Porifera, Verongida, Aplysinidae) from the Brazilian coast with description of eight new species. Zootaxa 1609:1–51. https://doi.org/10.11646/zootaxa.1610.1.zootaxa.1609.1.1

Pita, L., Hoeppner, M.P., Ribes, M., Hentschel, U., 2018. Differential expression of immune receptors in two marine sponges upon exposure to microbial-associated molecular patterns. Sci. Rep. 8, 16081. https://doi.org/10.1038/s41598-018-34330-w

Posadas, N., Baquiran, J.I.P., Nada, M.A.L., Kelly, M., Conaco, C., 2021. Microbiome diversity and host immune functions influence survivorship of sponge holobionts under future ocean conditions. ISME J. 1–10. https://doi.org/10.1038/s41396-021-01050-5

Rabinowitz, C., Douek, J., Weisz, R., Shabtay, A., Rinkevich, B., 2006. Isolation and characterization of four novel thraustochytrid strains from a colonial tunicate. Indian J. Mar. Sci. 35, 341–350.

Radwan, M., Hanora, A., Khalifa, S., Abou-El-Ela, S.H., 2012. Manzamines: A potential for novel cures. Cell Cycle 11, 1765–1772. https://doi.org/10.4161/cc.20135

Raghukumar, S., 2002. Ecology of the marine protists, the labyrinthulomycetes (thraustochytrids and labyrinthulids). Eur. J. Protistol. 38, 127–145. https://doi.org/10.1078/0932-4739-00832

Raghukumar, S., 2008. Thraustochytrid marine protists: Production of PUFAs and other emerging technologies. Mar. Biotechnol. 10, 631–640. https://doi.org/10.1007/s10126-008-9135-4

Redmond, A.K., McLysaght, A., 2021. Evidence for sponges as sister to all other animals from partitioned phylogenomics with mixture models and recoding. Nat. Commun. 12. https://doi.org/10.1038/s41467-021-22074-7

Ribeiro, B., Padua, A., Barno, A., Villela, H., Duarte, G., Rossi, A., Da Costa Fernandes, F., Peixoto, R., Klautau, M., 2021. Assessing skeleton and microbiome responses of a calcareous sponge under thermal and pH stresses. ICES J. Mar. Sci. 78, 855–866. https://doi.org/10.1093/icesjms/fsaa231
Richelle-Maurer, E., Gomez, R., Braekman, J.C., Van De Vyver, G., Van Soest, R.W.M., Devijver, C., 2003. Primary cultures from the marine sponge *Xestospongia muta* (Petrosiidae, Haplosclerida). J. Biotechnol. 100, 169–176. https://doi.org/10.1016/S0168-1656(02)00251-1
Rinkevich, B., 1999. Cell cultures from marine invertebrates: obstacles, new approaches and recent improvements. Prog. Ind. Microbiol. 70, 133–153. https://doi.org/10.1016/S0079-6352(99)80107-6
Rinkevich, B., 2005. Marine Invertebrate Cell Cultures: New Millennium Trends. Mar. Biotechnol. 7, 429–439. https://doi.org/10.1007/s10126-004-0108-y
Rinkevich, B., Blisko, R., Ilan, M., 1998. Further steps in the initiation of cell cultures from embryos and adult sponge colonies. Vitr. Cell. Dev. Biol. - Anim. 34, 753–756. https://doi.org/10.1007/s11626-998-0028-7
Rinkevich, B., Rabinowitz, C., 1993. In vitro culture of blood cells from the colonial protochordate *Botryllus schlosseri*. Vitr. Cell. Dev. Biol. - Anim. 29, 79–85. https://doi.org/10.1007/BF02634375
Rosado-Rodríguez, G., E, S.-M., SL, M.-R., E, O., 2019. Cultivable yeasts associated with demosponges from Puerto Rico. Stud. Fungi 4, 153–161. https://doi.org/10.5943/sif/4/1/18
Rozas, E.E., Albano, R.M., Lôbo-Hajdu, G., Müller, W.E.G., Schröder, H.C., Custódio, M.R., 2011. Isolation and cultivation of fungal strains from in vitro cell cultures of two marine sponges (Porifera: Halichondrida and Haplosclerida). Brazilian J. Microbiol. 42, 1560–1568. https://doi.org/10.1590/S1517-83822011000400043
Rozas, E.E., Mendes, M.A., Nascimento, C.A.O., Espinosa, D.C.R., Oliveira, R., Oliveira, G., Custodio, M.R., 2017. Bioleaching of electronic waste using bacteria isolated from the marine sponge *Hymeniacidon heliophila* (Porifera). J. Hazard. Mater. 329, 120–130. https://doi.org/10.1016/j.jhazmat.2017.01.037
Schippers, K.J., Sipkema, D., Osinga, R., Smidt, H., Pomponi, S.A., Martens, D.E., Wijffels, R.H., 2012. Cultivation of Sponges, Sponge Cells and Symbionts. Achievements and Future Prospects, in: Becerro, M.A., Uriz, M.J., Maldonado, M., Turon, X. (Eds.), Advances in Sponge Science: Physiology, Chemical and Microbial Diversity, Biotechnology. pp. 273–337. https://doi.org/10.1016/B978-0-12-394283-8.00006-0
Schmittmann, L., Franzenburg, S., Pita, L., 2021. Individuality in the Immune Repertoire and Induced Response of the Sponge *Halichondria panicea*. Front. Immunol. 12, 1–13. https://doi.org/10.3389/fimmu.2021.689051
Shinn, A.P., Pratoomyot, J., Bron, J.E., Paladini, G., Brooker, E.E., Brooker, A.J., 2015. Economic costs of protistan and metazoan parasites to global mariculture. Parasitology 142, 196–270. https://doi.org/10.1017/S0031182014001437
Simpson, T.L., 1984. The Cell Biology of Sponges. Springer New York, New York, NY. https://doi.org/10.1007/978-1-4612-5214-6
Spanova, M., Daum, G., 2011. Squalene - biochemistry, molecular biology, process biotechnology, and applications. Eur. J. Lipid Sci. Technol. 113, 1299–1320. https://doi.org/10.1002/ejlt.201100203
Sperling, E.A., Robinson, J.M., Pisani, D., Peterson, K.J., 2010. Where's the glass? Biomarkers, molecular clocks, and microRNAs suggest a 200-Myr missing Precambrian fossil record of siliceous sponge spicules. Geobiology 8, 24–36. https://doi.org/10.1111/j.1472-4669.2009.00225.x
Steinert, G., Stauffer, C.H., Aas-Valleriani, N., Borchert, E., Bhushan, A., Campbell, A., De Mares, M.C., Costa, M., Gutleben, J., Knobloch, S., Lee, R.G., Munroe, S., Naik, D., Peters, E.E., Stokes, E., Wang, W., Einarsdóttir, E., Sipkema, D., 2018. BluePharmTrain: Biology and Biotechnology of Marine Sponges, in: Grand Challenges in Biology and Biotechnology. pp. 505–553. https://doi.org/10.1007/978-3-319-69075-9_13
Stokes, N.A., Ragone Calvo, L.M., Reece, K.S., Burreson, E.M., 2002. Molecular diagnostics, field validation, and phylogenetic analysis of Quahog Parasite Unknown (QPX), a pathogen of the hard clam *Mercenaria mercenaria*. Dis. Aquat. Organ. 52, 233–247. https://doi.org/10.3354/dao052233
Suryanarayanan, T.S., 2012. The diversity and importance of fungi associated with marine sponges. Bot. Mar. 55, 553–564. https://doi.org/10.1515/bot-2011-0086
Taylor, M.W., Radax, R., Steger, D., Wagner, M., 2007. Sponge-Associated Microorganisms: Evolution, Ecology, and Biotechnological Potential. Microbiol. Mol. Biol. Rev. 71, 295–347. https://doi.org/10.1128/MMBR.00040-06
Thakur, N.L., Müller, W.E.G., 2004. Biotechnological potential of marine sponges. Curr. Sci. 86, 1506–1512.
Thomas, T., Moitinho-Silva, L., Lurgi, M., Björk, J.R., Easson, C., Astudillo-García, C., Olson, J.B., Erwin, P.M., López-Legentil, S., Luter, H., Chaves-Fonnegra, A., Costa, R., Schupp, P.J., Steindler,

L., Erpenbeck, D., Gilbert, J., Knight, R., Ackermann, G., Victor Lopez, J., Taylor, M.W., Thacker, R.W., Montoya, J.M., Hentschel, U., Webster, N.S., 2016. Diversity, structure and convergent evolution of the global sponge microbiome. Nat. Commun. 7, 11870. https://doi.org/10.1038/ncomms11870

Turner, E.C., 2021. Possible poriferan body fossils in early Neoproterozoic microbial reefs. Nature 596, 87–91. https://doi.org/10.1038/s41586-021-03773-z

Usher, K.M., 2008. The ecology and phylogeny of cyanobacterial symbionts in sponges. Mar. Ecol. 29, 178–192. https://doi.org/10.1111/j.1439-0485.2008.00245.x

Vaca, I., Faúndez, C., Maza, F., Paillavil, B., Hernández, V., Acosta, F., Levicán, G., Martínez, C., Chávez, R., 2013. Cultivable psychrotolerant yeasts associated with Antarctic marine sponges. World J. Microbiol. Biotechnol. 29, 183–189. https://doi.org/10.1007/s11274-012-1159-2

van Soest, R.W.M., Boury-Esnault, N., Vacelet, J., Dohrmann, M., Erpenbeck, D., de Voogd, N.J., Santodomingo, N., Vanhoorne, B., Kelly, M., Hooper, J.N.A., 2012. Global diversity of sponges (Porifera). PLoS One 7. https://doi.org/10.1371/journal.pone.0035105

Webster, N.S., Taylor, M.W., 2012. Marine sponges and their microbial symbionts: Love and other relationships. Environ. Microbiol. 14, 335–346. https://doi.org/10.1111/j.1462-2920.2011.02460.x

Webster, N.S., Thomas, T., 2016. Defining the Sponge Hologenome. MBio 7, 1–14. https://doi.org/10.1128/mBio.00135-16.Invited

Worden, A.Z., Follows, M.J., Giovannoni, S.J., Wilken, S., Zimmerman, A.E., Keeling, P.J., 2015. Rethinking the marine carbon cycle: Factoring in the multifarious lifestyles of microbes. Science. 347, 12575941–125759410. https://doi.org/10.1126/science.1257594

Wörheide, G., Solé-Cava, A.M., Hooper, J.N.A., 2005. Biodiversity, molecular ecology and phylogeography of marine sponges: Patterns, implications and outlooks. Integr. Comp. Biol. 45, 377–385. https://doi.org/10.1093/icb/45.2.377

Zhang, F., Blasiak, L.C., Karolin, J.O., Powell, R.J., Geddes, C.D., Hill, R.T., 2015. Phosphorus sequestration in the form of polyphosphate by microbial symbionts in marine sponges. Proc. Natl. Acad. Sci. 112, 4381 LP – 4386. https://doi.org/10.1073/pnas.1423768112

Zhang, X., Cao, X., Zhang, W., Yu, X., Jin, M., 2003. Primmorphs From Archaeocytes-Dominant Cell Population of the Sponge *Hymeniacidon perleve*: Improved Cell Proliferation and Spiculogenesis. Biotechnol. Bioeng. 84, 583–590. https://doi.org/10.1002/bit.10811

CAPÍTULO 2

Addressing the neglected associations between sponges and protists: The Porifera microeukaryome

Gabriel Nascimento-Silva[1*]; Cristiane Cassiolato Pires Hardoim[2]; Márcio Reis Custódio[1]

[1] Department of Physiology, Institute of Biosciences, University of São Paulo (USP), Brazil

[2] Institute of Biosciences, São Paulo State University (UNESP), Brazil

***Corresponding author:** Gabriel Nascimento-Silva (gabriel5.silva@usp.br)

ABSTRACT

Sponges are basal metazoans known for their broad capacity to associate with microorganisms. Historically, the bacterial and archaeal communities of these animals are the best known, followed by the fungi. While these three fractions are intensively studied given their importance in the physiology of the animal as well as their biotechnological potential, the Porifera microeukaryome remains poorly know. For many years the culture-dependent approach dominated the investigations of sponge-protist interactions. With the maturation of the omics techniques, these associations could be visualized at other equally important scales. Of the few existing studies, there is a strong tendency in the literature to focus on interactions between sponges and photosynthesizing taxa such as dinoflagellates and diatoms. However, there are studies that dissect on interactions with other less common groups. In addition, there are bottlenecks and inherent biases of using primer pairs and pipelines in the most commonly used metabarcoding studies. In this review, we propose the term microeukaryome to refer exclusively to protists associated with an animal host. It is also observed that in the context of microeukaryome the biotechnological potentials are still largely unexplored. Thus, this review addresses the issues underlying the associations between Porifera and protists.

Keywords: symbiosis; biotechnological potential; holobiome; culture-dependent; culture-independent

INTRODUCTION

The association between organisms is an ancestral and vital feature of the eukaryotic condition (Douglas, 2014). This intimate dependence is seen in the theories of origin of mitochondria, plastids and other organelles (López-Garcia et al. 2017). Such an associative tendency is something that permeates most evolutionary lineages of Eukarya. In the group comprising animals (Metazoa) this is well observed in the marine environment (Wilkinson, 1987; Castro, 1988; Sarà et al. 1998; Dubilier et al. 2008). In this context, being the first metazoans to diverge (Redmond and McLysaght, 2021) sponges have been widely studied in a variety of areas. In investigations of the interactions between microorganisms and animals, Porifera stand out as model animals (Hentschel et al. 2006; Taylor et al. 2007; Webster and Taylor 2012; Thomas et al. 2016). Initially, sponge-associated microorganisms were studied from a morphological viewpoint (Vacelet and Donadey, 1977; Wilkinson 1978a, 1978b, 1978c, Wilkinson et al. 1984) which provided the basis for this area of study. With the advent and popularization of the omics sciences the communities of associated microorganisms, especially bacteria and archaea, have been further explored. This has provided advances such as the characterization of an enriched phylum of bacteria discovered in marine sponge samples (Poribacteria - Fieseler et al. 2004; Podell et al. 2019). Although omics approaches are applied with relative success to the symbioses of these ancient animals, there are still several gaps in scientific knowledge. Within the enormous biodiversity of the Porifera microbiome there is still a fraction that is little discussed. Fungi and other microeukaryotes form a significant portion of the associated microbiome biodiversity (Taylor et al. 2007; Suryanarayanan, 2012). In other invertebrates such as corals, these other symbionts can play an essential role in maintaining the animal's homeostasis as well as disrupting it (Ainsworth et al. 2017). In recent years sponge-associated fungi have gained some relevance mainly with regard to biotechnological applications (Suryanarayanan, 2012; Indraningrat et al. 2016). However, our understanding of the role of microeukaryotes was and still is greatly hampered by the focus on bacterial and archaeal portions of the microbiome (Caron et al. 2009; Keeling and del Campo, 2017). Furthermore, when it comes to the association between Porifera and unicellular eukaryotes many of the records are focused on a few taxa such as diatoms (Cox and Larkum, 1983; Bavestrello et al, 2000; Regoli et al. 2004; Moitinho-Silva et al. 2017)

and dinoflagellates (Garson et al. 1998; Schönberg and Loh, 2005; Schönberg and Suwa, 2007; Müller et al. 2007; Achlatis et al. 2019).

Overall, the current understanding of unicellular eukaryotic microorganisms has grown. Expeditions such as Tara Oceans shed light on this much under-explored groups (de Vargas et al. 2015). There is also a focus on the questions related to biomass. It is estimated that marine unicellular eukaryotes can make up approximately 2 gigatons of carbon (Bar-On et al. 2018). Such immensity of microorganisms from a functional-ecological point of view becomes indispensable for the various biogeochemical cycles of the planet (Worden et al. 2015). Considering the importance of protists in aquatic environments in general, we bring in this text a narrative analysis of the relevant scientific literature. We seek to demonstrate that marine and freshwater sponges have associations with various protist lineages and that these have important functions (Figure 2.1). For instance, processes such as photosynthesis are performed by diatoms, dinoflagellates and green algae, where there is an exchange of photosynthates for the protection and use of nitrogen wastes. Other phenomena such as syntrophy are reasonable to expect within a complex community. In addition to that, the production of secondary metabolites is also one of the main services provided by these unicellular eukaryotes. Compounds such as antimicrobials, fatty acids, and alkaloids produced by these microorganisms can contribute to the biochemical profile of the sponge (Indraningrat et al. 2016; Zhu et al. 2019; de Kluijver et al. 2021). Also, mechanisms such as top-down pressure exerted on the bacterial, archaeal, fungal and viral community modulate the structure and composition of these assemblages.

Here we present the taxa that have already been reported and discuss the major obstacles in researching this important fraction of the sponge microbiome. We understand that the definitions of eukaryome made by Lukeš and colleagues (2015) and del Campo and colleagues (2020) are more wide-ranging than we intend to address. Therefore, we define the term microeukaryome as the fraction of the microbiome that comprises only single-celled eukaryotes known as protists. Unicellular and multicellular fungi have not been included in this definition because we consider the term mycobiome/mycobiota to encompass this diversity (Peay et al. 2016), it is already used in the context of spongology and recently reviewed (Bovio et al. 2020; Cohen et al. 2021). From this definition we have also excluded any macroeukaryotes such as animals, plants or macroalgae. In addition, we present a concise discussion of the

possible physiological roles of these associated unicellular eukaryotes and possible lines of investigation that may contribute to the solution of the problems presented.

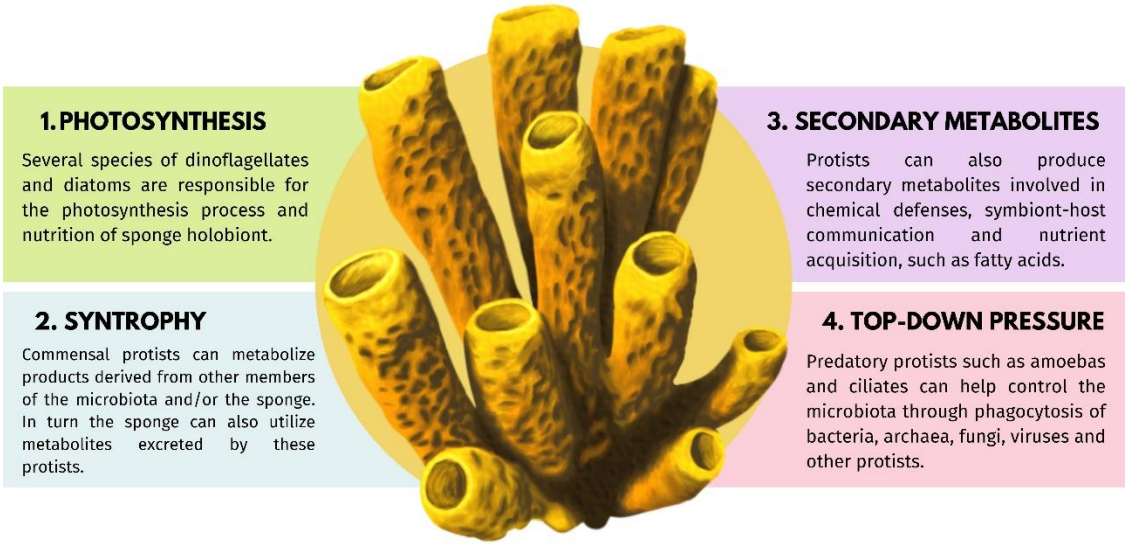

Figure 2.1. Putative microbial services provided by the Porifera microeukaryome. Based on Steinert et al., 2018.

A BRIEF DISCUSSION ON THE STUDY AND PHYLOGENY OF EUKARYOTES

Before discussing the diversity of protists associated with sponges, we should pay special attention to the diversity of eukaryotes in general. Much is known about macroscopic lineages such as animals, fungi and plants. However, considering the myriad of habitats on the planet we will see that in addition to bacteria, archaea and viruses, protists are essential in several of these spaces (Worden et al. 2015). In general, there are two main problems in the study of unicellular eukaryotes: cell size and lineage diversity (Keeling and del Campo, 2017). The first concerns the inherent difficulty in visualizing, culturing, and studying microorganisms. Although methodological advances have mitigated these problems there are still obstacles to overcome. One such major challenge is the culture and isolation of strains from which we frequently only have sequence fragments. The second major problem in protistology refers to the diversity of each strain itself. This characteristic is still difficult to be fully understood even with the current molecular tools and bioinformatics that we have. This is mainly due to the absence of reference sequences with which we can compare microeukaryotic sequences obtained to generate more robust assemblies (Dawson and Hagen, 2009;

Keeling and del Campo, 2017). Furthermore, in the field of protistology there is a strong tendency to apply the same metabolic-functional inference-based strategies used in Bacteria and Archaea (Keeling and del Campo, 2017). Such an approach creates some methodological problems, since microeukaryotes in general have a relative metabolic homogeneity compared to the other two domains (Keeling and del Campo, 2017). In this context, morphology and behavior can be more informative features than inferences based only on sequences of DNA and RNA (Keeling and del Campo, 2017; Keeling, 2019). Regarding the relationships between lineages, it can be said that the phylogeny of the group is highly dynamic (Baldauf, 2003; Keeling et al. 2005; Roger and Simpson, 2009; Hampl et al. 2009; Adl et al. 2019; Burki et al. 2020). For simplicity, we adopt here a version made from the detailed review by Adl et al. (2019) and Burki et al. (2020), since it is beyond the scope of this review to discuss the positioning of specific groups. In a comprehensive overview of the major evolutionary lineages within the tree of eukaryotes (Figure 2.2), one can see the division into two major groups: Amorphea and Diaphoretickes. In the former there is the clades of Amoebozoa, Nucletmycea and Holozoa, which have as their best-known members the amoebas, true fungi and animals, respectively. Importantly, this hypothesis incorporates recent evidence demonstrating the paraphyly of Excavata that are now divided into clades such as Metamonada and Discoba (Adl et al. 2019; Burki et al. 2020). In Diaphoretickes, on the other hand, are the photosynthesizing organisms. It contains part of the former taxon Excavata, represented by algae of the taxon Cryptista. Still in Diaphoretickes there are Archeoplastida, comprising the red algae Rhodophyceae, the green algae Chloroplastida, and Embryophyta, if land plants are included. The SAR supergroup is monophyletic, made up of three other major clades: Stramenopiles, Alveolata and Rhizaria. To facilitate the understanding of their associations with Porifera, Alveolata is represented here divided into smaller groups. From the limited literature on the associations of Porifera and microeukaryotes, we observed two major trends on the study of the biodiversity of sponge-associated microorganisms. The first concerns research that was able to study these associations through direct observations and classical culture and isolation methods. Another more recent approach makes use of molecular methods and bioinformatics to analyze large quantities of biological samples.

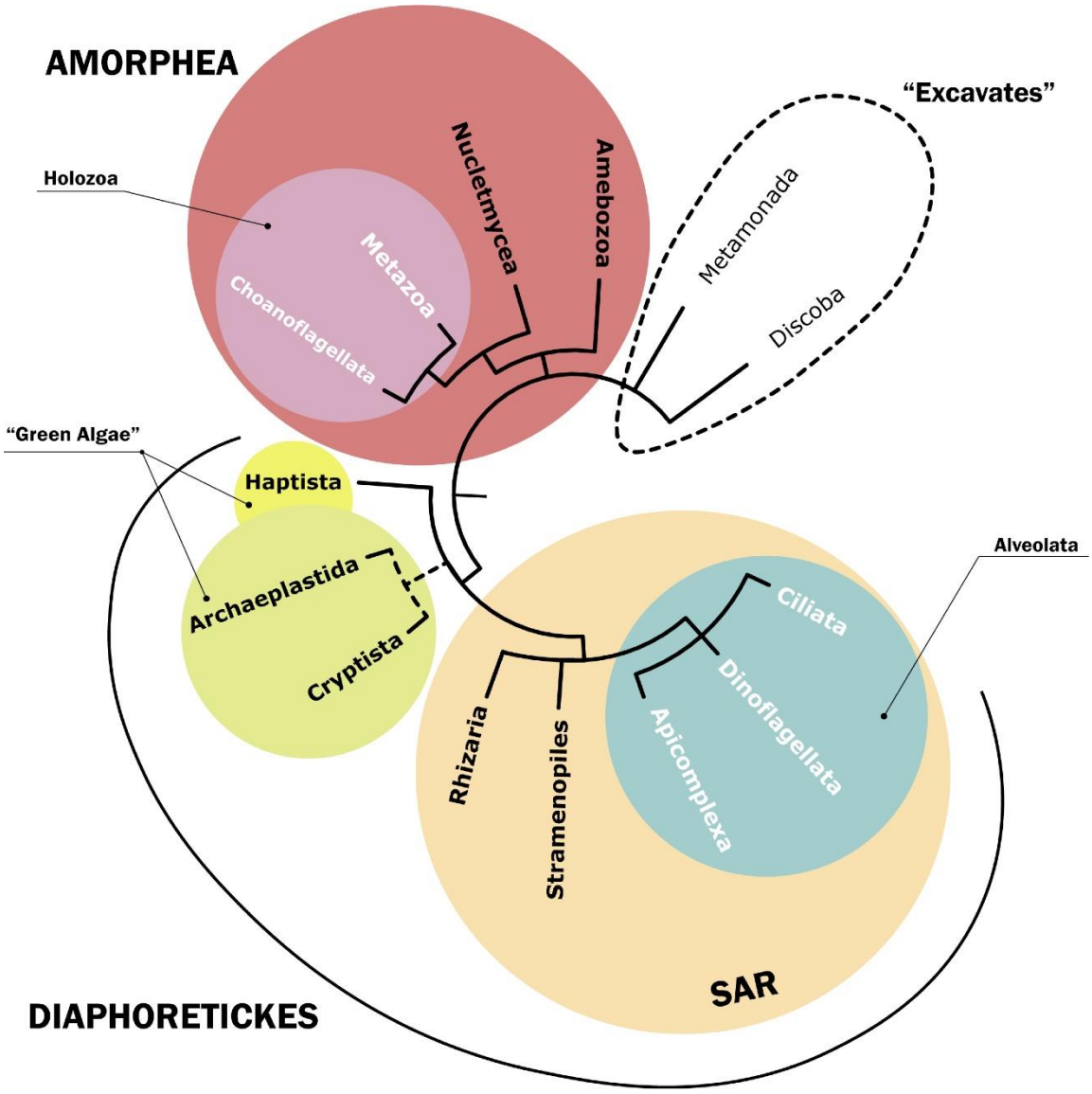

Figure 2.2. Simplified scheme of the main evolutionary lineages of Eukarya based on Adl et al. (2019) and Bulki et al. (2020). Amorphea is the group that includes true animals and fungi. Within this clade we have here as representatives of Holozoa the protists of Choanoflagellata as a sister group to the animals or Metazoa. In turn, Diaphoretickes is an extremely diverse supergroup. Within it is the SAR supergroup, consisting of Stramenopiles, Alveolata and Rhizaria. There is also Archaeplastida, which contains part of the diversity of multicellular and unicellular protists, as well as land plants. This group and the Cryptista protists have widely debated monophyly. The 'green algae' are a paraphyletic group that is generally composed of representatives of these two groups plus Haptista. Excavata, here represented by the Metamonada and Discoba clades is no longer considered monophyletic and these groups have uncertain positioning. There are more protist lineages that are not represented in this figure.

