UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTUTO DE BIOTECNOLOGIA
PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

IDENTIFICAÇÃO DE PEPTÍDEOS BIOATIVOS DE UMA AMOSTRA BRASILEIRA DE KEFIR, E SEU POTENCIAL ANTI-ALZHEIMER EM *DROSOPHILA MELANOGASTER*

Aluna: Serena Mares Malta

Orientador: Prof. Dr. Carlos Ueira-Vieira

Coorientador: Prof. Dr. Murilo Vieira da Silva

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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ATA DE DEFESA - PÓS-GRADUAÇÃO

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Data:	Trinta de setembro de dois mil e vinte e um	Hora de início:	09:00h	Hora de encerramento:	09:40h
Matrícula do Discente:	11922GBI011				
Nome do Discente:	Serena Mares Malta				
Título do Trabalho:	Identificação de peptídeos bioativos de uma amostra brasileira de Kefir, e seu potencial anti-alzheimer em *Drosophila melanogaster.*				
Área de concentração:	Genética				
Linha de pesquisa:	Biologia Molecular e Celular.				
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Aos trinta dias do mês de setembro de dois mil e vinte e um, às 09:00 horas, reuniu-se via web conferência pela plataforma *Cisco Webex Meetings*, 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 Fernanda Gobbi Amorim. 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):

(A) PROVADO.

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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COMISSÃO EXAMINADORA

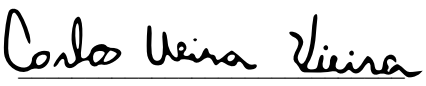
Presidente: Prof. Dr. Carlos Ueira-Vieira

Examinadores: Dra. Ana Paula Mendes-Silva

Dra. Fernanda Gobbi Amorim

Data da Defesa: 30/09/2021

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



Prof. Dr. Carlos Ueira-Vieira

i stand
on the sacrifices
of a million women before me
thinking
what can i do
to make this mountain taller
so the women after me
can see farther

legacy – rupi kaur

DEDICATÓRIA

Para minha mãe Silézia, cuja contribuição jamais poderá ser mensurada. Essa conquista é nossa.

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A todos que direta ou indiretamente contribuíram para realização desse trabalho,

Gratidão.

SUMÁRIO

Apresentação

A doença de Alzheimer (DA) é uma forma de demência mais comum que afeta principalmente idoso acima de 65 anos. É descrita como um declínio cognitivo progressivo que afeta as funções intelectuais e básicas levando os pacientes a situações de dependência. A hipótese principal da DA baseia-se na via amiloidogênica e na formação de placas senis. Estudos comprovam que fatores como disbiose podem contribuir com o desenvolvimento das características patológicas e, portanto, abordagens com probióticos – como o kefir- visando a recuperação do equilíbrio da microbiota tem se mostrado promissoras.

Diante disso, o Capítulo I descreve a doença de Alzheimer e sua relação com a disbiose e outros fatores, o uso de probióticos como terapias e a utilização de *Drosophila melanogaster* como ferramenta para estudos relacionados a DA. No Capítulo II exploramos a fração peptídica do kefir e seu potencial anti-Alzheimer em *D. melanogaster.*

CAPÍTULO I

Fundamentação teórica

1. Doença de Alzheimer

A doença de Alzheimer (DA), é caracterizada por uma diminuição da capacidade intelectual, que afeta principalmente idosos levando a situações de incapacidade dos mesmos, e considerada a forma mais comum de demência, responsável por 60-70% dos casos diagnosticados [1]. De acordo com a Organização Mundial da Saúde, há aproximadamente 50 milhões de casos já conhecidos, e está previsto um aumento de um milhão de casos anuais até 2050. O impacto socioeconômico causado por essa doença se torna uma questão de saúde pública, uma vez que está previsto um custo de 1,1 trilhão de dólares somados aos bilhões já gastos até então[2].

Descrita como um declínio cognitivo progressivo, a doença afeta primeiramente a capacidade de formação de memória recente, e evolui de modo a prejudicar também as habilidades motoras e de orientação espacial [3]. Essas características prejudicam a qualidade de vida dos pacientes e contribuem para torná-los dependentes de cuidados constantes. A DA apresenta diferentes manifestações, de modo que pode afetar indivíduos com menos de 60 anos, no que é considerada sua forma precoce, bem como indivíduos acima de 65 anos, sendo essa a maneira tardia e mais comum, a diferença entre a idade dos indivíduos acometidos está diretamente relacionada a aspectos genéticos específicos [4].

1.1 Características patológicas

Embora, seja muito comum sua incidência em idosos, a DA é considerada um transtorno mental e não apenas um processo de envelhecimento. Apesar de que certas características morfológicas são acentuadas em idosos que a possuem, a patologia é caracterizada por marcadores que permitem uma melhor identificação e diagnóstico. Anatomicamente a DA se caracteriza pela proeminente atrofia nas regiões frontais, temporais e parietais que podem ser identificadas a partir de imagens obtidas por Tomografia Computadorizada (TC) e Ressonância Magnética (RM)[5].

Alterações no volume e estrutura cerebral são consideradas normais durante o envelhecimento, portanto um certo grau de atrofia já é esperado. Em pessoas com DA esse declínio que ocorre por meio de perda neuronal causa uma atrofia global anormal e acentuada em algumas regiões específicas associadas com o comprometimento das funções cognitivas resultando nos efeitos sintomáticos da doença [6].

Diferentes hipóteses [7] explicam os mecanismos característicos da doença de Alzheimer, como hipótese colinérgica [8,9], toxicidade pela proteína Tau (GU;LIU, 2020; MURALIDAR et al., 2020), e a hipótese amiloidogênica [12,13], sendo essa última a mais abordada entre elas. A hipótese amiloidogênica descreve o processamento da proteína precursora de amilóide (APP) por duas vias distintas sendo elas a amiloidogênica e a não amiloidogênica. Pela primeira via, a clivagem da APP é realizada pela enzima α-secretase gerando os fragmentos sAPPα e C83 (uma porção C-terminal da APP com 83 aminoácidos). Na segunda via, a proteólise da proteína precursora é catalisada pela enzima β-secretase e os resultados são fragmentos sAPPβ e fragmento C99 (composto por 99 aminoácido). Ambos os fragmentos C83 e C99 são clivados pela enzima γ-secretase originando os produtos: fragmentos intracelulares de APP e fragmento P3 (oriundos de C83), e fragmentos extracelulares e peptídeo Aβ completo (oriundos de C99), sendo que Aβ pode apresentar tamanhos que variam entre 40-42 aminoácidos [12,14]

Ambas as vias estão presentes naturalmente nos seres humanos, porém, em situação de desbalanço, onde a primeira clivagem é predominantemente executada pela enzima β-secretase , o aumento desses fragmentos Aβ, resulta no acúmulo dos mesmos e formação de placas beta amiloides que tem como consequência a perda de sinapses e a morte de neurônios [15,16].

A enzima β-secretase também conhecida como BACE (*Beta-site APP Cleaving Enzyme*), possui dois homólogos em humanos (BACE-1 e BACE-2). Ambos são proteases da família da pepsina (possuem sítio de aspartil protease), e se diferem quanto a composição de seus aminoácidos e locais onde se encontram. Já se sabe que a BACE-1 desempenha um papel crítico na doença de Alzheimer atuando diretamente no processo de produção do peptídeo Aβ citado anteriormente. Por esse motivo, constitui-se um alvo para terapias farmacológicas que visam retardar a produção e acúmulo de Aβ [15–17].

Importante ressaltar que as hipóteses representam apenas um recorte das vias complexas que caracterizam a patologia. Leva-se em conta também que outros fatores estão presentes e que diferentes mecanismos podem acontecer simultaneamente nos indivíduos [18]. Sob esse aspecto, a via amiloidogênica se correlaciona com o estresse oxidativo [19] e também com enzimas da via colinérgica [20]. É sabido que o aumento nos níveis das espécies reativas de oxigênio (ROS) e o desequilíbrio entre sua produção e a defesa antioxidante, como também a homeostase anormal de metais bioativos como Ferro (Fe), Cobre (Cu), Zinco (Zn)

entre outros influenciam no aumento do estresse oxidativo e produção de radicais livres favorecendo a agregação dos peptídeos Aβ [21,22].

Outro possível fator que contribui na agregação dos peptídeos Aβ e indução de uma maior neurotoxicidade é a enzima acetilcolinesterase [23]. Sua inibição é alvo de diferentes fármacos disponíveis no mercado, que visam promover o aumento da disponibilidade de acetilcolina para melhoria das sinapses dos neurônios colinérgicos, mas pode também ter efeito mesmo que indireto na via amiloidogênica [24,25].

Além das teorias já consolidadas, outros aspectos como perda da homeostase da microbiota intestinal, são considerados no que tange a seus efeitos e sua relação com a doença de Alzheimer. Diferentes estudos apontam a relação do eixo cérebro-intestino e a influência da disbiose na patologia [26–28].

