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ESTER LOPES DE MELO

Kefir associado ao gérmen de soja: estudos físico-químico e farmacológico *in vivo* (atividades ansiolítica e antidepressiva)

Macapá
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Orientador: Prof. Dr. José Carlos Tavares Carvalho

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Dedico este trabalho...

A Deus,

Aos meus pais, por todo amor, carinho, dedicação e apoio dado por toda minha vida, juntamente com as minhas irmãs.

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SUMÁRIO

LISTA DE FIGURAS E ANEXOS

SÍMBOLOS, SIGLAS E ABREVIATURAS

ACTH	Hormônio adrenocorticotrófico
ADT	Antidepressivos tricíclicos
CRH	Hormônio liberador de corticotrofina
DSM-V	Diagnostic and Statistical Manual of Mental Disorders
EPS	Exopolissacarídeos de dextrano
ERα	Receptor estrogênico α
ERβ	Receptor estrogênico β
EtOH	Etanol
FSK	Solução fermentativa de Kefir
GABA	Ácido gama-aminobutírico
HPA	Hipotálamo-hipófise-adrenal
IMAO	Inibidores da monoamino-oxidase
ISRS	Inibidores seletivos da recaptação de serotonina
KSG	Kefir associado ao gérmen de soja
LABs	Bactérias ácido lácticas
OMS	Organização Mundial da Saúde
SFK	Solução fermentativa de kefir
SNC	Sistema nervoso central
SNE	Sistema nervoso entérico
TGI	Trato gastrointestinal

Kefir associado ao gérmen de soja: estudos físico-químico e farmacológico *in vivo* (atividades ansiolítica e anti-depressiva)

RESUMO

Introdução: A microbiota que coloniza naturalmente o trato gastrointestinal, apresenta um importante papel na ligação do eixo-cérebro intestino, desempenhando um papel significativo na saúde mental e comportamental do indivíduo. Estudos demonstram que o uso de probióticos, tal como o kefir, e alimentos contendo isoflavonas, desencadeiam respostas ansiolítica e antidepressiva. **Objetivo:** este estudo teve como objetivo realizar a padronização físico-química da associação do kefir ao gérmen de soja e, avaliar as atividades ansiolítica e antidepressiva dessa associação em modelos in vivo. **Metodologia:** O kefir foi preparado nas concentrações de 40 g/L de açúcar mascavo, 10 g/L de gérmen de soja e 60 g/L de kefir, utilizando o produto fermentado do 4º dia (FSK). Foi quantificado as isoflavonas (daidzeína, gliciteína e genisteína) presentes no kefir associado ao gérmen de soja (KSG) por cromatografia liquida de alta eficiência. A liberação de isoflavonas agliconas do KSG no trato gastrointestinal (fase gástrica, intestinal e cólon) foi avaliado pelo modelo de dissolução in vitro e, os efeitos ansiolítico e antidepressivo do KSG foram avaliados através dos método de ansiedade e depressão induzida por pentilenotetrazol (PTZ) em camundongos Swiss e, em modelos de ansiedade e depressão em zebrafish (Danio rerio). **Resultados e discussões:** no 4º dia de fermentação no KSG, a concentração das isoflavonas daidzeína, gliciteína e genisteína foram de 11.74, 40.30 e 0.81 µg/mL, respectivamente, e, no gérmen de soja, as concentrações foram de: 9.44, 26.50 e 0.78 µg/mL respectivamente. Foi demonstrado no estudo de liberação in vitro com o KSG, que na fase gástrica houve maior liberação de isoflavonas e redução nas fases intestinal e de cólon. Nos ensaios utilizando camundongos a administração por via oral do KSG desencadeou melhora das condições experimentais de ansiedade e depressão, produzindo efeitos ansiolítico e antidepressivo e aumento da atividade locomotora. Nos ensaios com zebrafish, no teste de scototaxis (light-dark test) o tratamento com KSG (400 mg/kg) e FSK (2 µL/kg), prolongaram a permanência dos animais no compartimento branco, quando comparado aos outros grupos. O tratamento com o KSG (200 mg/kg) apresentou resultado semelhante a buspirona. No teste do tanque novo, os animais tratados com FSK (2 µL/kg), permaneceram mais tempo no topo do aquário, comparado aos outros grupos. Entretanto, o grupo KSG (200 e 400 mg/kg) apresentou efeito similar ao grupo fluoxetina. **Conclusões:** Neste estudo, foi comprovado que a liberação do conteúdo de isoflavonas da aglicona no processo fermentativo é dependente do tempo de fermentação do kefir, e os resultados obtidos nos experimentos comportamentais em camundongos sugerem que o tratamento por 30 dias com KSG produziu efeitos ansiolítico e antidepressivo nas condições experimentais utilizadas, acompanhadas do aumento da atividade locomotora e, nos experimentos em zebrafish, o conjunto de dados dos padrões comportamentais obtidos nos diferentes experimentos demonstrou que o KSG e o FSK apresentam efeitos ansiolítico e antidepressivo.

