UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE BIOTECNOLOGIA
PÓS GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

KEFIR COMO PROBIÓTICO E FONTE DE METABÓLITOS: UM ESTUDO EM *DROSOPHILA MELANOGASTER* MODELO DA DOENÇA DE ALZHEIMER

Aluno: Letícia Leandro Batista

Orientador: Prof. Dr. Carlos Ueira-Vieira

UBERLÂNDIA - MG
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Dissertação apresentada à Universidade Federal de Uberlândia como parte dos requisitos para obtenção do Título de Mestre em Genética e Bioquímica (Área Genética)

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Aos vinte e nove dias do mês de dezembro de dois mil e vinte, às 09:50 horas, reuniu-se via web conferência pela plataforma Google Meet, em conformidade com a Portaria nº 36, de 19 de março de 2020 da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES e Resolução de nº 06/2020 do Conselho de Pesquisa e Pós-graduação pela Universidade Federal de Uberlândia, a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em Genética e Bioquímica, assim composta: Professores Doutores: Carlos Ueira Vieira (Orientador), Ana Paula Mendes Silva e Gabriela Venturini da Silva. A participação dos dois últimos se deu por epístola. Iniciando os trabalhos o (a) presidente Dr (a). Carlos Ueira Vieira apresentou a Comissão Examinadora e o candidato(a), agradeceu a presença dos participantes, e concedeu ao Discente a palavra para a exposição do seu trabalho. A duração da apresentação do Discente e o tempo de arguição e resposta foram conforme as normas do Programa. A seguir o senhor(a) presidente procedeu a leitura das epístolas enviadas pelos membros da banca. Em seguida os membros presentes, passaram a arguir o(a) candidato(a). Ultimada a leitura das epístolas e a arguição, que se desenvolveu dentro dos termos regimentais, a Banca, em sessão secreta, atribuiu o resultado final, considerando o(a) candidato(a):

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Esta defesa de Dissertação de Mestrado é parte dos requisitos necessários à obtenção do título de Mestre. O competente diploma será expedido após cumprimento dos demais requisitos, conforme as normas do Programa, a legislação pertinente e a regulamentação interna da UFU. Nada mais havendo a tratar foram encerrados os trabalhos. Foi lavrada a presente ata que após lida e achada conforme foi assinada pela Banca Examinadora.

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ALUNO: Letícia Leandro Batista

COMISSÃO EXAMINADORA

Presidente: Prof. Dr. Carlos Ueira-Vieira

Examinadores: Dra. Ana Paula Mendes-Silva

Dra. Gabriela Venturini da Silva

Data da Defesa: 29/12/2020

As sugestões da Comissão Examinadora e as Normas do PGGB para o formato da Dissertação/Tese foram contempladas

Prof. Dr. Carlos Ueira-Vieira

Wenn einer allein träumt, ist es nur ein Traum. Wenn viele gemeinsam träumen, ist das der Anfang einer neuen Wirklichkeit

DEDICATÓRIA

Dedico este trabalho à minha avó, **Osmária de Fátima Rodrigues Batista**, pelo apoio incansável. Obrigado por acreditar em mim. Esta conquista é nossa.

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APRESENTAÇÃO

A Doença de Alzheimer (DA) é a principal causa de demência entre idosos, gerando declínio cognitivo e consequente desgaste emocional e co-dependência. A DA é tradicionalmente caracterizada pela formação de placas senis pela via amiloidogênica e, estudos recentes indicam que a disbiose como um fator importante para sua patologia. Para superar a disbiosie, o uso de probióticos – como o kefir – tem mostrado um grande potencial como alternativa terapêutica para a Doença de Alzheimer.

Desta forma, no Capítulo I, descrevemos a Doença de Alzheimer – seus mecanismos, relação com a microbiota e o uso emergente de probióticos como agentes terapêuticos – e tais aspectos na *Drosophila melanogaster* como organismo modelo. Já no Capítulo II, utilizamos *D. melanogaster* como organismo modelo para exploramos os efeitos do kefir como probiótico e fonte de metabólitos, agindo como modulador da microbiota e da via amiloidogênica.

Capítulo I

Fundamentação Teórica

1. Doença de Alzheimer

Segundo a Organização Mundial de Saúde, a demência é uma das maiores causas de dependência e incapacidade entre idosos, atingindo cerca de 50 milhões de pessoas mundialmente. Suas características vão além do envelhecimento comum, englobando grande perda de funções cognitivas – como memória e habilidade de realizar tarefas rotineiras. Até 2050, são previstos um milhão de novos casos de demência por ano, resultado em um custo de 1,1 trilhões de dólares em saúde pública ao redor do mundo (Alzheimer's Association, 2020; LIVINGSTON et al., 2020).

A Doença de Alzheimer (D.A.) é uma doença neurodegenerativa, responsável por cerca de 70% dos casos de demência (LIVINGSTON et al., 2020). Seus principais sintomas englobam mudanças comportamentais – incluindo apatia e depressão –, deteriorações motoras e cognitivas como perda de memória recente, dificuldade em tomar decisões e confusão em relação ao tempo ou espaço (Alzheimer's Association, 2020).

Atualmente, cerca de 800 milhões de pessoas são maiores de 60 anos e estima-se que este número chegue a 2 bilhões até 2050 (WASAY et al., 2016). Estudos indicam que indivíduos acima de 70 anos possuem 10% a mais de risco de desenvolver D.A., enquanto que para idosos acima de 85 anos, este aumento é de 45% (WILLIAMSON; GOLDMAN; MARDER, 2009). Desta forma, o aumento da expectativa de vida global – assim como os impactos físicos, sociais, psicológicos e econômicos da D.A. – fazem com que esta seja uma doença cada dia mais relevante.

1.1 Neuropatologia

Neuropatologicamente a D.A. é caracterizada por uma alta densidade de placas senis e presença de emaranhados neurofibrilares – causados pelo acúmulo de precipitados de peptídeo β-amiloide e de proteína Tau hiperfosforilada, respectivamente (BLOOM, 2014). O conjunto destes fenômenos gera perda de conexões intracerebrais – e do cérebro com outros órgãos –, de funções sinápticas e também de células neurais (SERRANO-POZO et al., 2011).

Apesar de seu mecanismo patogênico exato ainda não ter sido identificado, há evidência de que o processo amiloidogêncio (de formação de peptídeos β-amilóide e do consequente acúmulo de placas senis) é um fator fundamental na causa e progressão da Doença de Alzheimer (LANE; HARDY; SCHOTT, 2018)

A presença de placas senis é natural – apesar de ausente durante a juventude – e pode ser observada em indivíduos cognitivamente intactos. Assim, a patologia é caracterizada de forma quantitativa. Placas senis são formadas primariamente por peptídeos β-amiloides derivados da clivagem da proteína precursora de β-amiloide (APP), componente do metabolismo celular em geral, expressa em todos tecidos e células nucleadas. Esta proteína pode ser processada proteoliticamente pela via amiloidogênica ou não amiloidogênica, sendo a primeira alvo de uma das principais hipóteses sobre o desenvolvimento da D.A. (CASTELLANI; PERRY, 2013)

A via não amiloidogênica (não produzindo peptídeos β-amiloide) é constituída pela clivagem da APP pela enzima α-secretase, gerando um fragmento C-terminal da APP de 83 aminoácidos. Há então processamento pela γ-secretase, gerando um fragmento intracelular da APP e um fragmento P3, ou β-amiloide truncado (por haver retenção do componente amiloidogênico). Tais produtos não culminam no desenvolvimento da D.A. (HARDY, J; SELOKOE, 2002)

Por sua vez, a via amiloidogênica é caracterizada pela clivagem da APP pela enzima β-secretase, gerando um fragmento N-terminal (extracelular) da APP de 99 aminoácidos. A enzima γ-secretase também cliva este fragmento, gerando um fragmento intracelular da APP e o peptídeo β-amiloide completo. Este peptídeo pode apresentar tamanhos variados – sendo que os mais relevantes para D.A. possuem 40 e 42 aminoácidos – e se oligomeriza e fibriliza, gerando placas β-amiloides que são depositadas no cérebro (HARDY, J; SELOKOE, 2002)

A enzima β-secretase, também conhecida como BACE (*β-Site APP Cleaving Enzyme*) é uma aspartil protease de membrana que possui dois homólogos que se diferem na localização tecidual e celular, BACE-1 e BACE-2, sendo que apenas a primeira atua na produção de peptídeo β-amiloide. Por estar centralmente envolvida na via amiloidogênica e ser um fator limitante, a BACE-1 é um grande alvo para terapias para a D.A. (CASTELLANI; PERRY, 2013).