1.2 Disbiose na Doença de Alzheimer

A microbiota intestinal, que corresponde a grande maioria da população microbiana humana, é constituída por diversos microrganismos entre eles bactérias, leveduras, arquéias, vírus e protozoários [29] que são capazes de influenciar a fisiologia e o desenvolvimento do hospedeiro, e podem contribuir de forma positiva na homeostase do sistema imunológico, desempenhar funções metabólicas essenciais e atuar como fonte de nutrientes [30]. Apesar de sua composição ser variável em cada indivíduo em função de diversos fatores extrínsecos como dieta, estilo de vida e consumo de antibióticos, alguns gêneros se mantêm presente como um consenso entre os indivíduos, tais como Actinobacteria (como *Bifidobacterium*), Cyanobacteria e *Lactobacilus* [29,30].

A microbiota é passível de mudanças em sua composição ao longo da vida, de modo que a tendência é que haja uma diminuição do número de espécies com a progressão do envelhecimento[31] . Manter o equilíbrio dessa diversidade é fundamental para o que se define como uma microbiota de qualidade ou microbiota saudável, e a disbiose (desequilíbrio entre as espécies presentes) apresenta sérios riscos à saúde uma vez que todas as reações favoráveis são equilíbrio dependente [30].

A interação da microbiota intestinal com o cérebro através do eixo intestino-cérebro ou ampliado com “eixo microbiota-intestino-cérebro” ocorre pela possibilidade de interação entre sistema nervoso e intestino, e por substâncias químicas que atravessam a barreira hematoencefálica [32,33]. Os neurotransmissores emitidos são capazes de interagir com as

bactérias como também produtos oriundos da ação dos microrganismos, podem atingir os neurônios centrais e influenciar sua atividade [31,33,34].

Nesse contexto, a disbiose se relaciona diretamente com disfunções no cérebro. Atinge funções do sistema nervoso influenciando na função cognitiva e comportamento, contribui com a neuroinflamação e estresse oxidativo [34], e está associada a diversas patologias de caráter neurodegenerativo tais como doença de Parkinson, Esclerose Múltipla e Alzheimer [29,33,35]. Na DA a disbiose tem como efeito a inibição mecanismos necessários para a eliminação de peptídeos Aβ, promovendo um aumento da agregação dos mesmos, e também influencia na produção direta do peptídeo contribuindo para o dano neuronal [36–38].

Diante disso, possíveis abordagens terapêuticas para patologias como Doença de Alzheimer, baseia-se no reestabelecimento do equilíbrio microbiano principalmente mediante a aplicação de probióticos [39,40].

2. Kefir como abordagem terapêutica

Os probióticos, são microrganismos não patogênicos capazes de causar efeitos benéficos na saúde do hospedeiro [41]. Auxiliam na recomposição da microbiota intestinal e por consequência, é efetivo para diminuir os efeitos da disbiose [42,43]. Nesse contexto o Kefir destaca-se por ser um simbiótico [44,45] (caracterizado como probiótico e prebiótico) de origem caucasiana, amplamente utilizado na nutrição humana [46].

Comumente, o kefir é cultivado em leite, podendo ele ser originado de diferentes animais, mas também pode ser cultivado em água. Sua composição microbiológica é uma associação de bactérias e leveduras, principalmente as do gênero Lactobacillus e Saccharomyces [47]. Embora esses sejam os gêneros mais presentes, a microbiota do kefir é variável em função do substrato, origem, forma de armazenamento, proporção entre grão e substrato [48–50].

Visualmente, o kefir é constituído por um aglomerado de vários grãos de tamanho médio de 3 – 5 mm de diâmetro e aspecto gelatinoso com coloração branca – amarelada. Sua microbiota complexa é confinada em uma matriz formada por polissacarídeos e proteínas e que lhe confere o aspecto descrito [47,51].

Estudos indicam que os probióticos possuem propriedades anti-inflamatórias, anti-hipertensiva e auxiliam no sistema imunológico[42,52–55]. Em estudos na qual o uso de probióticos consistiam uma abordagem terapêutica para doença de Alzheimer, os resultados

obtidos demonstraram um efeito positivo pois o tratamento foi capaz de melhorar as funções cognitivas, inibir a agregação de placas β-amiloides, reduzir a neuroinflamção e produzir compostos que auxiliam na diminuição do estresse oxidativo [56–59]. O kefir apresenta todas essas funções como também atividade antialergênica [60–62]. O processo de fermentação, pode gerar moléculas e peptídeos com propriedades bioativas que podem ser úteis como terapias em diferentes doenças [63].

2.1 Peptídeos bioativos

Peptídeos bioativos (PB) são sequências de aproximadamente 2- 20 aminoácidos unidos por ligação covalente, criptografados em proteínas chamadas de proteínas precursoras. São inicialmente inativos quando encriptados e podem se classificar como peptídeos endógenos (naturalmente liberados durante o processamento de alimentos ou pela digestão gastrointestinal) e exógenos (a liberação se dá através de síntese química ou por hidrólise das proteínas precursoras com o uso de enzimas proteolíticas) [64,65].

Os processos fermentativos são uma forma de obtenção desses PB e podem ser realizados por microrganismos em culturas como o kefir [66]. No kefir, a fermentação do leite por bactérias do ácido lático é atraente por gerar alimentos funcionais a baixo custo, além de apresentarem efeitos positivos no organismo. Os produtos podem ser variáveis em detrimento do grau de hidrólise, diretamente relacionado com o tempo de fermentação, os tipos de microrganismos e a fonte proteica [65].

A atividade dos PB está diretamente relacionada com a sequência de aminoácidos que o compõe. São considerados reguladores biologicamente ativos e possuem propriedades anti-hipertensivas, antioxidante, antimicrobiana, antidiabética e, portanto, podem ser utilizados na prevenção e tratamento de diferentes doenças e na composição de alimentos nutracêuticos [64,67]. Os substratos que dão origem a esses peptídeos podem ser tanto de origem vegetal como milho, soja, arroz, quanto animal como leite e carne [64,65].

Nos últimos anos, a aplicação de probióticos e a busca por sequências de PB para aplicações terapêuticas aumentou consideravelmente [68], diante disso, aplicações de técnicas de análise de proteopeptidômica como espectrometria de massa [60] e modelagem molecular, torna-se uma ferramenta de grande auxílio nesse processo, bem como o uso de organismos modelos tal qual *Drosophila melanogaster.*

3. Drosophila melanogaster

A *Drosophila melanogaster*, popularmente conhecida como mosca da fruta, é um inseto da família do Drosophilidae, com tamanho aproximado de 2-4 mm [69]. Possui apenas 4 pares de cromossomos, e seu genoma já está completamente sequenciado e, portanto, conhecido [70]. Entre as vantagens no seu uso como organismo modelo, a *Drosophila* se destaca por possuir um ciclo de vida curto, baixo custo de manutenção, facilidade de manejo e anatomia simples [71].

D. melanogaster apresenta um sistema nervoso complexo (aproximadamente 200.000 neurônios) e capacidade de aprendizado e memória [72]. As semelhanças funcionais e estruturais com humanos permitem que os estudos realizados com esse organismo produza insights nos níveis bioquímicos, moleculares e comportamentais no estudo de patologias de caráter neurodegenerativo[73].

Como modelo de DA, as *Drosophilas* permitem o estudo de diferentes recortes da doença, expressão de genes relacionados aos processos patológicos, e fenótipos complexos relacionados a patologia [74]. No aspecto da produção de peptídeos Aβ, a Dmel possui ortólogos de todos os componentes das vias amiloidogenica e não amiloidogênica (dAPPl ou dAPP-like, e enzimas α, β e γ- secretases), no entanto o produto do gene dAPPl não possui similaridade significativa dentro da região do Aβ da APP humana, e seus níveis de produção do corresponde β-secretase (BACE) são muito baixos [74–76].

Porém, utilizando engenharia genética é possível realizar a expressão dos genes humanos codificantes de APP e BACE-1 na *D. melanogaster*l. O ortólogo de γ-secretase endógeno é capaz de clivar os produtos das sequências exógenas portanto, gerando fragmentos idênticos aos presentes em humanos e consequentemente têm-se a mimetização fenotípica da DA sem carecer da expressão do gene precursor dessa enzima [77,78].

A construção do modelo de DA, frequentemente se dá através do uso do sistema UAS/Gal4. Originalmente encontrado em levedura, o sistema funciona de forma bipartida de modo que sua ativação completa se dá a partir do cruzamento entre duas linhagens [79]. Uma contém o elemento Gal4 (driver) que direciona o local específico para expressão do gene de interesse, e a outra contém o elemento UAS e os genes de interesse (BACE-1 e APP humanos) [75,80]. O resultado é uma F1 que contém os genes associados a UAS sob um padrão de expressão direcionado por Gal4 [81].

4. Objetivo

Utilizar os produtos do kefir e não somente o *in natura* pode ampliar as possiblidades de tratamento e potencializar seu benefícios já que suas frações podem não desempenhar exatamente as mesmas funções do produto, como sugere o trabalho de Batista et al., (2021)[82] que comparou os efeitos dos metabólitos vs kefir bruto. Uma vez provado o benefício das formulações probióticas, e o destaque do kefir como possível agente terapêutico, ainda não se têm descrito uma aplicação de seus peptídeos como agente terapêutico da DA, portanto, o objetivo desse trabalho é abordar os efeitos dos peptídeos bioativos provenientes de kefir utilizando *Drosophila melanogaster* como organismo modelo da Doença de Alzheimer.