Palavras-Chave: *Kefir. Validação. Quantificação. Isoflavonas. Gérmen de soja. Eixo cérebro-intestino. Depressão. Ansiedade. Microbiota intestinal.*

Agradecimentos: CNPq, CAPES, UNIFAP.

Kefir associated with soy germ: physical-chemical and pharmacological studies *in vivo* (anxiolytic and anti-depressant activities)

ABSTRACT

Introduction: The microbiota that naturally colonizes the gastrointestinal tract, plays an important role in the connection of the gut-brain axis, playing a significant role in the individual's mental and behavioral health. Studies show that the use of probiotics, such as kefir, and foods containing isoflavones, trigger anxiolytic and antidepressant responses. Objective: this study aimed to carry out the physical-chemical standardization of the association of kefir with soy germ and to evaluate the anxiolytic and antidepressant activities of this association in in vivo models. **Methodology:** The kefir was prepared in concentrations of 40 g/L of brown sugar, 10 g/L of soy germ and 60 g/L of kefir, using the fermented product of the 4th day (FSK). The isoflavones (daidzein, glycitein and genistein) present in the kefir associated with soy germ (KSG) were quantified by high performance liquid chromatography. The release of KSG aglycone isoflavones in the gastrointestinal tract (gastric, intestinal and colon phase) was assessed by the in vitro dissolution model and the anxiolytic and antidepressant effects of KSG were assessed using the pentylenetetrazole-induced anxiety and depression (PTZ) methods in Swiss mice and in models of anxiety and depression in zebrafish (*Danio rerio*). **Results and discussions:** on the 4th day of fermentation in FSK, the concentration of isoflavones daidzein, glycitein and genistein were 9.44, 26.50 and 0.78 µg/ml, respectively, and in KSG, the concentrations were: 11.74, 40.30 and 0.81 µg/ml, respectively. It was demonstrated in the in vitro release study with KSG, that in the gastric phase there was a greater release of isoflavones and a reduction in the intestinal and colon phases. In tests using mice, oral administration of KSG triggered improvement in experimental conditions of anxiety and depression, producing anxiolytic and antidepressant effects and increased locomotor activity. In the zebrafish tests, in the scototaxis test (light-dark test) the treatment with KSG (400 mg/kg) and FSK (2 µl/kg), prolonged the animals' stay in the white compartment, when compared to the other groups. Treatment with KSG (200 mg/kg) showed a result similar to buspirone. In the test of the new tank, the animals treated with FSK (2 µl/kg), remain longer on top of the aquarium, compared to the other groups. However, the KSG group (200 and 400 mg/kg) had a similar effect to the fluoxetine group. **Conclusions:** In this study, it was proved that the release of the isoflavone content of the aglycone in the fermentation process is dependent on the time of kefir fermentation, and the results obtained in the behavioral experiments in mice suggest that the treatment for 30 days with KSG produced anxiolytic and antidepressant effects in experimental conditions used, accompanied by increased locomotor activity and, in zebrafish experiments, the data set of behavioral patterns obtained in the different experiments demonstrated that KSG and FSK have anxiolytic and antidepressant effects.

Keywords: *Kefir. Validation. Quantification. Isoflavones. Soy germ. Brain-intestine axis. Depression. Anxiety. Intestinal microbiota.*

Acknowledgements: CNPq, CAPES, UNIFAP.

1 INTRODUÇÃO

Recentes pesquisas demonstraram que a microbiota intestinal, não apenas auxilia na digestão de alimentos, como também possui uma importante função eixo cérebro-intestino, mostrando que a disfunção, afeta na propensão de várias doenças, podendo influenciar até no comportamento e as emoções. A disbiose altera a permeabilidade intestinal, contribuindo para liberação de endotoxinas, alterando o eixo hipotálamo-hipófise-adrenal. Demonstrando assim, importante envolvimento com doenças como a ansiedade e depressão.