Os emaranhados neurofibrilares presente na D.A e sua neurotoxicidade se originam da hiperfosforilação da proteína Tau, uma MAP (proteína associada a microtúbulos) altamente solúvel, encontrada em baixos níveis em neurônios e outras células do sistema nervoso. As funções desta proteína é a estabilização de microtúbulos e a regulação de transportes axonais, permitindo a estruturação de neurônios e o transporte de nutrientes e proteínas para as células. Na D.A. a proteína Tau é hiperfosforilada, e por não ser funcional,

não contribui para a organização do citoesqueleto, levando à morte neuronal (HARDY, J; SELOKOE, 2002).

Estudos indicam que além das teorias clássicas de patogênese de hiperfosforilação da proteína Tau e da via amiloidogênica, a Doença de Alzheimer é na verdade uma doença multifatorial. Há indícios principalmente da contribuição da disbiose – desequilíbrio na microbiota do hospedeiro – em sua patologia (BHATTACHARJEE; LUKIW, 2013; BONFILI et al., 2017; SUN et al., 2020).

1.1.1 Disbiose na Doença de Alzheimer

A microbiota é composta por trilhões de microorganismos presentes no trato gastrointestinal, que participam de inúmeros processos relativos à saúde do hospedeiro, como absorção de minerais, síntese de vitaminas e extração de energia e nutrientes dos alimentos (LEE; HASE, 2014).

Sua interação com o cérebro se dá através do *gut-brain-axis* (trato cérebro-intestinal), cujo estudo tem contribuído para investigar causas de estados de cerebrais saudáveis e patológicos (SUN et al., 2020). O *gut-brain-axis* é constituído pela microbiota intestinal; dos sistemas nervosos entérico, parassimpático, simpático e central; de conexões neuroendócrinas, citocinas, neuropeptídeos, moléculas sinalizadoras e vias humorais (KOLOSKI et al., 2012).

Com o envelhecimento, há uma diminuição na diversidade microbiana intestinal natural. A quantidade de espécies comensais é diminuída – como *Bacterióides*, *Bifidobacteria* e *Lactobacilli* – enquanto que espécies patogênicas e oportunistas passam por um aumento relativo (NAGPAL et al., 2018)

A disbiose atinge funções do sistema nervoso central por um sistema de *feedback,* através de desbalanço na homeostase energética, modulação na secreção de ácidos graxos de cadeia curta e na produção de neurotransmissores – como ácido gama-aminobutírico (GABA), N-metil D-Aspartato (NMDA), e serotonina (BHATTACHARJEE; LUKIW, 2013; NOBLE; HSU; KANOSKI, 2017).

Alterações na microbiota foram observadas em modelos da Doença de Alzheimer em *Drosophila melanogaster*, ratos e até mesmo em pacientes humanos (CHEN et al., 2020; LIU et al., 2019; WU et al., 2017). Tais alterações podem contribuir para o dano neuronal de uma forma dependente de peptídeo β-amiloide pela produção direta do peptídeo – e aceleração de seu acúmulo – ou pela inibição de mecanismos necessários para a eliminação

de β-amiloide no cérebro (ERICKSON et al., 2012; GAO et al., 2019; MORALES et al., 2010).

Além de fatores diretamente relacionados à via amiloidogênica, a disbiose também está relacionada com a perda de plasticidade e funções de células nervosas; aumento da permeabilidade da barreira hematoencefálica; e aumento do estresse oxidativo e processos inflamatórios, que podem acelerar a ocorrência de neurodegeneração na D.A. (BRANISTE et al., 2014; LUCA et al., 2019; MARIZZONI et al., 2020). O estresse oxidativo estimula o aumento da APP, da disfunção mitocondrial e da hiperfosforilação da proteína Tau (JIANG; SUN; CHEN, 2016; KIM et al., 2015). Por sua vez, processos inflamatórios aumentam a ineficiência de fagocitose do peptídeo β-amiloide pela microglia (SPANGENBERG; GREEN, 2017).

Desta forma, a modulação da microbiota – buscando recuperação da disbiose – pode ser uma abordagem terapêutica para a Doença de Alzheimer, principalmente através do uso de probióticos (WESTFALL et al., 2017).

1.2 Probióticos como abordagem terapêutica

Probióticos são definidos como microorganismos vivos que são benéficos para saúde do hospedeiro quando ingeridos em quantidades adequadas (BRAVO et al., 2012). Seu uso é bem definido no tratamento de doenças relacionadas ao trato gastrointestinal – como casos de intolerância à lactose, diarreia e efeitos colaterais gerados por antibióticos.

Além disso, probióticos tem mostrado um grande potencial terapêutico ou profilático contra doenças neurodegenerativas. Isso se dá por atuarem reinstaurando o equilíbrio da microbiota e na sua participação no metabolismo hospedeiro (AKBARI et al., 2016; MARTIROSYAN; LEEM, 2019).

O efeito do uso de probióticos na Doença de Alzheimer tem sido investigado em animais modelos e também em humanos (AKBARI et al., 2016; KAUR et al., 2020; LEBLHUBER et al., 2018). Há evidências de atuarem na diminuição da agregação de placas senis e na restauração de vias proteolíticas neuronais, atenuando os níveis de declínio cognitivo (BONFILI et al., 2017; LEI; VACY; BOON, 2016). Desta forma, investigar diferentes formulações probióticas que sejam benéficas para a DA é o próximo passo (WESTFALL et al., 2017).

1.2.1 Kefir

Um probiótico promissor para terapia contra a D.A. é o kefir, um probiótico oriundo da região do Cáucaso. Considerado como o iogurte do século XXI, o kefir é composto por bactérias e leveduras – mais frequentemente isolados os gêneros *Lactobacillus, Leuconostoc, Kluyveromyces, Pichia* e *Saccharomyces* (SCHNEEDORF; ANFITEATRO, 2004) (PLESSAS et al., 2017). Ao fermentar seu substrato – o leite – tais microorganismos produzem metabólitos como ácidos orgânicos, dióxido de carbono, etanol e peptídeos (HSIEH et al., 2012).

O kefir tem como característica particular os grãos de kefir – estruturas compostas de proteínas e polissacarídeos que confinam sua microbiota complexa. Os grãos de kefir podem ser descritos como uma massa irregular – branca-gelatinosa ou levemente amarela – com consistência elástica e tamanho entre 0,3 e 5 cm de diâmetro (BENGOA et al., 2019). Os grãos de kefir são compostos aproximadamente por 83% de água, 4-5% de proteínas e 9-10% de um polissacarídeo chamado kefiran (ABRAHAM; DE ANTONI, 1999).

A composição microbiana do kefir é variável de acordo como a origem geográfica, forma de armazenamento, o tipo de leite utilizado, razão entre grãos/leite utilizada e a temperatura de fermentação (BARAO et al., 2019; LONDERO et al., 2012; NIELSEN; GÜRAKAN; UNLÜ, 2014; ROSA et al., 2017). Mais de 50 espécies diferentes de bactérias e leveduras já foram encontradas em grãos de kefir (BOURRIE; WILLING; COTTER, 2016; PRADO et al., 2015). Sua composição é majoritariamente de bactérias ácido-lácticas (LAB), seguido por bactérias acéticas (AAB) e leveduras, que interagem de maneira simbiótica (DONG et al., 2018).

Durante o processo de fermentação, compostos funcionais são gerados, com ação antioxidante, antialergênica, antitumoral, antimicrobiana e anti-inflamatória (AMORIM et al., 2019; CENESIZ et al., 2008; CHEN et al., 2015; COTÂRLEȚ et al., 2019; DINIZ, R.O.; PERAZZO F.F.; CARVALHO, J.C.T.*; SCHNEENEDORF, 2003; KIM et al., 2019; RODRIGUES et al., 2005).

O kefir é especialmente promissor na terapia contra a Doença de Alzheimer. Seu uso de kefir foi capaz de atenuar D.A. induzida por lipopolissacarídeos em ratos, através da modulação de processos inflamatórios (ANWAR et al., 2018, 2019). Ademais, em pacientes

de D.A., sua ingestão melhorou déficits cognitivos, níveis de estresse oxidativo e diminuiu dano à celulas vermelhas (TON et al., 2020).