5. Referências

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CAPÍTULO II

Identification of bioactive peptides from a Brazilian kefir sample, and their anti-Alzheimer potential in *Drosophila melanogaster*

Abstract

Alzheimer's disease (AD) is the most common form of dementia in the elderly, affecting cognitive, intellectual, and motor functions. Different hypotheses explain the mechanisms of the AD, such the amyloidogenic hypothesis. Moreover, this disease is multifactorial, and several studies have shown that dysbiosis and oxidative stress influence its pathogenesis. Knowing that kefir is a probiotic used in therapies to restore dysbiosis and that the bioactive peptides present in it have antioxidant capacity, we explored its biotechnological potential as a source of molecules capable of modulating the amyloidogenic pathway and reducing oxidative stress, contributing to the treatment of AD. For that, we used *Drosophila melanogaster* model for AD (AD-like flies). Identification of BP in the kefir sample was made by proteomics and peptidomics analysis, followed by *in vitro* analysis of antioxidant and acetylcholinesterase inhibition potential. Flies were treated and their motor performance, brain morphology, and oxidative stress evaluated. Finally, we performed molecular docking between the peptides found and the main pathology-related proteins in the flies. The results showed that the fraction with the higher level of peptide concentration was positive for the parameters evaluated. In conclusion, these results revealed that kefir fractions have therapeutic potential for the treatment of AD.

Keywords: Alzheimer’s Disease, Dysbiosis, Bioactive peptides, *Drosophila melanogaster*, Kefir

1 Introduction

Alzheimer's Disease (AD) is a multifactorial progressive pathology and the most common form of dementia[1]. It compromises distinct cognitive abilities, such as learning and memory, motor performance, and visual-spatial function[2]. In 2019, the estimate was 1.5 million deaths from the disease, and by 2015 global cases are estimated to reach 152.8 million[3]. It is neuropathological characterized by lesions and atrophy in brain regions and the main hypothesis for its development is the aggregation of beta-amyloid peptides[4]. Those peptides are generated through the amyloidogenic pathway, in which the amyloid precursor protein (APP) is cleaved by a β-secretase (BACE) followed by a γ- secretase[4–6].

Recent studies indicate that AD's pathology is directly correlated to the influence of dysbiosis in the gut-brain axis. This phenomenon induces inflammatory and oxidative responses beyond influencing β-amyloid aggregation – strong features of AD[7,8]. In this context, previous studies have demonstrated a positive effect of probiotics in AD's animal models and patients[9,10].

Among probiotic formulations tested on AD, an interesting one is kefir. It is constituted by a group of symbiotic bacteria and yeasts. Its species and ratio can vary in respect to its geographical origin and substrate – usually cow milk – allowing distinct fermentation products along time[11,12]. Previous study shows that Kefir's metabolites significantly improved both behavioral and pathological features of a *Drosophila melanogaster* model for AD. It decreased the presence of amyloid aggregation and improved the fly's motor capacity[13]. However, kefir has incredibly rich additional molecules, as bioactive peptides, still to be further explored in AD.

D. melanogaster has been extensively used to bring a clearer understanding of the neurobiology of AD and possible treatments[14,15]. It has favorable genetic characteristics and toolboxes, complex behavior, and a simplified nervous system and gut-brain axis – although remarkably similar to higher organisms. It is a unique model for exploring probiotic treatments to attenuate Alzheimer's symptoms[16–19].

In order to provide a better understanding of the effect of Kefir's bioactive peptides in AD's pathology, we treated the AD-like flies with kefir and evaluated behavior and oxidative stress markers.

2 Results

2.1 Kefir proteome and peptidome

Protein fractions (> and < 10 kDa) were analyzed by proteome approaches. When the peptides were analyzed using database "milk AND bovine" a similar profile of peptides from milk proteins was found in both fractions. Except for α-S1-casein that was found only in fraction > 10 kDa (Fig. 1a and 1b).

The peptidome analysis was made by a mass spectrometer of < 10 kDa fraction without digestion with trypsin. Peptides derived from α-S1-casein, β-casein and only one from β-lactoglobulin were found from the main milk protein (Fig. 1c). Peptides from *Lactobacillus* and *Acetobacter* databases were also found. However, in this work we focus on peptides encrypted only on milk proteins.

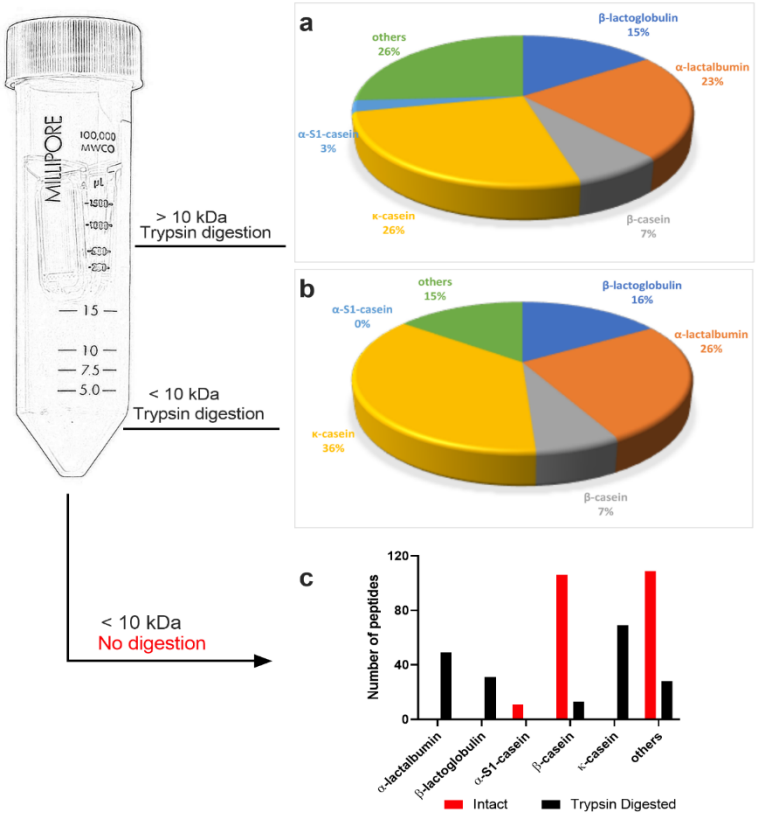

Figure 1. Proteome and peptidome of the kefir sample. (a) Proteomic analysis of the >10 kDa fraction digested with trypsin and relation of the present proteins. (b) Proteomic analysis of the <10 kDa fraction digested with trypsin and relation of the present proteins. (c) Peptidomic analysis of the <10 kDa non-digested fraction and quantity of peptides found that are derived from milk.

2.2 *In silico* prediction of bioactivities

The prediction of physicochemical parameters and bioactivities of peptides from "milk AND bovine" database and *de novo* analysis showed a large number of peptides with activity of antioxidant, immunomodulatory, ACE-inhibitor and others (Supplementary Table 1)

2.3 Kefir fractions *in vitro* analysis

As a first step to analyze the diversity of kefir water-soluble fractions, its fractions were tested for their acetylcholinesterase inhibition and antioxidative capacity *in vitro*.

Ascorbic acid was used as a positive control of the FRAP test[20] and as a pure compound it reflected the highest antioxidant compound. When the sample was compared, was possible to observe an increase in antioxidant in WSF, > 10 kDa, and a higher value in < 10 kDa (peptide enrichment fraction).

In the FRAP assay, all tested fractions (WSF and peptides >10kDa and <10kDa) demonstrated an antioxidant capacity on converting Fe^{3+} into Fe^{2+} (Fig. 2a). With an increase in filtering and purification processes, the antioxidant capacity increased, with the <10kDa/peptidic fraction having the best performance ($p< 0.5$) (Fig. 2a).

In the acetylcholinesterase inhibition assay, the fractions showed a similar pattern, with the peptidic fraction <10kDa showing the highest inhibitory activity ($p< 0.5$) (Fig. 2b). However, the WSF fraction did not inhibit acetylcholinesterase.