A ansiedade e depressão são doenças mentais que interferem na saúde e bem estar dos indivíduos, provocando tanto mudanças físicas como psicológicas. Essas doenças tem afetado um crescente número de pessoas ao longo dos anos, no qual, segundo dados da Organização Mundial de Saúde, 300 milhões de pessoas são afetadas com depressão, enquanto 264 milhões de pessoas, sofrem com ansiedade. Elas podem provocar perda de interesse, prazer, tristeza, medo, tensão, agitação, cefaleia e palpitação. Isso ocorre porque tanto a depressão quanto a ansiedade afetam neurotransmissores, como o distúrbio da atividade monoaminérgica e alteração nos sistemas gabaérgico, serotoninérgico, dopamínicos, neuropeptidícos, respectivamente. De acordo com as recentes descobertas, observou que a inclusão de determinados alimentos e probióticos, auxiliam na redução de sintomas da ansiedade e depressão.

O grão da soja se destaca por ser considerado um alimento funcional, e por seu alto valor nutricional e proteico. Os componentes responsáveis por grande parte dos seus benefícios são as isoflavonas que são fitoestrógenos, estas possuem atuação similar ao estrogênio e apresentam benefícios aplicáveis ao tratamento e prevenção de doenças

cardiovasculares, câncer de cólon, mama e próstata, osteoporose, ansiedade e depressão, como também reduzir sintomas causados durante a fase da menopausa.

O kefir por possuir microrganismos vivos benéficos à saúde, é considerado um produto probiótico. É uma bebida elaborada a partir da fermentação do seu substrato que pode ser o açúcar mascavo ou leite, possui baixo custo e fácil manipulação. Seu consumo regular, ajuda na regulação da microbiota intestinal, estimulando o crescimento de microrganismos benéficos e na inibição dos microrganismos prejudiciais, além de atuar melhorando o sistema imunológico, e no alívio dos sintomas da ansiedade e depressão.

Desta forma, este trabalho propôs avaliar a atividade ansiolítica e antidepressiva do kefir associado ao gérmen de soja, e quantificar as isoflavonas agliconas liberadas durante o processo fermentativo e após o processo gastrointestinal simulado. Logo, a elaboração deste trabalho pode ser utilizada como base para outras pesquisas com incorporação de isoflavonas com a perspectiva de criação de um probiótico.

2 OBJETIVOS

3.1 OBJETIVO GERAL

Realizar o estudo físico-quimico da associação do kefir ao gérmen de soja e, avaliar as atividades ansiolítica e antidepressiva dessa associação em modelos *in vivo*.

3.1 OBJETIVOS ESPECÍFICOS

a) Associar o gérmen de soja na cultura do kefir;

b) Avaliar o teor das isoflavonas agliconas do kefir associado ao gémen de soja, durante o processo fermentativo;

c) Avaliar *in vitro* a liberação das isoflavonas agliconas do kefir associado ao gémen de soja no trato gastrointestinal, considerando as fases gástrica, intestinal e cólon;

d) Avaliar as atividades ansiolítica e antidepressiva do kefir associado ao gérmen de soja em camundongos Swiss e em *Danio rerio* (Zebrafish).

3 REFERENCIAL TEÓRICO

3.1 EIXO CÉREBRO-INTESTINO

A ligação bidirecional do eixo cérebro-intestino apresenta como destaque principal a microbiota intestinal (KRAIMI et al., 2019). O eixo cérebro-intestino apresenta uma múltipla ligação que se conecta com vários sistemas que contribuem com o equilíbrio do organismo (MENDEZ-FIGUEROA et al., 2019). Uma das principais comunicações é através do Sistema Nervoso Central (SNC), ele possui uma importante e conhecida atividade sobre o intestino, auxiliando em diversas funções como a motilidade, secreção de mucina, produção hormonal, fluxo sanguíneo, entre outras (SILVESTRE, 2016). Já o Sistema Nervoso Entérico (SNE), deriva da crista neural, e se liga ao SNC através do nervo vago, ele possui a maior rede de neurônios do sistema nervoso periférico e autônomo, possuindo cerca de 600 milhões de neurônios e junto à comunidade microbiana, mediadores endócrinos e aos seus metabólitos, possuem a capacidade de modular o SNC (SILVESTRE, 2016; MENDEZ-FIGUEROA et al., 2019; MOULTON et al., 2019).