Porém, uma comparação entre o uso do kefir como probiótico e de seus metabólitos na D.A. ainda não foi descrito na literatura. Para tal, é necessário utilizar animais modelo como *D. melanogaster.*

2. *Drosophila melanogaster*

D. melanogaster, também conhecida como a mosca da fruta, é um invertebrado amplamente utilizado para estudar a patogênese e possíveis tratamentos para doenças neurodegenerativas como a D.A (LENZ et al., 2013; MCGURK; BERSON; BONINI, 2015) Há várias vantagens em seu uso: anatomia simples, curto ciclo de vida, baixo custo de manutenção e fácil manejo (YAMAGUCHI; YOSHIDA, 2018). Apesar de simples, *D. melanogaster* é um organismo modelo robusto para D.A.: possibilita a observação de placas senis, anormalidades morfológicas externas, mudanças neuroanatômicas dramáticas e déficits na habilidade motora e memória (DESHPANDE; GOGIA; SINGH, 2019). Além disso, *D. melanogaster* tem seu genoma inteiro é sequenciado, e 70% de seus genes são relacionados à genes de doenças humanas (BIER, 2005; LENZ et al., 2013; SINGH; IRVINE, 2012).

Em relação à D.A., a *D. melanogaster* possui homólogos de vários genes necessários para a via amiloidogênica. Exemplos são o gene APP-like (APPL), similar ao APP (FOSSGREEN et al., 1998; STRUHL; GREENWALD, 1999; WASCO et al., 1992) uma α-secretase chamada Kuzbanian (kuz), e uma β-secretase (dBACE) (CARMINE-SIMMEN et al., 2009; GREEVE et al., 2004). dBACE é expresso em neurônios e axons e é necessário para sobrevivência de células da glia, além de ser capaz de clivar APPL, produzindo amiloide neurotóxico. Porém, APPL não possui o domínio específico que geraria o peptídeo β-amiloide de 42 aminoácidos encontrado em humanos (LUO; TULLY; WHITE, 1992). Desta forma, a expressão de APP e BACE humanas em *D. melanogaster* é necessária para gerar um modelo da via amiloidogênica.

Várias ferramentas permitem a expressão de genes exógenos na mosca de fruta, com controle temporal e espacial. Por exemplo, o sistema transgênico GAL-4/UAS – derivado de leveduras – é ativado pelo cruzamento entre duas linhagens. Uma expressa Gal4 (*driver*), responsável por direcionar o gene de interesse para um tecido específico, enquanto a segunda

contém o elemento UAS (*Upstream Ativation Sequence* – que atua *responder,* modulando a transcrição do gene de interesse. Desta forma, a prole resultante irá expressar o gene ligado ao UAS sob um padrão de expressão dirigido por Gal4 (ELLIOTT et al, 2008).

Com esta ferramenta, APP e BACE humanas podem ser expressas em *D. melanogaster* de forma pan-neural – utilizando o driver elav-Gal4. Tais moscas demonstram alta produção de peptídeos B-amiloide de 42 aminoácidos, assim como perdas sinápticas e defeitos comportamentais – resultado consistente com efeitos encontrados em mamíferos modelos de D.A. (CHAKRABORTY et al., 2011; MHATRE et al., 2014).

Ademais, assim como em humanos, a microbiota da *D. melanogaster* influencia e é influenciada pelo seu hospedeiro (LESPERANCE; BRODERICK, 2020). Sua comunidade microbiana é menos complexa que a presente em mamíferos, sendo composta de 5-20 espécies (majoritariamente do gênero *Lactobacillus* e *Acetobacter*) (BRODERICK; LEMAITRE, 2012).

Mesmo sendo necessário levar em conta tais diferenças, a *D. melanogaster* tem sido utilizada na investigação de efeitos mediados pela mudança na microbiota (CLARK et al., 2015; COMBE et al., 2014; SHIN et al., 2011). Estudos indicam que a disbiose também pode ser observada em moscas modelo da D.A.. *Lactobacilus* – o principal gênero presente na microbiota da *D. Melanogaster* – e se encontra diminuído em moscas AD-like (LEE; HASE, 2014). Além, a reposição de tal microorganismo atenuam a patologia desta doença (TAN et al., 2020; WESTFALL; LOMIS; PRAKASH, 2018).

Desta forma, o modelo para Doença de Alzheimer em *Drosophila melanogaster* possui grande capacidade principalmente para avaliação de probióticos específicos, assim como seus metabólitos (CLARK; WALKER, 2018; PANDEY; NICHOLS, 2011; YEATES; SARKAR; KANGO-SINGH, 2019).

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Capítulo II

Kefir microorganisms and metabolites in a fly model for Alzheimer's Disease

Kefir microorganisms and metabolites in a fly model for Alzheimer's Disease

Resumo

A Doença de Alzheimer (DA) é a principal causa de demência entre idosos mundialmente, gerando declínio cognitivo e consequente desgaste emocional e co-dependência. A DA é tradicionalmente caracterizada pela formação de placas senis pela via amiloidogênica, e estudos recentes indicam que a disbiose como um fator importante para sua patologia. Para superar a disbiosie, o uso de probióticos – como o kefir – tem mostrado um grande potencial como alternativa terapêutica para a Doença de Alzheimer. Desta forma, neste trabalho o kefir foi explorado como probiótico e fonte de metabólitos, agindo como modulador da microbiota e da via amiloidogênica. Para tal avaliação, *Drosophila melanogaster* expressando os principais genes desta via, foi utilizada como modelo (moscas AD-like). A composição da microbiota do kefir foi determinada através do sequenciamento de 16S rRNA, e seus metabólitos foram obtidos através de partição líquido-líquido com hexano, diclorometano, acetato de etila e n-butanol, em order crescente de polaridade. Após tratamento, moscas AD-like foram avaliadas em termos de sobrevida, habilidade de escalada e morfologia do olho. Moscas tratadas com kefir mostraram melhora tanto em sua habilidade de escalada quanto em sua sobrevida, enquanto que moscas tratadas com frações apolares melhoraram o primeiro parâmetro e, as tratadas com frações polares melhoraram o segundo. Por fim, mostramos que tanto o kefir quanto suas frações são potenciais fontes terapêuticas contra a DA, atuando na modulação de vias relacionadas à amiloidogênese e disbiose.

Palavras-chave: *Drosophila melanogaster*, Doença de Alzheimer, Kefir; Probióticos; Disbiose

Abstract

Alzheimer's Disease (AD) is the most common cause of dementia among elderly individuals worldwide, leading to a strong motor-cognitive decline and consequent emotional distress and codependence. It is traditionally characterized amyloidogenic pathway formation of senile plaques, and recent studies indicate that dysbiosis is also an important factor in AD's pathology. To overcome dysbiosis, probiotics – as kefir – have shown to be a great therapeutic alternative for Alzheimer's disease. In this present work, we explored kefir as a probiotic and a metabolite source as a modulator of microbiome and amyloidogenic pathway, using a *Drosophila melanogaster* model for AD (AD-like flies). Kefir microbiota composition was determined through 16S rRNA sequencing, and its metabolites were obtained through liquid-liquid partitioning with hexane, dichloromethane, ethyl acetate and n-butanol. After treatment, flies had its survival, climbing ability and eye morphology analyzed. Kefir treated flies improved both their climbing ability and survival rate, whereas flies treated with non-polar fractions improved the first and, the ones treated with polar fractions improved the second. In conclusion, we show that both kefir and its fractions may be promising therapeutic source against AD, through modulating amyloidogenic related pathways and gut dysbiosis.

Key-words: *Drosophila melanogaster*, Alzheimer's Disease, Kefir; Probiotics; Disbiosis

Introduction

Alzheimer's Disease (AD) is the most common cause of dementia among elderly individuals worldwide, leading to a strong cognitive decline and consequent emotional distress and codependence (1). Its pathophysiology is multifactorial, but traditionally characterized by senile plaques production and deposit through the amyloidogenic pathway. In this pathway, the amyloid precursor protein (APP) is cleaved by the β-secretase enzyme, generating an Aβ peptide of 40 and 42 aminoacids (2), which oligomerizes and fibrilizes, causing senile plaques and leading to synapse degeneration (3).

Recent studies indicate that dysbiosis – characterized by host gut-microbiome disbalance – plays a big role in AD's pathology (4–6). These alterations may contribute to neuronal damage by inhibiting pathways related to Aβ clearance, or directly by improving this peptides production or accumulation (7–9).

To overcome dysbiosis, probiotics have shown to be a great therapeutic alternative for Alzheimer's Disease (10). Within this approach, kefir – natural probiotic drink constituted by symbiotic bacteria and yeasts – has been used (11–13). It uses milk as a substrate, producing metabolic molecules with health improving effects, as antioxidant and anti-inflammatory properties (14–21). Kefir administration has shown to be able to attenuate AD effect both in rats, through inflammatory process modulations (22,23), and in AD patients, improved cognitive function and lowering both oxidative stress levels and red cells damage (24).