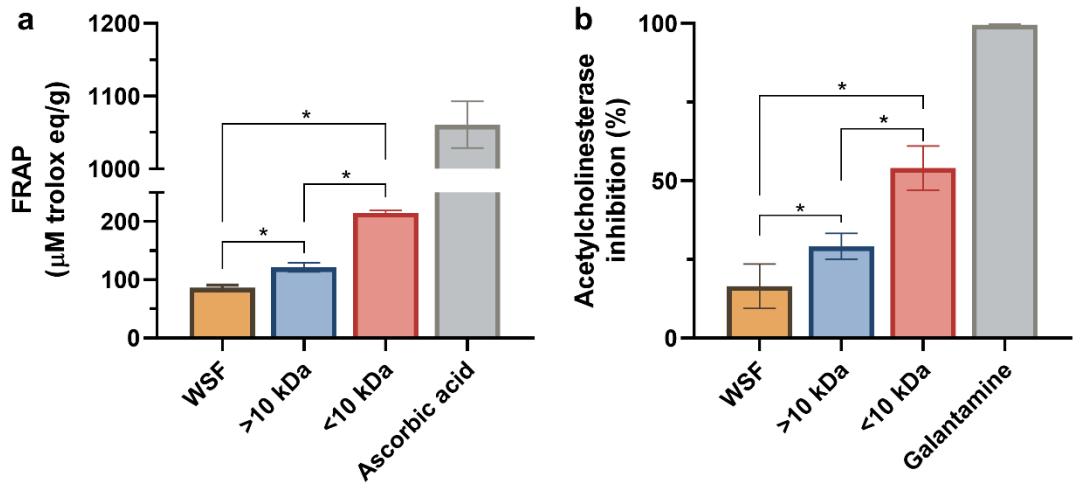

Figure 2. In vitro analysis of the effects of Kefir fractions. (a) Total antioxidant capacity by FRAP method of Fe3+ reduction analysis. Fraction <10kDa shows higher antioxidant action compared to

the others (* p< 0.5), but not as high as ascorbic acid used as control. (b) Acetylcholinesterase enzyme inhibition capacity, among the kefir fractions, <10kDa stands out as the highest inhibition capacity (* indicates p<0.5).

2.4 *Drosophila melanogaster* AD-model

After the promising activity of the fractions in the preliminary *in vitro* studies, we used the fractions in a D. melanogaster model for Alzheimer's Disease (AD-like). This model is based on the expression of human BACE and APP – mimicking the amyloidogenic pathway in the fruit fly's brain.

To ensure that the AD-model model is suitable for the fractions' evaluation, we evaluated its motor ability, performed ß-amyloid quantification, and histopathological analysis. For those assays, elav-Gal4/+ flies were used as a control.

To evaluate a decline in motor reflex behavior – linked to neurodegeneration – a negative geotaxis assay was used. As expected, 10-13 days old flies presented impaired motor performance when compared to the control genotype ($p< 0.01$) and to 5-8 days old flies ($p< 0.001$) (Fig. 3a).

Using the amyloid binding capacity of Thioflavin T[21], we quantified the amyloid relative levels in 10-13 days old flies ($n = 30$). AD-like flies had a higher level of ß-amyloid than control flies ($p< 0.01$, Fig. 3b).

Finally, the brain of 10-13 days old flies underwent histopathological analysis to evaluate its neurodegeneration index ranging from 0 to 5, as observed in elav-Gal4 (Fig.1d) and AD-like flies (Fig. 1e), in accordance with previous studies[13,22]. Results point to higher neurodegeneration in AD-like compared to control ($p< 0.5$, Fig. 3c).

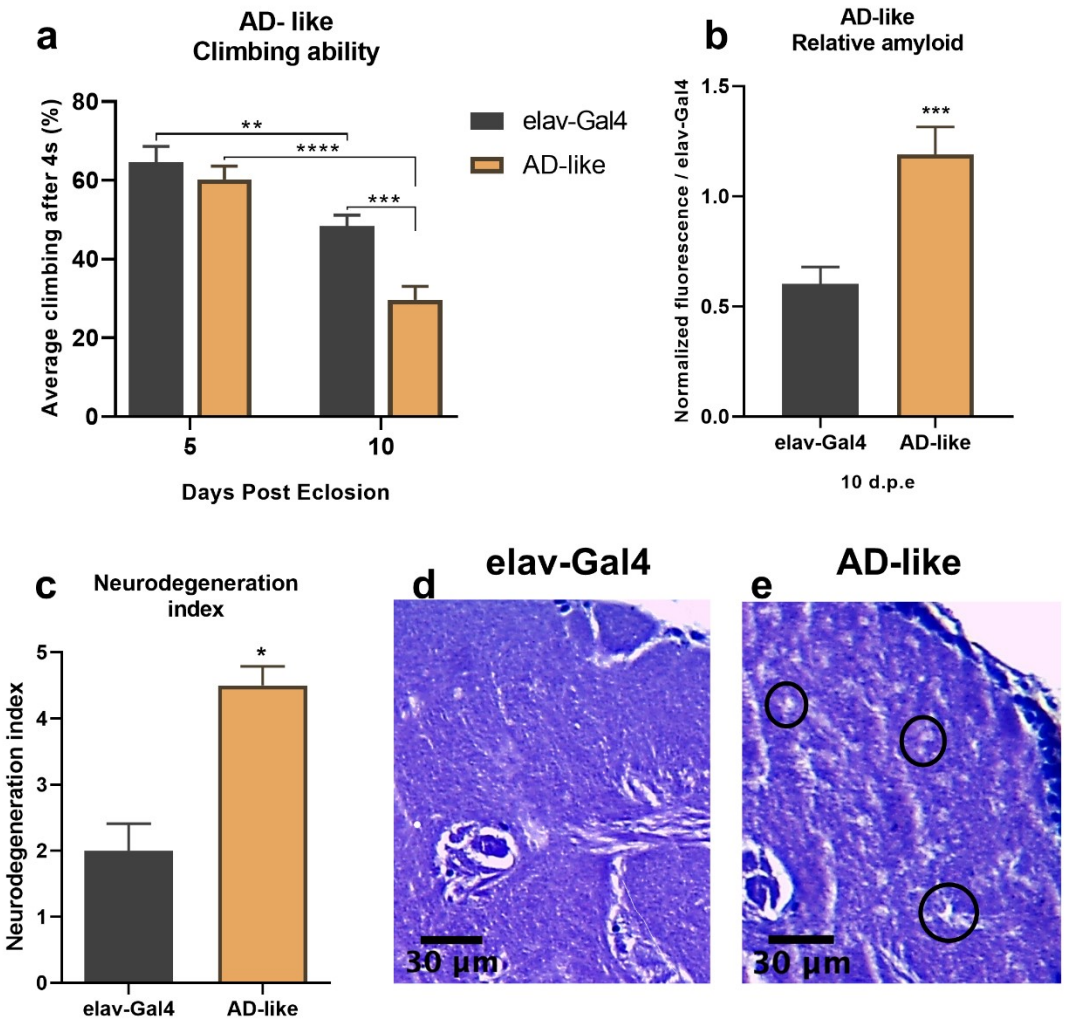

Figure 3. Validation of the Alzheimer's model. (a) Climbing ability. AD-like flies show lower motor ability than control genotype at 10-13 days post eclosion ($n = 90$ in each genotype). (b) Quantification of amyloid by the Thioflavin-T aggregation method. Alzheimer genotype flies show a higher amount of amyloid compared to the control at 10-13 days post eclosion. (n = 30 for each genotype). (c) Neurodegeneration index of elav and ad-like flies based on histopathological analysis, focusing on vacuolar lesions. Indices range from 0-5 with 0 indicating no lesions, and 5 indicating a neurodegenerative phenotype. Data are presented as mean ± SEM, and significance values represented as ** $p < 0.0001$, * $p < 0.001$, ** $p < 0.01$. Illustrative histopathology images of the elav (d) and ad-like (e) genotypes.

2.5 Peptides fraction from kefir can modulate the AD-model

Our previous work demonstrated a positive effect of kefir *in natura* and its compounds in this AD-like model[13]. On the RING assay, treated flies had an improvement on climbing ability starting at 10 days after treatment (and therefore 10-13 days after eclosion) when treated with WSF ($p < 0.5$) e <10 kDa ($p < 0.01$) fractions at 0.25 mg/mL (Fig. 4a) e compared to non-treated flies. The treatment with fractions at 0.5 mg/mL did not show

any improvements in climbing (Fig. 4b). But in order to analyze the fraction effect in other parameters, following up assays were done with flies at 10-13 days after eclosion, and treated with > 10 kDa and < 10kDa fractions at 0.25 and 0.5 mg/mL.