O trato gastrointestinal (TGI) possui um ecossistema complexo contendo milhares de microrganismos, incluindo bactérias, archaea, protozoa, vírus, de diferentes espécies presentes em uma proporção de 1:1 com as células humanas (MOHAMMADI et al., 2018). Cada pessoa possui a sua microbiota, variando de acordo com seu estilo de vida, alimentação, vícios e ambiente. As condições fisiológicas, como peristaltismo, acidez, influenciam quantidade, espécies e localização dos microrganismos (MENDEZ-FIGUEROA et al., 2019). Anteriormente, quando não possuía o conhecimento acerca da relevância da microbiota intestinal, tratavam as bactérias alí presentes como organismos comensais, com

a mera função de decomposição dos alimentos consumidos (BUTLER; CRYAN; DINAN, 2019; CHEUNG et al., 2019). No entanto, hoje sabe-se que a interação destes microrganismos com as células humanas possui uma função fundamental na manutenção da imunidade e homeostasia (MUKHTAR; NAWAZ; ABID, 2019). Estudos realizados observaram que a composição da microbiota intestinal residente possui influencia diante de alterações na ansiedade, memória, cognição e atividade locomotora, e até em doenças neurológicas (MOHAMMADI et al., 2018; ZHENG et al., 2019).

A comunicação do SNE com o TGI é modulada através de uma inervação intrínseca, em companhia dos seus dois plexos Auerbach (externo) e Meissner (interno), eles possuem diferentes funções que se complementam, o primeiro é responsável pela regulação dos músculos lisos, como o peristaltismo no intestino, enquanto que o segundo é responsável pela secreção e absorção (SILVESTRE, 2016; MUKHTAR; NAWAZ; ABID, 2019).

O intestino e cérebro se comunicam através de sinapses dos neurônios do nervo vago presentes no epitélio intestinal, que por meio de produtos gerados pela microbiota intestinal, influenciam nas respostas imunológicas, como citocinas e quimiocinas, e ativação de células enteroendócrinas (KRAIMI et al., 2019; BERTOTTO; CATRON; TAL, 2020). O eixo hipotálamo-hipófise-adrenal (HPA), atua sob condições de estresse, ocorrendo a liberação do hormônio liberador de corticotropina (CRH) e vasopressina, sinalizando a glândula pituitária para liberação de Hormônio adrenocorticotrófico (ACTH), no qual influencia na produção do hormônio cortisol, pelas glândulas supra-renais (BERTOTTO; CATRON; TAL, 2020; LASHERAS et al., 2020). Elevados níveis de citocina pró-inflamatóra, geram aumento de permeabilidade intestinal, provocando maior possibilidade de entrada de endotoxinas (AUDET, 2019; LASHERAS et al., 2020).

O eixo hipotálamo-hipófise-adrenal apresenta atuação nas células epiteliais do tgi, e interação com a microbiota intestinal (BERTOTTO; CATRON; TAL, 2020). Em estudos

realizados com camundongos livres de germes, criados em ambiente estéril foi observado altos níveis de ACTH e cortisol, demonstrando alto nível de estresse nestes animais, no entanto a hiperatividade do eixo HPA pôde ser alterada positivamente pela reconstituição com *Bifidobacterium infantis* (WEI et al., 2019; FUNG, 2020; LASHERAS et al., 2020).

Pesquisas realizadas nos últimos anos mostraram que a ingestão de probióticos e alimentos adequados, possuem um importante papel na organização da composição da microbiota intestinal, os alimentos e bebidas contribuem com moléculas neuroendócrinas e precursores que afetam tanto a fisiologia do hospedeiro, como também a viabilidade e função microbiana, como exemplo dado a ausência de organismos comensais que produzem metabólicos como ácidos graxos de cadeia curta, leva a um estado eubiotico, colaborando em alterações na microbiota intestinal, e cooperando na manifestação de doenças, como observado na cirrose, doença de Parkinson, autismo e infecção por *Clostridium difficile* e ansiedade e depressão (BAJAJ, 2019; LYTE, 2019; REID, 2019).

3.2 ANSIEDADE E DEPRESSÃO

A depressão maior ou transtorno depressivo maior é um estado clínico de caráter crônico incapacitante, multifatorial que interfere no bem estar pessoal, social e profissional do indivíduo (SILVA; MENEZES; DE SÁ, 2016). A prevalência, segundo o a Organização Mundial de Saúde (OMS), é de mais de 300 milhões de pessoas afetadas com a doença, com maior prevalência do o sexo feminino (CORREIA; SANTOS; SORAL, 2018; WALLACE et al., 2019). O número de atingidos no Brasil é em torno de 11,5 milhões de pessoas (5,8% da população) (GONÇALVES et al., 2018). E apresentam como fator de risco da depressão, traumas de infância e de vida, histórico familiar, personalidade, experiências de vida (FERNANDES et al., 2018).