As *Drosophila melanogaster* shares a similar yet simpler central nervous system in relation to mammals, its use in investigating neurodegenerative diseases has been incredibly valuable (25–28). Plus, it has been suggested to be an interesting model for exploring gut-brain-axis interactions within these diseases (29,30). Studies have shown that probiotic treatment attenuates AD effects (30), but no study has investigated probiotics metabolites on AD model.

This way, in this study we explored kefir effects – as a probiotic and from its metabolites – in *D. melanogaster* to expressing human BACE and APP.

Materials and methods

Kefir preparation

Kefir grains were obtained through donation in Uberlândia, Brazil. The fermented product – kefir – was obtained by inoculating kefir grains (4% m/v) in pasteurized whole cow milk. The fermentation process went for 24 hours at room temperature in a glass container covered with cloth to avoid contamination. Then, kefir grains were filtered, and the fermentation product was used for treatments. Exceeding kefir grains were inoculated in milk with 20% glycerol and were kept at -20ºC for further experiments.

Next-generation sequencing library preparation

Kefir grains together with its fermented product had its genomic DNA purified according to the BGI Americas in-house protocol. DNA integrity was tested by 1% agarose gel electrophoresis, and sample concentration was tested using Qubit Fluorometer (Invitrogen).

For library construction, 30ng of DNA sample and fusion primer were used to configure PCR for 16S-v4 regions (BGI Americas in-house protocol). After PCR, Agencourt AMPure XP beads (DNA/bead ratio of 1) were used to purify the DNA, which was dissolved in elution buffer. The library was qualified using the Agilent 2100 bioanalyzer (Agilent Technologies), and sequenced paired-end on the Hiseq 2500 (Ilumina), using the MiSeq-PE250 sequencing strategy (MiSeq Reagent Kit).

To obtain more accurate and reliable results, raw data was pre-processed by removing: reads with a lower average quality of 20 over 25 bp, based on the phred algorithm (31); trimmed reads with less than 75% of their original length; reads contaminated by adapters (with 15 bp overlapped); and reads with low complexity (with 10 consecutive same base). Plus, if the two paired-end reads overlapped (minimum of 15bp overlap), the consensus sequence was generated by FLASH (Fast Length Adjustment of Short reads, v1.2.11) (32).

Data analysis for sequencing

To analyze community patterns, all clean the tags were clustered to OUT (Operational Taxonomic Unit) using USEARCH (v7.0.1090) (33). The tags were clustered into OTU with a 97% threshold by using UPARSE (34), and OTU unique representative sequences were obtained. Chimeras were then filtered out by using UCHIME(v4.2.40) (35). The 16S rDNA were screened for chimeras by mapping to Gold Database (v20110519) (36) and to UNITE(v20140703) (37).

All tags were mapped to each OTU representative sequences using USEARCH GLOBAL, and taxonomically classified using Ribosomal Database Project (RDP) Classifier v.2.2 (38), using 0.6 confidence values as cutoff. Bacterial 16S rDNA were annotated using Greengene database (v201305) (39) and fungal 18S rDNA using Silva database (v119) (40). The sequences were also BLAST searched against the National Center for Biotechnology Information (NCBI) nucleotide collection to cross-check previously assigned taxonomy. Species were qualified when query cover was 100%.

Kefir methanolic extraction and liquid-liquid partitioning

After complete fermentation, kefir was frozen at -20ºC overnight and then lyophilized (L101, Liobras, SP, Brazil) for three days. Lyophilized material (100g) was solubilized with 150 mL of methanol 80% for 15 minutes. The liquid part was obtained through filter paper separation, and followed to liquid-liquid partitioning. In increasing polarity order, hexane, dichloromethane, ethyl acetate and n-butanol solvents were used. For each solvent, 200 mL were used and the process was repeated four times. The extractive solvents were removed using a rotary evaporator (Buchi Rotavapor R-210, Flawil, Switzerland) and the remaining liquid was removed by leaving the solutions in a chemical hood for a week. After that, resulting fractions were frozen overnight and lyophilized to remove the remaining water.

D. melanogaster stocks and genetics

Flies were reared with standard cornmeal medium (soy powder 0,01%, glucose 7,2%, agar 0,6%, cornmeal 0,073%, yeast 0,018%, nipagin 0,06% and acid solution 0,05% m/v) and kept in a 12-12 hour light/dark cycle incubator, at 25ºC. Flies stocks were obtained from Bloomington Stock Center: W1118 (stock number, 3605), UAS-BACE-1, UAS-APP (33797), elav-GAL4 (458), and GMR-GAL4 (1104).

AD-like flies

To generate AD-like flies, individuals from elav-Gal4 and UAS-BACE,UAS-APP strains were anesthetized with ether ethyl and sorted according to sex. Elav-Gal4 female virgins – with a visible meconium – and UAS-BACE,UAS-APP males and were crossed. The resulting F1 was sorted through while in pupae stage: the ones exhibiting tubby phenotype were discarded. This steps ensured the resulting individuals would be elav-Gal4; ;UAS-BACE,UAS-APP, here addressed as AD-like flies.

Treatments

For all assays, unless specifically said, AD-like flies were treated at 0-3 days after eclosion. Treatment food was prepared by adding 2 mL of freshly-prepared kefir or its fractions (hexane, dichloromethane, ethyl acetate or n-butanol) to 1g of enriched mashed potato medium (75% instant mashed potato, 15% yeast extract, 9,3% glucose and 0,07% nipagin). Food was changed every 2 days to ensure fresh treatment exposure. Beyond, water (control) and Tween80 0.01% (Sigma) (vehicle) were tested.

Survival assay

In order to evaluate its survival rate, male and female flies were treated as previously described and dead flies were counted every two days for 15 days. A total of 90 flies from

each genotype and treatment were assayed. The mean lifespan was calculated through the Kaplan-Meier test on GraphPad Prism 8.0.2 software.

Rapid iterative negative geotaxis (RING) assay

For climbing assays, groups of 30 male AD-like flies of each treatment (tested in triplicate) were transferred to clean vials and put in a custom 12-vials holder. Flies had its behavior accessed 5 and 10 days after treatment. Before testing, flies were exposed to light and kept in a silent environment for 20 minutes, in order to acclimate. The holder was then hit three times in the bench and the flies were given 4 seconds to climb 5 cm. This was repeated five times. The procedure was recorded and the video analyzed using QuickTime Player 7.7.9 software. The average climbing percentage was calculated as the percentage of flies of each group that reached the 5 cm mark after 120 frames that the holder touched the bench.

Light and scanning electron microscopy of the flies' eye

To overexpress human BACE and APP in the fly compound eye, UAS-BACE,UAS-APP males were crossed with GMR-GAL4 female virgins, as described before. The resulting GMR-Gal4>UAS-BACE,UAS-APP were kept from embryo stage on vials containing treatment food (or standard food with water, for positive control), as stated previously. As a negative control for the degenerative phenotype, GMR-GAL4>W1118 were used. One day old flies were anesthetized with ethyl ether and had its eyes observed and photographed with a stereomicroscope (Nikon SMZ745) equipped with an Integrated 2.3 Mega-Pixel DFK 23UX236 (Sony) camera.

After observation, flies were kept in 70% ethanol until usage. For scanning electron microscopy (SEM), flies were dried at room temperature and metalized with 10nm of gold. The outer surface morphology of the compound eye was visualized by a Zeiss EVO MA10 microscope operated at 5kV.

Statistical Analysis

Obtained data distribution for each analyzed group within each experiment was evaluated as either parametric or non-parametric through the D'Agostino&Pearson test. Groups were compared through a t test with a stablished significance level of $P < 0.05$. Analysis were performed using the software GraphPad Prism 8.

Results

Microorganisms found in kefir

A total of 180,388 paired-end V4-16S raw reads were obtained from kefir grains together with its fermentation product. After pre-processing, 143,961 reads were kept, with an average length of 252 bp. From those, all sequences were clustered with representative sequences, and a 97% sequence identity cut-off was used. From this, five Operational Taxonomic Units (OTUs) were generated. Sequences were BLAST against the NCBI nucleotide collection (Table 1). *Lactobacillus kefiranofaciens* was the most present strain, having 21.96% of read abundance.

Table 1 – 16S-v4 read abundance and taxonomic units

Taxonomy	**Read Abundance (Total)**	**Read Abundance (%)**
Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; *Lactobacillus; Lactobacillus kefiranofaciens*	31,980	21.96
Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; *Lactobacillus; Lactobacillus kefiri*	294	0.20
Bacteria; Proteobacteria; Alphaproteobacteria;Rhodospirillales; Acetobacteraceae; *Acetobacter; Acetobacter fabarum*	261	0.17
Bacteria; Firmicutes; Bacilli; Lactobacillales;Streptococcaceae; *Lactococcus; Lactococcus lactis*	7	0.004
Bacteria; Proteobacteria; Alphaproteobacteria;Rickettsiales	2	0.001

Lactobacillus kefiri was the second most present species – totalizing 0.2% of read abundance – and *Acetobacter fabarum* had 0.17% of read abundance. Traces of *Lactococcus lactis* (0.004%) and Rickettsiales order (0.001%) were also found. From the OTUs it was possible to investigate phylogeny of these microorganisms (Figure 1).