THE MICROEUKARYOME DIVERSITY FROM DIRECT OBSERVATIONS TO CULTURE-DEPENDENT APPROACHES

The culture-dependent approaches have some advantages over its counterpart. The main one is that it allows the elucidation of features not visualized in gene sequences such as structure and behavior (Keeling and Campo, 2017). However, it is

limited to a few microorganisms, implying a low resolution of the biodiversity of host-associated microeukaryotes in a manner similar to "the great plate count anomaly" first described by Staley and Konopka (1985). Despite the enormous biodiversity of microorganisms on the planet, only a small portion can be grown in the laboratory for diverse reasons (Epstein, 2013; Cross et al. 2019) one of them being the symbiont-host interdependence itself.

There are few studies that actively seek to understand in detail the nature of sponge-protist associations. The culture and isolation of these microeukaryotes in the vast majority of cases occurs indirectly. In primary host cell cultures, for example, contaminant protists are one of the main problems. They benefit from the dysbiosis caused by the stress of host cell reorganization and proliferate. Because protists are diverse, it is difficult to control the growth of their populations, and many host cultures become non-viable after a short time of establishment (Rinkevich, 1999). The almost constant presence of these microorganisms is one of the major obstacles to be overcome for the establishment of axenic cell culture strains of Porifera and several other marine invertebrates. Here we attempt to synthesize from the literature the main records of isolations and culture of non-fungal microeukaryotes associated with marine and freshwater sponges.

Amoebozoa

Amoebas are present in diverse habitats ranging from soils, mangroves, lakes and oceans (Rodríguez-Zaragoza, 1994; Rogerson and Gwaltney, 2000; Schulz et al. 2015). Along with other protists they are important predators of other microorganisms such as bacteria, archaea and viruses. The taxon Amoebozoa is characterized by amoeboid movement, which consists of altering cell conformation through pseudopod extensions and contractions (Adl et al. 2019). Common in primary Porifera cell cultures, they are reported as persistent contaminants, where they assume opportunistic behavior (Rinkevich, 1999). There are few reports of associations between amoebae and sponges and, as for the vast majority of unicellular eukaryotes, the diversity of associations is poorly studied. The amoeba *Neoparamoeba aestuarina* (Discosea) was isolated from dissociated tissues of *Clathrina aurea*, *Polymastia janeirensis* (Klautau et al. 1993, misidentified as sponge cells) and *Chelonaplysilla erecta* (Custodio et al. 1995). These microorganisms have already been isolated from other algae, invertebrates and fish, where they appear to play negative effects, such as Amoebic Gill Disease in marine

farmed fishes (Dyková et al. 2007; Dyková et al. 2008). In another work with adult tissues and embryos of *Negombata* spp. several species of amoebae proliferated, but were not identified (Rinkevich et al. 1998, Rinkevich, 1999). It is still uncertain whether the presence of amoebas in sponges characterizes some kind of parasitism or their proliferation is a result of opportunistic behavior. The amoebae, in general, are free-living and from this we can infer that the persistence of these microorganisms in cultures is due to the abundance of bacteria that the sponges harbor. It is possible that they play the role of predators of the microbial community (Figure 2.1), therefore assisting in the population control. Such a function is reported in other symbiotic systems, such as corals (Ainsworth et al. 2017) and plants (Gao et al. 2019).

"Green algae"

The term "green algae" is the common name for several unicellular and multicellular photosynthesizing eukaryotic organisms. Despite the lack of phylogenetic consensus this group comprises some organisms of the taxon Archeoplastida, Cryptista and Haptista (Simpson et al. 2017, Adl et al. 2019). Multicellular organisms are represented by macroalgae and land plants, but a significant part of the diversity is composed by unicellular microorganisms. With an emphasis on the latter, it is noted that these microeukaryotes are relatively common as epibionts and endobionts of Porifera. Some of these associations have been used as a model for understanding the general process of establishing intracellular symbioses, such as in the freshwater sponge *Ephydatia muelleri* and its *Chlorella*-like symbionts (Hall et al. 2021; Geraghty et al. 2021). From the interaction between sponges and algae, several benefits can be derived for both participants, the main ones being obtaining oxygen, nutrients and recycling of metabolic wastes (Trautman and Hinde, 2002).

One of the most studied associations is the symbiosis between the freshwater sponge *Spongilla lacustris* and the algae of the genus *Chlorellae*. The greenish color of this sponge is primarily due to their association with these algae. The first morphological works that sought to characterize this symbiosis date back to the 1910s and 1920s (Williamson, 1979; Saller, 1989). Also called zoochlorellae these algae are often found in the tissues of adult sponges, within the vacuoles of archaeocytes and other cell types as well as in the gemmules (Williamson, 1979; Saller, 1989). The presence of the symbionts protists in reproductive structures is evidence of the vertical transmission between generations. Still in the freshwater environment, in Lake Baikal,

the symbiosis between *Lubomirskia baicalensis* and endosymbiont green algae of the genus *Mychonastes* was described (Chernogor et al. 2013). Through the method of cultivation of primmorphs of this animal it was possible to isolate and subsequently cultivate these symbionts. Contrary to the trend observed for other freshwater sponge species, *Lubomirskia baicalensis* is perennial even in the face of harsh Siberian winter conditions, maintaining its association with its symbionts (Müller et al. 2009; Chernogor et al. 2013). It is suggested that *Mychonastes* plays an important role in the nutritional homeostasis of its host. The sponge would provide a safe and resourceful environment (shelter from predation and metabolic wastes) for the proliferation of the protist, which reciprocates through photosynthates and derivatives such as oxygen (Chernogor et al. 2013). It is worth noting that the sponge-algae association is maintained even during the organizational change of the host tissues into primmorphs. This intimate symbiosis might be one of the main responsible for the long-term maintenance of primmorphs under laboratory conditions - over 10 months - without the addition of nutrients in the culture medium (Chernogor et al. 2011; Chernogor et al. 2013). Another interesting symbiosis occurs between the marine sponge *Tethya seychellensis* and the cenocytic green algae *Ostreobium* sp. (Gaino and Sarà, 1994). Algae of this group are commonly found in association with other sessile invertebrates such as ascidians (Lambert et al. 1996), barnacles (Feussner et al. 2004), corals and gorgonians (del Campo et al. 2017). Organized in a globular shape, sponges of the genus *Tethya* have large siliceous spicules bundles arranged radially along their body. Such organization favors the growth of the associated algae from the cortex to the most nuclear region of the animal's body (Gaino and Sarà, 1994). It was speculated that, because they are mostly arranged around the spicular bundles of the host, the algae benefit from a light conduction system similar to an optical fiber (Gaino and Sarà, 1994). The transport of photoactive radiation by spicules was corroborated more than a decade later using *Tethya aurantium* (Brümmer et al. 2008).

SAR: Stramenopiles

The Stramenopiles, like the other SAR groups, constitute a very diverse lineage. Some of its more derived representatives are photosynthesizing while the more basal forms are heterotrophic. This group has as its main characteristic the presence of two tubular flagella in at least one of the stages of its life cycle (Adl et al. 2019). In Stramenopiles are contained well-known groups such as the "brown algae"

(Phaeophyceae) and the diatoms (Diatomea). The diatoms are one of the groups that have associations with various invertebrates, including intimate symbioses with marine sponges. There are records of the association between some unidentified tropical sponges, possibly *Prianos* cf. *melanos* (now accepted as *Batzella melanus*) and *Spirastrella* aff. *decumbens*, and the diatoms of the genus *Nitzschia* (Cox and Larkum, 1983; Cox et al. 1985). Although there is no physiological approach to this association, the sponge-diatomaceae relationship appears to be stable, as it has been observed in more than one locality (Cox and Larkum, 1983). A large number of these protists have been reported living extracellularly in the mesohyl of the sponge, with their frustules intact. Antarctic sponges, on the other hand, host a large diversity of diatoms in their mesohyl, just below the pinacoderm (Gaino et al. 1994, Bavestrello et al. 2000). In 17 Antarctic sponge species, diatoms of the genus *Fragilariopsis*, *Achnanthes* and *Pseudogomphonema* were the most commonly found, followed by some species of centric diatoms (Bavestrello et al. 2000). Although more evidence is still needed, it is hypothesized that these microeukaryotes strengthen the cortex of the animal and possibly serve as nutrition in the harsh Antarctic conditions (Gaino et al. 1994; Cerrano et al. 2004). In exchange, these endobiont diatoms appear to benefit from metabolic wastes of the host, adopting a myxotrophic strategy that allows survival in low or no light situations (Bavestrello et al. 2000).

In addition to photosynthesizing organisms, there are also associations between Porifera and heterotrophic stramenopiles. Of this subgroup, the interactions of sponges with the Labyrinthulomycetes, a small group of saprophytic, fungi-like microeukaryotes, stand out. Overall, their relationships with marine invertebrates and other organisms tend to be negative, with significant impacts to host fitness (Raghukumar, 2002; Schärer et al. 2007; Gast et al. 2008). However, it is likely that these microorganisms are opportunistic, benefiting from an eventual lack of regulation of the microbiota by the host. Yet, there is evidence that they are important at times of stress, such as in bleaching events on stony corals of the genus *Fungia* (Kramarsky-Winter et al. 2006). From the few studies that exist on the interaction of these protists with sponges it is noted that they can be isolated from tissues derived both from healthy and decaying specimens (Raghukumar, 2002). They are also recurrently isolated from primary cell cultures, probably benefiting from the dysbiosis caused by the tissue dissociation. With rapid proliferation, these unicellular eukaryotes can be mistaken for

well-established Porifera cultures (Rinkevich, 1999; Richelle-Maurer et al. 2003). Recently, different strains were isolated from primmorphs of *Hymeniacidon heliophila* and *Haliclona melana* (Nascimento-Silva et al. pers. obs.) and although investigations on this association are still in early stages some hypotheses are already discussed. It is possible that these microorganisms adopt endo- and exosymbiont strategies, similarly to diatoms. Their ectoplasmic projections, a synapomorphy of the group, may facilitate the uptake of metabolic wastes from the host, as well as particulate and dissolved organic matter. They are recognized producers of polyunsaturated fatty acids (PUFAs), carotenoids, polysaccharide exudates, squalene, and enzymes with varied metabolic activities (Marchan et al. 2018). PUFAs are important metabolites that are used in the structure of cell membranes and as precursors to immunomodulators and could possibly be utilized by the host sponge of these protists.

SAR: Alveolata

Alveolata is a monophyletic group consisting of three major groups (Apicomplexa, Dinoflagellata, and Ciliophora) (Adl et al. 2019). It comprises microorganisms with diverse life habits. One of the best known, mainly for its negative impacts on host fitness, is the Apicomplexa. This lineage has as its most distinguishable feature a cellular structure known as the apical complex (Katris et al. 2014). However, this direction to the study of parasites may be the result of a strong bias as there is an unexpected biodiversity yet to be explored (Saffo et al. 2010; Oborník et al. 2012; del Campo et al. 2020; Keeling et al. 2021). Previous studies of isolation and identification of Apicomplexa from Porifera tissues or cell cultures are unknown to us. In contrast, there are records of Porifera-Ciliophora and Porifera-Dinoflagellata interactions as a focus. The taxon Ciliophora is widely studied in the free-living context (Simpson et al, 2017). They have nuclear dimorphism as their main characteristic, possessing two cell nuclei: a macronucleus and a micronucleus. In addition, many representatives of this group have large amounts of cilia (Simpson et al. 2017; Adl et al. 2019). In the recently described Lake Baikal demosponge, *Swartschewskia khanaevi*, the presence of sessile epibiont ciliates of the genus *Lagenophrys* was observed (Bukshuk and Maikova, 2020). It is possible that the proliferation of these protists, along with diatoms that also make up the microeukaryome of these animals, is favored by the particular morphology of the sponge's aquiferous system (Bukshuk and Maikova, 2020). In environmental stress events which usually result in tissue necrosis and possible mass mortality for sponges,

ciliates can play important roles. In a study of *Petrosia ficiformis* populations it was observed that these ciliates consume the degenerated tissues of the sponges, allowing the animals to recover from a necrosis event (Cerrano et al. 2001). Dinoflagellata in turn has as its main characteristic the presence of two eukaryotic flagella in the mobile stage of the life cycle (Adl et al. 2019) and are recognized as symbionts of invertebrates. In terms of Porifera-Dinoflagellata interaction one of the most studied associations occurs between excavating sponges of the genus *Cliona* and *Symbiodinium* (Hill et al. 2011). This association can be destabilized in a manner similar to what occurs in coral-*Symbiodinium* symbioses, as was reported for the first record of mass bleaching in clionid sponges in 2015 (Hill et al. 2016). There is even evidence of vertical transfer of these symbionts between generations of *Cliona* sp. (Mariani et al. 2001; Mies et al. 2017). Although this is the best-known association, there are other investigations that are worth highlighting. Through an ultrastructural and morphological approach, it was suggested that one Haplosclerida, collected on the Great Barrier Reef and identified as *Haliclona* sp., contains a dinoflagellate very similar to *Symbiodinium microadriaticum* in its tissues (Garson et al. 1998). In Lake Baikal, the sponge *Lubomirskia baicalensis* and a dinoflagellate species of the genus *Gymnodinium* have a well-characterized symbiosis, mainly from a physiological point of view. Besides providing the hosts with primary metabolites, such as glycerol, there is evidence of their participation in the acclimatization of sponges (Müller et al. 2007; Müller et al. 2009). During the winter, Lake Baikal is covered by a layer of ice and it is at this time that a higher metabolic activity related to the reproduction of these sponges is observed (Müller et al. 2007). It has been identified that these symbiont dinoflagellates produce a secondary metabolite called okadaic acid. This molecule interacts with the host HSP70 system, increasing the expression of heat-shock proteins and consequently contributing to the survival of these animals in the harsh winter conditions of Lake Baikal (Müller et al. 2007). It is interesting to note that the interaction between sponges and protists that produces okadaic acid are studied since the 1980's. Okadaic acid was first isolated from associated microorganisms of two sponges: *Halichondria okadai* and *Halichondria melanadocia* (Tachibana et al. 1981). This molecule, classified as an ionophore, presented a number of characteristics that attracted strong academic and commercial interest over time (Bialojan and Takai, 1988; Fernandez et al. 2012; Kamat et al. 2014).

SAR: Rhizaria

Representatives of Rhizaria form a morphologically diverse group. These microorganisms have as their main characteristic thin pseudopodia, which display varying patterns, from simple extracellular projections to anastomosed and branched (Simpson et al. 2017; Adl et al. 2019). Because of its great diversity, there is no universal set of morphological characteristics that support the group, but phylogenetic relationships among the taxa can be obtained from molecular studies (Simpson et al. 2017). Within Rhizaria are three other prominent groups: Cercozoa, Radiolaria, and Foraminifera, the last one with the most records of isolation from Porifera tissues. Foraminifera are important in biogeochemical cycles, as they have shells or tests composed of calcium carbonate or other materials (Simpson et al. 2017). They are often epibionts of sponges, but there are also records of being inside the body of these animals (Granville and Nordmann, 1971; Linke and Lutze, 1993; Mazzoli-Dias et al. 2007). In a survey of the Faroe Islands archipelago, these protists were found in the pinacoderm of all studied sponges of the family Geodiidae (Klitgaard, 1995). The most common species were *Cibicides refulgens* and *Hyrrokkin sarcophaga*, the latter described as a parasite of corals and bivalves (Schleinkofer et al. 2021). Histological approaches have shown that in the sponges *Isops phlegraei* (now accepted as *Geodia phlegraei*) and *Stelleta normani,* the foraminifera *H. sarcophaga* can dissolve part of the cortex of the animal (Cedhagen, 1994). In turn the host's spicules are used by the protist, which deposits them on its external part. The outcome of this interaction is a negative impact on the sponge, left with scars where the spicules were removed (Cedhagen, 1994). Despite these observations, further investigation of the physiology of this interaction is still needed. It is possible that *H. sarcophaga*, like other protists, utilize the sponge body for reproduction and ease of obtaining nutrients, as they have been found at different stages of development within the hosts (Lintner et al. 2021). The association between foraminifera and sponges appears to be an old one. Several species of foraminifera already known to science and two new species - *Placopsilina spongiphila* and *Ramulina siphonifera* - have been identified in fossil sponge reefs (Guilbault et al. 2006). Another interesting aspect of Foraminifera-Porifera interaction is starred by the giant Mediterranean Sea foraminifera *Spiculosiphon oceana*. Through a meticulous organization of siliceous sponge spicules, this organism builds its test or

outer shell and assumes a feeding strategy and morphology very similar to carnivorous sponges (Maldonado et al. 2013).

THE MICROEUKARYOME DIVERSITY FROM CULTURE-INDEPENDENT APPROACHES

The use of omics approaches has completely transformed our understanding of the microbial ecology of multiple sponge species, allowing a more comprehensive view. However, the level of detail of the sponge-protist association from a functional-symbiotic point of view is still low. This approach does not allow the inference of morphological and behavioral characteristics which are important for the study of unicellular eukaryotes (Keeling and Campo, 2017; Keeling, 2019). In contrast it is possible to know in detail the abundance, richness, interactions and composition of entire communities of microorganisms associated with a given host in a generalized or group-specific manner. Considering the Porifera microeukaryome, this approach started to be successfully applied only in the last decade, usually with sets of nonspecific primers for the 18S rRNA gene such as those listed in Table 2.2. One of the first studies applied pyrosequencing, now an almost unused technology, to study the communities of fungi and unicellular eukaryotes associated with 11 sponges from the Chinese sea (He et al. 2014). In these species the diversity of protists was higher than that of fungi, with 721 unique Operation Taxonomic Units (OTUs) being obtained. Alveolata and Radiolaria were found in all investigated species and the protist sequences observed were significantly different from those in seawater, implying a host control on the composition of this community (He et al. 2014). In Antarctic sponges, diatoms (Diatomea) and dinoflagellates (Dinophyceae) are predominant in the microeukaryome (Rodríguez-Marconi et al. 2015). Although dominated by photosynthesizing microorganisms, heterotrophic dinoflagellates (Syndiniales) also constitute a significant portion of the protist community (Rodríguez-Marconi et al. 2015). However, in these two studies the authors did not use biological replicates and apparently did not normalize the data prior to alpha diversity metrics. Also, only Rodriguez-Marconi and collaborators included seawater samples in their experimental design. The approaches used in these studies directly impacts the understanding of the microeukaryotic community associated with these sponges, making comparisons difficult (Hardoim et al. 2021). On the other side, these investigations have gathered further evidence that, just as occurs in the bacterial and archaeal communities, the protists associated with sponges

can also possess host specificity. Nevertheless, this is not a consensus in the area. In a comparison between the microbiome of four sponge species (*Dysidea avara*, *Dysidea etheria*, *Aplysina aerophoba* and *Aplysina cauliformis*) no host specificity of the microeukaryome was observed (De Mares et al. 2017). Nonetheless, in this work there was a large variability in diversity among the samples of each species, possibly stemming from low sample numbers as pointed out by the authors. In the microeukaryome of these four sponges the vast majority of reads could not be classified taxonomically. Of those that were classified, Arthropoda and Annelida followed by Choanoflagellida (protists) have the highest relative abundances (De Mares et al. 2017). A similar trend was seen when analyzing the microeukaryome of six sponge species native to the Penghu Archipelago in Taiwan. Much of the microeukaryotic community of these animals appears to be derived from the surroundings, primarily seawater. Again, many taxa could not be taxonomically classified, indicating an unexplored diversity. Dinoflagellates (Dinophyceae and Syndiniales) were the most abundant group, but green algae and radiolarians were also present in remarkable proportions (Cleary, 2019). Interestingly, in the sponge *Acanthostylotella cornuta* it was observed a high relative abundance of Metamonada, basal heterotrophic eukaryotes that are usually grazers of bacterial communities, but with parasitic representatives (Adl et al. 2019). In a more recent study (Hardoim et al. 2021), again the question of microeukaryome specificity was raised. Although the previous studies are not directly comparable given methodological divergences, the investigation of the microeukaryotic community of three sympatric species from South America indicates that the hosts directly act on the selection of microorganisms. The marine sponges *Aplysina caissara*, *Aplysina fulva*, and *Tedania ignis* have a high diversity of microeukaryotes that could not be taxonomically classified, indicative of microbial novelty (Hardoim et al. 2021). Of the identifiable taxa, the photosynthesizing groups were the most abundant, dominated by macroscopic green (Phragmoplastophyta) and red (Florideophycidae) algae, followed by diatoms (Diatomea).

The second type of investigation, which considers only one or a few protists taxa, has also been used for Porifera. For example, in the freshwater sponges *Baikalospongia intermedia*, *Baikalospongia recta* and *Lubomirskia incrustans* a great diversity of dinoflagellates (Alveolata: Dinoflagellata) were found (Annenkovaa et al. 2011). Phylogenetic analyses in this work showed that the 18S rRNA of two groups of

dinoflagellates were repeatedly found in the total DNA extracted from these sponges. The protists belong to the order Suessiales, a group comprising endosymbiont dinoflagellates known to be associated with a wide range of marine invertebrates, and may be a potential symbiont of the sponges in this ecosystem (Annenkovaa et al. 2011). However, many of these works are early stages that have not yet delved into the physiology of the association. One use of omics sciences that goes further and attempts to integratively understand association function and metabolism is the work of Moitinho-Silva and colleagues (2017). Within the marine sponge *Cymbastela concentrica* there is a complex network of metabolic integration where diatoms, bacteria, archaea, and the host cells themselves are the key players. The results of this work show that there is a transfer of organic nitrogen compounds between these participants, which supports the nitrogen cycle within the holobiont. A comprehensive list of several taxa of unicellular eukaryotes associated with sponges can be found in Table 2.1.

THE HMA/LMA DICHOTOMY APPLIED TO PROTISTS AND THE SPECIFICITY OF THE MICROEUKARYOME

The terms *High Microbial Abundance* (HMA) and *Low Microbial Abundance* (LMA) were created to represent patterns of association between sponges and prokaryotes (Hentschel et al. 2003; Gloeckner et al. 2014). HMA-type sponges are defined as those that have around 10^8 to 10^{10} associated bacterial cells, while LMA have orders of magnitude to smaller around 10^5 to 10^6, closer to seawater (Hentschel et al. 2003; Gloeckner et al. 2014). However, there still remain several questions that either urgently need answers or still require further evidence. One of them concerns the presence of this pattern in relation to the communities of protists and fungi associated with sponges. So far, we are unaware of investigations that seek to understand if it is possible to observe this same HMA and LMA pattern when it comes to unicellular eukaryotes. Another interesting question is to observe if the diversity is the same among the different groups of Porifera and if there is a specificity of this community. This investigative line has been seen in recent work such as the one comparing the microeukaryotic community of the LMA sponges *Dysidea avara* and *Dysidea etheria* and the HMA sponges *Aplysina aerophoba* and *Aplysina cauliformis* (De Mares et al. 2017). In this work the authors noted that the protist sequences found in these species

failed to compose a sponge-specific cluster like the associated Bacteria and Archaea (De Mares et al. 2017). It is worth pointing out that the sponge-specific clusters and sponge-coral clusters with which the sequences of De Mares and collaborators were compared are based on only 95 eukaryotic sequences of 18S rRNA, most belonging to Metazoa and Fungi (Simister et al. 2012). Today the scientific community has access to a large number of eukaryotic sequences from both protists and fungi, which allows for a higher resolution of these clusters. On the other hand, the use of integrative approaches points to the ability of the host to regulate this microeukaryome by deliberately selecting symbionts (Hardoim et al. 2021). Added to this, through culture-dependent approaches, evidence is accumulating that there are sponge-enriched microeukaryotes in a similar way to what is observed for Bacteria and Archaea.

When considering this problematic in a physiological dimension, LMA sponges have less diversity of bacterial phyla related to important metabolic cycles such as nitrogen and phosphorus metabolism (Ribes et al. 2012; Gloeckner et al. 2014). Therefore, it would be interesting to investigate whether this need can be supplied by microeukaryotes that also possess such metabolizing capabilities. Also, genes related to polyketide synthases are often absent in LMA sponges, but widely distributed and diverse in HMA organisms (Hochmuth et al. 2010). Given the importance of these genes for the host primary and secondary metabolism (Fisch et al. 2009; Kaluzhnaya et al. 2021) it is possible that other microorganisms are producing these compounds, as may be the case of Labyrinthulomycetes (Morabito et al. 2019), dinoflagellates and/or haptophytes (Kohli et al. 2016), which have polyketide synthases-like pathways. It would also be interesting to investigate if the microeukaryotic community functions redundantly or complementarily to the bacterial community associated with HMA sponges, producing or consuming compounds of interest to the holobiont along the lines of the work of Moitinho-Silva and collaborators (2017).

THE PROBLEMS OF SEQUENCING AND ANALYSIS OF THE PROTISTS ASSOCIATED WITH PORIFERA

When dealing with the ocean of biological information there is a certain tendency to little question the assumptions and functioning of the main bioinformatics methods and tools used and the biases introduced by them (Keeling and Campo, 2017).

In this section we briefly discuss some of the main problems underlying the sequencing and analysis process of the sponge microeukaryome.

The choice of 18S rRNA primers

The most widely used gene for investigating microeukaryotic communities in various contexts has been the 18S rRNA (Pawlowski, 2014). In general, the primers used for sequencing specific regions of this gene produce good results. An example is the consistent use of these sequences and protocols in large projects such as BioMarKs/Biodiversa (Guillou et al. 2013), TARA Ocean Consortium (Pierella Karlusich et al. 2020), The Marine Microbial Eukaryote Transcriptome Sequencing Project (Keeling et al. 2014) and Earth Microbiome Project (Thompson et al. 2017). However unicellular eukaryotes, being very close phylogenetically to their hosts tend to be undersampled, with many of the reads obtained derived from the host animal (del Campo et al. 2019). Therefore, our reduced understanding of the Porifera microeukaryome may be a reflection of this methodological limitation. In the work of De Mares and collaborators (2017) even with the application of a deep sequencing methodology, about 85% of the reads belonged to the host. Also, in He and collaborators (2014) about 79% of all reads were from sponges and only 0.03% belonged to protists. This represents a huge data loss that has profound impact on our understanding of the microbiome of these animals. An alternative approach to diversity analysis is to use primers that only amplify the 18S rRNA gene of a specific group of protists of interest (Annenkova et al. 2011; Bass et al. 2015; Wecker et al. 2015; Hall et al. 2021). Another method consists of designing primers that do not amplify the copy of the gene of interest present in the host, something already applied for invertebrates such as insects (Waidele et al. 2017) and corals (Clerissi et al. 2018; Hume et al. 2018). For some one-off studies, such as ascertaining whether or not a particular group is present in sponge-derived samples, this may be a valid strategy. Although this procedure is not feasible for understanding biodiversity at the community level, it can be useful in the study of diseases caused by protists as a form of diagnosis, as is already widely used for several invertebrates (Gruebl et al. 2002; Liu et al. 2009; Pollock et al. 2011; Ríos et al. 2020).

Of the few more complete solutions, we can list three main strategies, each with its advantages and disadvantages (del Campo et al. 2020). The first one is to increase the sequencing depth in order to access the microeukaryotic fraction of the microbiome.