Os parâmetros que a Diagnostic and Statistical Manual of Mental Disorders (DSM-V) abrange para diagnóstico de depressão são, estado deprimido, perda de interesse e prazer (anedonia), culpa, baixa concentração e energia, distúrbios de sono, alterações psicomotoras e peso, pensamentos sobre morte ou suicídio. Essa doença apresenta três diferentes níveis de intensidade, variando de acordo com a quantidade de sintomas que o indivíduo expressa semanalmente. Depressão menor, contendo dois a quatro sintomas por duas ou mais semanas, incluindo o estado deprimido ou anedonia. Distimia apresentando três ou quatro sintomas, incluindo estado deprimido, por no mínimo dois anos. Depressão maior: cinco ou mais sintomas no período de duas semanas ou mais, incluindo estado deprimido ou anedonia (GONÇALVES et al., 2018).

A depressão apresenta uma fisiopatologia complexa, e não totalmente esclarecida, os primeiros fármacos que surgiram na década de 50, a partir da teoria das monoaminas proposto por Schildkraut no qual se entendia que a depressão sucedia devido um distúrbio da atividade monoaminérgica envolvendo insuficiente quantidade de neurotransmissores de monoamina, entre eles serotonina, norepinefrina. Essa teoria criou força a partir da análise dos efeitos clínicos positivos dos fármacos observando diminuição dos sintomas de depressão enquanto que os fármacos bloqueadores da síntese de norepinefrina e serotonina alteravam negativamente o humor. Diante disso, os tratamentos farmacológicos para depressão são voltados para deficiência destes neurotransmissores e receptores, como os Inibidores da monoamino-oxidase (IMAO), Antidepressivos tricíclicos (ADT) e os inibidores seletivos da recaptação de serotonina (ISRS) (GERHARD; WOHLEB; DUMAN, 2016; SILVA; MENEZES; DE SÁ, 2016; WALLACE et al., 2019).

Já a ansiedade é uma condição crônica e incapacitante na qual o indivíduo apresenta estado emocional dirigido para o futuro possuindo percepção de não poder controlar ou até mesmo prever situações de perigo, levando a uma alteração da resposta comportamental, física e fisiológica e com uma constante e desproporcional preocupação, medo, tensão,

agitação, cefaleia, palpitação, aumento das atividade do sistema nervoso autônomo, dos quais aumento da pressão arterial, frequência cardíaca, tônus muscular entre outras alterações. Este comportamento está associado a experiências vivenciadas comprometendo a vida social, profissional e o bem-estar do paciente, comprometendo cerca de 264 milhões de pessoas em todo o mundo, segundo dados da OMS e destes, 18 milhões de ansiosos no Brasil (COUTINHO et al., 2017; FREITAS; CALAIS; CARDOSO, 2018; MELO et al., 2018; VIANA FILHO et al., 2018).

Diversas vias de neurotransmissão fazem parte da mediação da ansiedade, principalmente, os sistemas gabaérgico e serotoninérgico assim como os dopamínicos, neuropeptidícos, entre outros, além de ativar o eixo HPA (BRAGA, 2010; DOS SANTOS SAMPAIO et al., 2018). A fisiopatologia da ansiedade não está totalmente elucidada, até mesmo, com menos informações que a depressão, no entanto os principais fármacos utilizados para ansiedade incluem benzodiazepínicos, barbitúricos e composto z, eles todos operam mediante via receptor $GABA_A$. O GABA (ácido gama-aminobutírico) é um neurotransmissor inibitório do SNC, que possui três receptores, no entanto o $GABA_A$ é o que está presente em maior quantidade. Ele apresenta três subunidades, α, β e γ, no qual os ansiolíticos benzodiazepínicos, barbitúricos e composto z, interagem em diferentes sítios destas subunidades do GABA (SILVA; MENEZES; DE SÁ, 2016).

Outros grupos farmacológicos também são efetivos no tratamento de ansiedade, como antidepressivos ISRS, inibidores da recaptação de serotonina/norepinefrina, tricíclicos e inibidores da monoamino-oxidase, buspirona, atuando como agonista de receptor de serotonina, fármacos epiléticos como a gabapentina, alguns antipisicóticos ativos, como a rispiridona e antagonistas beta-adrenégicos, assim como o propranolol (BRAGA, 2010; RANG et al., 2016).