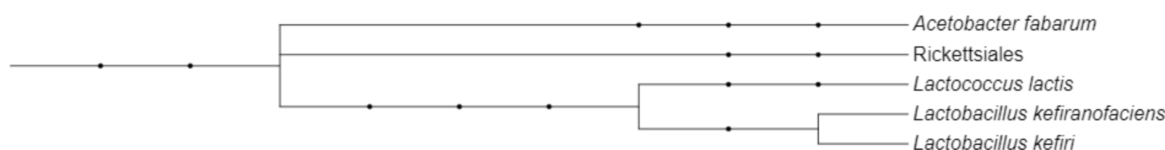

Figure 1. Phylogenetic tree based on 16S rRNA genes sequence of microbial groups found in kefir grains plus its fermentation product. Percentages in front of the taxa indicate read abundance.

Liquid-liquid partition yield

To distinguish between microbiome interactions and metabolite effects from kefir, a series of liquid-liquid partitioning was performed. From there, four organic fractions were obtained through liquid-liquid partitioning. Solvents were used with a increasing polarity order: hexane, dichloromethane, ethyl acetate and n-butanol. The yield of different organic fraction was listed on Table 2, based on 100 g of liophilized kefir were used as a starting point.

Table 2 – Liquid-liquid partitioning yield from kefir methanolic extract.

Fraction	Solvent	Obtained weight (g)	Yield (%)
Hex	Hexane	0.1334	0.5053
DCM	Dichloromethane	0.3634	1.3765
EtOAc	Ethyl acetate	0.8226	3.1159
ButOH	N-butanol	2.3012	8.7136

Extraction yield increased more polar solvents were used: n-butanol (ButOH) and ethyl acetate fraction (EtOAc) presented higher yields (8.71% and 3.11%) than dichloromethane (DCM) and hexane fractions (Hex) (1.37% and 0.50% respectively).

Survival assay

Survival rate after kefir treatment

To verify if kefir microorganisms and metabolites effect on AD-like flies, we first investigated its effect on its survival. Control AD-like flies (elav-Gal4>UAS-BACE,UAS-APP), fed with non-treated food – displayed a high mortality rate (only 40.4% of the flies were alive) within first two weeks of life. Kefir treatment improved the survival rate of these flies (P < 0.0001) – 64.71% of kefir treated flies were still alive in the same period.

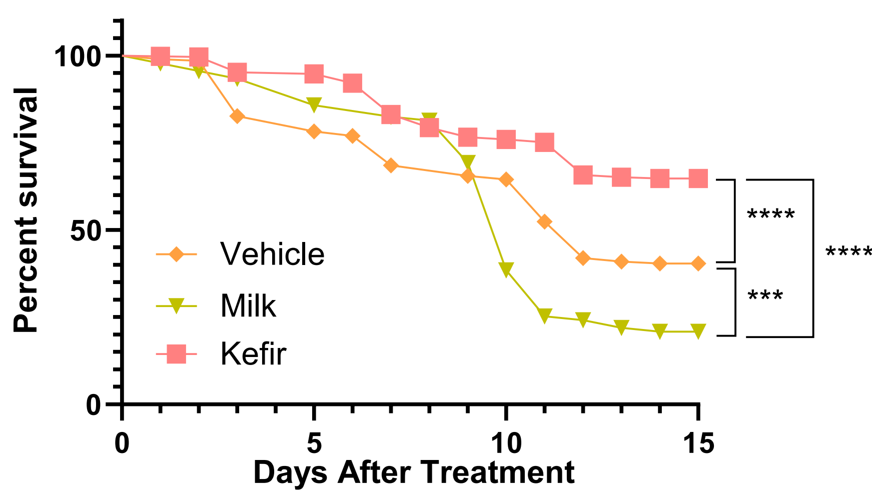

Figure 2. Survival rate of non-treated, kefir and milk-treated AD-like flies (n ≥ 90 in each group). The statistical significance is indicated as *** for P < 0.001 and **** for P < 0.0001 (log-rank, Mantel–Cox test).

This improvement was due to properties exclusive to kefir, as flies treated with non-fermented milk had a lower survival rate than control (P < 0.001), with only 20.8% of live flies within 15 days of treatment.

Survival rate after fractions treatment

To check if this improvement in survival rate could also be due to metabolites – or if it was exclusive to microbiome interactions. AD-like flies treated with kefir derived fractions had its survival compared to flies treated with Tween80 0.01% (used as a vehicle) For each fraction, three concentrations were used: 0.1, 0.25 and 0.5mg/mL (Figure 3).

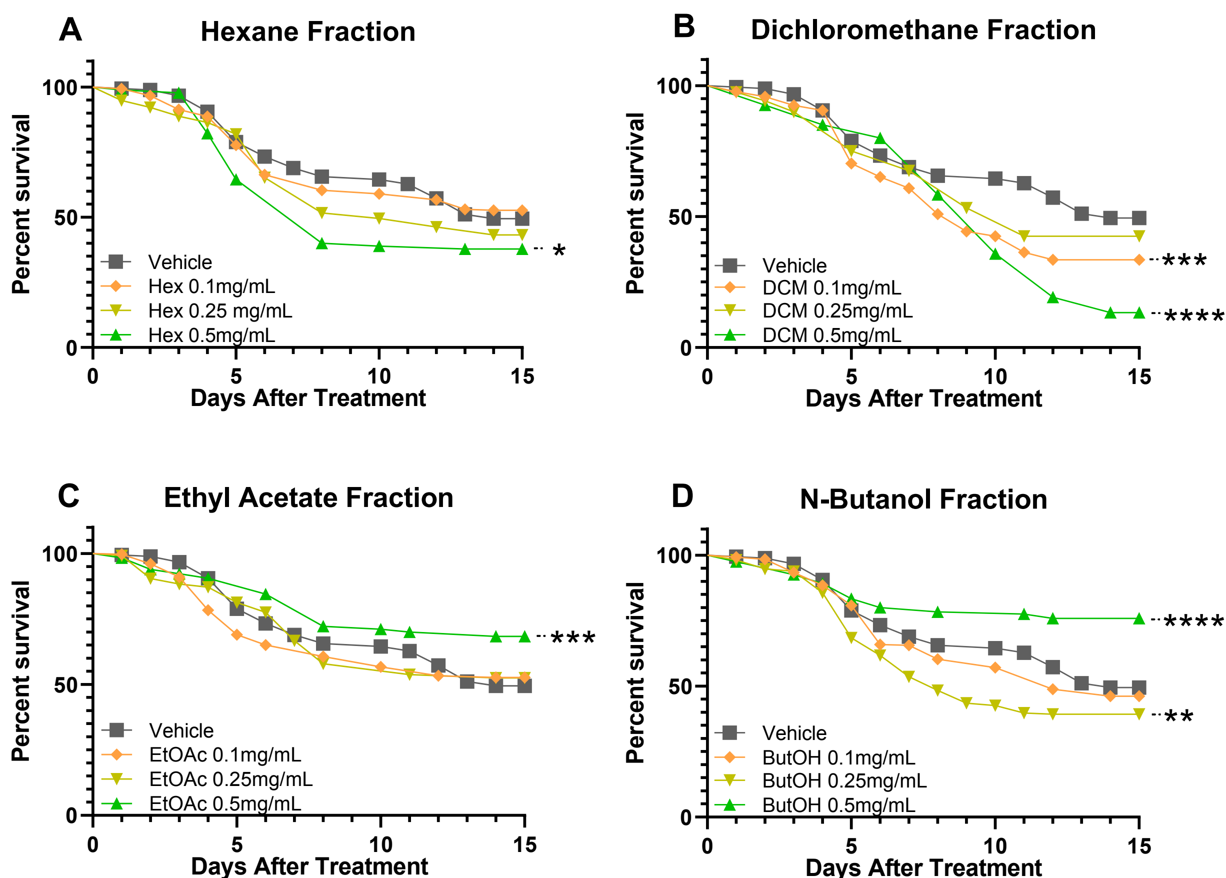

Figure 3. Survival rate of AD-like flies after treatment with (A) hexane, (B) dichloromethane, (C) ethyl acetate or (D) n-butanol fractions from kefir's methanolic extract. ($n \geq 90$ in each group). The statistical significance is indicated as * for $P < 0.05$, ** for $P < 0.01$, *** for $P < 0.001$, and **** for $P < 0.0001$ (log-rank, Mantel–Cox test).