However, this alternative is often not affordable for the vast majority of Porifera research laboratories given the still high costs of this process. Furthermore, as mentioned, deep sequencing, which can work very well for communities of prokaryotic organisms (Webster et al. 2010) can still be inefficient for protists (De Mares et al. 2017). The second option consists of using blocking primers for the host 18S rRNA gene. This method has already been successfully applied to investigate the microeukaryome of mammals (*Mammal_block_I-short_1391f* - https://earthmicrobiome.org/protocols-and-standards/18s/) and invertebrates such as corals (Clerissi et al. 2018), mollusks (Clerissi et al. 2020) and crustaceans (Liu et al. 2019). However, to date it is unknown to us if there are works that used blocking primers in metabarcoding studies of Porifera-associated microeukaryotic communities. This gap in knowledge needs to be filled by creating primers that prevent the amplification of the Porifera 18S rRNA gene at the phylum level or at the level of the four major classes: Demospongiae, Calcarea, Hexactinellida and Homoscleromorpha. The third option is the use of 'non-metazoan' primers (Bower et al. 2004, del Campo et al. 2019, del Campo et al. 2020; Minardi et al. 2021), *i.e.*, primers that amplify sequences from protists without amplifying those of the animal host. Although these primers show promising numbers as they capture only 2.6% of metazoan sequences, about 74% of these sequences still belong to Porifera (del Campo et al. 2019). This shows that, at least for the study of the protist communities of the Porifera phylum, these primers still do not fully solve the host sequencing problem, but they configure one of the best options. Table 2.2 provides a summary of the main primers used in the investigation of the communities of unicellular eukaryotes associated with sponges and other studies as well as their percentage of 18S rRNA gene coverage. It is possible that the solution to this problem comes from the combination of these strategies: the use of primers blocking the 18S rRNA gene from Porifera in addition to non-metazoan primers may be effective in reducing reads derived from the metazoan host. The adoption of this approach will most likely reveal a previously unknown diversity and provide important insights into how the sponge functions as a holobiont.

The choice of the bioinformatics pipeline

With the rapid advancement of bioinformatics, we have seen the emergence of numerous computational tools. Many of them offer totally new resources, opening possibilities of biological data exploration unknown until now. Others come to correct

flaws in older pipelines that, in face of new evidences, have lost scientific support for their use. Recently, these discussions gained strength within the context of bacterial and archaeal metabarcoding, mainly through the conceptual discussion between OTUs and ASVs (Amplicon Sequence Variants) (Callahan et al. 2017; Edgar, 2018; Prodan et al. 2020). Among these two concepts, OTUs were the first to be applied to microorganism sequence analysis. This approach is based on the idea of grouping sequences by relying on a reference library and using an arbitrary threshold, usually 97% similarity. In recent years, pipelines using an OTU-based approach have fallen into disuse given their difficulty in reproducibility and comparison with other studies (Callahan et al. 2017). In contrast, the use of ASVs as an atomic unit of analysis has been growing, and is even recommended for protist investigation (Forster et al. 2019). ASVs, also called zOTUs (zero-radius OTU) or exact sequence variants, do not use the arbitrary 97% similarity threshold. Instead, ASV methods are based on the assumption that real biological sequences are more observed than erroneous sequences (Callahan et al. 2017; Edgar, 2018). Furthermore, the use of ASVs over OTUs is indicated since they allow for increased sensitivity when investigating from community structures and patterns (Callahan et al. 2017; Needham et al. 2017; Forster et al. 2019). The ASVs allows much higher resolution than OTUs, generally being able to distinguish between different variants of the same species (Prodan et al. 2020). Furthermore, its use allows direct comparison between different studies, enabling new insights into the microbial community of interest. To date, the few published studies that addressed the Porifera microeukaryome used pipelines that had OTUs as the end product. Using this procedure may introduce a new layer of underrepresentation of microeukaryotic diversity, since there is already the whole issue of 18S rRNA gene host similarity. By not being dependent on a reference database (closed-reference OTU analysis) or using an approach such as *de novo* OTU analysis (Callahan et al. 2017), ASVs studies can be compared between sponges from different locations in a more appropriate way. The adoption of more comprehensive, robust and reproducible pipelines such as USEARCH-UNOISE3 or DADA2 can be helpful in this regard (Prodan et al. 2020).

BIOTECHNOLOGICAL APPLICATIONS OF THE PORIFERA MICROEUKARYOME

The interactions between humans and the organisms of the phylum Porifera are ancient (Voultsiadou, 2007; Pronzato and Manconi, 2008), and recently great attention has been paid to the production of bioactive compounds by the microbiota of these animals. The biotechnological potential of bacteria and fungi has become widely explored (*e.g.*, Thomas et al. 2010; Suryanarayanan, 2012; Vaca et al. 2013; Mehbub et al. 2016) including targeting the recent SARS-CoV2, responsible for the COVID-19 pandemic (Geahchan et al. 2021; Shady et al. 2021). However, possible applications of the sponge microeukaryome are still virtually unexplored. Unicellular eukaryotes have diverse evolutionary histories, complex molecular mechanisms, and a significant capacity to synthesize bioactive molecules (Vallesi et al. 2020). Within the sponges-protists associations, it is plausible to consider that primary metabolites such as amino acids, enzymes, photosynthates, fatty acids, and vitamins can be exchanged between the participating organisms. In turn, secondary metabolites are poorly known within this context. In other symbiont systems, these secondary metabolites can act from symbiont-host communication to even chemical defense against natural enemies of the holobiont (Kita et al. 2010; Shinzato et al. 2012; Lopanik, 2014). They are also important in the physiology of the animal, as the mentioned association of dinoflagellates with the freshwater sponge *Lubomirskia baicalensis* (Müller et al. 2007). In the investigation of Della Sala and collaborators (2014) using metagenomic data from *Plakortis halichondrioides* it was evidenced that protists can be a significant source of polyketides.

Being an area that is still expanding within Protistology itself, to date, natural product bioprospecting is focused on already well-known taxa such as diatoms and dinoflagellates. Many species from these groups are closely associated with marine and freshwater sponges. Another group that deserves to be highlighted within this line of research is the basal stramenopiles, since these microorganisms are capable of producing large quantities of metabolites. Carotenoids, squalene and polyunsaturated fatty acids are some of the natural products that can be obtained from these taxa and can be targets for bioprospecting (Marchan et al. 2018). Biotoxins produced by dinoflagellates and diatoms that cause severe negative effects in humans can be potential targets in drug discovery pipelines (Vallesi et al. 2020). Potent metabolites

such as gambierol (Cuypers et al. 2008), gymnodimine (Seki et al. 1995), yessotoxin (Alfonso et al. 2016) among others have been investigated as possible immunosuppressive, anticancer drugs, and with action capable of interrupting the progression of neurodegenerative and metabolic diseases (review in Assunção et al. 2017). It is important to note that often the interaction between the microorganism and its host is complex. Therefore, it is possible that secondary metabolites of interest are not produced by the symbiont separately, requiring a more integrative approach in their isolation and culture (Trench, 1993). Further investigation into this biochemical profile of Porifera considering the portion of associated unicellular eukaryotes is warranted. The adaptation of pipelines developed for bioprospection of fungi may be an interesting starting point for the discovery of molecules of biotechnological interest produced by protists associated with sponges.

CONCLUDING REMARKS

We still have a long way to go before we understand the major roles that protists play in the biology of Porifera. However, we already have an outline of how these interactions occur. Culture-dependent approaches and direct observations remain essential for the in-depth study of these relationships, as it allows visualization of features that cannot be inferred from the genetic material, such as behavior and morphology. On the other hand, culture-independent analyses, based on omics, may help in understanding the influences of these microorganisms on sponge functioning as well as on the composition of this microeukaryotic community. Importantly, further investigations should pay attention to the use of appropriate primers and pipelines to reduce the inherent biases of the method. Ideally, the combination of the two types of approaches can bring several new insights about the holobiont. Furthermore, although some of these relationships are not species specific, we can ask ourselves the question of the extent of these relationships. How beneficial are they? In what context are they neutral? They can become detrimental to one or both organisms involved? and when and under what conditions can this occur? Other topics worth mentioning are the interaction between the sponges and their associated protists with respect to particulate and dissolved organic matter as well as a better understanding of the contribution of these single-celled eukaryotes to the biochemical profile of the sponges. In summary, there are still many discoveries waiting to be made within the context of the much-neglected Porifera microeukaryome.

CRediT Author Statement

GN-S, CCPH and MRC contributed to the conceptualization of the review theme. GN-S and MRC contributed to the selection of appropriate literature. GN-S wrote the original draft and designed the figures. CCPH and MRC contributed to editing and reviewing the article. All authors approved the submitted version.

Acknowledgments

The authors would like to thank Ryan Stamp for providing us with the illustration of *Aplysina fulva*, used in Figure 2.1. This is a contribution of NP-BioMar (Research Center for Marine Biodiversity - USP).

Declaration of Competing Interest

The authors declare no competing interests.

Funding

This work was supported by the grant #2019/04707-8, São Paulo Research Foundation (FAPESP) and the Young Investigator Project grant #2016/17189-7 and Young Investigator fellowship #2017/10157-5, FAPESP.

Table 2.1. Comprehensive list of protists associated with marine and freshwater sponges.

Taxon	Protist ID or Most Abundant Protist Taxa	Sponge-host	Location	Study type	Reference
Amoebozoa	Non-identified amoeba	*Negombata* sp.	Red Sea (Israel)	Culture dependent (contaminant)	Rinkevich et al. (1998)
	Neoparamoeba aestuarina	*Clathrina aurea* *Polymastia janeirensis* *Chelonaplysilla erecta*	Rio de Janeiro Coast (Brazil)	Observation only Culture dependent	Klautau et al. (1993) Custodio et al. (1995)
"*Green Algae*"	*Chlorella*-like	*Ephydatia muelleri*	Richmond, Virginia (USA)	Culture dependent	Hall et al. (2021) Geraghty et al. (2021)
	Chlorellae	*Spongilla lacustris*	Massachusetts (USA) Sieg River (Germany)	Observation only Culture dependent	Williamson (1979) Saller (1989)
	Mychonastes sp.	*Lubomirskia baicalensis*	Lago Baikal (Russia)	Culture dependent	Chernogor et al. (2013)
	Ostreobium	*Tethya seychellensis*	Ari Atoll (Maldive Islands)	Observation only	Gaino and Sarà (1994)
Stramenopiles	*Nitzschia* spp. (Diatom)	*Batzella melanus* *Spirastrella* aff. *decumbens*	One Tree Island (Australia)	Observation only	Cox and Larkum (1983)
	Fragilariopsis spp. (Diatom) *Achnanthes* spp. (Diatom) *Pseudogomphonema* spp. (Diatom)	17 marine sponges species	Terra Nova Bay (Ross Sea)	Observation only	Bavestrello et al. (2000)
	Porannulus contentus (Diatom) *Thalassiosira* cf. *gracilis*, (Diatom) *Thalassiosira. Perpusilla* (Diatom) *Plagiotropis* sp. (Diatom)	*Mycale acerata*	Terra Nova Bay (Ross Sea)	Observation only	Cerrano et al. 2004
	Diatomea	*Aplysina caissara* *Aplysina fulva* *Tedania ignis*	São Paulo Coast (Brazil)	Culture independent	Hardoim et al. (2021)
	Labyrinthulomycetes	*Xestospongia muta*	Caribbean Sea (Curaçao)	Culture dependent (contaminant)	Richelle-Maurer et al. (2003)

		Negombata sp.	Red Sea (Israel)	Culture dependent (contaminant)	Rinkevich et al. (1998)
		Hymeniacidon heliophila *Haliclona melana*	São Paulo coast (Brazil)	Culture dependent	Nascimento-Silva et al. (pers. obs.)
Alveolata	*Lagenophrys* (Ciliophora)	*Swartschewskia khanaevi*	Lago Baikal (Russia)	Culture dependent	Bukshuk and Maikova (2020)
	Non-identified ciliates (Ciliophora)	*Petrosia ficiformis*	Island of Gallinara (Italy)	Observation only	Cerrano et al. (2001)
	Symbiodinium spp.	*Cliona* spp.	Florida Keys (USA) Heron Island (Australia) Okinawa (Japan)	Culture independent	Hill et al. (2011)
	Symbiodinium microadriaticum	*Haliclona* sp.	Heron Island (Australia)	Culture dependent	Garson et al. (1998)
	Gymnodinium sp.	*Lubomirskia baicalensis*	Lago Baikal (Russia)	Culture dependent	Müller et al. (2007) Müller et al. (2009)
	Dinophyceae (Dinoflagellata)	Several sponge species	Antarctic Sea São Paulo coast (Brazil)	Culture independent and Culture dependent	Rodríguez-Marconi et al. (2015) Moitinho-Silva et al. (2017) Hardoim et al. (2021)
	Syndiniales (Dinoflagellata)	Several sponge species	Antarctic Sea Penghu Archipelago (Taiwan)	Culture independent	Rodríguez-Marconi et al. (2015) Cleary (2019)
	Suessiales (Dinoflagellata)	*Baikalospongia intermedia* *Baikalospongia recta* *Lubomirskia incrustans*	Lake Baikal (Russia)	Culture independent	Annenkovaa et al. (2011)
Rhizaria	Radiolarians	Several sponge species	Chinese Sea (China) Penghu Archipelago (Taiwan)	Culture independent	He et al. (2014) Cleary (2019)
	*Cibicides refulgens (*Foraminifera*)* *Hyrrokkin sarcophaga (*Foraminifera*)*	*Geodia phlegraei* *Stelleta normani*	Faroe Islands Archipelago	Observation only	Cedhagen (1994) Klitgaard (1995)
	*Placopsilina spongiphila (*Foraminifera*)* *Ramulina siphonifera (*Foraminifera*)*	Fossil Sponge Reefs	British Columbia (Canada)	Observation only	Guilbault et al. (2006)
Choanoflagellata	Choanoflagellida	*Dysidea avara* *Dysidea etheria* *Aplysina aerophoba* *Aplysina cauliformis*	Mediterranean Sea (Spain)	Culture independent	De Mares et al. (2017)
Metamonada	Metamonads	*Acanthostylotella cornuta*	Penghu Archipelago (Taiwan)	Culture independent	Cleary (2019)

Table 2.2. Primers used to study the diversity of unicellular eukaryotes and the 18S rRNA coverage of sponges. Data obtained from the TestPrimer tool (Klindworth et al. 2013) against the SILVA 138 RefNR database.

Primer pairs	Coverage of 18S rRNA of Porifera (%)	Coverage of 18S rRNA of Eumetazoa (%)	Region of 18S rRNA	Forward Primer (5'-3')	Reverse Primer (5'-3')	Sequencing technology	Reference
Euk1Af / Euk516r	97.70%	68.50%	V1-V3	CTGGTTGATCCTGCCAG	ACCAGACTTGCCCTCC	Next Generation Sequencing (Illumina)	De Mares et al. (2017)
TAReuk454FWD1 / TAReukREV3	91.10%	69.40%	V4	CCAGCASCYGCGGTAATTCC	ACTTTCGTTCTTGATYRA	Next Generation Sequencing (Illumina)	Cleary (2019)
EUK581-F /EUK1134-R	72.80%	1.80%	V4	GTGCCAGCAGCCGCG	TTTAAGTTTCAGCCTTGCG	Next Generation Sequencing (Illumina)	Bower et al. (2004) tested in del Campo et al. (2019)
18SV1V2F/18SV1V2R **#a**	-	-	V1-V2	ACCTGGTTGATCCTGCCA	GTARKCCWMTAYMYTACC	Next Generation Sequencing (Illumina)	Clerissi et al. (2018)
1391f **#b** / EukBr	-	-	V9	GTACACACCGCCCGTC	TGATCCTTCTGCAGGTTCACCTAC	Next Generation Sequencing (Illumina)	Rodríguez-Marconi et al. (2015) Hardoim et al. (2021)
574*f/UNonMetDB	77.50%	1.90%	V4	CGGTAAYTCCAGCTCYV	CTTTAARTTTCASYCTTGCG	Next Generation Sequencing (Illumina)	Bass and del Campo (2020)

#a - The reverse primer 18SV1V2R has a number of 'wobbles' that cannot be interpreted and resolved using the TestPrime tool.

#b - The primer 1391f is a universal primer, used for the three domains Archaea, Bacteria and Eukarya. Such a range, even when used in conjunction with a Eukarya-specific primer, does not allow a reliable analysis of the results on TestPrimer tool.

REFERENCES

Achlatis, M., Schönberg, C.H.L., van der Zande, R.M., LaJeunesse, T.C., Hoegh-Guldberg, O., Dove, S., 2019. Photosynthesis by symbiotic sponges enhances their ability to erode calcium carbonate. J. Exp. Mar. Bio. Ecol. 516, 140–149. https://doi.org/10.1016/j.jembe.2019.04.010

Adl, S.M., Bass, D., Lane, C.E., Lukeš, J., Schoch, C.L., Smirnov, A., Agatha, S., Berney, C., Brown, M.W., Burki, F., Cárdenas, P., Čepička, I., Chistyakova, L., Campo, J., Dunthorn, M., Edvardsen, B., Eglit, Y., Guillou, L., Hampl, V., Heiss, A.A., Hoppenrath, M., ... Zhang, Q., 2019. Revisions to the Classification, Nomenclature, and Diversity of Eukaryotes. J. Eukaryot. Microbiol. 66, 4–119. https://doi.org/10.1111/jeu.12691

Ainsworth, T.D., Fordyce, A.J., Camp, E.F., 2017. The Other Microeukaryotes of the Coral Reef Microbiome. Trends Microbiol. 25, 980–991. https://doi.org/10.1016/j.tim.2017.06.007

Alfonso, A., Vieytes, M.R., Botana, L.M., 2016. Yessotoxin, a promising therapeutic tool. Mar. Drugs 14, 11–15. https://doi.org/10.3390/md14020030

Annenkova, N. V, Lavrov, D. V, Belikov, S.I., 2011. Dinoflagellates Associated with Freshwater Sponges from the Ancient Lake Baikal. Protist 162, 222–236. https://doi.org/10.1016/j.protis.2010.07.002

Assunção, J., Guedes, A., Malcata, F., 2017. Biotechnological and Pharmacological Applications of Biotoxins and Other Bioactive Molecules from Dinoflagellates. Mar. Drugs 15, 393. https://doi.org/10.3390/md15120393

Baldauf, S.L., 2003. The Deep Roots of Eukaryotes. Science. 300, 1703–1706. https://doi.org/10.1126/science.1085544

Bar-On, Y.M., Phillips, R., Milo, R., 2018. The biomass distribution on Earth. Proc. Natl. Acad. Sci. 115, 6506–6511. https://doi.org/10.1073/pnas.1711842115

Bass, D., Stentiford, G.D., Littlewood, D.T.J., Hartikainen, H., 2015. Diverse Applications of Environmental DNA Methods in Parasitology. Trends Parasitol. 31, 499–513. https://doi.org/10.1016/j.pt.2015.06.013

Bass, D., del Campo, J., 2020. Microeukaryotes in animal and plant microbiomes: Ecologies of disease? Eur. J. Protistol. 76, 125719. https://doi.org/10.1016/j.ejop.2020.125719

Bavestrello, G., Arillo, A., Calcinai, B., Cattaneo-Vietti, R., Cerrano, C., Gaino, E., Penna, A., Sara, M., 2000. Parasitic diatoms inside antarctic sponges. Biol. Bull. 198, 29–33. https://doi.org/10.2307/1542801

Bialojan, C., Takai, A., 1988. Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. Specificity and kinetics. Biochem. J. 256, 283–290. https://doi.org/10.1042/bj2560283

Bovio, E., Sfecci, E., Poli, A., Gnavi, G., Prigione, V., Lacour, T., Mehiri, M., Varese, G.C., 2020. The culturable mycobiota associated with the Mediterranean sponges *Aplysina cavernicola*, *Crambe crambe* and *Phorbas tenacior*. FEMS Microbiol. Lett. 366, 1–10. https://doi.org/10.1093/femsle/fnaa014

Bower, S.M., Carnegie, R.B., Goh, B., Jones, S.R.M., Lowe, G.J., Mak, M.W.S., 2004. Preferential PCR amplification of parasitic protistan small subunit rDNA from metazoan tissues. J. Eukaryot. Microbiol. 51, 325–332. https://doi.org/10.1111/j.1550-7408.2004.tb00574.x

Brümmer, F., Pfannkuchen, M., Baltz, A., Hauser, T., Thiel, V., 2008. Light inside sponges. J. Exp. Mar. Bio. Ecol. 367, 61–64. https://doi.org/10.1016/j.jembe.2008.06.036

Bukshuk, N.A., Maikova, O.O., 2020. A new species of baikal endemic sponges (Porifera, Demospongiae, Spongillida, Lubomirskiidae). Zookeys 2020, 113–130. https://doi.org/10.3897/zookeys.906.39534

Burki, F., Roger, A.J., Brown, M.W., Simpson, A.G.B., 2020. The New Tree of Eukaryotes. Trends Ecol. Evol. 35, 43–55. https://doi.org/10.1016/j.tree.2019.08.008

Callahan, B.J., McMurdie, P.J., Holmes, S.P., 2017. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. ISME J. 11, 2639–2643. https://doi.org/10.1038/ismej.2017.119

Caron, D.A., Worden, A.Z., Countway, P.D., Demir, E., Heidelberg, K.B., 2009. Protists are microbes too: A perspective. ISME J. 3, 4–12. https://doi.org/10.1038/ismej.2008.101

Castro, P., 1988. Animal symbioses in coral reef communities: a review. Symbiosis 5, 161–184.

Cedhagen, T., 1994. Taxonomy and biology of *Hyrrokkin sarcophaga* gen. et sp. n., a parasitic foraminiferan (Rosalinidae). Sarsia 79, 65–82. https://doi.org/10.1080/00364827.1994.10413549

Cerrano, C., Magnino, G., Sarà, A., Bavestrello, G., Gaino, E., 2001. Necrosis in a population of *Petrosia ficiformis* (Porifera, Demospongiae) in relation with environmental stress. Ital. J. Zool. 68, 131–136. https://doi.org/10.1080/11250000109356397

Cerrano, C., Calcinai, B., Cucchiari, E., Camillo, C.D., Totti, C., Bavestrello, G., 2004. The diversity of relationships between Antarctic sponges and diatoms: The case of *Mycale acerata* Kirkpatrick, 1907 (Porifera, Demospongiae). Polar Biol. 27, 231–237. https://doi.org/10.1007/s00300-003-0581-1

De Mares, M.C., Sipkema, D., Huang, S., Bunk, B., Overmann, J., van Elsas, J.D., 2017. Host Specificity for Bacterial, Archaeal and Fungal Communities Determined for High- and Low-Microbial Abundance Sponge Species in Two Genera. Front. Microbiol. 8, 1–13. https://doi.org/10.3389/fmicb.2017.02560

Chernogor, L.I., Denikina, N.N., Belikov, S.I., Ereskovsky, A. V., 2011. Long-Term Cultivation of Primmorphs from Freshwater Baikal Sponges *Lubomirskia baikalensis*. Mar. Biotechnol. 13, 782–792. https://doi.org/10.1007/s10126-010-9340-9

Chernogor, L., Denikina, N., Kondratov, I., Solovarov, I., Khanaev, I., Belikov, S., Ehrlich, H., 2013. Isolation and identification of the microalgal symbiont from primmorphs of the endemic freshwater sponge *Lubomirskia baicalensis* (Lubomirskiidae, Porifera). Eur. J. Phycol. 48, 497–508. https://doi.org/10.1080/09670262.2013.862306

Cleary, D.F.R., 2019. A comparison of microeukaryote communities inhabiting sponges and seawater in a Taiwanese coral reef system. Ann. Microbiol. 69, 861–866. https://doi.org/10.1007/s13213-019-01476-5

Clerissi, C., Brunet, S., Vidal-Dupiol, J., Adjeroud, M., Lepage, P., Guillou, L., Escoubas, J.M., Toulza, E., 2018. Protists within corals: The hidden diversity. Front. Microbiol. 9. https://doi.org/10.3389/fmicb.2018.02043

Clerissi, C., Guillou, L., Escoubas, J.M., Toulza, E., 2020. Unveiling protist diversity associated with the Pacific oyster *Crassostrea gigas* using blocking and excluding primers. BMC Microbiol. 20, 1–13. https://doi.org/10.1186/s12866-020-01860-1

Cohen, E.B.D., Ilan, M., Yarden, O., 2021. The culturable mycobiome of mesophotic *Agelas oroides*: Constituents and changes following sponge transplantation to shallow water. J. Fungi 7, 13–18. https://doi.org/10.3390/jof7070567

Cox, G., Larkum, A.W.D., 1983. A diatom apparently living in symbiosis with a sponge. Bull. Mar. Sci. 33, 943–945

Cox, G.C., Hiller, R.G., Larkum, A.W.D., 1985. An unusual cyanophyte, containing phycourobilin and symbiotic with ascidians and sponges. Mar. Biol. 89, 149–163. https://doi.org/10.1007/BF00392886

Cross, K.L., Campbell, J.H., Balachandran, M., Campbell, A.G., Cooper, C.J., Griffen, A., Heaton, M., Joshi, S., Klingeman, D., Leys, E., Yang, Z., Parks, J.M., Podar, M., 2019. Targeted isolation and cultivation of uncultivated bacteria by reverse genomics. Nat. Biotechnol. 37, 1314–1321. https://doi.org/10.1038/s41587-019-0260-6

Custodio, M.R., Imsiecke, G., Borojevic, R., Rinkevich, B., Rogerson, A., Müller, W.E.G., 1995. Evolution of Cell Adhesion Systems: Evidence for Arg-Gly-Asp-Mediated Adhesion in the Protozoan *Neoparamoeba aestuarina*. J. Eukaryot. Microbiol. 42, 721–724. https://doi.org/10.1111/j.1550-7408.1995.tb01623.x

Cuypers, E., Abdel-Mottaleb, Y., Kopljar, I., Rainier, J.D., Raes, A.L., Snyders, D.J., Tytgat, J., 2008. Gambierol, a toxin produced by the dinoflagellate *Gambierdiscus toxicus*, is a potent blocker of voltage-gated potassium channels. Toxicon 51, 974–983. https://doi.org/10.1016/j.toxicon.2008.01.004