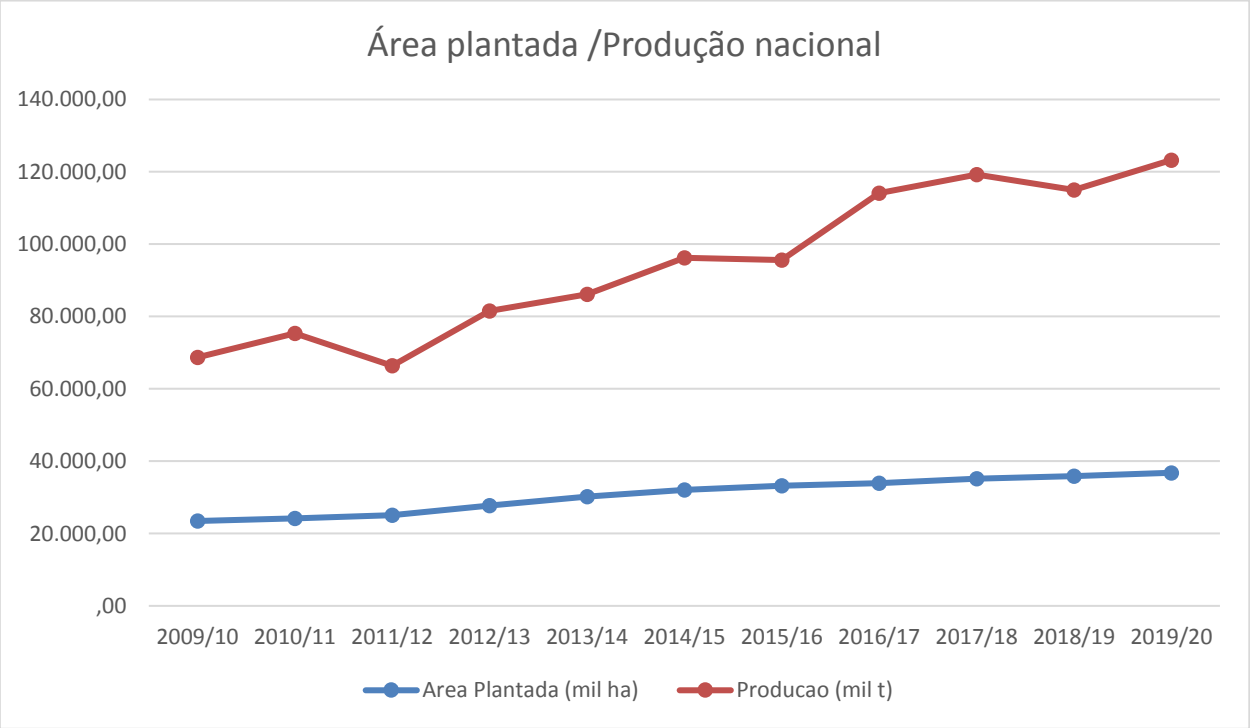
Pesquisas realizados para esclarecimento das teorias sobre o mecanismo da depressão, mostraram limitações terapêuticas, resultados incoerentes e conclusões equivocadas, assim outras vias e receptores e neurotransmissores vem sendo estudadas para melhor entender o desencadeamento da depressão (GERHARD; WOHLEB; DUMAN, 2016; RANG et al., 2016). Entre eles os neurotransmissores dopamina, glutamato, GABA e o eixo hipotalâmico-hipofisário-adrenal (EBADA, 2017). Ele é controlado pelas monoaminas, os neurônios hipotalâmicos que controlam a função hipofisária recebem aferências noradrenérgicas e serotoninérgica, liberando cortisol. Pacientes depressivos possuem altos níves de concentração plasmática de cortisol, como também outros homônios são afetados, como diminuição do homônio do crescimento e aumento da prolactina. Desta forma a hiperfunção de corticotrofina e a hipofunção das monoaminas está associada a depressão (RANG et al., 2016).

Os mecanismos de ação das diferentes classes de antidepressivos e ansiolíticos se assemelham, no entanto é crescente o número de pacientes que apresentam dificuldade no tratamento farmacológico, podendo levar semanas para surtir efeito ou apenas não ser eficaz, de tal forma que 40% dos pacientes apresentam resistência aos antidepressivos (KAUFLING, 2019). O primeiro relatório informando os benefícios dos probióticos foi publicado em 2011 pela OMS, logo depois, em 2005 o primeiro artigo propôs a utilização dos probióticos para redução dos sintomas da depressão, até então é crescente a quantidade de artigos publicados sobre esse assunto, surgindo em 2013 o termo “psicobióticos”, mostrando ser uma alternativa no tratamento adjuvante na depressão (NADEEM et al., 2019).

3.3 SOJA

A produção de soja apresenta como principal destaque da agricultura brasileira, sendo o principal produto agrícola da exportação, colocando o Brasil como o segundo maior produtor do grão e o maior exportador do mundo. Os três principais países produtores desta semente são os Estados Unidos, Brasil e Argentina, juntos possuem cerca de 70% da produção mundial da soja (CONAB, 2017). Na safra 2019/20 obteve a marca de 36.798 mil hectares de área de plantio, crescendo 2.5% em relação à safra anterior, como pode ser visto na Figura 1 (CONAB).