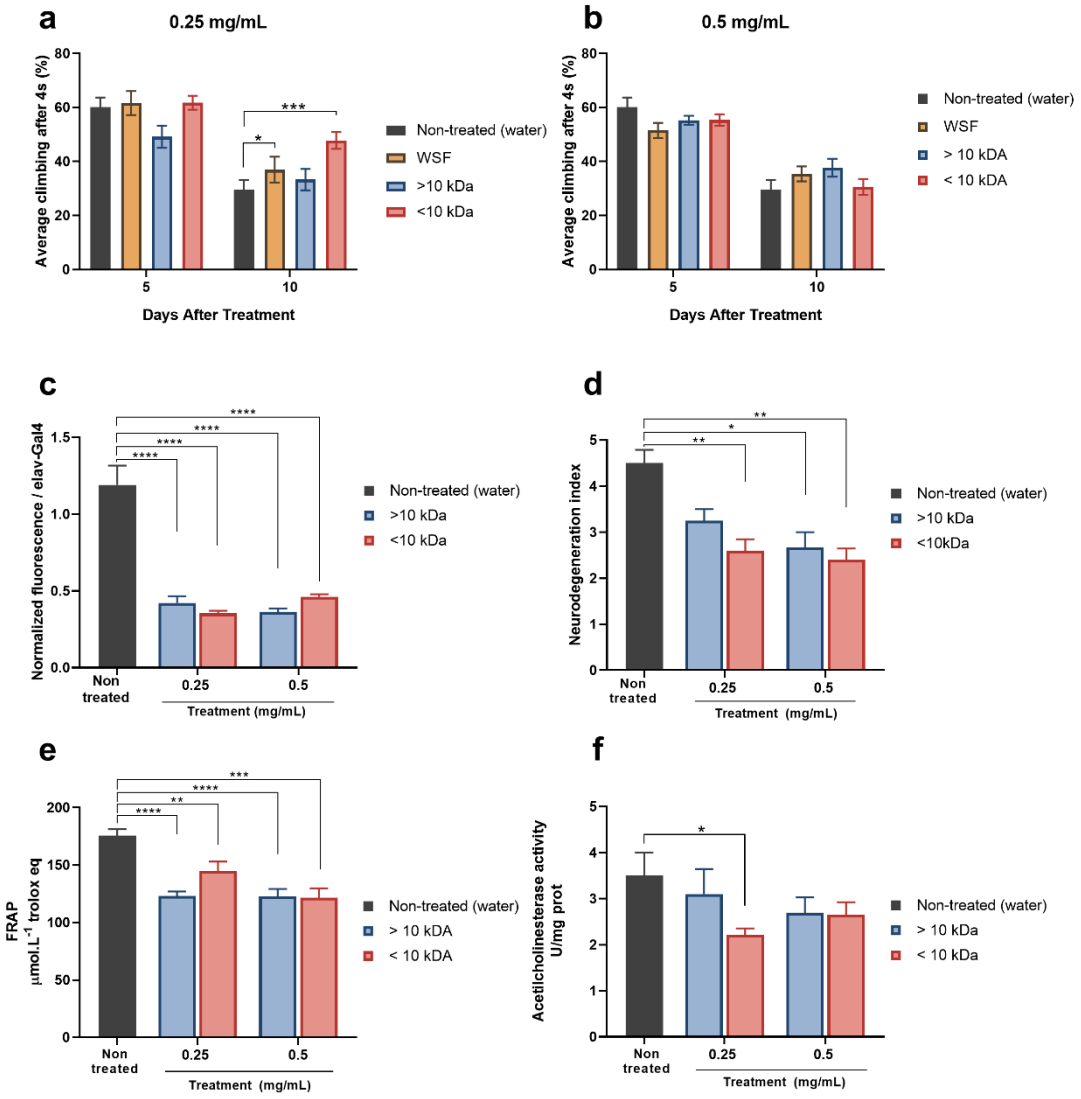

Figure 4. In vivo effects of Kefir fractions. (a) The climbing ability of AD-like flies after 5 and 10 days of treatment with the fractions at concentration 0.25 mg/mL, flies treated with WSF, and <10 kDa show an improvement in motor performance (n = 90). (b) The climbing ability of AD-like flies after 5 and 10 days of treatment with the fractions at concentration 0.5 mg/mL (n = 90). (c) Quantification of amyloid content by the Thioflavin T assay. Flies treated with all fractions at both concentrations showed a reduction in amyloid content compared to the control (untreated) at 10 days of treatment (n=30 for each treatment). (d) Index of neurodegeneration based on histopathological analysis according to vacuolar lesions (n=10 at 10 days of treatment). The results show a decrease in the index in all treated flies compared to the control. (e) Fe^{3+} reduction capacity by the FRAP method. Flies treated with the fractions show reduced antioxidant activity (n = 30 and 10 days of treatment). (f) Acetylcholinesterase activity. Only flies treated with the <10 kDa fraction at 0.25 mg/mL had

decreased acetylcholinesterase activity. Data are presented as mean + S.E.M. Statistically significant differences are indicated by * $p < 0.5$, ** $p < 0.1$, *** $p < 0.01$ and **** $p < 0.001$.

T-test analysis showed that both tested fractions demonstrated a significant result when administrated at 0.25 and 0.5 mg/mL. Flies treated with those samples showed a decrease in amyloid content (Fig. 4C), neurodegeneration index (Fig. 4d) and antioxidant activity (Fig. 4E) when compared with non-treated flies.

Exceptionally in the anti-acetylcholinesterase assay (Fig. 4f), only flies treated with the <10 kDa fraction (at 0.25 mg/mL) displayed a decrease in acetylcholinesterase activity when compared to control.

2.6 Molecular Docking

The peptides fraction from kefir <10kDa had antioxidant activity, improved climbing ability, decreased beta-amyloid levels and inhibited acetylcholinesterase activity. Molecular dockings were performed to verify the peptides present in the fraction identified in peptidomic assay in BACE, amyloid fibrils and acetylcholinesterase.

Nine peptides were used for prediction of 3D structure e docking. All peptides contributed for atomic contact energy to the global energy (ACE) below -6 in docking prediction for BACE, a Aβ (1-42) fibril and human acetylcholinesterase (Table 1). The best model for docking (peptide with the lowest ACE value for each protein) are showed in Fig. 5.

The peptide VPPFLQPEV was predicted as the best ligand for BACE. This peptide binds to an important flap that controls the enzyme activity by partially cover the substrate-binding cleft localized between the N- and C-terminus lobe (Fig. 5a and 5b).

The best predicted ligand for Aβ(1-42) fibril was the peptide VYPFPGPIPN. It can bind between the first and second β-strand region (Fig. 5c and 5d). This is a hydrophobic region, and the binding of the peptide in this region may turn it more hydrophobic (Fig. 5e).

The VYPFPGPIPN was also predicted as the best for human AChE. This enzyme has two binding sites: one is a peripheral anionic site (PAS) located at the entrance of the active gorge and the other is a catalytically active site (CAS) located at the base of the active-site gorge. The peptide binding in PAS (Fig. 5f and 5g).

Table 1: Results of *in silico* docking of putative bioactivities from main milk proteins

Source	Peptide Sequence	Peptide ranker	Protein	Docking (Kcal/mol)					
				BACE		β-Amyloid		AChE	
				Global energy	ACE	Global energy	ACE	Global energy	ACE
DataBase	YPFVPGLP	0.86	β-casein	-13.32	-6.17	-61.36	-19.58	--33.49	--9.77
	VYPFPGPI	0.84	β-casein	no predicted					
	VAPFPEVFG	0.77	α-S1-casein	-25.70	-7.90	-92.21	-16.35	-75.29	-12.79
	EMPFPK	0.76	α-S1-casein	no predicted					
	LVYPFPGPI	0.74	β-casein	-41.44	-10.85	-82.06	-20.24	-73.62	-14.66
	VYPFPGPIPN	0.72	β-casein	-37.34	-11.95	-78.37	-24.27	-70.68	-14.72
	SLPQNIPPLTQTPVVVPPFL	0.68	β-casein	-35.08	-7.61	-104.61	-20.23	-20.66	7.45
De novo	HQPHQPLPPT	0.62	β-casein	-23.83	-4.29	-50.56	-8.54	-57.54	-9.95
	VPPFLQPEV	0.53	β-casein	-44.55	-13.33	-96.41	-19.06	-57.70	-8.75