Dawson, S.C., Hagen, K.D., 2009. Mapping the protistan "rare biosphere." J. Biol. 8, 105.1-105.5. https://doi.org/10.1186/jbiol201

de Kluijver, A., Nierop, K.G.J., Morganti, T.M., Bart, M.C., Slaby, B.M., Hanz, U., de Goeij, J.M., Mienis, F., Middelburg, J.J., 2021. Bacterial precursors and unsaturated long-chain fatty acids are biomarkers of North-Atlantic deep-sea demosponges. PLoS One 16, 1–18. https://doi.org/10.1371/journal.pone.0241095

de Vargas, C., Audic, S., Henry, N., Decelle, J., Mahé, F., Logares, R., Lara, E., Berney, C., Le Bescot, N., Probert, I., Carmichael, M., Poulain, J., Romac, S., Colin, S., Aury, J.-M., Bittner, L., Chaffron, S., Dunthorn, M., Engelen, S., Flegontova, O., Guidi, L., Horák, A., … Velayoudon, D., 2015. Eukaryotic plankton diversity in the sunlit ocean. Science. 348, 1–11. https://doi.org/10.1126/science.1261605

del Campo, J., Pombert, J.-F., Šlapeta, J., Larkum, A., Keeling, P.J., 2017. The 'other' coral symbiont: *Ostreobium* diversity and distribution. ISME J. 11, 296–299. https://doi.org/10.1038/ismej.2016.101

del Campo, J., Pons, M.J., Herranz, M., Wakeman, K.C., del Valle, J., Vermeij, M.J.A., Leander, B.S., Keeling, P.J., 2019. Validation of a universal set of primers to study animal-associated

microeukaryotic communities. Environ. Microbiol. 21, 3855–3861. https://doi.org/10.1111/1462-2920.14733
del Campo, J., Bass, D., Keeling, P.J., 2020. The eukaryome: Diversity and role of microeukaryotic organisms associated with animal hosts. Funct. Ecol. 34, 2045–2054. https://doi.org/10.1111/1365-2435.13490
Della Sala, G., Hochmuth, T., Teta, R., Costantino, V., Mangoni, A., 2014. Polyketide Synthases in the Microbiome of the Marine Sponge *Plakortis halichondrioides*: A Metagenomic Update. Mar. Drugs 12, 5425–5440. https://doi.org/10.3390/md12115425
Douglas, A.E., 2014. Symbiosis as a General Principle in Eukaryotic Evolution. Cold Spring Harb. Perspect. Biol. 6, 1–13. https://doi.org/10.1101/cshperspect.a016113
Dubilier, N., Bergin, C., Lott, C., 2008. Symbiotic diversity in marine animals: the art of harnessing chemosynthesis. Nat. Rev. Microbiol. 6, 725–740. https://doi.org/10.1038/nrmicro1992
Dyková, I., Nowak, B., Pecková, H., Fiala, I., Crosbie, P., Dvoráková, H., 2007. Phylogeny of *Neoparamoeba* strains isolated from marine fish and invertebrates as inferred from SSU rDNA sequences. Dis. Aquat. Organ. 74, 57–65. https://doi.org/10.3354/dao074057
Dyková, I., Fiala, I., Pecková, H., 2008. *Neoparamoeba* spp. and their eukaryotic endosymbionts similar to *Perkinsela amoebae* (Hollande, 1980): Coevolution demonstrated by SSU rRNA gene phylogenies. Eur. J. Protistol. 44, 269–277. https://doi.org/10.1016/j.ejop.2008.01.004
Edgar, R.C., 2018. Updating the 97% identity threshold for 16S ribosomal RNA OTUs. Bioinformatics 34, 2371–2375. https://doi.org/10.1093/bioinformatics/bty113
Epstein, S.S., 2013. The phenomenon of microbial uncultivability. Curr. Opin. Microbiol. 16, 636–642. https://doi.org/10.1016/j.mib.2013.08.003
Fernandez, J., Candenas, M., Souto, M., Trujillo, M., Norte, M., 2012. Okadaic Acid, Useful Tool for Studying Cellular Processes. Curr. Med. Chem. 9, 229–262. https://doi.org/10.2174/0929867023371247
Feussner, K. -D., Skelton, P.A., South, G.R., Alderslade, P., Aalbersberg, W., 2004. *Ostreobium quekettii* (Ostreobiaceae: Chlorophyceae) invading the barnacle *Acasta* sp. (Pendunculata: Acastinae), endozoic in the octocoral *Rumphella suffruticosa* (Alcyonacea: Gorgoniidae) from Fiji, south pacific. New Zeal. J. Mar. Freshw. Res. 38, 87–90. https://doi.org/10.1080/00288330.2004.9517220
Fieseler, L., Horn, M., Wagner, M., Hentschel, U., 2004. Discovery of the novel candidate phylum "Poribacteria" in marine sponges. Appl. Environ. Microbiol. 70, 3724–3732. https://doi.org/10.1128/AEM.70.6.3724-3732.2004
Fisch, K.M., Gurgui, C., Heycke, N., van der Sar, S.A., Anderson, S.A., Webb, V.L., Taudien, S., Platzer, M., Rubio, B.K., Robinson, S.J., Crews, P., Piel, J., 2009. Polyketide assembly lines of uncultivated sponge symbionts from structure-based gene targeting. Nat. Chem. Biol. 5, 494–501. https://doi.org/10.1038/nchembio.176
Forster, D., Lentendu, G., Filker, S., Dubois, E., Wilding, T.A., Stoeck, T., 2019. Improving eDNA-based protist diversity assessments using networks of amplicon sequence variants. Environ. Microbiol. 21, 4109–4124. https://doi.org/10.1111/1462-2920.14764
Gaino, E., Sara, M., 1994. Siliceous spicules of *Tethya seychellensis* (Porifera) support the growth of a green alga: a possible light conducting system. Mar. Ecol. Prog. Ser. 108, 147–151. https://doi.org/10.3354/meps108147
Gaino, E., Bavestrello, G., Cattaneo-Vietti, R., Sarà, M., 1994. Scanning electron microscope evidence for diatom uptake by two Antarctic sponges. Polar Biol. 14, 55–58. https://doi.org/10.1007/BF00240273
Gao, Z., Karlsson, I., Geisen, S., Kowalchuk, G., Jousset, A., 2019. Protists: Puppet Masters of the Rhizosphere Microbiome. Trends Plant Sci. 24, 165–176. https://doi.org/10.1016/j.tplants.2018.10.011
Garson, M.J., Flowers, A.E., Webb, R.I., Charan, R.D., McCaffrey, E.J., 1998. A sponge/dinoflagellate association in the haplosclerid sponge *Haliclona* sp.: Cellular origin of cytotoxic alkaloids by Percoll density gradient fractionation. Cell Tissue Res. 293, 365–373. https://doi.org/10.1007/s004410051128
Gast, R.J., Moran, D.M., Audemard, C., Lyons, M.M., DeFavari, J., Reece, K.S., Leavitt, D., Smolowitz, R., 2008. Environmental distribution and persistence of Quahog Parasite Unknown (QPX). Dis. Aquat. Organ. 81, 219–229. https://doi.org/10.3354/dao01948
Geahchan, S., Ehrlich, H., & Rahman, M. A. (2021). The anti-viral applications of marine resources for COVID-19 treatment: An overview. Marine Drugs, 19(8). https://doi.org/10.3390/md19080409
Geraghty, S., Koutsouveli, V., Hall, C., Chang, L., Sacristan-Soriano, O., Hill, M., Riesgo, A., Hill, A., 2021. Establishment of Host–Algal Endosymbioses: Genetic Response to Symbiont Versus Prey in a Sponge Host. Genome Biol. Evol. 13, 1–16. https://doi.org/10.1093/gbe/evab252

Gloeckner, V., Wehrl, M., Moitinho-Silva, L., Gernert, C., Schupp, P., Pawlik, J. R., Lindquist, N. L., Erpenbeck, D., Wörheide, G., & Hentschel, U. (2014). The HMA-LMA Dichotomy Revisited: an Electron Microscopical Survey of 56 Sponge Species. The Biological Bulletin, 227(1), 78–88. https://doi.org/10.1086/BBLv227n1p78

Granville, R., Nordmann, E., 1971. Maastrichtian adherent foraminifera encircling clionid pores. Bull. geol. Soc. Denmark 20, 362–368.

Gruebl, T., Frischer, M.E., Sheppard, M., Neumann, M., Maurer, A.N., Lee, R.F., 2002. Development of an 18S rRNA gene-targeted PCR-based diagnostic for the blue crab parasite *Hematodinium* sp. Dis. Aquat. Organ. 49, 61–70. https://doi.org/10.3354/dao049061

Guilbault, J.P., Krautter, M., Conway, K.W., Barrie, J.V., 2006. Modern foraminifera attached to hexactinellid sponge meshwork on the west Canadian shelf: Comparison with jurassic counterparts from Europe. Palaeontol. Electron. 9, 1–48.

Guillou, L., Bachar, D., Audic, S., Bass, D., Berney, C., Bittner, L., Boutte, C., Burgaud, G., De Vargas, C., Decelle, J., Del Campo, J., Dolan, J.R., Dunthorn, M., Edvardsen, B., Holzmann, M., Kooistra, W.H.C.F., Lara, E., Le Bescot, N., Logares, R., Mahé, F., Massana, R., Montresor, M., Morard, R., Not, F., Pawlowski, J., Probert, I., Sauvadet, A.L., Siano, R., Stoeck, T., Vaulot, D., Zimmermann, P., Christen, R., 2013. The Protist Ribosomal Reference database (PR2): A catalog of unicellular eukaryote Small Sub-Unit rRNA sequences with curated taxonomy. Nucleic Acids Res. 41, 597–604. https://doi.org/10.1093/nar/gks1160

Hall, C., Camilli, S., Dwaah, H., Kornegay, B., Lacy, C., Hill, M.S., Hill, A.L., 2021. Freshwater sponge hosts and their green algae symbionts: a tractable model to understand intracellular symbiosis. PeerJ 9, e10654. https://doi.org/10.7717/peerj.10654

Hampl, V., Hug, L., Leigh, J.W., Dacks, J.B., Lang, B.F., Simpson, A.G.B., Roger, A.J., 2009. Phylogenomic analyses support the monophyly of Excavata and resolve relationships among eukaryotic "supergroups." Proc. Natl. Acad. Sci. 106, 3859–3864. https://doi.org/10.1073/pnas.0807880106

Hardoim, C.C.P., Lôbo-Hajdu, G., Custódio, M.R., Hardoim, P.R., 2021. Prokaryotic, Fungal, and Unicellular Eukaryotic Core Communities Across Three Sympatric Marine Sponges From the Southwestern Atlantic Coast Are Dominated Largely by Deterministic Assemblage Processes. Front. Microbiol. 12. https://doi.org/10.3389/fmicb.2021.674004

He, L., Liu, F., Karuppiah, V., Ren, Y., Li, Z., 2014. Comparisons of the Fungal and Protistan Communities among Different Marine Sponge Holobionts by Pyrosequencing. Microb. Ecol. 67, 951–961. https://doi.org/10.1007/s00248-014-0393-6

Hentschel, U., Fieseler, L., Wehrl, M., Gernert, C., Steinert, M., Hacker, J., Horn, M., 2003. Microbial Diversity of Marine Sponges, in: Progress in Molecular and Subcellular Biology. pp. 59–88. https://doi.org/10.1007/978-3-642-55519-0_3

Hentschel, U., Usher, K.M., Taylor, M.W., 2006. Marine sponges as microbial fermenters. FEMS Microbiol. Ecol. 55, 167–177. https://doi.org/10.1111/j.1574-6941.2005.00046.x

Hill, M., Allenby, A., Ramsby, B., Schönberg, C., Hill, A., 2011. *Symbiodinium* diversity among host clionaid sponges from Caribbean and Pacific reefs: Evidence of heteroplasmy and putative host-specific symbiont lineages. Mol. Phylogenet. Evol. 59, 81–88. https://doi.org/10.1016/j.ympev.2011.01.006

Hill, M., Walter, C., Bartels, E., 2016. A mass bleaching event involving clionaid sponges. Coral Reefs 35, 153. https://doi.org/10.1007/s00338-016-1402-7

Hochmuth, T., Niederkrüger, H., Gernert, C., Siegl, A., Taudien, S., Platzer, M., Crews, P., Hentschel, U., Piel, J., 2010. Linking chemical and microbial diversity in marine sponges: Possible role for Poribacteria as producers of methyl-branched fatty acids. ChemBioChem 11, 2572–2578. https://doi.org/10.1002/cbic.201000510

Hume, B.C.C., Ziegler, M., Poulain, J., Pochon, X., Romac, S., Boissin, E., de Vargas, C., Planes, S., Wincker, P., Voolstra, C.R., 2018. An improved primer set and amplification protocol with increased specificity and sensitivity targeting the *Symbiodinium* ITS2 region. PeerJ 2018, 1–22. https://doi.org/10.7717/peerj.4816

Indraningrat, A.A.G., Smidt, H., Sipkema, D., 2016. Bioprospecting sponge-associated microbes for antimicrobial compounds. Mar. Drugs 14, 1–66. https://doi.org/10.3390/md14050087

Kaluzhnaya, O.V., Lipko, I.A., Itskovich, V.B., 2021. PCR-screening of bacterial strains isolated from the microbiome of the *Lubomirskia baicalensis* sponge for the presence of secondary metabolite synthesis genes. Limnol. Freshw. Biol. 2021, 1137–1142. https://doi.org/10.31951/2658-3518-2021-A-2-1137

Kamat, P.K., Rai, S., Swarnkar, S., Shukla, R., Nath, C., 2014. Molecular and Cellular Mechanism of Okadaic Acid (OKA)-Induced Neurotoxicity: A Novel Tool for Alzheimer's Disease Therapeutic Application. Mol. Neurobiol. 50, 852–865. https://doi.org/10.1007/s12035-014-8699-4

Katris, N.J., van Dooren, G.G., McMillan, P.J., Hanssen, E., Tilley, L., Waller, R.F., 2014. The Apical Complex Provides a Regulated Gateway for Secretion of Invasion Factors in *Toxoplasma*. PLoS Pathog. 10, e1004074. https://doi.org/10.1371/journal.ppat.1004074

Keeling, P.J., Burger, G., Durnford, D.G., Lang, B.F., Lee, R.W., Pearlman, R.E., Roger, A.J., Gray, M.W., 2005. The tree of eukaryotes. Trends Ecol. Evol. 20, 670–676. https://doi.org/10.1016/j.tree.2005.09.005

Keeling, P.J., Campo, J. del, 2017. Marine Protists Are Not Just Big Bacteria. Curr. Biol. 27, R541–R549. https://doi.org/10.1016/j.cub.2017.03.075

Keeling, P.J., Burki, F., Wilcox, H.M., Allam, B., Allen, E.E., Amaral-Zettler, L.A., Armbrust, E.V., Archibald, J.M., Bharti, A.K., Bell, C.J., Beszteri, B., Bidle, K.D., Cameron, C.T., Campbell, L., Caron, D.A., Cattolico, R.A., Collier, J.L., Coyne, K., Davy, S.K., … Worden, A.Z., 2014. The Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP): Illuminating the Functional Diversity of Eukaryotic Life in the Oceans through Transcriptome Sequencing. PLoS Biol. 12. https://doi.org/10.1371/journal.pbio.1001889

Keeling, P.J., 2019. Combining morphology, behaviour and genomics to understand the evolution and ecology of microbial eukaryotes. Philos. Trans. R. Soc. B Biol. Sci. 374, 20190085. https://doi.org/10.1098/rstb.2019.0085

Keeling, P.J., Mathur, V., Kwong, W.K., 2021. Corallicolids: The elusive coral-infecting apicomplexans. PLoS Pathog. 17, 1–8. https://doi.org/10.1371/journal.ppat.1009845

Kita, M., Ohno, O., Han, C., Uemura, D., 2010. Bioactive secondary metabolites from symbiotic marine dinoflagellates: symbiodinolide and durinskiols. Chem. Rec. 10, 57–69. https://doi.org/10.1002/tcr.200900007

Klautau, M., Custodio, M.R., Borojevic, R., 1993. Cell Cultures of Sponges *Clathrina* and *Polymastia*. Vitr. Cell. 29, 97–99.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glöckner, F.O., 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 41, 1–11. https://doi.org/10.1093/nar/gks808

Klitgaard, A.B., 1995. The fauna associated with outer shelf and upper slope sponges (Porifera, Demospongiae) at the Faroe Islands, northeastern Atlantic. Sarsia 80, 1–22. https://doi.org/10.1080/00364827.1995.10413574

Kohli, G.S., John, U., Van Dolah, F.M., Murray, S.A., 2016. Evolutionary distinctiveness of fatty acid and polyketide synthesis in eukaryotes. ISME J. 10, 1877–1890. https://doi.org/10.1038/ismej.2015.263

Kramarsky-Winter, E., Harel, M., Siboni, N., Ben Dov, E., Brickner, I., Loya, Y., Kushmaro, A., 2006. Identification of a protist-coral association and its possible ecological role. Mar. Ecol. Prog. Ser. 317, 67–73. https://doi.org/10.3354/meps317067

Lambert, G., Lambert, C.C., Waaland, J.R., 1996. Algal Symbionts in the Tunics of Six New Zealand Ascidians (Chordata, Ascidiacea). Invertebr. Biol. 115, 67–78. https://doi.org/10.2307/3226942

Linke, P., Lutze, G.F., 1993. Microhabitat preferences of benthic foraminifera—a static concept or a dynamic adaptation to optimize food acquisition? Mar. Micropaleontol. 20, 215–234. https://doi.org/10.1016/0377-8398(93)90034-U

Lintner, B., Lintner, M., Wollenburg, J., Wurz, E., Heinz, P., 2021. Foraminifera-sponge interactions – commensalism to parasitism in the Norwegian-Greenland Sea, in: EGU General Assembly. vEGU21, p. 1. https://doi.org/https://doi.org/10.5194/egusphere-egu21-12913

Liu, Q., Allam, B., Collier, J.L., 2009. Quantitative Real-Time PCR Assay for QPX (Thraustochytriidae), a Parasite of the Hard Clam (*Mercenaria mercenaria*). Appl. Environ. Microbiol. 75, 4913–4918. https://doi.org/10.1128/AEM.00246-09

Liu, C., Qi, R.J., Jiang, J.Z., Zhang, M.Q., Wang, J.Y., 2019. Development of a Blocking Primer to Inhibit the PCR Amplification of the 18S rDNA Sequences of *Litopenaeus vannamei* and Its Efficacy in *Crassostrea hongkongensis*. Front. Microbiol. 10, 1–15. https://doi.org/10.3389/fmicb.2019.00830

Lopanik, N.B., 2014. Chemical defensive symbioses in the marine environment. Funct. Ecol. 28, 328–340. https://doi.org/10.1111/1365-2435.12160

López-García, P., Eme, L., Moreira, D., 2017. Symbiosis in eukaryotic evolution. J. Theor. Biol. 434, 20–33. https://doi.org/10.1016/j.jtbi.2017.02.031

Lukeš, J., Stensvold, C.R., Jirků-Pomajbíková, K., Wegener Parfrey, L., 2015. Are Human Intestinal Eukaryotes Beneficial or Commensals? PLoS Pathog. 11, 7–12. https://doi.org/10.1371/journal.ppat.1005039

Maldonado, M., López-Acosta, M., Sitjá, C., Aguilar, R., García, S., Vacelet, J., 2013. A giant foraminifer that converges to the feeding strategy of carnivorous sponges: *Spiculosiphon oceana* sp. Nov. (Foraminifera, Astrorhizida). Zootaxa 3669, 571–584. https://doi.org/10.11646/zootaxa.3669.4.9

Marchan, L.F., Lee Chang, K.J., Nichols, P.D., Mitchell, W.J., Polglase, J.L., Gutierrez, T., 2018. Taxonomy, ecology and biotechnological applications of thraustochytrids: A review. Biotechnol. Adv. 36, 26–46. https://doi.org/10.1016/j.biotechadv.2017.09.003

Mariani, S., Piscitelli, M.P., Uriz, M.J., 2001. Temporal and spatial co-occurrence in spawning and larval release of *Cliona viridis* (Porifera: Hadromerida). J. Mar. Biol. Assoc. United Kingdom 81, 565–567. https://doi.org/10.1017/S0025315401004246

Mazzoli-dias, M., Ribeiro, S.M., Oliveira-silva, P., 2007. Foraminifera associated to the sponge *Mycale microsigmatosa* in Rio de Janeiro State , southeastern Brazil - an initial approach, in: Custódio, M.R., Lôbo-Hajdu, G., Hajdu, E., Muricy, G. (Eds.), Algae. Museu Nacional, Rio de Janeiro, pp. 439–442.

Mehbub, M.F., Perkins, M. V., Zhang, W., Franco, C.M.M., 2016. New marine natural products from sponges (Porifera) of the order Dictyoceratida (2001 to 2012); a promising source for drug discovery, exploration and future prospects. Biotechnol. Adv. 34, 473–491. https://doi.org/10.1016/j.biotechadv.2015.12.008

Mies, M., Sumida, P.Y.G., Rädecker, N., Voolstra, C.R., 2017. Marine Invertebrate Larvae Associated with *Symbiodinium*: A Mutualism from the Start? Front. Ecol. Evol. 5. https://doi.org/10.3389/fevo.2017.00056

Minardi, D., Ryder, D., Campo, J., Garcia Fonseca, V., Kerr, R., Mortensen, S., Pallavicini, A., Bass, D., 2021. Improved high throughput protocol for targeting eukaryotic symbionts in metazoan and eDNA samples. Mol. Ecol. Resour. 1–15. https://doi.org/10.1111/1755-0998.13509

Moitinho-Silva, L., Díez-Vives, C., Batani, G., Esteves, A.I.S., Jahn, M.T., Thomas, T., 2017. Integrated metabolism in sponge-microbe symbiosis revealed by genome-centered metatranscriptomics. ISME J. 11, 1651–1666. https://doi.org/10.1038/ismej.2017.25

Morabito, C., Bournaud, C., Maës, C., Schuler, M., Aiese Cigliano, R., Dellero, Y., Maréchal, E., Amato, A., Rébeillé, F., 2019. The lipid metabolism in thraustochytrids. Prog. Lipid Res. 76, 101007. https://doi.org/10.1016/j.plipres.2019.101007

Müller, W.E.G., Belikov, S.I., Kaluzhnaya, O. V., Perović-Ottstadt, S., Fattorusso, E., Ushijima, H., Krasko, A., Schröder, H.C., 2007. Cold stress defense in the freshwater sponge *Lubomirskia baicalensis*. FEBS J. 274, 23–36. https://doi.org/10.1111/j.1742-4658.2006.05559.x

Müller, W.E., Belikov, S.I., Kaluzhnaya, O. V, Chernogor, L., Krasko, A., Schröder, H.C., 2009. Symbiotic interaction between dinoflagellates and the demosponge *Lubomirskia baicalensis*: aquaporin-mediated glycerol transport. Prog. Mol. Subcell. Biol. 47, 145–170. https://doi.org/10.1007/978-3-540-88552-8_6

Needham, D.M., Sachdeva, R., Fuhrman, J.A., 2017. Ecological dynamics and co-occurrence among marine phytoplankton, bacteria and myoviruses shows microdiversity matters. ISME J. 11, 1614–1629. https://doi.org/10.1038/ismej.2017.29

Oborník, M., Modrý, D., Lukeš, M., Černotíková-Stříbrná, E., Cihlář, J., Tesařová, M., Kotabová, E., Vancová, M., Prášil, O., Lukeš, J., 2012. Morphology, Ultrastructure and Life Cycle of *Vitrella brassicaformis* n. sp., n. gen., a Novel Chromerid from the Great Barrier Reef. Protist 163, 306–323. https://doi.org/10.1016/j.protis.2011.09.001

Pawlowski, J., 2014. Protist Evolution and Phylogeny, in: ELS. Wiley, Chichester, UK. https://doi.org/10.1002/9780470015902.a0001935.pub2

Peay, K.G., Kennedy, P.G., Talbot, J.M., 2016. Dimensions of biodiversity in the Earth mycobiome. Nat. Rev. Microbiol. 14, 434–447. https://doi.org/10.1038/nrmicro.2016.59

Pierella Karlusich, J.J., Ibarbalz, F.M., Bowler, C., 2020. Phytoplankton in the Tara Ocean. Ann. Rev. Mar. Sci. 12, 233–265. https://doi.org/10.1146/annurev-marine-010419-010706

Podell, S., Blanton, J.M., Neu, A., Agarwal, V., Biggs, J.S., Moore, B.S., Allen, E.E., 2019. Pangenomic comparison of globally distributed Poribacteria associated with sponge hosts and marine particles. ISME J. 13, 468–481. https://doi.org/10.1038/s41396-018-0292-9

Pollock, F.J., Morris, P.J., Willis, B.L., Bourne, D.G., 2011. The Urgent Need for Robust Coral Disease Diagnostics. PLoS Pathog. 7, e1002183. https://doi.org/10.1371/journal.ppat.1002183

Prodan, A., Tremaroli, V., Brolin, H., Zwinderman, A.H., Nieuwdorp, M., Levin, E., 2020. Comparing bioinformatic pipelines for microbial 16S rRNA amplicon sequencing. PLoS One 15, e0227434. https://doi.org/10.1371/journal.pone.0227434

Pronzato, R., Manconi, R., 2008. Mediterranean commercial sponges: Over 5000 years of natural history and cultural heritage. Mar. Ecol. 29, 146–166. https://doi.org/10.1111/j.1439-0485.2008.00235.x

Raghukumar, S., 2002. Ecology of the marine protists, the labyrinthulomycetes (thraustochytrids and labyrinthulids). Eur. J. Protistol. 38, 127–145. https://doi.org/10.1078/0932-4739-00832

Redmond, A.K., McLysaght, A., 2021. Evidence for sponges as sister to all other animals from partitioned phylogenomics with mixture models and recoding. Nat. Commun. 12. https://doi.org/10.1038/s41467-021-22074-7

Regoli, F., Nigro, M., Chierici, E., Cerrano, C., Schiapparelli, S., Totti, C., Bavestrello, G., 2004. Variations of antioxidant efficiency and presence of endosymbiotic diatoms in the Antarctic Porifera *Haliclona dancoi*. Mar. Environ. Res. 58, 637–640. https://doi.org/10.1016/j.marenvres.2004.03.055

Ribes, M., Jiménez, E., Yahel, G., López-Sendino, P., Diez, B., Massana, R., Sharp, J.H., Coma, R., 2012. Functional convergence of microbes associated with temperate marine sponges. Environ. Microbiol. 14, 1224–1239. https://doi.org/10.1111/j.1462-2920.2012.02701.x

Richelle-Maurer, E., Gomez, R., Braekman, J.C., Van De Vyver, G., Van Soest, R.W.M., Devijver, C., 2003. Primary cultures from the marine sponge *Xestospongia muta* (Petrosiidae, Haplosclerida). J. Biotechnol. 100, 169–176. https://doi.org/10.1016/S0168-1656(02)00251-1

Rinkevich, B., Blisko, R., Ilan, M., 1998. Further steps in the initiation of cell cultures from embryos and adult sponge colonies. Vitr. Cell. Dev. Biol. - Anim. 34, 753–756. https://doi.org/10.1007/s11626-998-0028-7

Rinkevich, B., 1999. Cell cultures from marine invertebrates: obstacles, new approaches and recent improvements. Prog. Ind. Microbiol. 70, 133–153. https://doi.org/10.1016/S0079-6352(99)80107-6

Ríos, R., Aranguren, R., Gastaldelli, M., Arcangeli, G., Novoa, B., Figueras, A., 2020. Development and validation of a specific real-time PCR assay for the detection of the parasite *Perkinsus olseni*. J. Invertebr. Pathol. 169, 1–8. https://doi.org/10.1016/j.jip.2019.107301

Rodríguez-Marconi, S., De la Iglesia, R., Díez, B., Fonseca, C.A., Hajdu, E., Trefault, N., 2015. Characterization of Bacterial, Archaeal and Eukaryote Symbionts from Antarctic Sponges Reveals a High Diversity at a Three-Domain Level and a Particular Signature for This Ecosystem. PLoS One 10, e0138837. https://doi.org/10.1371/journal.pone.0138837

Rodríguez-Zaragoza, S., 1994. Ecology of Free-Living Amoebae. Crit. Rev. Microbiol. 20, 225–241. https://doi.org/10.3109/10408419409114556

Roger, A.J., Simpson, A.G.B., 2009. Evolution: Revisiting the Root of the Eukaryote Tree. Curr. Biol. 19, R165–R167. https://doi.org/10.1016/j.cub.2008.12.032

Rogerson, A., Gwaltney, C., 2000. High numbers of naked amoebae in the planktonic waters of a mangrove stand in southern Florida, USA. J. Eukaryot. Microbiol. 47, 235–241. https://doi.org/10.1111/j.1550-7408.2000.tb00042.x

Saffo, M.B., McCoy, A.M., Rieken, C., Slamovits, C.H., 2010. *Nephromyces*, a beneficial apicomplexan symbiont in marine animals. Proc. Natl. Acad. Sci. U. S. A. 107, 16190–16195. https://doi.org/10.1073/pnas.1002335107

Saller, U., 1989. Microscopical aspects on symbiosis of *Spongilla lacustris* (Porifera, Spongillidae) and green algae. Zoomorphology 108, 291–296. https://doi.org/10.1007/BF00312161

Sarà, M., Bavestrello, G., Cattaneo-vietti, R., Cerrano, C., 1998. Endosymbiosis in sponges: Relevance for epigenesis and evolution. Symbiosis 25, 57–70.