Some fractions had a toxic for effect on AD-like flies, since they displayed a lower survival rate in relation to vehicle-fed ones, in the analyzed period. This happened for flies treated with Hex 0.5mg/mL ($P < 0.05$), DCM 0.1 and 0.5mg/mL ($P < 0.001$ and $P < 0.0001$, respectively), plus ButOH 0.25mg/mL ($P < 0.01$).

Despite that, survival rate was improved on flies treated with EtOAc 0.5mg/mL ($P < 0.001$) and ButOH 0.5mg/mL ($P < 0.0001$). These fractions improved AD-like flies survival rate as much as the kefir treatment (Figure 4).

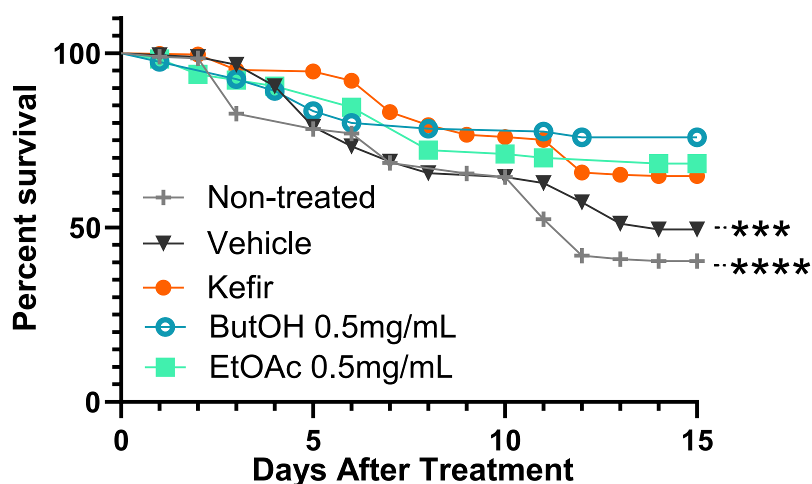

Figure 4. Percent survival comparison of AD-like flies treated with ButOH and EtOAc 0.5mg/mL to kefir. (n ≥ 90 in each group). The statistical significance is indicated as *** for P < 0.001 and **** for P < 0.0001 (log-rank, Mantel–Cox test).

Rapid Iterative Negative Geotaxis (RING) Assay

The Rapid Iterative Negative Geotaxis (RING) assay was used as a measurement for motor reflex decline related to neurodegeneration, in here observed as the fly climbing ability. As a proof of principle, untreated AD-like flies had its motor reflex tested and compared to a control genotype (elav-Gal4) at 5-8, 10-13, and 15-18 days post eclosion (Figure 5).

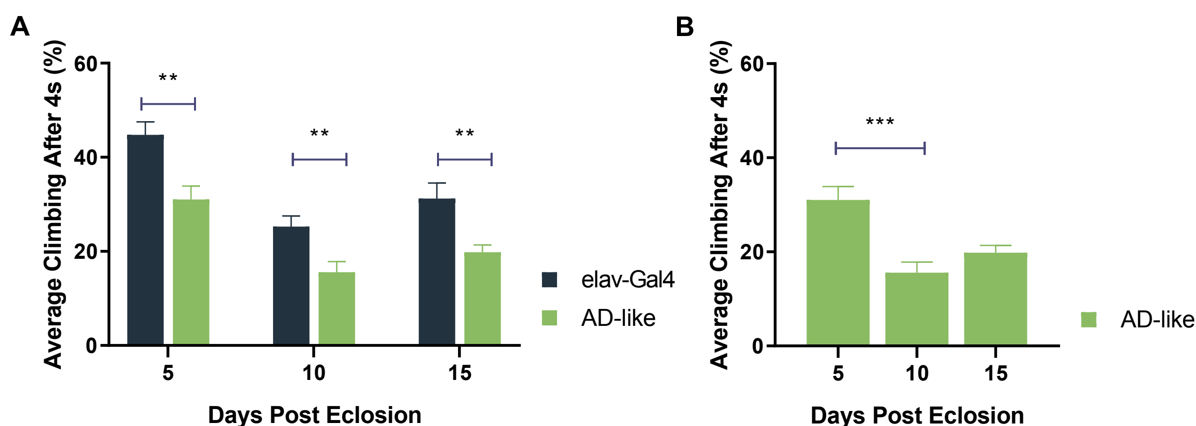

Figure 5. (A) AD-like flies presented a lower climbing ability than the control genotype at 5-8, 10-13, and 15-18 days post eclosion. (B) AD-like flies present climbing ability decrease when comparing flies at 5-8 and 10-13-days post eclosion. Data are shown as the mean ±

S.E.M. (n ≥ 180 in each genotype). The statistical significance is indicated as ** for $P < 0.01$ and *** for $P < 0.001$ (Unpaired two-tailed t-test).

AD-like flies presented a decline in climbing ability already at 5-8 days post eclosion (d.p.e) (Figure 5A, $P < 0.01$) in relation to control elav-Gal4 flies, which persisted at 10-13 and 15-18 d.p.e. ($P < 0.01$). When looking at AD-like flies climbing ability though time, there is a significative decay in climbing ability when comparing 5-8 to 10-13 d.p.e. flies ($P < 0.001$), but there is no change from 10-13 to 15-18 d.p.e. flies. (Figure 5B). Therefore, treated AD-like flies will only be assayed on the first two trial ages. These results confirm both that the RING assay can be used to distinguish neurodegenerative flies, and that AD-like flies show the expected motor reflex decline related to neurodegeneration – being suitable for treatment testing.

Climbing ability after kefir treatment

At 5 days after treatment (d.a.t.) AD-like flies treated with kefir displayed a higher climbing ability than both control ($P < 0.0001$) and flies treated with milk ($P < 0.001$) (Figure 6).

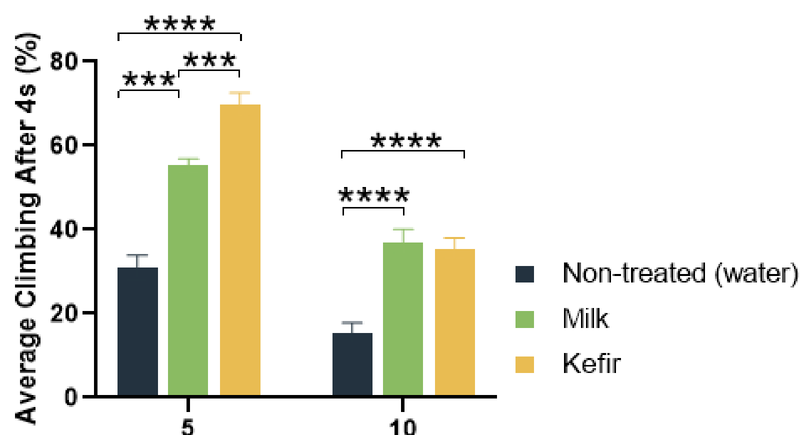

Figure 6. AD-like flies climbing ability after kefir and milk treatment at 5 and 10 days after treatment (d.a.t.). Flies treated with kefir performed better than control at both tested ages, and was better than milk-treated flies at 5 d.a.t., but at 10 d.a.t. milk and kefir treated flies perform the same. Data are shown as the mean ± S.E.M. n ≥ 90 in each group. The statistical

significance is indicated as *** for P < 0.001, and **** for P < 0.0001 (Unpaired two-tailed t-test).

At 10 d.a.t. this improvement is still present in relation to control flies (P < 0.0001), but not in relation to milk treated flies.

Climbing ability after fractions treatment

To check for improvements in climbing ability caused only by kefir metabolites, we submitted flies treated with fractions (Hex, DCM, EtOAc and ButOH, at 1, 0.5 and 0.25mg/mL) to the RING assay. Tween 0.01% was used as a vehicle for all fractions. Flies treated only with vehicle showed no change in its climbing ability when compared to control flies (fed with non-treatment food) both at 5 and 10 d.a.t. (data not shown). At 5 d.a.t., treatment with at least one concentration of each fraction improved AD-like flies climbing ability when compared with the ones treated only with vehicle (Figure 7).