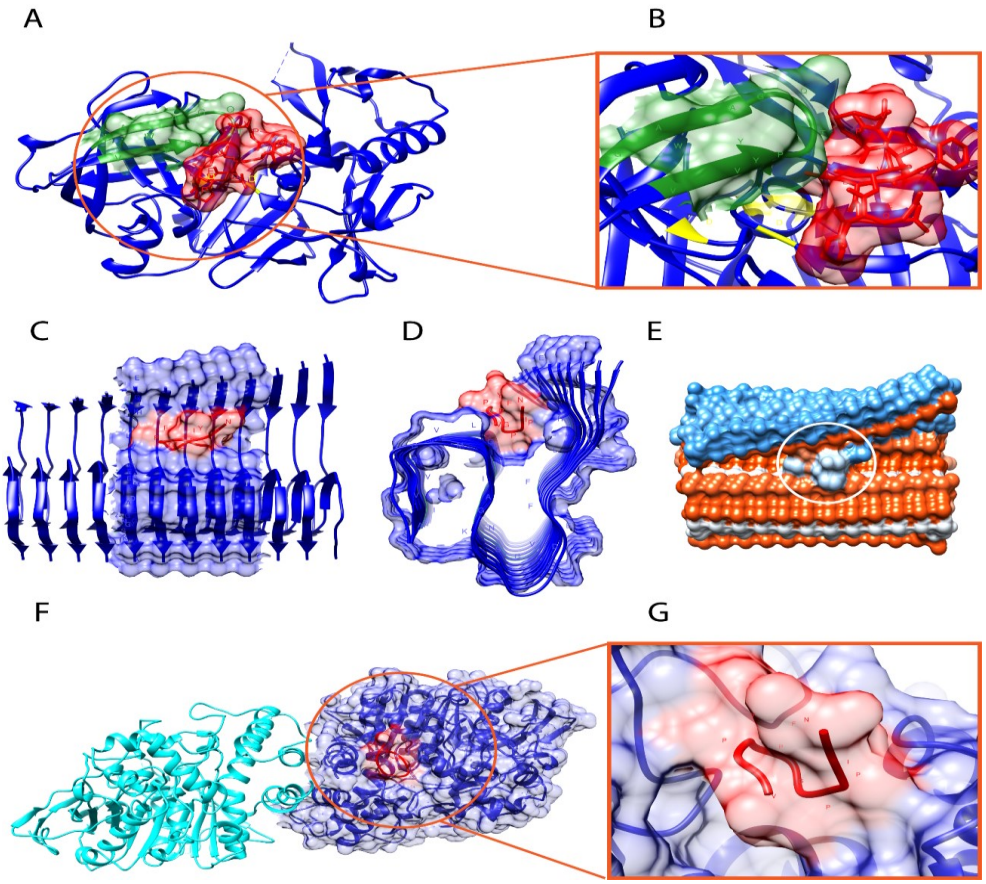

Figure 5. Molecular docking analysis of VPPFLQPEV and VYPFPGPIPN peptides and their interaction with BACE-1, βA and Acetylcholinesterase. (a) Prediction of the interaction of VPPFLQPEV peptide with the enzyme BACE-1. (b) Zoom of figure A. (c) and (d) Prediction of the interaction of VYPFPGPIPN peptide with β-amyloid plaques between first and second β-strand regions. (e) Hydrophobic region of interaction of the VYPFPGPIPN peptide with the β-amyloid plaque. (f) Prediction of the interaction of VYPFPGPIPN peptide in the peripheral anionic site of the Acetylcholinesterase enzyme. (g) Zoom of figure F.

3 Discussion

Kefir is a broadly used probiotic, characterized as a symbiotic agglomerate of bacteria and yeasts. It is popularly used as complementary treatment for many diseases[23–26]. Throughout milk's fermentation, kefir microorganisms present a unique protein metabolism process, resulting in bioactive molecules[27]– among those peptides. Kefir grains composition varies according to geographic location and fermentation parameters[11,28,29] which is thought to imply variation also on its metabolites and peptides. Even though there is variability, some core bacteria and yeast remain consistent across samples, which allows comparison throughout different studies[12]. Our group already characterized the effects of raw kefir and its organic fractions – as well as its metabolome – in improving DA-like flies[13]. This present work is a complementary effort, investigating the effects of kefir

peptides in DA-like flies, as well as its proteomic and peptidomic characterization. Additionally, it is the first case to our knowledge that a group characterizes both peptides and metabolites from a single kefir grain sample.

In the present work, the focus was in the peptidome due to displaying the intact bioactive peptides present in the sample. The fermentation generated peptides were identified through a proteomic analysis, and, similar results were described by other research groups[30,31], which supports kefir's composition consistency despite its particular variations across samples.

As we used cell free protein to produce the proteomic profile, the peptidases found using databases from *Lactobacillus* and *Acetobacter* could be from cell disrupted (intracellular) from fermentation or by novel secretory protein degradation system[32]. Further assays would be needed to resolve this question.

Our *in silico* analysis indicates that the major part of the peptides generated from our kefir sample during fermentation display several bioactive activities which characteristics have been described in other milk-derived peptides[33,34]. Here we focus on its putative acetylcholinesterase inhibition and antioxidant property as well BACE inhibitor and putative amyloids fibrils bindings

Those characteristics are especially instigating for AD since it is a multifactorial disease[35] and researchers have turned their attention to developing multi-target drugs to inhibit multiple factors involved in AD, including protein misfolding and associated AB aggregation, t aggregation, metal dyshomeostasis, oxidative stress and the decrease AChE levels. Even though the proposed model is based on the amyloidogenic hypothesis, we should expect alteration in other metabolic processes. Previous studies showed the relation between amyloid aggregation and both oxidative stress[36] and acetylcholinesterase activity[37], during the AD's progression.

Oxidative stress related to AD's neuropathological manifestations implies an increased level of reactive oxygen species (ROS)[38,39] and in the abnormal homeostasis of bioactive metals[40]. Otherwise, acetylcholinesterase acts on acetylcholine conversion, which is related to the cholinergic cascade and cholinergic neuron loss in the AD's pathology[41].

Targeting those processes, antioxidant compounds (e.g. resveratrol) have shown a role in AD prevention[42] or as a support treatment[43]. Furthermore, many AD drugs inhibit acetylcholinesterase activity, aiming to increase the brain's acetylcholine levels[44]. However, those drugs have a limited effect and generate collateral effects[45].

Therefore, with a positive prediction from the *in silico* investigation, we screened the effect of the fractions *in vitro*. Both antioxidant and anti-acetylcholinesterase properties were confirmed using the FRAP and Acetylcholinesterase Inhibition assay. To our best knowledge, this is the first work to report the anti-acetylcholinesterase activity of a kefir-produced molecule.

In order to verify the effects of a kefir-produced molecule *in vivo*, we used the *D. melanogaster* AD-like model. For that, we first displayed a confirmation of the model – in which the human BACE and APP are overexpressed in the fly neurons by the pan-neural driver (elva-Gal4).

In the performed assays, the flies displayed a motor deficit starting at 10-13 d.p.e, which was correlated to an increase in amyloid content – demonstrated by amyloidogenic quantification and severe neurodegeneration index on histopathological analysis. This has been previously described for a similar model[13,46,47] which indicates our model's suitability.

With a validated model, we treated the AD-like flies with kefir fractions. Flies treated with WSF and <10 kDa fraction (both at 0.25 mg/mL) displayed a better climbing ability in relation to non-treated flies. However, the <10 kDa had an apparent higher influence in this process, probably due to its higher peptide concentration – due to serial filtering processes.

To investigate this effect, molecular dockings were performed for potentially bioactive peptides both with AChE and BACE1 as targets. The AChE has two binding sites: one is a peripheral anionic site (PAS) located at the entrance of an active-site gorge, and the catalytically activity site (CAS) located at the base of active-site gorge[48] and ligands of PAS or CAS can inactivate this enzyme[49]. The binding of VAPFPEVFG peptide in PAS could be enough to promote the inhibition of acetylcholinesterase. Our docking data is only a tentative to prospect kefir peptides characteristics and effects. The real effects observed *in vivo* AD-like model were promoted by a complex mix of of peptides.

The BACE1 molecule is formed by three portions: an extracellular N-terminal domain, a transmembrane domain and a C-terminal cytosolic domain. Its cleavage site is between the N- and C-terminal regions, characterized by the aspartate catalytic dyad (Asp32 e Asp228, highlighted in yellow on Fig. 5 a-b)[50].

Near this region there is a flexible flap (highlighted in green in Fig. 5 a-b), perpendicular to the active site. This flexible flap can obtain either an open or closed conformation, and that way, help or obstruct the access of a molecule to the enzyme active

site[51,52]. Between the flap and the active site there is also a space on BACE1 structure, this is the region to be reached by the molecule so that it can reach the catalytic dyad[53].

By analyzing the 3D structure of BACE1 and the peptide VPPFLQPEV (Table 1 - docking of smaller global energy value, Figure 5 a-b), it was possible to verify that it did not interact with the BACE1 active site. Despite that, this peptide interacts with the ALCA, being able to alternate its conformation, localized strategically between the big N- and C-terminal portions of BACE1. The peptide position could block the access area of other molecules – as substrates – to the enzyme active site, inhibiting its action. As a consequence, BACE1 could lose its capacity of cleaving APP – and consequently reducing the Aβ-peptides production and amyloid plaques accumulation. This way, it could be possible to decrease the progress of the neurodegeneration through the amyloidogenic pathway.

The amyloidogenic pathway results in the production of Aβ-peptides of distinct sizes, depending on the γ-secretase cleavage region. Amongst those, the 42 amino acids peptide (Aβ42) is the most neurotoxic[54,55]. Based on that, we also evaluated the molecular docking between the bioactive peptide VYPFPGPIPN and the amyloid plaques generated by the Aβ42 peptide.

This peptide has a unique conformation composed by three β-sheets that cover the residues 12-18 (β1), 24-33 (β2) and 36-40 (β3)[56]. This tertiary fold is responsible for this peptide's toxicity and aggregation, which results in the β-amyloid plaques formation in AD. With the molecular docking analysis between the peptide VYPFPGPIPN and amyloid plaques (Fig. 5 c-e), we observed an interaction among those molecules, and a consequent conformational change in the amyloid plaque – which is highlighted by the opening of its tertiary folding (Fig. 5e). This alteration in β-amyloid plaque conformation could reduce its toxicity, neutralizing the negative effects of its aggregation and therefore attenuate the Alzheimer's Disease effects.