Schärer, L., Knoflach, D., Vizoso, D.B., Rieger, G., Peintner, U., 2007. Thraustochytrids as novel parasitic protists of marine free-living flatworms: *Thraustochytrium caudivorum* sp. nov. parasitizes *Macrostomum lignano*. Mar. Biol. 152, 1095–1104. https://doi.org/10.1007/s00227-007-0755-4

Schleinkofer, N., Evans, D., Wisshak, M., Büscher, J.V., Fiebig, J., Freiwald, A., Härter, S., Marschall, H.R., Voigt, S., Raddatz, J., 2021. Host-influenced geochemical signature in the parasitic foraminifera *Hyrrokkin sarcophaga*. Biogeosciences 18, 4733–4753. https://doi.org/10.5194/bg-18-4733-2021

Schönberg, C.H.L., Loh, W.K.W., 2005. Molecular identity of the unique symbiotic dinoflagellates found in the bioeroding demosponge *Cliona orientalis*. Mar. Ecol. Prog. Ser. 299, 157–166. https://doi.org/10.3354/meps299157

Schönberg, C.H.L., Suwa, R., 2007. Why bioeroding sponges may be better hosts for symbiotic dinoflagellates than many corals, in: Custodio, M.R., Lôbo-Hajdu, G., Hajdu, E., Muricy, G. (Eds.), Porifera Research: Biodiversity, Innovation and Sustainability. Museu Nacional, Rio de janeiro, pp. 569–580.

Schulz, F., Tyml, T., Pizzetti, I., Dyková, I., Fazi, S., Kostka, M., Horn, M., 2015. Marine amoebae with cytoplasmic and perinuclear symbionts deeply branching in the Gammaproteobacteria. Sci. Rep. 5, 13381. https://doi.org/10.1038/srep13381

Seki, T., Satake, M., Mackenzie, L., Kaspar, H.F., Yasumoto, T., 1995. Gymnodimine, a new marine toxin of unprecedented structure isolated from New Zealand oysters and the dinoflagellate, *Gymnodinium* sp. Tetrahedron Lett. 36, 7093–7096. https://doi.org/10.1016/0040-4039(95)01434-J

Shady, N.H., Hayallah, A.M., Mohamed, M.F.A., Ghoneim, M.M., Chilingaryan, G., Al-Sanea, M.M., Fouad, M.A., Kamel, M.S., Abdelmohsen, U.R., 2021. Targeting 3CLpro and SARS-CoV-2 RdRp by *Amphimedon* sp. Metabolites: A Computational Study. Molecules 26, 3775. https://doi.org/10.3390/molecules26123775

Shinzato, C., Hamada, M., Shoguchi, E., Kawashima, T., Satoh, N., 2012. The Repertoire of Chemical Defense Genes in the Coral *Acropora digitifera* Genome. Zoolog. Sci. 29, 510. https://doi.org/10.2108/zsj.29.510

Simister, R.L., Deines, P., Botté, E.S., Webster, N.S., Taylor, M.W., 2012. Sponge-specific clusters revisited: a comprehensive phylogeny of sponge-associated microorganisms. Environ. Microbiol. 14, 517–524. https://doi.org/10.1111/j.1462-2920.2011.02664.x

Simpson, A.G.B., Slamovits, C.H., Archibald, J.M., 2017. Protist Diversity and Eukaryote Phylogeny, in: Archibald, J.M., Simpson, A.G.B., Slamovits, C.H., Margulis, L., Melkonian, M., Chapman, D.J., Corliss, J.O. (Eds.), Handbook of the Protists. Springer International Publishing, Cham, pp. 1–21. https://doi.org/10.1007/978-3-319-32669-6_45-1

Staley, J., Konopka, A., 1985. Measurement of *in situ* Activities of Nonphotosynthetic Microorganisms in Aquatic and Terrestrial Habitats. Annu. Rev. Microbiol. 39, 321–346. https://doi.org/10.1146/annurev.micro.39.1.321

Steinert, G., Stauffer, C.H., Aas-Valleriani, N., Borchert, E., Bhushan, A., Campbell, A., De Mares, M.C., Costa, M., Gutleben, J., Knobloch, S., Lee, R.G., Munroe, S., Naik, D., Peters, E.E., Stokes, E., Wang, W., Einarsdóttir, E., Sipkema, D., 2018. BluePharmTrain: Biology and Biotechnology of Marine Sponges, in: Grand Challenges in Biology and Biotechnology. pp. 505–553. https://doi.org/10.1007/978-3-319-69075-9_13

Suryanarayanan, T. S. (2012). The diversity and importance of fungi associated with marine sponges. Botanica Marina, 55(6), 553–564. https://doi.org/10.1515/bot-2011-0086

Tachibana, K., Scheuer, P.J., Tsukitani, Y., Kikuchi, H., Van Engen, D., Clardy, J., Gopichand, Y., Schmitz, F.J., 1981. Okadaic Acid, a Cytotoxic Polyether from Two Marine Sponges of the Genus *Halichondria*. J. Am. Chem. Soc. 103, 2469–2471. https://doi.org/10.1021/ja00399a082

Taylor, M.W., Radax, R., Steger, D., Wagner, M., 2007. Sponge-Associated Microorganisms: Evolution, Ecology, and Biotechnological Potential. Microbiol. Mol. Biol. Rev. 71, 295–347. https://doi.org/10.1128/mmbr.00040-06

Thomas, T.R.A., Kavlekar, D.P., LokaBharathi, P.A., 2010. Marine Drugs from Sponge-Microbe Association—A Review. Mar. Drugs 8, 1417–1468. https://doi.org/10.3390/md8041417

Thomas, T., Moitinho-Silva, L., Lurgi, M., Björk, J.R., Easson, C., Astudillo-García, C., Olson, J.B., Erwin, P.M., López-Legentil, S., Luter, H., Chaves-Fonnegra, A., Costa, R., Schupp, P.J., Steindler, L., Erpenbeck, D., Gilbert, J., Knight, R., Ackermann, G., Victor Lopez, J., Taylor, M.W., Thacker, R.W., Montoya, J.M., Hentschel, U., Webster, N.S., 2016. Diversity, structure and convergent evolution of the global sponge microbiome. Nat. Commun. 7, 11870. https://doi.org/10.1038/ncomms11870

Thompson, L.R., Sanders, J.G., McDonald, D., Amir, A., Ladau, J., Locey, K.J., Prill, R.J., Tripathi, A., Gibbons, S.M., Ackermann, G., Navas-Molina, J.A., Janssen, S., Kopylova, E., Vázquez-Baeza, Y., González, A., Morton, J.T., Mirarab, S., Xu, Z.Z., Jiang, L., … Zhao, H., 2017. A communal catalogue reveals Earth's multiscale microbial diversity. Nature 551, 457–463. https://doi.org/10.1038/nature24621

Trautman, D.A., Hinde, R., 2002. Sponge/Algal Symbioses: A Diversity of Associations, in: Seckbach, J. (Ed.), Symbiosis: Mechanisms and Model Systems. Springer Netherlands, Dordrecht, pp. 521–537. https://doi.org/10.1007/0-306-48173-1_33

Trench, R.K., 1993. Microalgal-invertebrate symbioses: a review. Endocytobiosis Cell Res. 9, 135–175.

Vaca, I., Faúndez, C., Maza, F., Paillavil, B., Hernández, V., Acosta, F., Levicán, G., Martínez, C., Chávez, R., 2013. Cultivable psychrotolerant yeasts associated with Antarctic marine sponges. World J. Microbiol. Biotechnol. 29, 183–189. https://doi.org/10.1007/s11274-012-1159-2

Vacelet, J., Donadey, C., 1977. Electron microscope study of the association between some sponges and bacteria. J. Exp. Mar. Bio. Ecol. 30, 301–314. https://doi.org/10.1016/0022-0981(77)90038-7

Vallesi, A., Pucciarelli, S., Buonanno, F., Fontana, A., Mangiagalli, M., 2020. Bioactive molecules from protists: Perspectives in biotechnology. Eur. J. Protistol. 75, 125720. https://doi.org/10.1016/j.ejop.2020.125720

Voultsiadou, E., 2007. Sponges: An historical survey of their knowledge in Greek antiquity. J. Mar. Biol. Assoc. United Kingdom 87, 1757–1763. https://doi.org/10.1017/S0025315407057773

Waidele, L., Korb, J., Voolstra, C.R., Künzel, S., Dedeine, F., Staubach, F., 2017. Differential Ecological Specificity of Protist and Bacterial Microbiomes across a Set of Termite Species. Front. Microbiol. 8, 1–13. https://doi.org/10.3389/fmicb.2017.02518

Webster, N.S., Taylor, M.W., Behnam, F., Lücker, S., Rattei, T., Whalan, S., Horn, M., Wagner, M., 2010. Deep sequencing reveals exceptional diversity and modes of transmission for bacterial sponge symbionts. Environ. Microbiol. 12, 2070–2082. https://doi.org/10.1111/j.1462-2920.2009.02065.x

Webster, N.S., Taylor, M.W., 2012. Marine sponges and their microbial symbionts: love and other relationships. Environ. Microbiol. 14, 335–346. https://doi.org/10.1111/j.1462-2920.2011.02460.x

Wecker, P., Fournier, A., Bosserelle, P., Debitus, C., Lecellier, G., Berteaux-Lecellier, V., 2015. Dinoflagellate diversity among nudibranchs and sponges from French Polynesia: Insights into associations and transfer. C. R. Biol. 338, 278–283. https://doi.org/10.1016/j.crvi.2015.01.005

Wilkinson, C.R., 1978a. Microbial associations in sponges. I. Ecology, physiology and microbial populations of coral reef sponges. Mar. Biol. 49, 161–167. https://doi.org/10.1007/BF00387115

Wilkinson, C.R., 1978b. Microbial associations in sponges. II. Numerical analysis of sponge and water bacterial populations. Mar. Biol. 49, 169–176. https://doi.org/10.1007/BF00387116

Wilkinson, C.R., 1978c. Microbial associations in sponges. III. Ultrastructure of the *in situ* associations in coral reef sponges. Mar. Biol. 49, 177–185. https://doi.org/10.1007/BF00387117

Wilkinson, C.R., 1987. Significance of microbial symbionts in sponge evolution and ecology. Symbiosis 4, 135–145

Wilkinson, C.R., Garrone, R., Vacelet, J., 1984. Marine sponges discriminate between food bacteria and bacterial symbionts: electron microscope radioautography and *in situ* evidence. Proc. R. Soc. London. Ser. B. Biol. Sci. 220, 519–528. https://doi.org/10.1098/rspb.1984.0018

Williamson, C.E., 1979. An Ultrastructural Investigation of Algal Symbiosis in White and Green *Spongilla lacustris* (L.) (Porifera: Spongillidae). Trans. Am. Microsc. Soc. 98, 59. https://doi.org/10.2307/3225940

Worden, A.Z., Follows, M.J., Giovannoni, S.J., Wilken, S., Zimmerman, A.E., Keeling, P.J., 2015. Rethinking the marine carbon cycle: Factoring in the multifarious lifestyles of microbes. Science. 347, 12575941–125759410. https://doi.org/10.1126/science.1257594

Zhu, J., Liu, Y., Liu, Z., Wang, H., Zhang, H., 2019. Bioactive nitrogenous secondary metabolites from the marine sponge genus *Haliclona*. Mar. Drugs 17, 1–13. https://doi.org/10.3390/md17120682

CAPÍTULO 3

Isolation and characterization of Labyrinthulomycetes associated with *in vitro* cell cultures of two tropical marine sponges

Gabriel Nascimento-Silva; Márcio Reis Custódio

Department of Physiology, Institute of Biosciences, University of São Paulo, Brazil

ABSTRACT

The marine microorganisms of the order Labyrinthulomycetes (Phylum Stramenopiles) have a microfungal-like morphology, osmotrophic metabolism, and saprotrophic habits. They can be found free-living, but also in association with algae, vertebrates and invertebrates as parasites, commensals or mutualists. Labyrinthulomycetes also are one of the main obstacles for the establishment of cell lines of marine invertebrates, with several reports of contamination by these microorganisms in cell cultures of sponges and other organisms. Using culture-dependent approaches, we aimed to investigate the nature of the association between these protists and two tropical marine sponges. By adapting different methods, we successfully isolated two different strains belonging to the Thraustochytrida and one to the Labyrinthulida family from cell cultures of the sponges *Hymeniacidon heliophila* and *Haliclona melana*. The general morphology of the isolated protists was characterized and aspects of its metabolic features and fatty acid profiles were also assessed. Our results indicate that the isolates are different from each other at morphological and biochemical levels. In particular, one strain obtained from *H. melana* has great biotechnological potential. We propose that these microeukaryotes may be associated with Porifera in a commensal or mutualistic manner. This study is the first systematic investigation of the nature of the association between marine sponges and Labyrinthulomycetes, but other approaches are needed for a deeper understanding of this relationship.

Keywords: protists; Stramenopiles; microbiota; culture-dependent; Porifera; microeukaryome

INTRODUCTION

Sponges are known to have associations with multiple phyla of bacteria, archaea, fungi, virus and unicellular eukaryotes (Webster and Taylor, 2012). In certain species up to 40% of the biomass may be composed of these associated microorganisms (Webster and Thomas, 2016). This microbiota is directly related to the physiology of these animals and has been the focus of several studies in recent decades (*e.g.* Hentschel et al. 2006; Taylor et al. 2007; Schmitt et al. 2007; 2012; Webster and Taylor, 2012; Webster et al. 2016). Most of these works focus on the portion of the microbiome composed of bacteria and archaea (Margot et al. 2002; Moitinho-Silva et al. 2017; Steinert et al. 2020), although other groups such as fungi (Suryanarayanan, 2012; Henriquez et al. 2014; Naim et al. 2017) and viruses (Pascelli et al. 2020; Nguyen et al. 2021) have gained attention in recent years. Despite important advances in the study of non-bacterial microorganisms, most of the knowledge is related only to identification, and their role in the holobiont is still unknown.

Other sponge-associated microorganisms that deserve to have their relationship with the host studied in more detail are the protists (He et al. 2014; Rodríguez-Marconi et al. 2015; De Mares et al. 2017). Protists are a paraphyletic group composed single-celled microeukaryotes that includes multiple evolutionary lineages (Pawlowski, 2014; Adl et al. 2019), with a wide diversity of forms and metabolisms. They occupy various ecological niches (Worden et al. 2015; Leray and Knowlton, 2016), as both free-living and associated with other organisms forming a symbiotic continuum (del Campo et al. 2020). One of these groups, common to the microbiome of diverse organisms, is the Stramenopile (Tan, 2008; Siboni et al. 2010; Padmakumar et al. 2012; He et al. 2014; Ceja-Navarro et al. 2021). This group includes the marine microorganisms of the order Labyrinthulomycetes, which have a microfungal-like morphology and osmotrophic metabolism, with saprotrophic habits being a common feature (Raghukumar, 2002; Marchan et al. 2018). They can live in association with algae, vertebrates and invertebrates as parasites, commensals or mutualists, but also can be found as free-living forms (Raghukumar, 2002; 2008). Despite their simple morphology, these microorganisms possess a large metabolic repertoire, which enable a wide variety of biotechnological applications given their different capabilities (Bongiorni et al. 2005; Yamasaki et al. 2006; Raghukumar, 2008; Taoka et al. 2009; Chang et al. 2015; Merkx-Jacques et al. 2018). They are also known as producers of polyunsaturated fatty acids

(PUFAS) such as docosahexaenoic and eicosapentaenoic acids, as well as carotenoids, squalene and polysaccharide exudates (Aki et al. 2003; Raghukumar, 2008; Gupta et al. 2016; Aasen et al. 2016). As parasites these protists cause significant economic impacts, since they can affect invertebrates of human consumption (Gast et al. 2008; Kvingedal et al. 2006; Shinn et al. 2015)

Labyrinthulomycetes are also one of the main obstacles for the establishment of cell cultures of different marine invertebrates (Rinkevich, 1999). There are reports of contamination by these microorganisms in primary culture of sponges, cnidarians, crustaceans, mollusks, echinoderms, tunicates and urochordates (Rinkevich, 1999, 2005; Rabinowitz et al. 2006; Han et al. 2013, Nowotny et al. 2021). Although ubiquitous, this group is so obscure that some researchers have misidentified the vegetative forms of these Stramenopiles as cells from the host animals (ascidians - Rinkevich and Rabinowitz, 1993; sponges - Richelle-Maurer et al. 2003). Regarding Porifera, the nature of the interaction between these protists and sponges is poorly known, and most records refer only to its presence in primary cell cultures or in high-throughput sequencing studies. There are few reports of in-depth study of these protists isolated from healthy or decaying sponges (Höhnk and Ulken, 1979) and the understanding of sponge-associated microeukaryotes is still limited to omics approaches that describe their diversity. Their constant appearance, even after the cultures are established and seemingly axenic, indicates a closer relation than only contamination and deserves further investigation.

Therefore, in this work, we report the first isolations of Labyrinthulomycetes from established *in vitro* cell cultures (primmorphs) of the tropical marine sponges *Hymeniacidon heliophila* and *Haliclona melana*. Three different isolates were obtained using a culture-dependent approach, and its general morphology and some metabolic features were characterized. The polyunsaturated fatty acid profiles (PUFAs) of the protists were also evaluated. Finally, we discuss the nature of the association between these microeukaryotes and Porifera in light of the new evidence obtained.

MATERIALS AND METHODS

Collection of *Hymeniacidon heliophila* and *Haliclona melana*

The marine demosponges *Hymeniacidon heliophila* (Suberitidae) and *Haliclona melana* (Haplosclerida) were collected by freediving or taken from intertidal areas at Praia Grande (23°49'22.5"S, 45°24'57.4"W), São Sebastião Channel, SP - Brazil. We collected those organisms at different times: July of 2019; February and September of 2020, and March 2021. The animals were maintained in running seawater tanks at the Center for Marine Biology of the University of São Paulo (CEBIMar/USP) until the moment of transportation to the laboratory in São Paulo, SP. At the laboratory, the specimens were acclimated to seawater tanks previously assembled with the pH, salinity, and temperature from the local of collection.

In vitro culture of marine sponges

The sponges collected were dissociated in artificial seawater without calcium and magnesium ions and supplemented with EDTA (CMFSW+E: 460 mM NaCl, 7 mM Na_2SO_4, 10 mM KCl, 10 mM, HEPES, 2.5 mM EDTA, pH 8.2) according to Custodio et al. (1998). The individuals were cleaned from epibionts and debris and after that were cut in fragments of approximately 2 mm. The resulting fragments were placed in 50 mL tubes with CMFSW+E for 30 minutes in gentle shaking and the supernatant was discarded. After that, 50 mL of CMFSW+E was added to the tube and was put into agitation for one hour. In the end, the dissociated cells in the supernatant were filtered through a nylon mesh (100 µm) and centrifuged at 250 x g for 10 minutes. The pellet was resuspended in sterile-filtered (0.22 µm) natural seawater and supplemented with penicillin-streptomycin (PS, 0.2 mg/mL) for removal of transient bacteria. The resulting suspension was placed in 6-well culture plates at a concentration of 1.5 x 10^6 cells/mL and the culture medium was changed daily on the first week, then each three days. Formed primmorphs were picked and transferred to new plates to avoid eventual transient fungal and protist contamination present in the initial suspension (Custodio *et al.* 1998).

Isolation and maintenance of Labyrinthulomycetes associated with primmorphs

We use the baiting technique (Raghukumar, 2002) and a low nutrient media. The baiting technique consists of using the saprobic metabolism of those protists and utilizes recalcitrant substrates like pollen and keratinized material to isolate them (Raghukumar, 2008, Caamaño et al. 2017, Boro et al. 2018). Here we use autoclaved cysts of *Artemia* sp. and spongin fibers of *Aplysina fulva* as bait to Labyrinthulomycetes cells, which adhere to the outer surface of those materials. For the culture medium, we use a combination of Yeast Extract, Tryptone, and Dextrose (YTD) in a concentration of 0.01%. We placed previously autoclaved cysts or thin sections of spongin skeleton mesh (approximately 5 mm in diameter) in 6-well culture plates with sterile-filtered natural seawater supplemented with PS as previously described. Subsequently, three to five primmorphs of *Hymeniacidon heliophila* or *Haliclona melana* were added to each well. The 6-well plates were placed in a dark chamber and left at room temperature (24º C ± 2º C) and observed daily in the phase-contrast inverted microscope (Nikon TE300). Around four days, unicellular eukaryotic cells could be observed adhering to the surface of the cyst or the spongin fibers. These baits were then picked and transferred to plates containing YTD-agar (2%) and subcultured until axenic cultures were obtained.

Morphology of the isolates of Labyrinthulomycetes

We use light microscopy for morphological characterization of these protists. In YTD-broth we cultivated the microorganisms in 6-well plates over a period of two weeks, for which daily observations were made. The same was done in solid medium, with plates with thin layers of YTD-agar (2%) to enable detailed observations in inverted microscopy. For standardization, all the measurements of these isolates were taken after one week of cultivation in YTD-agar. Images were taken on a Nikon TE300 inverted microscope with a Nikon D5100 attached. Measurements of morphological characteristics such as average cell diameter (n = 100 cells) and colony size range were obtained using Fiji/ImageJ (Schindelin et al. 2012).

Fatty acid profiles

For the characterization of fatty acid profiles, we grew the isolates for 12 days in YTD-broth. Extraction of total lipids from these cultures was done using the

chloroform-methanol based method (Folch et al. 1957). Methylation was conducted using acetyl chloride (Christie and Han, 2010). The methyl esters were analyzed on a gas chromatograph (Scion 436) equipped with a flame ionization detector (FID), autosampler (CP 8410) and capillary column of 0.25 µm thickness, 0.25 mm internal diameter and 30 m length (CP Wax). The carrier gas was hydrogen with a linear velocity of 1.4 mL/min cm/s. The column temperatures were programmed as 170 ºC for 1 minute, ramped at 2.5 ºC per minute up to 240 ºC and a final hold time of 5 minutes. The injector had a temperature of 250 ºC while the FID was 260 ºC. Identification of the fatty acids was done by comparing the retention times with the retention times obtained from the commercial standard (Sulpeco 37 Component FAME Mix). The data are presented as a percentage of total fatty acids based on the area analysis of each peak.

Metabolic activities of the isolates against different substrates

The activities of cellulases, proteases and lipases of the isolates were tested using qualitative assays on agar plates. Each experiment was done in triplicate using 30 µL of inoculum (around 0.75 x 10^5 cells to 1 x 10^5 cells) from each protist culture. The same procedure was applied for the control plates. For investigation of cellulolytic activity, we applied the carboxymethylcellulose (CMC) degradation assay (modified from Nagano et al. 2011) using YTD-agar (1.5%) supplemented with CMC (0.5% w/v). After five days the plates were stained with a solution of Congo red (0.1% w/v) for 20 minutes at room temperature and then washed twice with a 1M NaCl solution. Cellulolytic activity was demonstrated by the presence of a yellowish halo around the inoculums. For the protease activity assay (Devasia and Muraleendharan, 2012), YTD-agar supplemented with commercial powdered milk (1% w/v) was used. The protease activity was demonstrated by the presence of a clear halo around the inoculums. The activity test for lipases was based on the pH decrease caused by the action of these enzymes (Liu et al. 2014), using YTD-agar supplemented with commercial olive oil (1% v/v), phenol red (0.01% w/v) and $CaCl_2$. The decrease in pH caused by the release of fatty acids into the medium was indicated by the yellow coloration. The plates were incubated at room temperature (24ºC ± 2ºC) and observed up to 14 days after the beginning of the experiments

RESULTS

Efficiency of the bait method for isolation

Recalcitrant materials such as *Artemia* sp. cysts and spongin fibers derived from the skeleton of the sponge *Aplysina fulva* allowed the isolation of three different strains of Labyrinthulomycetes. Four to five days after the primmorphs were added to 6-well plates containing sterile-filtered natural seawater supplemented with PS and the baits, the cells of these protists adhered to the surface of these materials (Fig. 3.1A-B). After transferring the cysts and spongin fibers to the YTD-agar plates, within five days of incubation at 25°C the first colonies of Labyrinthulomycetes began to grow. There were no notable differences in the efficience of the two baits in recoverig the protists.

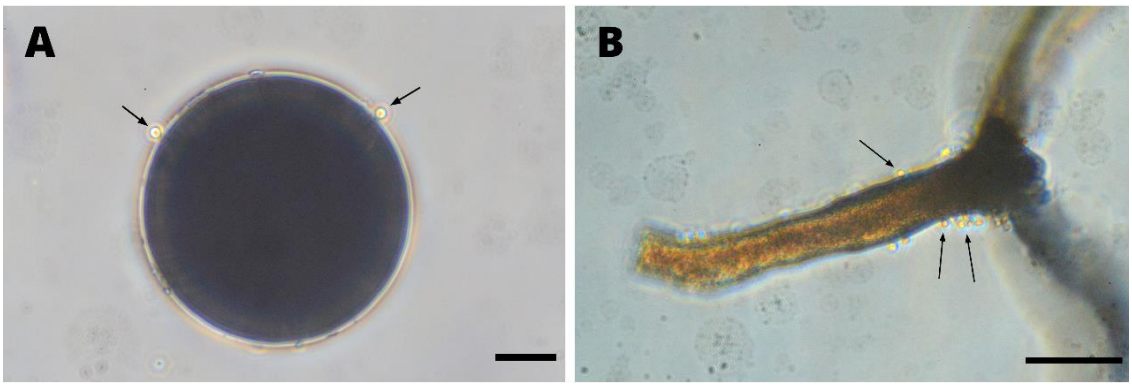

Figure 3.1. Isolation of protists using the bait method (phase contrast microscopy). (**A**) Autoclaved *Artemia* sp. eggs and (**B**) *Aplysina fulva* spongin skeleton with Labyrinthulomycetes cells (black arrows) attached to its surface after four days of incubation (scale bars: 50 µm).

Protists isolated from *Hymeniacidon heliophila*

For *Hymeniacidon heliophila* we successfully isolated one strain of Labyrinthulomycetes from primary culture cells using both *Artemia* sp. cysts and spongin skeleton sections as bait. After 5-day incubation of YTD-agar plates, we have confirmed the growing of only one morphotype, named HYM1, which were subcultured until obtaining axenic cultures. The HYM1 cells when in YTD-agar have gregarious grow, with the colonies found on the surface of the agar with regular rounded borders (Fig. 3.2A). The colony sizes are highly variable and ranges from 100 mm to 360 µm but we found few colonies up to 540 µm. Around the colony was observed the secretion hyaline substances yet to be identified. We also observed slim ectoplasmic projections,

characteristic of Labyrinthulomycetes species (Fig. 3.2A). In YTD-broth at least two cell morphotypes over its life cycle were detected. One of them is smaller in size while the other is the vegetative cell, which has a well-developed ectoplasmic net (Fig. 3.2B). The vegetative (*viz*. mature) cells have an average size of 15.8 µm ± 3.4 µm. The observations made over two weeks indicate that HYM1 does not have a flagellated form (zoospore), common to the Thraustochytrida family (Adl et al. 2019). This isolate seems to reproduce asexually by multiple cellular divisions. In this process the sporangia can grow up to 27 µm and its cytoplasm is filled by child cells, ultimately released by the disruption of the sporangia cell membrane and the wall.