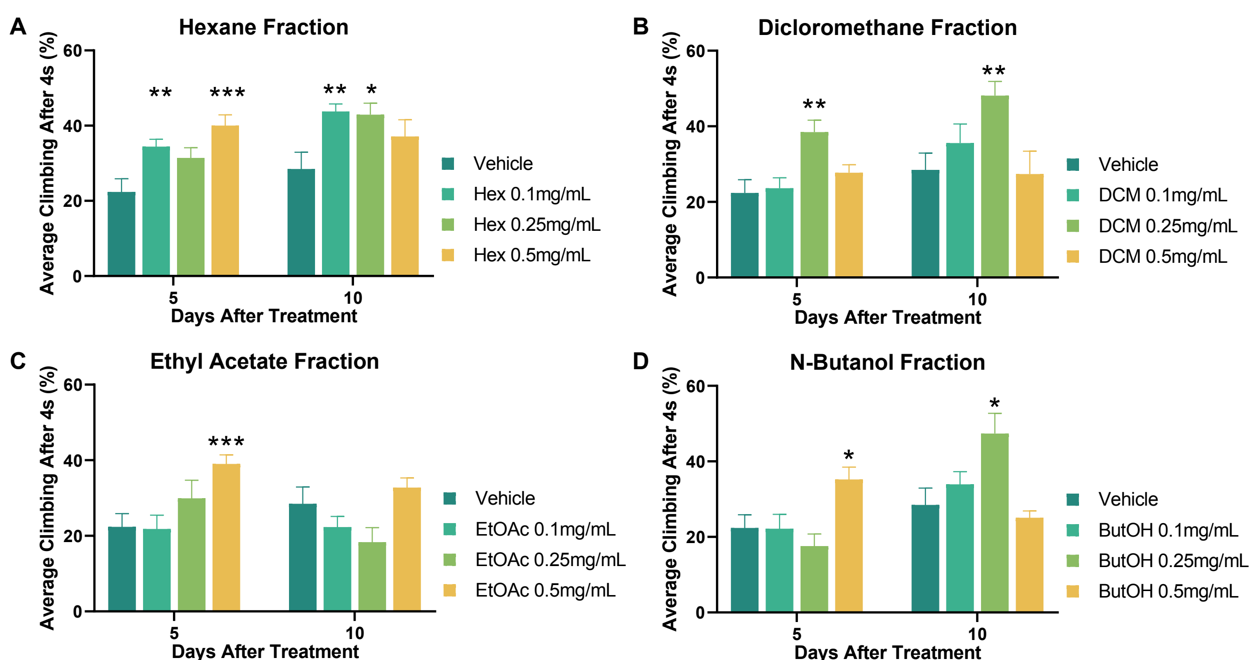

Figure 7. AD-like flies climbing ability after 5 and 10 days of treatment with Tween 0.01% (used as a vehicle) or either (A) hexane, (B) dichloromethane, (C) ethyl acetate or (D) n-butanol fractions at 0.1, 0.25 and 0.5 mg/mL. Data are shown as the mean ± S.E.M. (n ≥ 90

in each treatment). The statistical significance is indicated as * indicates P < 0.05, ** P < 0.01 and *** P < 0.001 (Unpaired two-tailed t-test).

Flies treated with DCM fraction performed the best climbing ability when treated with a 0.25mg/mL concentration (P < 0.01), while for both EtOAc and ButOH the best concentration was 0.5mg/mL (P < 0.001 and P < 0.05, respectively). Two concentrations of Hex fraction increased AD-like flies climbing ability: 0.1 (P < 0.01) and 0.5mg/mL (P < 0.001), being the last the most efficient.

At 10 d.a.t., EtOAc-treated flies did not differ on its climbing ability from the vehicle-fed ones. Flies treated with Hex 0.1mg/mL and DCM 0.25mg/mL maintained a better climbing ability performance than the ones treated only with vehicle (P < 0.01). Also, flies treated with ButOH and Hex at 0.25mg/mL had a higher motor reflex behavior than the ones fed only with vehicle (P < 0.05) – rather than 0.5mg/mL, which performed better at 5 d.a.t. for both fractions.

Highest performing fraction treatments were compared to kefir (fermented product) both at 5 and 10 d.a.t. (Figure 8).

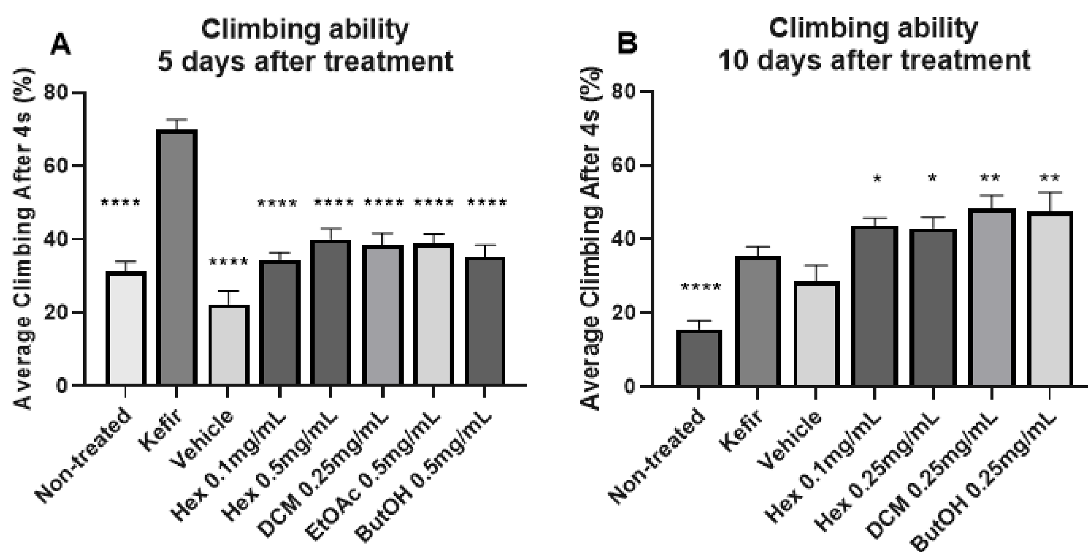

Figure 8. Average climbing ability of flies treated with fractions and kefir, as well as control and vehicle after (A) 5 or (B) 10 days after treatment. Data are shown as the mean ± S.E.M. n ≥ 90 in each treatment. The statistical significance is indicated in relation to kefir group as * indicates P < 0.05, ** P < 0.01 and *** P < 0.001 (Unpaired two-tailed t-test).

At 5 d.a.t., kefir-treated flies had a higher climbing ability than the ones treated with fractions ($P < 0.0001$). At 10 d.a.t., all treatments that improved AD-like flies climbing ability performed better than kefir-treated flies ($P < 0.05$ for both Hex 0.1 and 0.25mg/mL, $P < 0.01$ for DMC and ButOH 0.25 mg/mL).

Light and scanning electron microscopy of the flies' eye

For further insights into the effects of the better performing fractions in previous assays (Hex 0.1mg/mL, DCM 0.25mg/mL, EtOAc 0.5mg/mL and ButOH 0.25mg/mL) were tested into embryo stage flies, in order to attempt to recover GMR-Gal4 > UAS-BACE,UAS-APP degenerative eye phenotype. For this, the eyes of treated flies were observed using scanning electron microscope (SEM) (Figure 9).

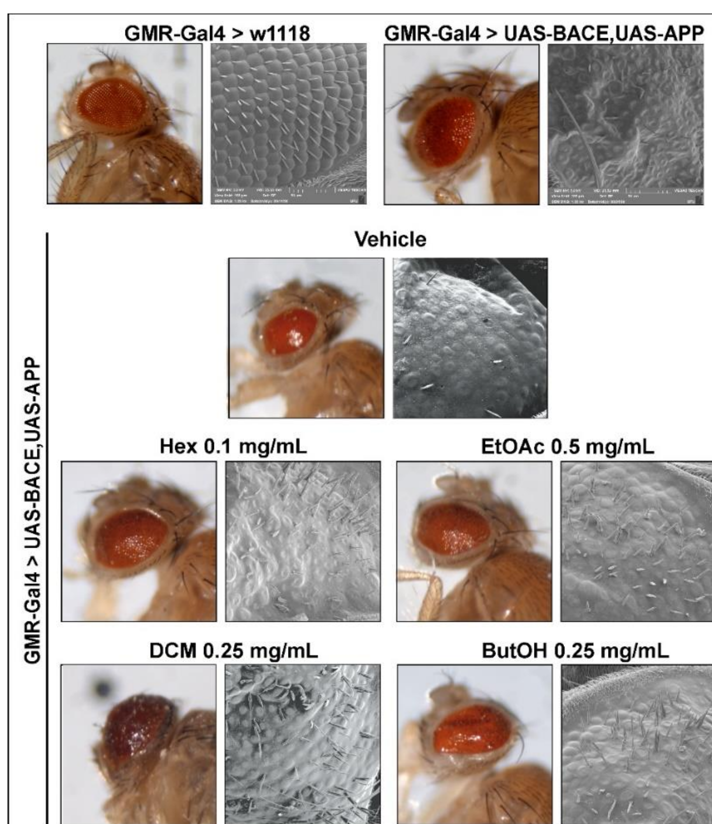

Figure 9. Drosophila eye development observed by light microscope and scanning electron microscope of (A) wild-type phenotype (GMR-Gal4 > w^{1118}), (B) rough eye phenotype associated with GMR > BACE,APP after treatment with (D) Hex 0.1 mg/mL, (E) EtOAc 0.5 mg/mL, (F) DMC 0.25 mg/mL or (G) ButOH 0.25 mg/mL.