The present study extends the existing literature providing evidence that peptides derived from cow milk kefir can modulate Alzheimer phenotype in AD-like by decreasing the brain's β-amyloid relative level. Consequently, it decreases the neuronal tissue damage, improves motor ability, and decreases acetylcholinesterase activity. In summary, our study was able to identify bioactive peptides present in kefir (fraction <10 kDa) and predict their potential to be used as alternative adjuvant treatment of Alzheimer Disease and be used as a drug prototype.

4 Methods

4.1 Samples

Kefir grains were obtained as a donation from the local population in Uberlandia, Brazil inoculated (4% w/v) in UHT whole cow milk for fermentation process and left for 24 hours at room temperature in a glass container. A genetic fingerprint was previous published by our group[13]. The fermented product was collected by removing the grains by filtration and after that, was centrifuged at 4°C, 4900 x*g* for 10 minutes. The resulting supernatant went through a series of filtering processes, where we obtained the three fractions that were further tested.

The pellet was discarded and the supernatant re-centrifuged, generating kefir's water-soluble fraction (WSF). This was then filtered using a vacuum pump, passed through a 0.22 um membrane. Finally, this filtrated passed through a 10 kDa Amicon column, separating peptides by size: bigger than 10kDA (referred to as >10kDa) and smaller than 10kDA (<10kDa).

4.2 Proteopeptidomics

As preparation for shotgun proteomics, the samples were reduced with 100 mM dithiothreitol (DTT) and alkylated with 0.5 M iodoacetamide. Samples were then digested with trypsin (0.01 μg/μL) – except for the fraction of < 10 kDa peptides, obtained from Amicon filtering. The desalination step was performed with ZipTips C18 (Millipore, Billerica, MA, United States).

Shotgun proteomics and peptidomics were performed on the Liquid Chromatography-Electrospray Ionization-Quadrupole-Time of Flight-Mass Spectrometry (6520B LC-ESI-Q-TOF-MS) from Agilent. AdvanceBio Peptide Mapping column (Agilent) was used (2.1 mm internal diameter, 10 cm long, and 2.7 μm particles). In the mobile phase, water (A) and acetonitrile (B) were acidified with formic acid (0.1% v/v), under the gradient: 2% B (0 min), 2% B (10 min), 15% B (40 min), 50% B (150 min), 70% B (200 min), 98% B (220 min), 98% B (300 min), 100% B (301 min) and 100% B (400 min) at 400 μL/min. For ionization, a nebulizer pressure of 45 psi was used, with drying gas at 8L / min (325 °C). 4KV energy was applied to the capillary.

Spectrum Mill software (Agilent) was used for data analysis, using the following Uniprot databases: 1) "Milk and Bovine" (69,760 results in February 2021; 2)

"Lactobacillus" (1,250,616 results in February 2021) and 3) "Acetobacter" (185,991 results in February 2021). Carbamidomethylation was set as fixed with and. Maximum missed cleavages were selected in two for trypsin. The precursor mass error and the fragments at 20 ppm, product mass tolerance at 50 ppm, and maximum ambiguous precursor charge at 3.

For *de novo* analysis, the Sherenga module of the Spectrum Mill software (Agilent) was used.

4.3 *In silico* physicochemical parameters, toxicity, and bioactivity prediction of obtained peptides

ToxinPred (https://webs.iiitd.edu.in/raghava/toxinpred/algo.php) was used to calculate each characterized peptides physicochemical characteristics and expected toxicity level, while the Peptide Property Calculator from Innovagen (http://www.innovagen.com/proteomics-tools) analyze its solubility. For bioactivity prediction, PeptideRanker (http://distilldeep.ucd.ie/PeptideRanker/) was used. Finally, the Milk Bioactive Peptide Database (http://mbpdb.nws.oregonstate.edu/) was used to determine the origin of peptides from milk protein and their putative biological function. The peptides with a rank above 0.5 were considered as potential bioactivity.

4.4 *In vitro* fraction evaluations

4.4.1 Total Antioxidant activity

Total antioxidant capacity was evaluated through the FRAP assay (Ferric Reducing Antioxidant Power Assay)[20]. This method consists in evaluating the compound's capacity of reducing Fe^{3+} to Fe^{2+}. Kefir's fractions (WSF, and both Amicon resultants) were solubilized in distilled water at 500 µg/mL. Ascorbic acid was used in the same concentration as a positive control and sodium acetate buffer was used as blank.

For the assay, 250 µL of FRAP reagent (10X Sodium acetate 0.3M, TPTZ (2,3,5-Triphenyltetrazolium chloride) 10 mM, 1 vol. Ferric Chloride 20 mM) were mixed with 10 µL of each sample and 25 µL of MiliQ Water. The reaction went for 6 minutes at 37°C. The respective absorbances were measured at 593 nm in a spectrophotometer.

Each sample's antioxidative capacity was determined by the construction of an analytical curve built with Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid).

4.4.2 Acetylcholinesterase inhibition assay

The acetylcholinesterase inhibition assay was based on the one described by Rhee (2001)[57] .In the assay three Tris-HCl buffers were used: I) standard Tris-HCl buffer (pH 8); II) with Bovine serum albumin 1% w/v; and III) with NaCl 0.6% w/v and MgCl 0.4% w/v. The enzyme was diluted (0.2 U/mL) on Buffer I.

The solutions used were prepared in the following way: the enzyme was diluted to 0.2 U/mL in buffer I, DTNB 0.1% diluted in tampon III, acetylcholine iodide solution (substrate) 0.4% v/v diluted in Milli-Q water, and inhibitor (positive control) Galantamine diluted in Milli-Q water.

For the assay, 25 µL of each sample were added to 125 µL of DTNB solution, 50 µL of Buffer II, 25 µL of acetylcholine iodide solution, and 25 µL of acetylcholinesterase solution. In a spectrophotometer, the absorbance was measured at 405nm for 20 minutes at 30°C. Acetylcholinesterase inhibition was calculated using the following equation:

Inhibition (%) = [(100 -(Sample abs.- Blank abs.) / (Blank abs.)] x 100

4.5 *In vivo* experiments

4.5.1 *Drosophila melanogaster* genetics

Fly's stocks were obtained from the Bloomington Stock Center: W1118 (stock number 3605), UAS-BACE-1,UAS-APP (#33797), elav-GAL4 (#5146) and kept in an incubator at 25 degrees in a 12:12 hours dark and light cycle. Unless otherwise stated, flies were reared in 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% w/v).

AD-like flies – expressing human BACE and APP pan neuronally – were obtained by crossing elav-GAL4 and UAS-BACE,UAS-APP flies. Resulting pupa was selected according to its phenotype – without any genetic marker, as tubby – ensuring that the resulting flies' genotype is elav-Gal4/+; ;UAS-BACE, UAS-APP/+ (AD-like model).

4.5.2 Treatments

Fly treatment was administered via food and began at 0-3 days post eclosion. All kefir samples were solubilized at 0.5 and 0.25 mg/mL. From those, 5mL were mixed to 1g of enriched mashed potato medium (75% instant mashed potato, 15% yeast extract, 9.3% glucose, and 0.07% nipagin). Treatment medium was changed every other day to ensure

fresh exposure to the samples. Control flies were fed with enriched potato powder medium solubilized with distilled water only. For all experiments, only male flies were used.

4.5.3 Rapid iterative negative geotaxis assay (RING)

Rapid iterative negative geotaxis assay (RING) was adapted from Gargano (2009)[58] and used to evaluate the effect of different types of treatment on flies' locomotor ability. To ensure AD-like phenotype, these flies were compared to control (elav-Gal4). Groups of 30 AD-like flies (in triplicate) of each treatment (WSF, 0.22um, and both Amicon conditions) were transferred to clean vials (9.5 cm x 2.5cm) and put in a custom vials holder. Flies had their behavior accessed 5 and 10 days after treatment. Before testing, flies were exposed to light and kept in a silent environment for 20 minutes, to acclimate. For the assay, the holder was hit three times on 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 (4 seconds) that the holder touched the bench.

4.5.4 Total antioxidant activity in vivo

To evaluate total antioxidant activity for each treatment, the modified FRAP method was used. 10 fly heads (in triplicate) after 10 days of treatment were homogenized with PBS and centrifuged (2 min, 1000x*g* at 4°C). For the assay, 10 µL of each supernatant were mixed with 50 µL of FRAP reagent 1:1 in distilled water. Antioxidant activity was evaluated as previously described for *in vitro* analysis.

4.5.5 Acetylcholinesterase activity assay

For the assay, fly head homogenized solutions were made as described above. 25µL of the solution was added to 50 µL of Tris-HCl Buffer (Bovine serum albumin 1% w/v), 125 µL of DNTB and 25 µL of acetylcholine iodine solution and 25 µL of Acetylcholinesterase (0.2 U/mL) (based on Ellman's protocol)[59]. The sample's absorbance was measured at 405nm at 30°C for 20 minutes with a 30s interval.