Protists isolated from *Haliclona melana*

For *Haliclona melana* we successfully isolated two different strains of Labyrinthulomycetes from primary culture cells after five to seven days incubation period, named HAL1 and HAL2. The former was isolated using only *Artemia* sp. cysts as bait, while the latter was isolated using both the crustacean cysts and the spongin sections. In YTD-agar plates, HAL1 form colonies that have size range from 85 µm to 374 µm and have regular rounded borders (Fig. 3.2C). In YTD-broth, the cells of this isolate organize itself in aggregates of variable sizes (Fig. 3.2D). On these conditions, the cells of HAL1 are fusiform and have an average length of 7.7 µm + 1.2 µm. Its projections are visible when the cells have adhered to the substratum and show a well-developed ectoplasmic network (Fig. 3.2D). The HAL1 isolate is the only strain observed in this study to show the characteristic flagellate form (zoospore), found in very early stages of the culture, indicating that HAL1 is a member of Labyrinthulida (Adl *et al.* 2019). The HAL2 isolate has some similarities with the HYM1 isolate from *Hymeniacidon heliophila*. Our observations also indicate that HAL2 does not have a flagellated form, evidencing that strain belongs to Thrausthochytrida and might related to HYM1. In YTD-agar the colonies of HAL2 have a variable size, ranging from 178 µm to 637 µm, and very irregular rounded borders, a differential to the other strains (Fig. 3.2E). In YTD-Broth the cells are spherical and have the characteristic ectoplasmic network of Labyrinthulomycetes. Just as in other isolates we can find microcolonies or individual cells (Fig. 3.2F). On these conditions, the main morphological difference

between the strains HYM1 and HAL2 isolates is that vegetative cells of HAL2 have a smaller diameter when compared to HYM1 cells, with an average of 11.4 µm + 1.6 µm.

Fatty acid profile of the isolates

After 12 days of growth, we obtained sufficient biomass for fatty acid analysis (HYM1: 192 mg; HAL1: 169 mg; HAL2: 116 mg, each per 500 mL of YTD). The analysis showed that each of the isolates has an individual profile, with different ratios to each other and the presence of unique fatty acids (Table 3.1). It can be seen that all strains have large amounts of unsaturated fatty acids, more specifically docosahexanoic acid or DHA (C22:6n3) which makes up approximately 40% of the total lipids of each isolate. Other types of fatty acids are also present in notable proportions. In all three isolates palmitic acid (C16:0) is the second most abundant fatty acid, and in HAL1 it makes up 27% of the total. Importantly, pentadecanoic acid (C15:0), an odd-chain fatty acid, is also found in significant proportions in strain HYM1 (10%). We also observed the presence of arachidonic acid (C20:4n6) in large amounts (12.78%) only in isolate HAL2.

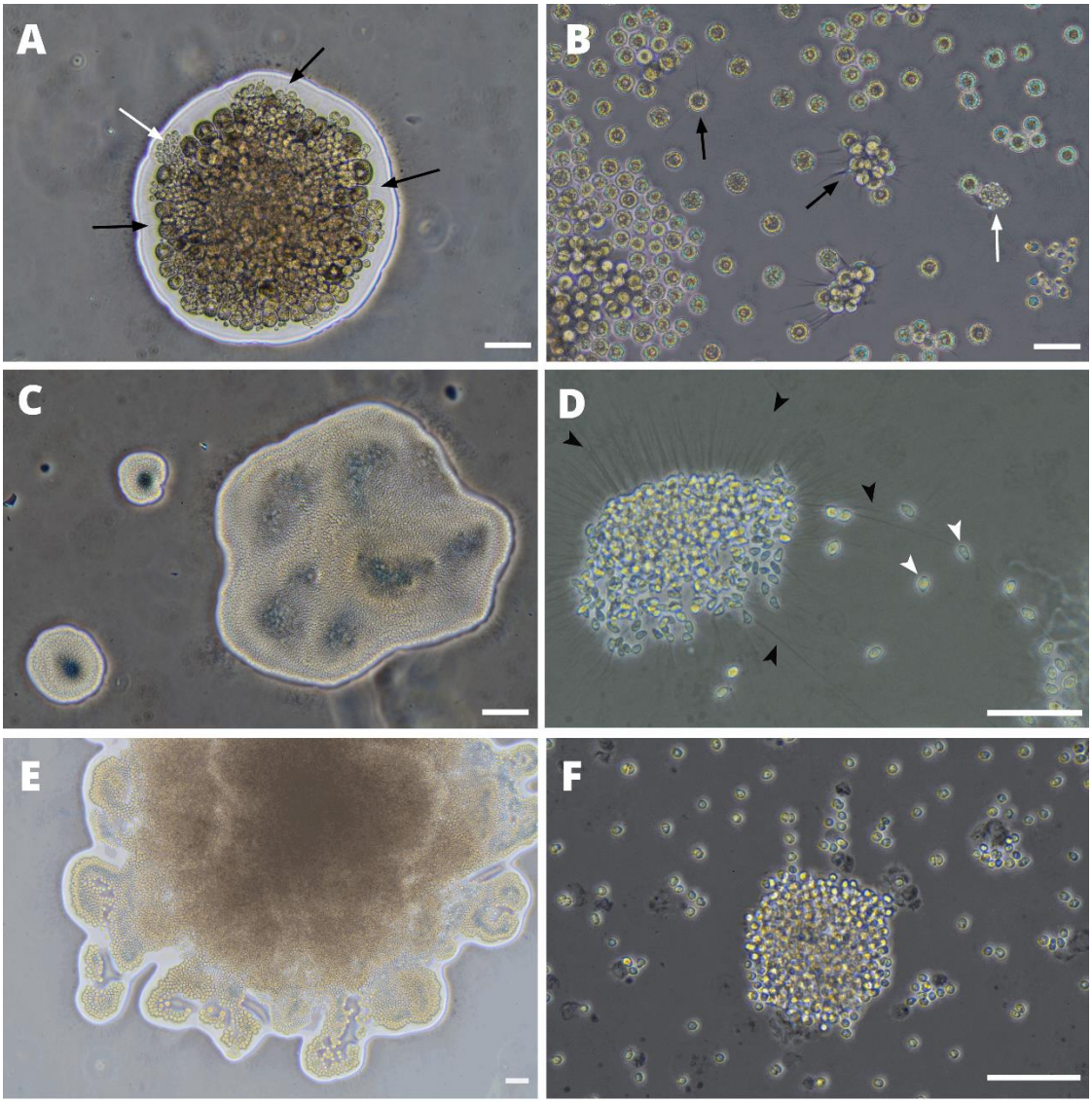

Figure 3.2. The strains HYM1, HAL1 and HAL2 (phase contrast light microscopy). (**A**) Colony of HYM1 in YTD-agar medium (**B**) Vegetative cells of HYM1 in YTD-Broth. Black arrows indicate mature cells with well-developed ectoplasmic projections, while white arrows indicate sporulation. (**C**) Colonies of the strain HAL1 and. (**D**) Colonies of HAL1 in YTD-Broth, it is possible to observe the ectoplasmic net (black arrowheads) connecting individual cells (white arrowheads). (**E**) HAL2 in YTD-agar medium (**F**) Colonies of HAL2 and individual cells in YTD-Broth. Each isolated cell is intertwined by an ectoplasmic net (not visible) (scale bars: 50 µm).

Table 3.1. Fatty acid profile of the three cultures of Labyrinthulomycetes isolated from marine sponges (% of total fatty acids). *nd: non-detected.*

Fatty Acids (%)		**HYM1**	**HAL1**	**HAL2**
Saturated Fatty Acids:				
	C13:0	5,36%	1,41%	2,99%
	C14:0	1,92%	0,68%	1,30%
	C15:0	10,01%	3,33%	6,75%
	C16:0	17,85%	26,84%	14,89%
	C17:0	5,75%	4,55%	2,92%
	C18:0	6,92%	5,70%	7,59%
	C20:0	1,26%	0,77%	1,30%
	C22:0	*nd*	*nd*	0,98%
Unsaturated Fatty Acids:				
	C18:1n9	2,92%	0,93%	3,45%
	C18:2n6	0,65%	0,36%	*nd*
	C18:3n6	0,85%	0,34%	0,83%
	C16:1	1,39%	3,36%	*nd*
	C20:4n6	*nd*	*nd*	12,78%
	C20:5n3	0,97%	*nd*	3,76%
	C22:4n6	1,29%	9,90%	0,59%
	C22:5n3	*nd*	0,56%	*nd*
	C22:5n6	1,12%	*nd*	*nd*
	C22:6n3	41,75%	41,28%	39,88%

Qualitative evaluation of the metabolic activities of the isolates

We found that none of the isolates (HYM1, HAL1 and HAL2) showed the halo indicating CMC hydrolysis. However, all isolates presented proteolytic activity, with the halo formation time varying among them. Only HAL1 showed degradation of the substrate after five days of experiment, while it took approximately 12 days before the first signs of degradation appeared in HYM1 and HAL2. Also, only HAL1 showed evidence of lipases at the end of the five-day experiment. Further observations 14 days later showed that HAL2 also has lipases, but HYM1 did not show any evidence of metabolic activity in this assay. The results for these experiments are summarized in Table 3.2 and illustrated in Figure 3.3.

Table 3.2. Evaluation of the metabolic activity against three types of substrates by the isolated protists. *nd: non-detected*

Isolates	Cellulases	Proteases	Lipases
HYM1	*nd*	+	*nd*
HAL1	*nd*	++	++
HAL2	*nd*	+	+

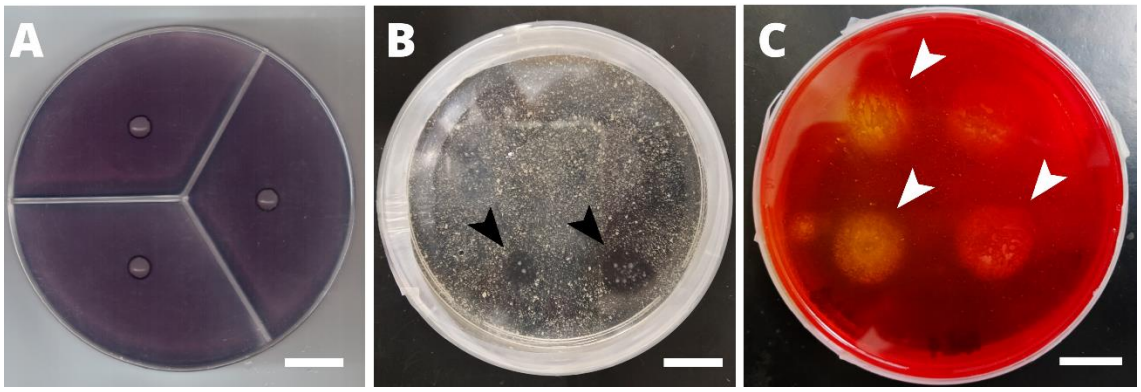

Figure 3.3. Metabolic activity assays performed with the HAL1 strain isolated from the marine sponge *Haliclona melana*. In the cellulase detection assay (**A**) no halo of substrate degradation was observed. However, in the protease detection assay (**B**) a transparent halo was formed around the inocula, indicating activity (black arrowheads). For the lipase detection assay (**C**) a strong metabolic activity was observed on this substrate, as indicated by the yellowish halos (white arrowheads) (scale bars: 15 mm).

DISCUSSION

Labyrinthulomycetes are saprophytic microeukaryotes that have been found in association with several animals, plants and algae, assuming epibiont or endobiont strategies (Raghukumar, 2002). They are often considered opportunists that benefit from physiological disruptions of their host, implying a decrease in its biological fitness (Raghukumar, 2002; Marchan et al. 2018). In marine plants and algae, they can cause diseases that lead to major environmental impacts, as is the case of "wasting disease", caused by a labyrinthulid (Muehlstein et al. 1991). In animals these protists can mainly be found in marine invertebrates. The free-living platyhelminth *Macrostomum lignano* is severely affected by infection of the thraustochytrid *Thraustochytrium caudivorum*, which in extreme cases can cause severe lesions on the posterior part and even the death of the animal (Schärer et al. 2007). Mollusks are also severely affected by association with Labyrinthulomycetes. In bivalves of commercial interest such as *Mercenaria*

mercenaria, a thraustochytrid known as *Quahog Parasite Unknown* (QPX) was primarily responsible for mass mortality of these organisms in Asia and North America (Gast et al. 2008). In contrast, while the negative effects of parasitism are well evidenced, the study of commensal and mutualistic relationships is more complex, with few records. Some studies suggest that there is a commensal relationship between thraustochytrids and the sea urchin *Lytechinus variegatus* (Wagner-Merner et al. 1980); and with the salp *Pegea confoederata* (Raghukumar and Raghukumar, 1999), but more evidence is still needed to support these hypotheses. In cnidarians, especially corals, when subjected to environmental stresses such as bleaching events, association with these protists may be beneficial (Kramarsky-Winter et al. 2006).

The knowledge about the association between Labyrinthulomycetes and Porifera is quite limited. An investigation conducted in the late 1970s was the first research on the interaction between these protists and sponges (Höhnk and Ulke, 1979). In this work was reported the presence of Labyrinthulomyecetes species *Ulkenia visurgensis* and *Thraustochytrium multirudimentale* (now accepted as *Oblongichytrium multirudimentale*) in healthy and decaying tissues of *Geodia cydonium*, *Verongia* sp. (now accepted as *Aplysina* sp.) and *Ircinia fasciculata* (Höhnk and Ulke, 1979). Recently it has been shown through high-throughput sequencing approaches that these microeukaryotes can also be found in tissue samples from healthy sponges (De Mares et al. 2017). Our results, which depart from a culture-dependent approach, corroborate these observations.

Morphological comparisons between the three strains show a great diversity in size. While HYM1 cells can reach up to 27 µm before sporulating, HAL2 generally reach only a maximum size of 20 µm. For HAL1 we observed a maximum size of 10.2 µm. The shapes can also be varied, as seen between the spherical shapes of HYM1 and HAL2 compared to the fusiform or spindle-shaped HAL1. Despite some similarities, there are several features that support each isolate as a distinct species, seen primarily in the morphology of the colonies when on YTD-agar. Our observations place the strains HYM1 and HAL2 as members of the Thraustochytrida family, given the absence of zoospore, the common mobile phase of many Stramenopiles (Adl et al. 2019). In contrast, the HAL1 strain has several features that place it in the *Labyrinthulida* family, like the presence of zoospore and the spindle-shaped vegetative cells (Adl et al. 2019). However, a more detailed investigation using molecular methods is necessary to

confirm these observations and determine the species. Biochemical profiles are also indicative of the individuality of each isolate, and fatty acids have been applied as a biochemical marker for classification of these microorganisms (Marchan et al. 2018). The proportions of FAs found in this work are in agreement with the profiles observed in several isolates of Labyrinthulomycetes (Gupta et al. 2016; Caamaño et al. 2017) and HYM1, HAL1 and HAL2 have different fatty acid ratios despite being grown under the same conditions. This is well demonstrated by the expressive amount of arachidonic acid (C20:4n6) in HAL2, a compound absent in HYM1 despite both probably belonging to the same family. The metabolic activities of each of the isolates bring interesting perspectives on the biotechnological potential of these protists isolated from marine sponges. In special, HAL1 showed considerable production of lipases and proteases in the preliminary tests and is a strong candidate for future investigations. The absence of CMC degradation activities could be explained both by the lack of the enzymatic apparatus and the sensitivity of the qualitative assay employed. This was observed by Liu et al. (2014), who studying the presence of proteases in 60 isolates only detected these enzymes in quantitative assays, due to the low production. The different metabolic activities of the isolates also support the individuality of each one of them. While HAL1 and HAL2 were able to degrade both lipids and proteins, HYM1 showed only proteolytic activities.

The gathered evidence supports that there is a long-lasting association between the marine sponges *Hymeniacidon heliophila* and *Haliclona melana* and these protists. Our findings suggests that this interspecific relationship is commensal or mutualistic. During field collections at Praia Grande over two years no signs of disease caused by thraustochytrids, as reported for other invertebrates such as gorgonians (Burge et al. 2012) and cephalopods (Polglase, 2019), were observed in any sponge. Further evidence supporting this hypothesis is that HYM1, HAL1 and HAL2 strains were specifically isolated from the same sponge species at different times, demonstrating a long-lasting association. Importantly, the culture of sponge cells as primmorphs allowed the elimination of the vast majority of transiently associated microorganisms, such as those in the surface or in the aquiferous system. As manipulable 3D structures, the primmorphs can be easily picked from one plate, rinsed several times in sterile medium and transferred to new plates. Associated with this, the long cultivation periods in oligotrophic media, supplemented with antibiotics, contribute to the reduction of

environmental contamination. In our understanding, the proliferation of Labyrinthulomycetes on primary cell cultures is not evidence of parasitism. We can attribute this event to the dysbiosis caused by the dissociation and reorganization of the cells in primmorphs. During this process, cells containing the microorganisms can be damaged and the sponge probably loses the ability to control the associated microbiota, which then overgrow its host. However, the hypothesis that the Labyrinthulomycetes growing in the primary cultures of these sponges come from the environment cannot be completely disregarded yet. In view of the planktonic and benthic distribution of the various species of these microeukaryotes (Raghukumar, 2002), it is plausible to assume that the sponges may have filtered and phagocytosed these cells. Then, when tissue reorganization occurs to form primmorphs, the still viable protists can proliferate under these new conditions. Experiments using In Situ Hybridization (ISH) (Stokes et al. 2002), associated with environmental collections to determine the specificity of each strain, may be interesting to elucidate this question.

In view of the results obtained and in the absence of clear evidences supporting the parasitism of these microorganisms on the studied marine sponges, we can discuss the roles of the Labyrinthulomycetes in this association. Considering this association of a positive nature for at least one of those involved we can cite precedents in other animals that have similar life habits, such as corals. In the hard corals of the genus *Fungia* sp. (Kramarsky-Winter et al. 2006; Harel et al. 2008) and *Favia* sp. (Siboni et al. 2010) several strains of Labyrinthulomycetes are consistently isolated from the mucus of these animals. In induced bleaching experiments these protists can supply the energy needs of the host when these are not met by symbiont algae. In the absence of *Symbiodinium* sp. the production of polyunsaturated fatty acids and/or carotenoids may function as an important carbon source for the coral, implying its survival (Kramarsky-Winter et al. 2006). Thus, it is plausible that in Porifera these protists may perform similar functions for the host. Considering the diversity of microorganisms that inhabit the tissues of sponges, it is possible that they perform redundant functions to other microorganisms such as synthesis and/or recycling of organic substances. In contrast, the benefits that Labyrinthulomycetes can obtain from their association with marine sponges include protection from the holobiont and access to and metabolization of particulate and dissolved organic matter as observed for other microorganisms (Bart et al. 2020). Considering that bactivory was already recorded in Labyrinthulomycetes

(Raghukumar, 1992) it is also possible that these microorganisms are benefiting from the predation of Porifera-associated bacteria, exerting a top-down pressure. In corals, Labyrinthulomycetes may function as important regulators of bacterial communities present in hosts (Ainsworth et al. 2017). Furthermore, considering the trophic strategies of these microeukaryotes and the complex microorganism-host interactions, it is also plausible that syntrophic interactions (Fiore et al. 2020) occur between the microbial communities of *Hymeniacidon heliophila* and *Haliclona melana*.

CONCLUSIONS

This work is the first to systematically characterize Labyrinthulomycetes isolated from primary Porifera cell cultures. It is also the first to use spongin from *Aplysina fulva*, a highly recalcitrant material, for the isolation of these protists. Through a culture-dependent approach it was possible to demonstrate that each sponge has distinct species of associated Labyrinthulomycetes. The analyses indicate that the three isolates from *Hymeniacidon heliophila* and *Haliclona melana* cell cultures are different from each other at morphological and biochemical levels. In view of the evidence obtained, is highly probable that these protists may be associated with Porifera in a commensal or mutualistic manner. The biotechnological potential of these strains is worth investigating in depth, especially HAL1 which possesses enzymatic activities that may be of commercial interest. As next steps, it is necessary to identify the species of the isolates by 18S rRNA sequencing. Also, it is important to investigate in detail the physiology of this association and the ways in which these protists are acquired. The mechanism of production and transport of PUFAs by these protists in context of the association may be an interesting starting point for investigation through proteomics and metabolomics approaches.

ACKNOWLEDGMENTS

The authors would like to thank the Prof. Renata Guimarães Moreira-Whitton and the technician Vagner Alberto for all help and support with fatty acids analysis. This is a contribution of NP-BioMar (Research Center for Marine Biodiversity - USP).

COMPETING INTERESTS

The authors declare no competing interests.

FUNDING

This study was supported by the grant #2019/04707-8, São Paulo Research Foundation (FAPESP).

REFERENCES

Aasen, I.M., Ertesvåg, H., Heggeset, T.M.B., Liu, B., Brautaset, T., Vadstein, O., Ellingsen, T.E., 2016. Thraustochytrids as production organisms for docosahexaenoic acid (DHA), squalene, and carotenoids. Appl. Microbiol. Biotechnol. 100, 4309–4321. https://doi.org/10.1007/s00253-016-7498-4

Adl, S.M., Bass, D., Lane, C.E., Lukeš, J., Schoch, C.L., Smirnov, A., Agatha, S., Berney, C., Brown, M.W., Burki, F., Cárdenas, P., Čepička, I., Chistyakova, L., Campo, J., Dunthorn, M., Edvardsen, B., Eglit, Y., Guillou, L., Hampl, V., Heiss, A.A., Hoppenrath, M., James, T.Y., Karnkowska, A., Karpov, S., Kim, E., Kolisko, M., Kudryavtsev, A., Lahr, D.J.G., Lara, E., Le Gall, L., Lynn, D.H., Mann, D.G., Massana, R., Mitchell, E.A.D., Morrow, C., Park, J.S., Pawlowski, J.W., Powell, M.J., Richter, D.J., Rueckert, S., Shadwick, L., Shimano, S., Spiegel, F.W., Torruella, G., Youssef, N., Zlatogursky, V., Zhang, Q., 2019. Revisions to the Classification, Nomenclature, and Diversity of Eukaryotes. J. Eukaryot. Microbiol. 66, 4–119. https://doi.org/10.1111/jeu.12691

Ainsworth, T.D., Fordyce, A.J., Camp, E.F., 2017. The Other Microeukaryotes of the Coral Reef Microbiome. Trends Microbiol. 25, 980–991. https://doi.org/10.1016/j.tim.2017.06.007

Aki, T., Hachida, K., Yoshinaga, M., Katai, Y., Yamasaki, T., Kawamoto, S., Kakizono, T., Maoka, T., Shigeta, S., Suzuki, O., Ono, K., 2003. Thraustochytrid as a potential source of carotenoids. J. Am. Oil Chem. Soc. 80, 789. https://doi.org/10.1007/s11746-003-0773-2

Bart, M.C., de Kluijver, A., Hoetjes, S., Absalah, S., Mueller, B., Kenchington, E., Rapp, H.T., de Goeij, J.M., 2020. Differential processing of dissolved and particulate organic matter by deep-sea sponges and their microbial symbionts. Sci. Rep. 10, 1–13. https://doi.org/10.1038/s41598-020-74670-0

Bongiorni, L., Pusceddu, A., Danovaro, R., 2005. Enzymatic activities of epiphytic and benthic thraustochytrids involved in organic matter degradation. Aquat. Microb. Ecol. 41, 299–305. https://doi.org/10.3354/ame041299

Boro, M.C., Harakava, R., Pires-Zottarelli, C.L.A., 2018. Labyrinthulomycota from Brazilian mangrove swamps and coastal waters. Bot. Mar. 61, 65–74. https://doi.org/10.1515/bot-2017-0052

Burge, C.A., Douglas, N., Conti-Jerpe, I., Weil, E., Roberts, S., Friedman, C.S., Harvell, C.D., 2012. Friend or foe: The association of Labyrinthulomycetes with the Caribbean Sea fan *Gorgonia ventalina*. Dis. Aquat. Organ. 101, 1–12. https://doi.org/10.3354/dao02487

Caamaño, E., Loperena, L., Hinzpeter, I., Pradel, P., Gordillo, F., Corsini, G., Tello, M., Lavín, P., González, A.R., 2017. Isolation and molecular characterization of *Thraustochytrium* strain isolated from Antarctic Peninsula and its biotechnological potential in the production of fatty acids. Brazilian J. Microbiol. 48, 671–679. https://doi.org/10.1016/j.bjm.2017.01.011

Ceja-Navarro, J.A., Wang, Y., Ning, D., Arellano, A., Ramanculova, L., Yuan, M.M., Byer, A., Craven, K.D., Saha, M.C., Brodie, E.L., Pett-Ridge, J., Firestone, M.K., 2021. Protist diversity and community complexity in the rhizosphere of switchgrass are dynamic as plants develop. Microbiome 9, 1–18. https://doi.org/10.1186/s40168-021-01042-9

Christie, W.W., Han, X., 2012. Isolation of fatty acids and identification by spectroscopic and related techniques, in: Christie, W.W., Han, X. (Eds.), Lipid Analysis: Isolation, Separation, Identification and Lipidomic Analysis. Woodhead Publishing, pp. 181–211. https://doi.org/https://doi.org/10.1533/9780857097866.181

Custodio, M.R., Prokic, I., Steffen, R., Koziol, C., Borojevic, R., Brümmer, F., Nickel, M., Müller, W.E.G., 1998. Primmorphs generated from dissociated cells of the sponge *Suberites domuncula*: A model system for studies of cell proliferation and cell death. Mech. Ageing Dev. 105, 45–59. https://doi.org/10.1016/S0047-6374(98)00078-5

De Mares, M.C., Sipkema, D., Huang, S., Bunk, B., Overmann, J., van Elsas, J.D., 2017. Host Specificity for Bacterial, Archaeal and Fungal Communities Determined for High- and Low-Microbial Abundance Sponge Species in Two Genera. Front. Microbiol. 8, 1–13. https://doi.org/10.3389/fmicb.2017.02560

del Campo, J., Bass, D., Keeling, P.J., 2020. The eukaryome: Diversity and role of microeukaryotic organisms associated with animal hosts. Funct. Ecol. 34, 2045–2054. https://doi.org/10.1111/1365-2435.13490

Fiore, C.L., Jarett, J.K., Steinert, G., Lesser, M.P., 2020. Trait-Based Comparison of Coral and Sponge Microbiomes. Sci. Rep. 10, 1–16. https://doi.org/10.1038/s41598-020-59320-9

Folch, J., Lees, M., Stanley, G.H.S., 1957. A Simple Method for The Isolation and Purification of Total Lipides from Animal Tissues. J. Biol. Chem. 226, 497–509. https://doi.org/10.1016/S0021-9258(18)64849-5

Gast, R.J., Moran, D.M., Audemard, C., Lyons, M.M., DeFavari, J., Reece, K.S., Leavitt, D., Smolowitz, R., 2008. Environmental distribution and persistence of Quahog Parasite Unknown (QPX). Dis. Aquat. Organ. 81, 219–229. https://doi.org/10.3354/dao01948

Gupta, A., Singh, D., Byreddy, A.R., Thyagarajan, T., Sonkar, S.P., Mathur, A.S., Tuli, D.K., Barrow, C.J., Puri, M., 2016. Exploring omega-3 fatty acids, enzymes and biodiesel producing thraustochytrids from Australian and Indian marine biodiversity. Biotechnol. J. 11, 345–355. https://doi.org/10.1002/biot.201500279