GMR-Gal4 > w^{1118} flies (negative control) showed wild-type eye morphology, with no interference in ommatidia or bristles organization (Figure 9A). Untreated GMR > BACE,APP flies (positive control) presented ommatidia atrophy and lack of bristles (Figure 9B).

GMR>BACE,APP embryos treated with kefir did not develop, and therefore it was not possible to analyze its effect on recovering eye degeneration (data not shown). Embryos treated with vehicle (Tween 0.01%) developed into flies with more ommatidia then the positive (degenerative) control, and a low number of bristles were present (Figure 9C).

After Hex 0.1mg/mL treatment, ommatidia morphology and bristles presence was slightly improved on the eye's margins and not present reaching the eye center (Figure 9D). DCM 0.25mg/mL treatment did not show ommatidia recuperation and even though bristles number increased, there was no improvement on its organization (Figure 9F).

Both EtAOc 0.5mg/mL and ButOH 0.25mg/mL treatment increased relative bristles presence and organization, as well as generated a mild improvement in ommatidia organization on the eye's margins, being lower reaching the eye center (Figure 9E and G). Still, most ommatidial bristles were still absent and the size of ommatidia reduced. This way, all tested fractions failed to suppress completely the eye degeneration phenotype observed in GMR > BACE,APP flies.

Discussion

Kefir microbiota can vary depending on geographical origin, storage, used milk, grain-milk ratio and fermentation temperature (41–44). Therefore, characterizing our kefir sample bacterial microbiota – on composition and abundance – was the first step. This was done using next-generation sequence (NGS), in which the prokaryotic 16S ribosomal DNA gene (v4 region) was analyzed. Using BLAST, four main taxonomic units were confirmed: *Lactobacillus kefiranofaciens* and *Lactobacillus kefiri* being the most present in kefir, followed by *Lactococcus lactis* and *Acetobacter fabarum*. A similar profile has also been shown in kefir samples from France, Ireland and the United Kingdom (45), Belgium (46), Malaysia (47), Italy (48) and Brazil (49).

After bioinformatic analysis, one of the taxonomic units was assigned to the Rickettsiales order. When BLAST against the NCBI nucleotide collection, the best

alignment was with an uncultured bacterium clone (MH977830.1), with 97% query cover and 7 bp difference. This difference might indicate the presence of a new putative specie in our sample.

Regarding diversity, the number of found operational taxonomic units (OTU) was low comparing to other studies. So far, 400-600 OTUs have been documented from kefir samples, comprising uncultivable microorganisms, sub-dominant populations, and late-growing species. (50). This might be due to 16S rRNA sequencing limitation in resolution and precision at lower taxonomic levels. Even though, this technique allows reliable genus identification, it can misguide species identification due to annotation disagreements across different reference databases (51–53). This can be overcome with the use of shotgun whole-metagenome sequencing (WGS), which targets all genes in the microbiome rather than just 16S rRNA genes. With this method, taxonomic assignment of species could be done more reliably, obtaining a better species-level characterization of kefir microbiota (54).

Kefir's health improving effects have been demonstrated *in natura* (15,22,55–58), but also from metabolites present in its cell free fraction (59–62) and purified peptides (63–67). However, cell free fractions may contain peptides, also showing effects due to it.

No previous studies have explored the effects of a peptide and cell free fraction of kefir, or compared its effects to the *in natura* version. To do so, we performed a methanol extract of its fermented product, which not only kills the microorganisms but also precipitates the peptides (68). Since kefir has a big microbiological diversity – which reflets in a great number of secondary metabolites – we decided to partition this extract with four organic solvents, with increasing polarity. This way, we could easily obtain distinct classes of molecules and test the effect of each one of them in the amyloidogenic pathway.

Despite this approach is common with plants (69–73), is not normally done with probiotics. Few studies have performed liquid-liquid partitioning of microorganisms' metabolites, and the ones that did, used just one solvent, either chloroform or ethyl acetate (74,75).

To explore the effect of kefir – both *in natura* and of its metabolites fractions – we evaluated its capacity to improve AD-like flies survival and locomotor activity, as well as capacity to recover GMR>BACE,APP flies eye morphology.

Before testing, we first validated the used AD-like flies, which expressed APP and BACE in its central nervous system. When compared with a parental strain, these flies had its survival reduced already within 15 days of life and its climbing ability decreased. This

was also reported in previous works using AD-like flies, indicating the suitability of our model (76,77). Plus, GMR>BACE,APP flies presented the expected degenerative phenotype (78,79), with disorganized ommatidia and bristles.

Also, we wanted to be sure that the chosen vehicle wouldn't influence AD-like phenotype. Tween treatment displayed no effect on flies survival, climbing ability or eye morphology. This is important since other vehicles, as DMSO and PEG, have shown neuroprotective effects or even CSN modulation (80–84). This way, we knew that effects we saw from fractions was not biased.

Kefir has been shown to improve learning and memory (24), oxidative stress and inflammation (85) in AD patients. We also see this beneficial effect, since treatment with kefir *in natura* improved AD-like flies survival rate and climbing ability at 5 and 10 days after treatment. We hypothesize that this beneficial effect might be to a regulation in dysbiosis, since AD flies have a decrease in the *Lactobacillus* genus (86).

Beyond, it was not possible to evaluate kefir's effect on recovering eye degeneration of GMR>BACE,APP flies. This assay requires treatment in the embryo stage, and evaluation of adult eye, but after embryo exposure to kefir, no adult fly was obtained. This is due to kefir's interference in the fly's egg and larval development (87) . This way, kefir isn't suitable for embryo investigations in *D. melanogaster*, and adult stage treatment and evaluations should be preferred.

When evaluating kefir's metabolite, hexane and dichloromethane fractions – non-polar, with the lowest yield in liquid-liquid partitioning -, improved fly climbing ability when in lower concentrations (hex 0.1mg/mL and DCM 0.25mg/mL). These treatments were the only that improved this behavior in both tested ages, generating a better outcome than kefir at 10 d.a.t.. Flies treated with their highest tested concentration, though, showed a decrease in survival.

On the other hand, ethyl acetate and n-butanol fractions – polar, with the highest yield – improved fly survival in the highest tested concentration (0.5mg/mL). These fractions also showed a slight recovering of the eye phenotype in GMR>BACE,APP flies.

Due to fractions improving either climbing or survival – and kefir improving both – we hypothesize that two distinct pathways regulate each of these AD-like characteristics. To our knowledge, no study has used hexane, dichloromethane or n-butanol to partition bacterial metabolites. A recent study used ethyl acetate to partition *Lactobacillus plantarum* metabolites, which demonstrated an anti-inflammatory activity in mouse (74).

Since hexane is used to extract short-chain fatty acids (SCFAs), we hypothesize that this organic solvent's fraction includes these molecules. SCFAs are the largest metabolic group of fermentative probiotics (88), and markedly downregulated in AD models in *D. melanogaster* (89) and in mice (90). Plus, treatment with SCFAs inhibited Aβ aggregation *in vitro* (91). This way, we believe that these could have a role in improving AD-like flies climbing ability.

Despite that, to further investigate each fraction's effect and potential application, their molecular characterization through mass spectrometry is still needed. Plus, molecular and biochemical tests are needed in order to determine which pathways these molecules are affecting related to the amyloidogenic pathway.

In conclusion, this present work investigated the effect of kefir *in natura* and its metabolic fractions in the AD's amyloidogenic pathway. Kefir microbiota composition was determined through 16S sequencing, finding *Lactobacillus kefiranofaciens* as its most abundant species and detecting one yet unknown bacterial species. To our knowledge, this is the first report comparing the effect of a probiotic *in natura* and its metabolic fractions. Kefir treated flies improved both their climbing ability and survival rate, whereas flies treated with non-polar fractions improved the first, and the ones treated with polar fractions improved the second. Further studies are needed to investigate kefir's metabolite fractions composition, as well as which pathways are affected by these treatments.

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