Figura 1 – Estimativas anuais da área de plantação de soja no Brasil.



Fonte: O autor.

Pertencente à família Fabaceae, o grão da soja (*Glycine max* L. Meer.), apresenta aparência arredondada e coloração amarelada (ZAKIR; FREITAS, 2015), a sua semente possui um revestimento externo e dois cotilédones ao seu redor, e através da extração do

gérmen, também denominado hipocótilo, pode ser obtido as substâncias nutritivas e bioativas para produção de alimentos e suplementos (BARBOSA et al., 2006; KIM et al., 2013).

Há muitos anos a soja tem sido consumida pelos asiáticos, utilizando o grão como base para vários pratos, que fazem parte da cultura oriental como o caso do tofu, o tempeh e o leite de soja (WALLY-VALLIM et al., 2014). As sementes da soja estão presentes o ano todo na Coréia, China e Japão, contudo devido ser um alimento funcional, que além de nutrir proporciona efeitos benéficos à saúde, gerou interesse por parte do Ocidente (QUINHONE JÚNIOR; IDA, 2014; ZAKIR; FREITAS, 2015).

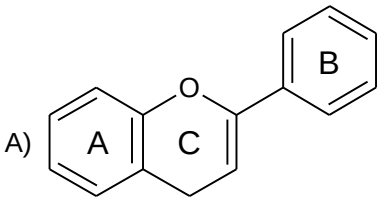
A soja tem recebido cada vez mais atenção de indústrias de alimentos e farmacêuticas, devido seus componentes nutritivos, suas atividades nutracêuticas e seus metabólitos gerados, como as saponinas, triterpenos, ácidos fenólicos, flavonóides, proteínas, ácidos graxos e as isoflavonas (KIM et al., 2020). No entanto, ressalta-se que a quantidade de nutrientes da soja depende do método empregado na criação do grão, sua genética, bem como todo seu processo de colheita, secagem e armazenamento, podendo afetar, tanto características bioquímicas como nutricionais e fração de metabólitos secundários presentes na sua semente (KIM et al., 2013; MESSINA, 2016; ROCHA; CARLOS; CASTRO, 2017).

3.4 ISOFLAVONAS

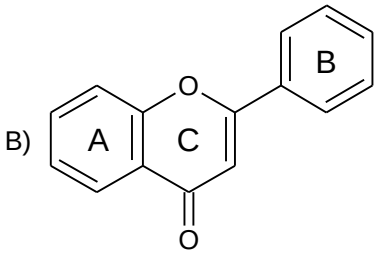
As isoflavonas fazem parte da família dos flavonoides, um grupo de metabólitos devivados de plantas (PEÑALVO, 2004). A síntese dos flavonóides ocorre a partir da fenilalanina, através da via metabólica do ácido chiquímico, com o ácido acético, e com as suas subsequentes reações, há a formação da chalcona, que a partir desta, é produzido diferentes formas de flavonoides (HUBER; RODRIGUEZ-AMAYA, 2008). A estrutura

química base dos flavonoides (Figura 2), apresenta um núcleo das flavonas, sendo este um anel pirano heterocíclico, ou uma pirona (C) entre dois anéis fenólicos (A e B) (HUBER; RODRIGUEZ-AMAYA, 2008). As isoflavonas originam-se via chalconas, assim como os diversos flavonoides elas possuem atividade estrogênica, antifúngica e antibacteriana (SIMÕES et al., 2007).

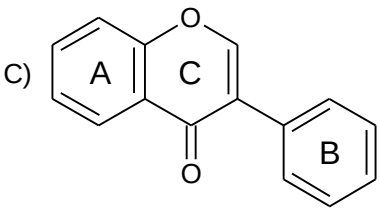
Figura 2 – Estrutura básica dos flavonóides (A), dos flavonóides com grupo carbonila (B) e das isoflavonas (C).



Estrutura básica dos flavonóides



Flavonóide com grupo carbonila



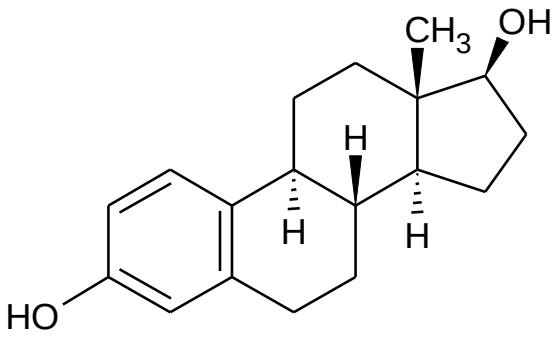
Isoflavona

Fonte: Elaborado pelo autor.