4.5.6 Amyloid quantification

The amyloid content was assessed using the Thioflavin T (ThT), a benzothiazole dye that exhibits enhanced fluorescence upon binding to amyloid fibrils. Fly heads of elav-Gal4 (control) and AD-like flies (pool of 10 heads each, in triplicate) were collected, sacrificed in liquid nitrogen, decapitated, and kept at –80°C until the next steps. Fly heads were then kept in ice and homogenized in PBS and centrifuged (2 min, 10000x*g* at 4°C). The supernatants were collected and used for amyloid quantification and Bradford protein dosage in technical triplicate. In a protocol modified from Westfall et al[60] supernatants were incubated with ThT working solution (20 μM) for 20 minutes under agitation. Fluorescence was measured at 450 nm excitation / 482 nm emission and normalized to ThT only samples. Total fluorescence was corrected to each sample's total protein content and to elav-Gal4 (control flies) fluorescence levels. This additional step was needed once *in vivo* samples could present autofluorescence.

4.5.7 Histopathological analysis

Ten flies of each treatment group, at the best performed concentration in previous experiments were used. All flies were analyzed at 10-13 d.p.e. and elav-Gal4 was used as a control. The flies had its head transferred to 4% formaldehyde in sodium phosphate buffer 0.1 M pH 7.2 for 16 h at 4 °C. The samples were then dehydrated in a graded ethanol series (70, 80, 90, and 95%) and transferred to methanol for 16 h at 4 °C. After, they were embedded in HistoResin (Leica) and 3 μm thick slices were stained with hematoxylin and eosin, analyzed, and photographed with a light photomicroscope. Neuropile images were used to calculate the neurodegenerative index - as normal to low, moderate, or severe - according to vacuolar lesions[13,22].

4.6 Molecular Docking

Putative bioactive components were used for docking analysis. The peptides 3D structures were created using PEP-FOLD 2.0. The Protein Data Bank (PDB) file from BACE (3TPJ), acetylcholinesterase (AChE – 3LII), 42-Residue Beta-Amyloid Fibril (2MXU), were retrieved from PDB. The docking between the peptides and these enzymes was performed by using PathDocking. The best model was chosen based both on global energy and atomic contact energy contribution to the global binding energy.

4.7 Statistical Analysis

Data analysis was performed using GraphPad Prism 8. A priori, we evaluated the normal distribution of the data by D'Agostino&Pearson test. Groups were compared through a t-test with an established significance level of $P < 0.05$

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Supplementary Material

Supplementary Table 1: Results of in silico prediction of physicochemical parameters and bioactivities from main milk proteins

Source	Peptide Sequence	Peptide ranker	Solubility	MM (Da)	pI	Net charge at pH 7	Function	Protein	IC50 (µM)	DOI
DataBase	YPFVPGLP	0.86	Poor	889.17	5.88	0.00	satiety immunomodulatory Anxiolytic ACE-inhibitory Antioxidant	β-casein	500	10.1039/c4fo00164h 10.1016/j.jff.2014.03.018 10.1007/BF02496839 10.3390/foods9080991
	VYPFPGPI	0.84	Poor	889.17	5.88	0.00	Antioxidant ACE-inhibitory PEP-inhibitory	β-casein	50	10.3390/foods9080991 10.3390/foods9080991 10.1271/bbb.56.976
	VAPFPEVFG	0.77	Poor	962.23	4.00	-1.00	ACE-inhibitory	α-S1-casein	650	10.1021/jf049510t
	EMPFPK	0.76	Good	747.98	6.35	0.00	ACE-inhibitory	α-S1-casein	650	10.1021/jf049510t
	LVYPFPGPI	0.74	Poor	1002.35	5.88	0.00	Antimicrobial ACE-inhibitory	β-casein	565.58	10.1016/S0958-6946(98)00048-X
	VYPFPGPIPN	0.72	Poor	1100.42	5.88	0.00	Antioxidant ACE-inhibitory	β-casein	6.2	10.1007/s00217-012-1894-5, 10.3390/antiox9020117 10.3168/jds.S0022-0302(05)73032-0
	SLPQNIPPLTQTPVVVPPFL	0.68	Poor	2157.89	5.88	0.00	ACE-inhibitory	β-casein	450	10.1128/AEM.00096-07
	VYPFPGPIPNS	0.54	Poor	1187.51	5.88	0.00	Antioxidant ACE-inhibitory	β-casein	6,2	10.1007/s00217-012-1894-5, 10.3390/antiox9020117
	NIPPLTQTPVVVPPF	0.49	Poor	1619.16	5.88	0.00	ACE-inhibitory	β-casein	450	10.1128/AEM.00096-07
	VVPPFLQPE	0.48	Poor	1025.34	4.00	-1.00	Antimicrobial ACE-inhibitory	β-casein	749	10.1017/S0007114511001085 10.3168/jds.S0022-0302(98)75878-3
	PVVVPPFLQPEV	0.42	Poor	1320.77	4.00	-1.00	Antimicrobial ACE-inhibitory	β-casein	749	10.1017/S0007114511001085 10.3168/jds.S0022-0302(98)75878-3
	SLPQNIPPLTQ	0.42	Poor	1207.56	5.88	0.00	DPP-IV Inhibitory	β-casein	205.2	10.1016/j.peptides.2016.03.005

	NIPPLTQTPVVVPPFLQPE	0.36	Poor	2086.75	4.00	-1.00	ACE-inhibitory	β-casein	450	10.1128/AEM.00096-07
	NIPPLTQTPVVVPPFLQPEV	0.36	Poor	2185.90	4.00	-1.00	ACE-inhibitory	β-casein	450	10.1128/AEM.00096-07
	IPPLTQTPVVVPPFLQPEV	0.35	Poor	2071.78	4.00	-1.00	ACE-inhibitory	β-casein	450	10.1128/AEM.00096-07
	TQTPVVVPPFL	0.33	Poor	1197.60	5.88	0.00	ACE-inhibitory	β-casein	749	10.3168/jds.S0022-0302(98)75878-3
	VVVPPFLQPEV	0.32	Poor	1223.64	4.00	-1.00	ACE-inhibitory	β-casein	749	10.3168/jds.S0022-0302(98)75878-3
	NIPPLTQTP	0.37	Poor	980.26	5.88	0.00	ACE-inhibitory	β-casein	17.3	10.1128/AEM.66.9.3898-3904.2000
	TPVVVPPFLQPEV	0.29	Poor	1421.89	4.00	-1.00	Antioxidant Antimicrobial ACE-inhibitory	β-casein	749	10.1016/j.foodchem.2010.05.029 10.1017/S0007114511001085 10.3168/jds.S0022-0302(98)75878-3
	TQTPVVVPPFLQPEV	0.17	Poor	1651.16	4.00	-1.00	Antioxidant	β-casein		10.1016/j.foodchem.2010.05.029
	VEELKPTPEGDLE	0.14	Good	1455.77	3.91	-4.00	Antimicrobial Antioxidant	β-lactoglobulin		10.1017/S0007114511001085 10.3390/ijms19071955
	SLPQNIPPLTQTP VVVPPFLQPEV	0.11	Poor	2611.45	4.00	-1.00	ACE-inhibitory	β-casein	144	10.3168/jds.S0022-0302(94)77026-0
De novo	AAEPVSLNGMF	0.77	Poor	1135.29	4.00	-1				
	HQPHQPLPPT	0.62	Poor	1151.29	6.92	0.2	Anti-inflammatory ACE-inhibitory	β-casein		10.3168/jds.2019-17976
	VPPFLQPEV	0.53	Poor	1025.21	4.00	-1		β-casein		10.1017/S0007114511001085
	VVPPFLQPEV	0.45	Poor	1124.35	4.00	-1	Antimicrobial	β-casein		
	NIPPLTQTPVVVPPFLQPE	0.36	Poor	2086.46	4.00	-1	ACE-inhibitory	β-casein		10.1128/AEM.00096-07
	TENKIPVYAFKLPV	0.33	Poor	1618.94	8.17	1		β-casein		
	VVVPPFLQPEV	0.32	Poor	1223.48	4.00	-1	Antimicrobial ACE-inhibitory	β-casein	749	10.1017/S0007114511001085 10.3168/jds.S0022-0302(98)75878-3
	NIPPLTQTP	0.30	Poor	980.13	5.52	0	ACE-inhibitory	β-casein	173.3	10.1128/AEM.66.9.3898-3904.2000

GAVDIPIAKQV	0.29	Good	1110.32	5.84	0		β-casein		
RNAIDFCLEQAG	0.29	Good	1336.49	4.37	-1.1		β-casein		
TPVVVPPFLQPEV	0.29	Poor	1421.70	4.00	-1	Antioxidant Antimicrobial ACE-inhibitory	β-casein	749	10.1016/j.foodchem.2010.05.029 10.1017/S0007114511001085 10.3168/jds.S0022-0302(98)75878-3
IPPLTQTPV	0.25	Poor	965.16	5.52	0	ACE-inhibitory	β-casein	173.3	10.1128/AEM.66.9.3898-3904.2000
TQTPVVVPPFLQPEV	0.17	Poor	1650.94	4.00	-1	Antioxidant	β-casein		10.1016/j.foodchem.2010.05.029
VEELKPTPEGDLEI	0.13	Good	1568.74	3.91	-4	Antioxidant Antimicrobial	β-lactoglobulin		10.3390/ijms19071955