Han, Q., Li, P., Lu, X., Guo, Z., Guo, H., 2013. Improved primary cell culture and subculture of lymphoid organs of the greasyback shrimp *Metapenaeus ensis*. Aquaculture 410–411, 101–113. https://doi.org/10.1016/j.aquaculture.2013.06.024

Harel, M., Ben-Dov, E., Rasoulouniriana, D., Siboni, N., Kramarsky-Winter, E., Loya, Y., Barak, Z., Wiesman, Z., Kushmaro, A., 2008. A new Thraustochytrid, strain Fng1, isolated from the surface mucus of the hermatypic coral *Fungia granulosa*. FEMS Microbiol. Ecol. 64, 378–387. https://doi.org/10.1111/j.1574-6941.2008.00464.x

He, L., Liu, F., Karuppiah, V., Ren, Y., Li, Z., 2014. Comparisons of the Fungal and Protistan Communities among Different Marine Sponge Holobionts by Pyrosequencing. Microb. Ecol. 67, 951–961. https://doi.org/10.1007/s00248-014-0393-6

Henríquez, M., Vergara, K., Norambuena, J., Beiza, A., Maza, F., Ubilla, P., Araya, I., Chávez, R., San-Martín, A., Darias, J., Darias, M.J., Vaca, I., 2014. Diversity of cultivable fungi associated with Antarctic marine sponges and screening for their antimicrobial, antitumoral and antioxidant potential. World J. Microbiol. Biotechnol. 30, 65–76. https://doi.org/10.1007/s11274-013-1418-x

Hentschel, U., Usher, K.M., Taylor, M.W., 2006. Marine sponges as microbial fermenters. FEMS Microbiol. Ecol. 55, 167–177. https://doi.org/10.1111/j.1574-6941.2005.00046.x

Höhnk, W., Ulken, A., 1979. Pilze aus marinen Schwämmen. Veröffentlichungen des Instituts für Meeresforsch. Bremerhaven 17, 199–204. https://doi.org/https://doi.org/10.2312/reports-ifm-bhv.1979.17.2.199

Kramarsky-Winter, E., Harel, M., Siboni, N., Ben Dov, E., Brickner, I., Loya, Y., Kushmaro, A., 2006. Identification of a protist-coral association and its possible ecological role. Mar. Ecol. Prog. Ser. 317, 67–73. https://doi.org/10.3354/meps317067

Kvingedal, R., Owens, L., Jerry, D.R., 2006. A new parasite that infects eggs of the mud crab, *Scylla serrata*, in Australia. J. Invertebr. Pathol. 93, 54–59. https://doi.org/10.1016/j.jip.2006.06.002

Lee Chang, K.J., Rye, L., Dunstan, G.A., Grant, T., Koutoulis, A., Nichols, P.D., Blackburn, S.I., 2015. Life cycle assessment: heterotrophic cultivation of thraustochytrids for biodiesel production. J. Appl. Phycol. 27, 639–647. https://doi.org/10.1007/s10811-014-0364-9

Leray, M., Knowlton, N., 2016. Censusing marine eukaryotic diversity in the twenty-first century. Philos. Trans. R. Soc. B Biol. Sci. 371, 20150331. https://doi.org/10.1098/rstb.2015.0331

Marchan, L.F., Lee Chang, K.J., Nichols, P.D., Mitchell, W.J., Polglase, J.L., Gutierrez, T., 2018. Taxonomy, ecology and biotechnological applications of thraustochytrids: A review. Biotechnol. Adv. 36, 26–46. https://doi.org/10.1016/j.biotechadv.2017.09.003

Margot, H., Acebal, C., Toril, E., Amils, R., Fernandez Puentes, J.L., 2002. Consistent association of crenarchaeal Archaea with sponges of the genus *Axinella*. Mar. Biol. 140, 739–745. https://doi.org/10.1007/s00227-001-0740-2

Merkx-Jacques, A., Rasmussen, H., Muise, D.M., Benjamin, J.J.R., Kottwitz, H., Tanner, K., Milway, M.T., Purdue, L.M., Scaife, M.A., Armenta, R.E., Woodhall, D.L., 2018. Engineering xylose metabolism in thraustochytrid T18. Biotechnol. Biofuels 11, 1–18. https://doi.org/10.1186/s13068-018-1246-1

Moitinho-Silva, L., Steinert, G., Nielsen, S., Hardoim, C.C.P., Wu, Y.C., McCormack, G.P., López-Legentil, S., Marchant, R., Webster, N., Thomas, T., Hentschel, U., 2017. Predicting the HMA-LMA status in marine sponges by machine learning. Front. Microbiol. 8, 1–14. https://doi.org/10.3389/fmicb.2017.00752

Muehlstein, L.K., Porter, D., Short, F.T., 1991. *Labyrinthula zosterae* sp. nov., the causative agent of wasting disease of eelgrass, Zostera marina. Mycologia 83, 180–191. https://doi.org/10.2307/3759933

Naim, M.A., Smidt, H., Sipkema, D., 2017. Fungi found in Mediterranean and North Sea sponges: How specific are they? PeerJ 2017, 1–19. https://doi.org/10.7717/peerj.3722

Nguyen, M., Wemheuer, B., Laffy, P.W., Webster, N.S., Thomas, T., 2021. Taxonomic, functional and expression analysis of viral communities associated with marine sponges. PeerJ 9, 1–21. https://doi.org/10.7717/peerj.10715

Nowotny, J.D., Connelly, M.T., Traylor-Knowles, N., 2021. Novel methods to establish whole-body primary cell cultures for the cnidarians *Nematostella vectensis* and *Pocillopora damicornis*. Sci. Rep. 11, 1–9. https://doi.org/10.1038/s41598-021-83549-7

Padmakumar, K.B., Cicily, L., Shaji, A., Maneesh, T.P., Sanjeevan, V.N., 2012. Symbiosis between the stramenopile protist *Solenicola setigera* and the diatom *Leptocylindrus mediterraneus* in the North Eastern Arabian Sea. Symbiosis 56, 97–101. https://doi.org/10.1007/s13199-012-0160-8

Pascelli, C., Laffy, P.W., Botté, E., Kupresanin, M., Rattei, T., Lurgi, M., Ravasi, T., Webster, N.S., 2020. Viral ecogenomics across the Porifera. Microbiome 8, 1–22. https://doi.org/10.1186/s40168-020-00919-5

Pawlowski, J., 2014. Protist Evolution and Phylogeny, in: eLS. Wiley, Chichester, UK. https://doi.org/10.1002/9780470015902.a0001935.pub2

Polglase, J.L., 2019. Cephalopod Diseases Caused by Fungi and Labyrinthulomycetes, in: Gestal, C., Pascual, S., Guerra, Á., Fiorito, G., Vieites, J.M. (Eds.), Handbook of Pathogens and Diseases in Cephalopods. Springer, Cham, Cham, pp. 113–122. https://doi.org/10.1007/978-3-030-11330-8_6

Rabinowitz, C., Douek, J., Weisz, R., Shabtay, A., Rinkevich, B., 2006. Isolation and characterization of four novel thraustochytrid strains from a colonial tunicate. Indian J. Mar. Sci. 35, 341–350.

Raghukumar, S., 2002. Ecology of the marine protists, the labyrinthulomycetes (thraustochytrids and labyrinthulids). Eur. J. Protistol. 38, 127–145. https://doi.org/10.1078/0932-4739-00832

Raghukumar, S., 2008. Thraustochytrid marine protists: Production of PUFAs and other emerging technologies. Mar. Biotechnol. 10, 631–640. https://doi.org/10.1007/s10126-008-9135-4

Raghukumar, S., Raghukumar, C., 1999. Thraustochytrid fungoid protists in faecal pellets of the tunicate *Pegea confoederata*, their tolerance to deep-sea conditions and implication in degradation processes. Mar. Ecol. Prog. Ser. 190, 133–140. https://doi.org/10.3354/meps190133

Richelle-Maurer, E., Gomez, R., Braekman, J.C., Van De Vyver, G., Van Soest, R.W.M., Devijver, C., 2003. Primary cultures from the marine sponge *Xestospongia muta* (Petrosiidae, Haplosclerida). J. Biotechnol. 100, 169–176. https://doi.org/10.1016/S0168-1656(02)00251-1

Rinkevich, B., 1999. Cell cultures from marine invertebrates: obstacles, new approaches and recent improvements. Prog. Ind. Microbiol. 70, 133–153. https://doi.org/10.1016/S0079-6352(99)80107-6

Rinkevich, B., 2005. Marine Invertebrate Cell Cultures: New Millennium Trends. Mar. Biotechnol. 7, 429–439. https://doi.org/10.1007/s10126-004-0108-y

Rinkevich, B., Rabinowitz, C., 1993. In vitro culture of blood cells from the colonial protochordate *Botryllus schlosseri*. Vitr. Cell. Dev. Biol. - Anim. 29, 79–85. https://doi.org/10.1007/BF02634375

Rodríguez-Marconi, S., De la Iglesia, R., Díez, B., Fonseca, C.A., Hajdu, E., Trefault, N., 2015. Characterization of Bacterial, Archaeal and Eukaryote Symbionts from Antarctic Sponges Reveals a High Diversity at a Three-Domain Level and a Particular Signature for This Ecosystem. PLoS One 10, e0138837. https://doi.org/10.1371/journal.pone.0138837

Schärer, L., Knoflach, D., Vizoso, D.B., Rieger, G., Peintner, U., 2007. Thraustochytrids as novel parasitic protists of marine free-living flatworms: *Thraustochytrium caudivorum* sp. nov. parasitizes *Macrostomum lignano*. Mar. Biol. 152, 1095–1104. https://doi.org/10.1007/s00227-007-0755-4

Schindelin, J., Rueden, C.T., Hiner, M.C., Eliceiri, K.W., 2015. The ImageJ ecosystem: An open platform for biomedical image analysis. Mol. Reprod. Dev. 82, 518–529. https://doi.org/10.1002/mrd.22489

Schippers, K.J., Sipkema, D., Osinga, R., Smidt, H., Pomponi, S.A., Martens, D.E., Wijffels, R.H., 2012. Cultivation of Sponges, Sponge Cells and Symbionts. Achievements and Future Prospects, in: Becerro, M.A., Uriz, M.J., Maldonado, M., Turon, X. (Eds.), Advances in Sponge Science: Physiology, Chemical and Microbial Diversity, Biotechnology. pp. 273–337. https://doi.org/10.1016/B978-0-12-394283-8.00006-0

Schmitt, S., Tsai, P., Bell, J., Fromont, J., Ilan, M., Lindquist, N., Perez, T., Rodrigo, A., Schupp, P.J., Vacelet, J., Webster, N., Hentschel, U., Taylor, M.W., 2012. Assessing the complex sponge microbiota: Core, variable and species-specific bacterial communities in marine sponges. ISME J. 6, 564–576. https://doi.org/10.1038/ismej.2011.116

Schmitt, S., Wehrl, M., Bayer, K., Siegl, A., Hentschel, U., 2007. Marine sponges as models for commensal microbe-host interactions. Symbiosis 44, 43–50.

Shinn, A.P., Pratoomyot, J., Bron, J.E., Paladini, G., Brooker, E.E., Brooker, A.J., 2015. Economic costs of protistan and metazoan parasites to global mariculture. Parasitology 142, 196–270. https://doi.org/10.1017/S0031182014001437

Siboni, N., Rasoulouniriana, D., Ben-Dov, E., Kramarsky-Winter, E., Sivan, A., Loya, Y., Hoegh-Guldberg, O., Kushmaro, A., 2010. Stramenopile microorganisms associated with the massive coral *Favia* sp. J. Eukaryot. Microbiol. 57, 236–244. https://doi.org/10.1111/j.1550-7408.2010.00469.x

Steinert, G., Busch, K., Bayer, K., Kodami, S., Arbizu, P.M., Kelly, M., Mills, S., Erpenbeck, D., Dohrmann, M., Wörheide, G., Hentschel, U., Schupp, P.J., 2020. Compositional and Quantitative

Insights into Bacterial and Archaeal Communities of South Pacific Deep-Sea Sponges (Demospongiae and Hexactinellida). Front. Microbiol. 11, 1–16. https://doi.org/10.3389/fmicb.2020.00716

Stokes, N.A., Ragone Calvo, L.M., Reece, K.S., Burreson, E.M., 2002. Molecular diagnostics, field validation, and phylogenetic analysis of Quahog Parasite Unknown (QPX), a pathogen of the hard clam *Mercenaria mercenaria*. Dis. Aquat. Organ. 52, 233–247. https://doi.org/10.3354/dao052233

Suryanarayanan, T.S., 2012. The diversity and importance of fungi associated with marine sponges. Bot. Mar. 55, 553–564. https://doi.org/10.1515/bot-2011-0086

Tan, K.S.W., 2008. New Insights on Classification, Identification, and Clinical Relevance of *Blastocystis* spp. Clin. Microbiol. Rev. 21, 639–665. https://doi.org/10.1128/CMR.00022-08

Taoka, Y., Nagano, N., Okita, Y., Izumida, H., Sugimoto, S., Hayashi, M., 2009. Extracellular Enzymes Produced by Marine Eukaryotes, Thraustochytrids. Biosci. Biotechnol. Biochem. 73, 180–182. https://doi.org/10.1271/bbb.80416

Taylor, M.W., Radax, R., Steger, D., Wagner, M., 2007. Sponge-Associated Microorganisms: Evolution, Ecology, and Biotechnological Potential. Microbiol. Mol. Biol. Rev. 71, 295–347. https://doi.org/10.1128/MMBR.00040-06

Wagner-Merner, D.T., Duncan, W.R., Lawrence, J.M., 1980. Preliminary Comparison of Thraustochytriaceae in the Guts of a Regular and Irregular Echinoid. Bot. Mar. 23, 95–98. https://doi.org/10.1515/botm.1980.23.2.95

Webster, N.S., Taylor, M.W., 2012. Marine sponges and their microbial symbionts: love and other relationships. Environ. Microbiol. 14, 335–346. https://doi.org/10.1111/j.1462-2920.2011.02460.x

Webster, N.S., Thomas, T., 2016. Defining the Sponge Hologenome. MBio 7, 1–14. https://doi.org/10.1128/mBio.00135-16.Invited

Worden, A.Z., Follows, M.J., Giovannoni, S.J., Wilken, S., Zimmerman, A.E., Keeling, P.J., 2015. Rethinking the marine carbon cycle: Factoring in the multifarious lifestyles of microbes. Science. 347, 12575941–125759410. https://doi.org/10.1126/science.1257594

Yamasaki, T., Aki, T., Shinozaki, M., Taguchi, M., Kawamoto, S., Ono, K., 2006. Utilization of Shochu distillery wastewater for production of polyunsaturated fatty acids and xanthophylls using thraustochytrid. J. Biosci. Bioeng. 102, 323–327. https://doi.org/10.1263/jbb.102.323

CAPÍTULO 4

Resultados, Discussão e Considerações Finais

4.1 Resultados e Discussão Geral

Para além das bactérias, arqueias, fungos e vírus, o microbioma de Porifera também é composto por eucariontes unicelulares (Taylor et al., 2007). Esses microrganismos são comumente conhecidos como "*protistas"* e compreendem uma grande diversidade de linhagens eucariontes (Adl et al., 2019). Essa fração do microbioma, ainda pouco explorada dentro da espongologia, foi discutida no **Capítulo 2** através de uma revisão da literatura científica da área. Nesse manuscrito abordamos diversas questões sobre o estudo dos protistas que estão associados às esponjas marinhas e dulciaquícolas. O termo "*Eukaryome*" foi recentemente cunhado para descrever todo o conjunto de eucariontes associado a um hospedeiro animal (Lukeš et al, 2015; del Campo et al., 2020). Contudo, essa definição também engloba fungos e outros organismos. Logo, a diversidade de protistas associada às esponjas foi aqui definida como "*Microeukaryome*" ou "*Microeucarioma*" visando uma maior precisão. Fungos unicelulares (*i.e.*, leveduras) não se encaixam nessa definição visto que possuem outros termos que os descrevem: "*Micobiota"* ou *"Micobioma"*. Historicamente, os protistas associados aos animais são estudados devido aos efeitos negativos ao fitness do hospedeiro (Lukeš et al, 2015; del Campo et al., 2020). Entretanto, essas relações podem ser bem mais complexas do que o simples parasitismo ou mutualismo e, além disso, também dependentes do cenário em que essas interações hospedeiro-microrganismo são observadas (del Campo et al., 2020). No contexto das culturas primárias de células de invertebrados marinhos esses microrganismos também são bem conhecidos. Existem diversos registros dos impactos dos protistas associados nos avanços dessa área (revisão em Rinkevich, 2005). Nas seções seguintes discutimos em maiores detalhes alguns pontos importantes que não foram abordados nos manuscritos apresentados anteriormente.

4.1.1 Aspectos do isolamento Labyrinthulomycetes associados à Porifera

Como apresentado nos capítulos anteriores, as culturas primárias de esponjas são frequentemente suplantadas pelo crescimento exacerbado de certas linhagens de protistas. Dentre esses, se destaca um ubíquo grupo de eucariontes unicelulares: os Labyrinthulomycetes. Através de abordagens cultura-dependentes, no **Capítulo 3**, investigamos a natureza da associação entre duas esponjas marinhas e os protistas desse táxon. O uso de primorfos derivados dos tecidos de *Hymeniacidon heliophila* e *Haliclona melana* e a aplicação de diferentes meios e técnicas permitiu o sucesso no isolamento desses protistas em mais de uma ocasião. Entretanto, além dos diversos meios e condições de cultura testadas (Tabela 1), foi necessária uma série de adaptações para tornar possível o isolamento.

Tabela 1. Meios testados para isolamento dos Labyrinthulomycetes associados às culturas primárias de esponjas marinhas *Hymeniacidon heliophila* e *Haliclona melana*.

Meio	Concentração	Crescimento	Condição	Temperatura	Inóculos	Tempo de Observação
DYNB	1%	-	Sólido/Líquido	24ºC ± 2ºC	*Hymeniacidon heliophila*	2 semanas
BHI	–	-	Sólido	24ºC ± 2ºC	*Hymeniacidon heliophila*	2 semanas
YTD-H	0,1%	+	Sólido/Líquido	24ºC ± 2ºC	*Hymeniacidon heliophila*	2 semanas
YTD	0,01%	++	Sólido/Líquido	24ºC ± 2ºC	*Hymeniacidon heliophila* e *Haliclona melana*	1 semana

Em um primeiro momento, a abordagem inicial mais promissora consistiu em usar o meio sólido e líquido denominado YTD-H modificado a partir de Harel et al. (2008). Observações periódicas mostraram que Labyrinthulomycetes começaram a se desenvolver nesse novo meio líquido entre 10 e 12 dias após a incubação das amostras. Contudo, uma grande quantidade de microrganismos contaminantes cresceu em conjunto, principalmente leveduras e fungos filamentosos (Figura 4.1 A-D). Foram feitas diversas tentativas subsequentes de isolamento por diluição seriada e micromanipulação, mas essas se mostraram ineficientes dado o grande número de

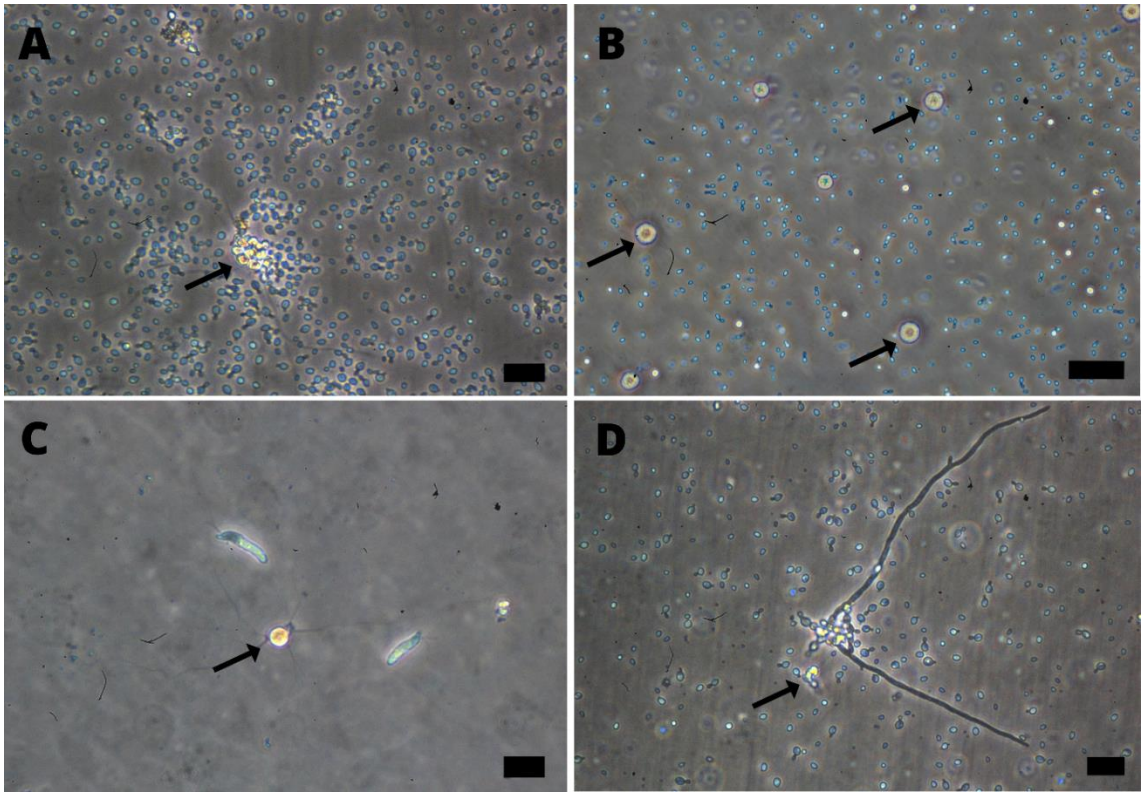

Figura 4.1. (A-D) Culturas em meio YTD1, as setas pretas indicam células vegetativas de Labyrinthulomycetes. Nota-se uma grande quantidade de contaminantes por fungos (A, B, D) e/ou protistas não-identificados (C). As contaminações apresentavam um crescimento exacerbado, inviabilizando o processo de isolamento dos protistas de interesse. Contraste de fase, Nikon TE300. Barras de escala = 10 µm.

contaminantes. Com o objetivo de diminuir a contaminação por fungos e outros microrganismos partimos da hipótese de que os protistas associados com as esponjas marinhas possuíam baixas necessidades energéticas. Essa hipótese tem como base as necessidades metabólicas de certas espécies bactérias simbiontes de Porifera, que preferem meios oligotróficos (Sipkema et al., 2011). Sendo assim, o meio YTD-H foi diluído dez vezes atingindo uma concentração de 0,01% (sendo denominado YTD), diminuindo a disponibilidade de nutrientes para outros organismos contaminantes. Desta forma, observamos que o crescimento desses microrganismos, principalmente de leveduras, ciliados e amebas, foi reduzido significativamente. Em conjunto com o meio oligotrófico a técnica de *baiting* se mostrou essencial para o processo de isolamento. Como mostrado no **Capítulo 3**, por serem envoltos por quitina a utilização de cistos de *Artemia* sp. como isca para esses protistas associados se mostrou uma estratégia bem sucedida. Também aplicamos com sucesso e de forma inédita, o uso de fragmentos do esqueleto de espongina oriundos da esponja *Aplysina fulva*. Em certas ordens de

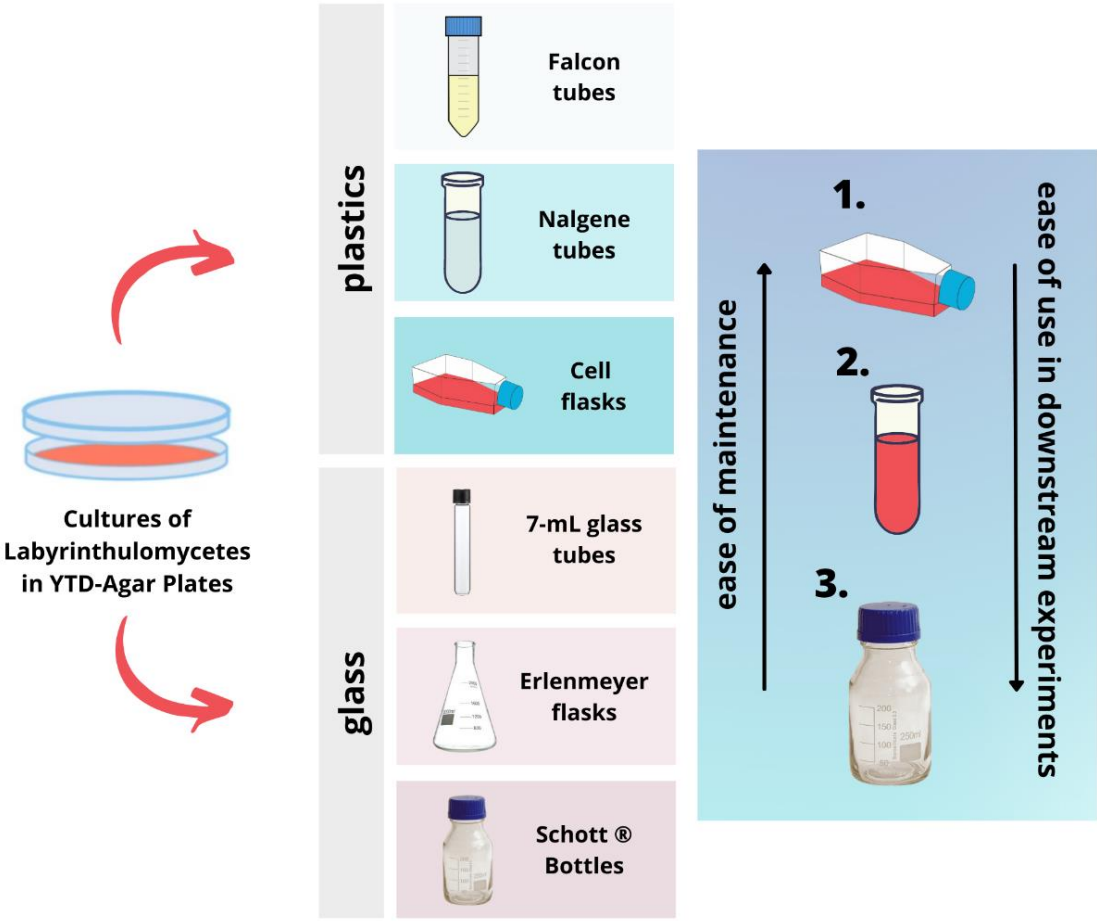

Figura 4.2. Frascos de cultivo testados para cada um dos isolados. Foram testados dois tipos de materiais: plástico e vidro. No geral, as culturas de Labyrinthulomycetes são melhor mantidas em garrafas de cultura celular. Essas apresentam maior facilidade de manipulação e visualização em microscópio, porém apresentam certas dificuldades de escalabilidade para experimentação. Tubos Nalgene se mostraram uma opção intermediária nesse contexto, por seu volume e possibilidade de reutilização (autoclaváveis). Por fim, as garrafas Schott possuem mais limitações, porém permitem uma grande obtenção de biomassa.

Demospongiae, espículas de sílica estão ausentes e o esqueleto é composto por redes de fibras de espongina (Ehrlich, 2019). A espongina possui colágeno em sua composição, mas é uma substância complexa e ainda pouco compreendida, porém sabe-se que é altamente resistente a tratamentos enzimáticos e apresenta grande potencial biotecnológico (Jesionowski et al. 2018; Ehrlich, 2019, Araújo et al. 2021).

Como apresentado no **Capítulo 3**, foram isolados com sucesso três cepas com características diferentes, duas delas obtidas a partir de *Haliclona melana*: HAL1 e HAL2; e uma isolada a partir de *Hymeniacidon heliophila*: HYM1. Apesar do nosso sucesso no isolamento, crescimento e manutenção desses microrganismos, a obtenção de uma massa de células para experimentos subsequentes se mostrou um desafio. Com

exceção de um dos isolados (HAL1), os demais (HYM1 e HAL2) se aderem fortemente aos frascos de cultura. Esse comportamento dificulta a obtenção de biomassa para experimentos que necessitam quantidades significativas de células. Apesar dos vários fracos de culturas testados e mesmo quando cultivadas sob agitação horizontal (60 - 100 RPM) as células seguiam a tendência de aderir à lateral e ao fundo do recipiente, limitando a quantidade de biomassa adquirida.

Uma das alternativas disponíveis para resolver esse problema consistiu no uso de garrafas graduadas (500 mL - Schott) contendo 450 mL de meio YTD e uma barra magnética. Nessas garrafas foi adicionado 1 mL dos inóculos e em seguida os frascos foram colocados em agitadores magnéticos. Essa configuração simula a agitação orbital de um biorreator, favorecendo a oxigenação do meio. Além disso, o constante movimento impede a adesão das células às paredes do frasco. Apesar de resolver o problema da adesão e obtenção de biomassa para experimentos subsequentes, o processo de manutenção dessa configuração se mostrou mais difícil do que as outras alternativas. A agitação magnética teve que ser cuidadosamente ajustada para evitar disrupção das células dos protistas, assim como são necessários cuidados extras para evitar contaminações. Por outro lado, apesar de não prover uma boa quantidade de biomassa, a utilização de garrafas de cultura celular de 25 cm² (Kasvi) se mostrou uma boa forma de manter os isolados por longos períodos de tempo. Além disso, o uso delas permite a detalhada documentação das características morfológicas das células em microscópio. Os tubos Nalgene configuram uma opção intermediária entre esses dois extremos. Um esquema dos métodos de cultivo testados assim como sua classificação no quesito manutenção e uso em experimentos subsequentes é sintetizado na Figura 4.2.

4.1.2 Potencial biotecnológico dos Labyrinthulomycetes associados às esponjas marinhas

Com a declaração de pandemia do SARS-CoV2, diversos institutos de pesquisa se mobilizaram para buscar soluções para combater o vírus. As esponjas marinhas e seus simbiontes já começaram a ser exploradas nesse sentido (Geahchan et al., 2021). Como discutido no **Capítulo 2**, o potencial biotecnológico dos eucariontes unicelulares ainda é pouco explorado. Os microrganismos pertencentes ao táxon Labyrinthulomycetes quando em cultura são conhecidos por secretarem uma série de

polissacarídeos (polissacarídeos extracelulares - PEs) com funções ainda pouco estudadas (Jain et al., 2005; Marchan et al., 2018). Uma função recentemente demonstrada é que esses PE podem ter funções antivirais contra enterovírus, retrovírus, adenovírus e citomegalovírus existindo inclusive patentes relacionadas (Raghukumar et al, 2014). Sabendo o pico de produção está associado à fase estacionária do crescimento desses protistas (Jain et al., 2005) nós cultivamos por um período de tempo maior do que o habitual (25 dias). A extração bruta usando ácido tricloroacético (adaptação de Bajpai et al., 2016) teve como produto final um precipitado esbranquiçado, indicativo da produção de PEs. No entanto, o extrato não pode ser caracterizado e a explorações das propriedades desses componentes deverão ainda ser realizadas em momentos futuros.

Além da produção desses polissacarídeos, os Labyrinthulomycetes têm um perfil de ácidos graxos bastante diverso, que vem sendo explorado significativamente nos últimos anos (Lee Chang et al., 2014; Aasen et al., 2016). Este interesse se deriva em boa parte da indústria nutracêutica, que tem buscado cada vez mais de alternativas economicamente mais viáveis ao ômega-3 derivado de peixes (Gupta e Gupta, 2020; Du et al., 2021). Assim, a produção de ácidos graxos polinsaturados por esses protistas tem sido alvo de engenharia genética, buscando potencializar a produção de ácido eicosapentaenoico (EPA) e ácido docosaexaenoico (DHA) (Allemann e Allen, 2018; Du et al., 2021). Da mesma forma, a indústria de aquicultura também tem se interessado no potencial desses protistas. Existem diversos estudos que demonstram os impactos positivos da suplementação de rações de peixes e camarões com os Labyrinthulomycetes. Esses protistas já foram usados na alimentação de espécies de interesse comercial como a tilápia-do-nilo (Sarker et al. 2016), salmão do Atlântico (Carter et al., 2003; Lee Chang et al., 2020) e o camarão-da-pata-branca (Allen et al., 2019). Aqui nós avaliamos o perfil dos ácidos graxos de cada um dos isolados obtidos das esponjas marinhas estudadas (**Capítulo 3 -** Table 3.1). No contexto apresentado acima, todos os três isolados apresentaram quantidades expressivas de DHA (C22:6n3), com valores em torno de 40% dos lipídeos totais extraídos. O EPA (C20:5n3) não foi observado em grandes quantidades em nenhuma das culturas. Por outro lado, o ácido palmítico (C16:0), um ácido graxo saturado usado como surfactante em cosméticos, aditivo alimentar e até na produção de biodiesel (Kalustian, 1985; Chen et al., 2015), foi a segunda maior classe detectada nos Labyrinthulomycetes estudados. Importante

salientar que as proporções de ácidos graxos observadas são altamente dependentes das condições de cultura, meios utilizados e tempo de experimento (Marchan et al., 2018). Em vista desses resultados, os três isolados se mostraram válidos para explorações subsequentes uma vez que cada um possui um perfil de ácidos graxos único.

Em nossos experimentos, através de abordagens qualitativas expusemos as culturas de Labyrinthulomycetes a três substratos diferentes (**Capítulo 3 -** Table 3.2). Os isolados obtidos não apresentaram nenhuma atividade metabólica contra carboximetilcelulose, indicando uma ausência de celulases. Isso vai de encontro com o que é visto na literatura, onde essa enzima geralmente está presente (Nagano et al., 2011; Liu et al., 2014; Jaseera et al., 2019). Uma possível explicação para ausência desse aparato é que a maioria dos isolados que possuem esse mecanismo foram obtidos de espécies vegetais, principalmente folhas de mangue caídas, e não de espécies animais como nesse trabalho. Por outro lado, observamos que proteases são comuns, estando presentes em todos os protistas isolados. Essas enzimas, conhecidas também por proteinases ou enzimas proteolíticas, compõem um amplo grupo que tem como função principal catalisar a hidrólise de proteínas e polipeptídios (Zhu et al., 2019). Diferem entre si em várias características como especificidade do substrato, sítio de atividade e mecanismo catalítico, pH e temperatura ideal e perfil de estabilidade (Zhu et al., 2019). A partir desses resultados podemos inferir que as proteases desses microrganismos diferem no tempo de produção assim como na quantidade produzida.

De maneira similar, detectamos a presença de lipases em dois dos isolados: HAL1 e HAL2. As lipases pertencem à classe das hidrolases e têm como função principal a catalisação da reação de hidrólise de triglicerídeos e ácidos graxos (Derewenda, 1994). Essas enzimas são muito utilizadas em processos industriais como nas indústrias de óleos e gorduras, indústria de alimentos, na fabricação de detergentes, no processamento de couro e papel, na indústria cosmética, além do biodiesel e biorremediação (Sarmah et al., 2017). Microrganismos marinhos, principalmente extremófilos, constituem uma fonte importante dessas enzimas, uma vez que as características particulares de seus ambientes permitem a evolução de lipases com funcionamentos únicos (Navvabi et al., 2018). Aqui observamos que HAL1 novamente mostrou maior efetividade na degradação do substrato. O isolado HAL2 demorou a manifestar a presença dessa enzima, um indicativo de que ou condições de cultura como

temperatura, salinidade e/ou pH não eram as mais adequadas; ou esse isolado tem pouca atividade metabólica em relação a esse substrato.

4.2 Considerações Finais e Perspectivas

Antes da pandemia de SARS-CoV2, as esponjas destinadas à experimentação eram mantidas em tanques do Centro de Biologia Marinha (CEBIMar/USP) até o momento do transporte resfriado (18ºC ± 2ºC) para o Laboratório de Biologia Celular de Invertebrados Marinhos (LabCel/USP) em São Paulo – SP. Durante a pandemia, com a indisponibilidade de recursos humanos e da infraestrutura do CEBIMar, tivemos que adaptar o protocolo de coleta. Nossa solução consistiu na manutenção simples em aquários mantidos por aeradores e água do mar local seguida do transporte resfriado no mesmo dia ou no máximo no dia seguinte. Ao pular a etapa de aclimatação ao cativeiro e triagem de esponjas doentes realizada no CEBIMar nós causamos um estresse significativo nesses animais. Vale ressaltar que as coletas de esponjas marinhas, assim como as de qualquer outro animal, devem ser feitas de maneira sustentável e consciente e por isso nós evitamos coletar grandes quantidades. Além disso, a aquisição periódica de esponjas também é justificada pela instabilidade do microbioma das esponjas em cativeiro (Schellenberg et al., 2020), que poderiam resultar em artefatos nas nossas investigações. Essa série de fatores influenciou diretamente o desenvolvimento deste trabalho durante a pandemia.

Um outro aspecto de nossa pesquisa que foi diretamente impactado pela pandemia foi a identificação das espécies de protistas. A morfologia e comportamento dos eucariontes unicelulares, apesar de ser importante para investigações aprofundadas (Keeling, 2019), muitas vezes não é suficiente para distinguir com clareza os táxons aos quais pertencem esses microrganismos. Geralmente é necessária uma abordagem molecular para a correta identificação taxonômica de certas linhagens de protistas (Chihi et al., 2022). Além disso, determinar a localização de microrganismos de interesse nos tecidos do animal hospedeiro é uma das principais formas de investigar a natureza da associação entre as duas partes (Williamson, 1977; Pfannkuchen et al., 2010; Nikolakakis et al., 2015). Contudo, em decorrência da pandemia, até o presente momento não foi possível realizar o sequenciamento do 18S rRNA. Etapas anteriores

tais quais extração do DNA das culturas de protistas e verificação de sua qualidade foram executadas. De maneira similar, também não foi possível avançar com os experimentos de hibridização *in situ* em decorrência de atrasos e/ou desabastecimento de reagentes específicos nos fornecedores. As amostras dos isolados e das esponjas aqui estudadas já foram devidamente preparadas e aguardam um momento propício que permita o uso dessa técnica.

A despeito destes problemas, neste trabalho nós abordamos uma fração do microbioma das esponjas muito pouco explorada: os protistas, com indicativos que estes podem desempenhar funções importantes no contexto do holobionte. Da grande diversidade de eucariontes unicelulares associados às esponjas marinhas percebemos que os Labyrinthulomycetes se destacam pela sua ubiquidade. O trabalho desenvolvido levanta a discussão sobre a importância e potencial do microeucarioma de Porifera e ao mesmo tempo dá o primeiro passo no estudo sistemático dos Labyrinthulomycetes associados às esponjas. Não encontramos evidências que suportem o parasitismo desses protistas, podendo estes estar associados de uma maneira comensal ou mutualística. Os resultados negativos dessa interação geralmente são produtos de disbioses, como ocorre em outros sistemas holobiontes.

Dos três isolados obtidos a partir dos primorfos das esponjas *Hymeniacidon heliophila* e *Haliclona melana*, HAL1 aparenta ser mais promissor para aplicações biotecnológicas, dado seu perfil de ácidos graxos e atividades metabólicas. Contudo, não podemos descartar o potencial dos outros isolados somente com essas abordagens iniciais. A presença de outras enzimas importantes para a indústria, tais como amilases, pectinases, xilanases, lacases, colagenases ou quitinases, ainda deve ser testada. Apesar dos impactos negativos da pandemia nesta pesquisa, espera-se que em breve a identificação taxonômica dos isolados e outras investigações pendentes sejam realizadas. Também é esperado que a partir dos resultados obtidos seja possível explorar com maior foco o potencial biotecnológico desses protistas assim como compreender a contribuição desses microrganismos no funcionamento de seu hospedeiro.

REFERÊNCIAS

Aasen, I.M., Ertesvåg, H., Heggeset, T.M.B., Liu, B., Brautaset, T., Vadstein, O., Ellingsen, T.E., 2016. Thraustochytrids as production organisms for docosahexaenoic acid (DHA), squalene, and carotenoids. Appl. Microbiol. Biotechnol. 100, 4309–4321. https://doi.org/10.1007/s00253-016-7498-4

Adl, S.M., Bass, D., Lane, C.E., Lukeš, J., Schoch, C.L., Smirnov, A., Agatha, S., Berney, C., Brown, M.W., Burki, F., Cárdenas, P., Čepička, I., Chistyakova, L., Campo, J., Dunthorn, M., Edvardsen, B., Eglit, Y., Guillou, L., Hampl, V., Heiss, A.A., Hoppenrath, M., James, T.Y., Karnkowska, A., Karpov, S., Kim, E., Kolisko, M., Kudryavtsev, A., Lahr, D.J.G., Lara, E., Le Gall, L., Lynn, D.H., Mann, D.G., Massana, R., Mitchell, E.A.D., Morrow, C., Park, J.S., Pawlowski, J.W., Powell, M.J., Richter, D.J., Rueckert, S., Shadwick, L., Shimano, S., Spiegel, F.W., Torruella, G., Youssef, N., Zlatogursky, V., Zhang, Q., 2019. Revisions to the Classification, Nomenclature, and Diversity of Eukaryotes. J. Eukaryot. Microbiol. 66, 4–119. https://doi.org/10.1111/jeu.12691

Allemann, M.N., Allen, E.E., 2018. Characterization and Application of Marine Microbial Omega-3 Polyunsaturated Fatty Acid Synthesis, in: Moore, B.S. (Ed.), Methods in Enzymology. Academic Press, pp. 3–32. https://doi.org/10.1016/bs.mie.2018.02.018

Allen, K.M., Habte-Tsion, H.M., Thompson, K.R., Filer, K., Tidwell, J.H., Kumar, V., 2019. Freshwater microalgae (*Schizochytrium* sp.) as a substitute to fish oil for shrimp feed. Sci. Rep. 9, 1–10. https://doi.org/10.1038/s41598-019-41020-8

Araújo, T.A.T., de Souza, A., Santana, A.F., Braga, A.R.C., Custódio, M.R., Simões, F.R., Araújo, G.M., Miranda, A., Alves, F., Granito, R.N., Yu, N., Renno, A.C.M., 2021. Comparison of Different Methods for Spongin-like Collagen Extraction from Marine Sponges (*Chondrilla caribensis* and *Aplysina fulva*): Physicochemical Properties and In Vitro Biological Analysis. Membranes. 11, 522. https://doi.org/10.3390/membranes11070522

Bajpai, V.K., Majumder, R., Rather, I.A., Kim, K., 2016. Extraction, isolation and purification of exopolysaccharide from lactic acid bacteria using ethanol precipitation method. Bangladesh J. Pharmacol. 11, 573–576. https://doi.org/10.3329/bjp.v11i3.27170

Carter, C.G., Bransden, M.P., Lewis, T.E., Nichols, P.D., 2003. Potential of Thraustochytrids to Partially Replace Fish Oil in Atlantic Salmon Feeds. Mar. Biotechnol. 5, 480–492. https://doi.org/10.1007/s10126-002-0096-8

Chang, K.J.L., Parrish, C.C., Simon, C.J., Revill, A.T., Nichols, P.D., 2020. Feeding whole thraustochytrid biomass to cultured atlantic salmon (*Salmo salar*) fingerlings: Culture performance and fatty acid incorporation. J. Mar. Sci. Eng. 8, 14–17. https://doi.org/10.3390/jmse8030207

Chen, W., Ma, L., Zhou, P. -P, Zhu, Y.-M, Wang, X.-P, Luo, X. -A, Bao, Z. -D, Yu, L. -J, 2015. A novel feedstock for biodiesel production: The application of palmitic acid from *Schizochytrium*. Energy 86, 128–138. https://doi.org/10.1016/j.energy.2015.03.110

Chihi, A., O'Brien Andersen, L., Aoun, K., Bouratbine, A., Stensvold, C.R., 2022. Amplicon-based next-generation sequencing of eukaryotic nuclear ribosomal genes (metabarcoding) for the detection of single-celled parasites in human faecal samples. Parasite Epidemiol. Control 17. https://doi.org/10.1016/j.parepi.2022.e00242

del Campo, J., Bass, D., Keeling, P.J., 2020. The eukaryome: Diversity and role of microeukaryotic organisms associated with animal hosts. Funct. Ecol. 34, 2045–2054. https://doi.org/10.1111/1365-2435.13490

Derewenda, Z.S., 1994. Structure and function of lipases. Adv. Protein Chem. 45, 1–52. https://doi.org/10.1016/s0065-3233(08)60637-3

Du, F., Wang, Y.Z., Xu, Y.S., Shi, T.Q., Liu, W.Z., Sun, X.M., Huang, H., 2021. Biotechnological production of lipid and terpenoid from thraustochytrids. Biotechnol. Adv. 48, 107725. https://doi.org/10.1016/j.biotechadv.2021.107725

Ehrlich, H., 2019. Enigmatic Structural Protein Spongin, in: Marine Biological Materials of Invertebrate Origin, Biologically-Inspired Systems. Springer International Publishing, Cham, pp. 161–172. https://doi.org/10.1007/978-3-319-92483-0_11

Geahchan, S., Ehrlich, H., Rahman, M.A., 2021. The anti-viral applications of marine resources for COVID-19 treatment: An overview. Mar. Drugs 19. https://doi.org/10.3390/md19080409

Gupta, J., Gupta, R., 2020. Nutraceutical Status and Scientific Strategies for Enhancing Production of Omega-3 Fatty Acids from Microalgae and their Role in Healthcare. Curr. Pharm. Biotechnol. 21, 1616–1631. https://doi.org/10.2174/1389201021666200703201014

Harel, M., Ben-Dov, E., Rasoulouniriana, D., Siboni, N., Kramarsky-Winter, E., Loya, Y., Barak, Z., Wiesman, Z., Kushmaro, A., 2008. A new Thraustochytrid, strain Fng1, isolated from the surface

mucus of the hermatypic coral *Fungia granulosa*. FEMS Microbiol. Ecol. 64, 378–387. https://doi.org/10.1111/j.1574-6941.2008.00464.x

Jain, R., Raghukumar, S., Tharanathan, R., Bhosle, N.B., 2005. Extracellular polysaccharide production by thraustochytrid protists. Mar. Biotechnol. 7, 184–192. https://doi.org/10.1007/s10126-004-4025-x

Jaseera, K. V., Kaladharan, P., Vijayan, K.K., Sandhya, S. V., Antony, M.L., Pradeep, M.A., 2019. Isolation and phylogenetic identification of heterotrophic thraustochytrids from mangrove habitats along the southwest coast of India and prospecting their PUFA accumulation. J. Appl. Phycol. 31, 1057–1068. https://doi.org/10.1007/s10811-018-1627-7

Jesionowski, T., Norman, M., Zółtowska-Aksamitowska, S., Petrenko, I., Joseph, Y., Ehrlich, H., 2018. Marine spongin: Naturally prefabricated 3D scaffold-based biomaterial. Mar. Drugs 16, 1–23. https://doi.org/10.3390/md16030088

Kalustian, P., 1985. Pharmaceutical and cosmetic uses of palm and lauric products. J. Am. Oil Chem. Soc. 62, 431–433. https://doi.org/10.1007/BF02541417

Keeling, P.J., 2019. Combining morphology, behaviour and genomics to understand the evolution and ecology of microbial eukaryotes. Philos. Trans. R. Soc. B Biol. Sci. 374, 20190085. https://doi.org/10.1098/rstb.2019.0085

Lee Chang, K.J., Nichols, C.M., Blackburn, S.I., Dunstan, G.A., Koutoulis, A., Nichols, P.D., 2014. Comparison of Thraustochytrids *Aurantiochytrium* sp., *Schizochytrium* sp., *Thraustochytrium* sp., and *Ulkenia* sp. for Production of Biodiesel, Long-Chain Omega-3 Oils, and Exopolysaccharide. Mar. Biotechnol. 16, 396–411. https://doi.org/10.1007/s10126-014-9560-5

Liu, Y., Singh, P., Sun, Y., Luan, S., Wang, G., 2014. Culturable diversity and biochemical features of thraustochytrids from coastal waters of Southern China. Appl. Microbiol. Biotechnol. 98, 3241–3255. https://doi.org/10.1007/s00253-013-5391-y

Lukeš, J., Stensvold, C.R., Jirků-Pomajbíková, K., Wegener Parfrey, L., 2015. Are Human Intestinal Eukaryotes Beneficial or Commensals? PLoS Pathog. 11, 7–12. https://doi.org/10.1371/journal.ppat.1005039

Marchan, L.F., Lee Chang, K.J., Nichols, P.D., Mitchell, W.J., Polglase, J.L., Gutierrez, T., 2018. Taxonomy, ecology and biotechnological applications of thraustochytrids: A review. Biotechnol. Adv. 36, 26–46. https://doi.org/10.1016/j.biotechadv.2017.09.003

Nagano, N., Matsui, S., Kuramura, T., Taoka, Y., Honda, D., Hayashi, M., 2011. The Distribution of Extracellular Cellulase Activity in Marine Eukaryotes, Thraustochytrids. Mar. Biotechnol. 13, 133–136. https://doi.org/10.1007/s10126-010-9297-8

Navvabi, A., Razzaghi, M., Fernandes, P., Karami, L., Homaei, A., 2018. Novel lipases discovery specifically from marine organisms for industrial production and practical applications. Process Biochem. 70, 61–70. https://doi.org/10.1016/j.procbio.2018.04.018

Nikolakakis, K., Lehnert, E., McFall-Ngai, M.J., Ruby, E.G., 2015. Use of hybridization chain reaction-fluorescent *in situ* hybridization to track gene expression by both partners during initiation of symbiosis. Appl. Environ. Microbiol. 81, 4728–4735. https://doi.org/10.1128/AEM.00890-15

Pfannkuchen, M., Schlesinger, S., Fels, A., Brümmer, F., 2010. Microscopical techniques reveal the in situ microbial association inside *Aplysina aerophoba*, Nardo 1886 (Porifera, Demospongiae, Verongida) almost exclusively consists of cyanobacteria. J. Exp. Mar. Bio. Ecol. 390, 169–178. https://doi.org/10.1016/j.jembe.2010.04.038

Raghukumar, S., Madhavan, H.N., Malathi, J., 2014. Extracellular polysaccharides from labyrinthulomycetes with broad-spectrum antiviral activities. WO2014045191A2.

Rinkevich, B., 2005. Marine Invertebrate Cell Cultures: New Millennium Trends. Mar. Biotechnol. 7, 429–439. https://doi.org/10.1007/s10126-004-0108-y

Sarker, P.K., Kapuscinski, A.R., Lanois, A.J., Livesey, E.D., Bernhard, K.P., Coley, M.L., 2016. Towards sustainable aquafeeds: Complete substitution of fish oil with marine microalga *Schizochytrium* sp. improves growth and fatty acid deposition in juvenile Nile tilapia (*Oreochromis niloticus*). PLoS One 11, 1–17. https://doi.org/10.1371/journal.pone.0156684

Sarmah, N., Revathi, D., Sheelu, G., Yamuna Rani, K., Sridhar, S., Mehtab, V., Sumana, C., 2018. Recent advances on sources and industrial applications of lipases. Biotechnol. Prog. 34, 5–28. https://doi.org/10.1002/btpr.2581

Schellenberg, J., Reichert, J., Hardt, M., Klingelhöfer, I., Morlock, G., Schubert, P., Bižić, M., Grossart, H.P., Kämpfer, P., Wilke, T., Glaeser, S.P., 2020. The Bacterial Microbiome of the Long-Term Aquarium Cultured High-Microbial Abundance Sponge *Haliclona cnidata* – Sustained Bioactivity Despite Community Shifts Under Detrimental Conditions. Front. Mar. Sci. 7. https://doi.org/10.3389/fmars.2020.00266

Sipkema, D., Schippers, K., Maalcke, W.J., Yang, Y., Salim, S., Blanch, H.W., 2011. Multiple approaches to enhance the cultivability of bacteria associated with the marine sponge Haliclona (gellius) sp. Appl. Environ. Microbiol. 77, 2130–2140. https://doi.org/10.1128/AEM.01203-10

Taylor, M.W., Radax, R., Steger, D., Wagner, M., 2007. Sponge-Associated Microorganisms: Evolution, Ecology, and Biotechnological Potential. Microbiol. Mol. Biol. Rev. 71, 295–347. https://doi.org/10.1128/MMBR.00040-06

Williamson, C.E., 1977. Fluorescence identification of zoochlorellae: A rapid method for investigating alga-invertebrate symbioses. J. Exp. Zool. 202, 187–193. https://doi.org/10.1002/jez.1402020207

Zhu, D., Wu, Q., Hua, L., 2019. Industrial Enzymes, in: Moo-Young, M. (Ed.), Comprehensive Biotechnology. Academic Press, pp. 1–13. https://doi.org/10.1016/B978-0-444-64046-8.00148-8

Apêndice I

Referências Detalhadas das Figuras

Figura 1.1. Fonte: Montagem do autor usando Fotografias Pinheiro, U.S.; Hajdu, E.; Custódio, M.R. (2007) (A-B); Van Soest et al. (2012) (C); N.J. de Voogd (D); G. Parent (E); NOAA Expedition (F); Van Soest et al. (2012) (G). Imagens reproduzidas através de licenças CC-BY 2.5, 3.0 e 4.0.

Figura 1.2: Fonte: Esquema do autor. Imagem adaptada através de licença CC-BY 3.0.

Figura 1.3: Fonte: Rix et al. (2020) - https://doi.org/10.1038/s41396-020-0706-3. Imagem adaptada através de licença CC-BY 4.0.

Figura 1.4: Fonte: Montagem do autor. Adaptado de Bennet et al. (2017) - https://doi.org/10.1007/978-3-319-28149-0_25 e Bailey, J. Craig. 2010. Stramenopiles - http://tolweb.org/Stramenopiles/2380/2010.04.28.

Figura 1.5: Fonte: Montagem do autor. (A-D) Bennet et al. (2017) e Iwata e Honda (2017). (E-G) Micrografias do autor (presente trabalho). (H) Boro et al. (2018) - doi.org/10.1515/bot-2017-005. (I) FioRito et al. (2016) - https://doi.org/10.1007/s00227-016-2944-5. (J) Micrografia de Celeste Leander. Imagens adaptadas através de licença CC-BY 3.0.

Figura 1.6: Fonte: Collier et al. (2017) - https://doi.org/10.1515/bot-2016-0133.

Figura 1.7: Fonte: Fotos do autor.

Figura 1.8: Fonte: Microfotografias do autor.

Figura 1.9: Fonte: Esquema do autor.

Figura 2.1: Fonte: Esquema do autor.

Figura 2.2: Fonte: Esquema do autor.

Figura 3.1: Fonte: Microfotografias do autor.

Figura 3.2: Fonte: Microfotografias do autor.

Figura 3.3: Fonte: Microfotografias do autor.

Figura 4.1: Fonte: Microfotografias do autor.

Figura 4.2: Fonte: Esquema do autor.