Nas plantas, as isoflavonas atuam como antifúngicos, antioxidantes e no equilíbrio hormonal dos vegetais (BEDANI; ROSSI, 2005). Em estudo realizado por Ma et al. (2018), demostrou que dose adequada de radiação UV-B estimulou o aumento de isoflavonas, porém com um tempo prolongado houve diminuição do teor. A atividade antioxidante das isoflavonas é atribuído a presença do grupo fenólico que atua removendo radicais livres (MEZA; MERCADO; BARRAZA, 2015).

As isoflavonas são compostos denominados fitoestrógenos, devido sua atividade estrogênica, no qual apresenta estrutura química favorável ao 17β-estradiol (Figura 3), e atuação nos receptores estrogênicos ERα e ERβ (DURU et al., 2018).

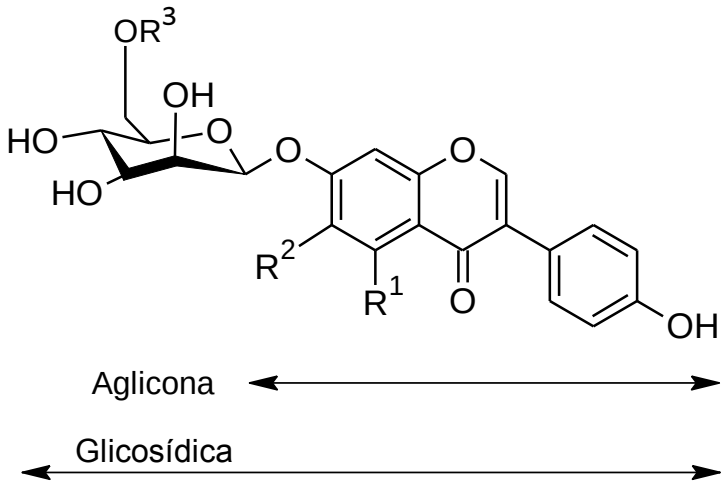
Figura 3 – Estrutura química do 17β-estradiol.



Fonte: Elaborado pelo autor.

As isoflavonas apresentam-se em doze compostos diferentes, divididos em quatro grupos, contendo três tipos cada (Figura 4): Agliconas (2%), daidzeína, genisteína e gliciteína. Glicosídicas (25%), nesta está presente um anel de glicose, daidzina, genistina e glicitina. Acetilglicosídeo (5%), acetildaidzina, acetilgenistina e acetilglicitina. Malonilglicosídeo (70-80%), malonildaidzina, malonilgenistina e malonilglicitina (PEÑALVO, 2004; KIM et al., 2020).

Figura 4 – Estrutura molecular das isoflavonas de soja.



Isoflavona	**R¹**	**R²**	**R³**
Daidzeína	H	H	
Genisteína	OH	H	
Gliciteína	H	OCH3	
Daidzina	H	H	H
Genistina	OH	H	H
Glicitina	H	OCH3	H
Acetildaidzina	H	H	COCH3
Acetilgenistina	OH	H	COCH3
Acetilglicitina	H	OCH3	COCH3
Malonildaidzina	H	H	COCH3COOH
Malonilgenistina	OH	H	COCH3COOH
Malonilglicitina	H	OCH3	COCH3COOH

Fonte: Elaborado pelo autor.

A proporção de isoflavonas nos grãos de soja sofre variação conforme alterações ambientais e genéticas, de forma que, a quantidade presente no eixo embrionário pode variar de 5 a 25 vezes mais que no cotilédone, enquanto que o invólucro da semente é desprovido de isoflavonas (QUINHONE JÚNIOR; IDA, 2014). Os fitoestrógenos mais comumente pesquisados e consumidos são as isoflavonas, sendo a genisteína a isoflavona que possui a maior atividade funcional dentre as isoflavonas (CARBONEL et al., 2011), e ser a isoflavona que possui a maior afinidade pelo receptor ERβ (XIAO et al., 2018).

3.4.1 Absorção e metabolismo das isoflavonas

As isoflavonas agliconas são efetivamente absorvidas, enquanto que as demais formas, são inicialmente hidrolisadas. A sua metabolização ocorre após sua hidrólise, absorção, para então ser difundidas no intestino, em seguida elas se ligam nos quilomícrons, e são transportadas para o sistema linfático e caem no sistema circulatório onde executam sua atividade metabólica ao se ligar no ERα e ERβ. Sua meia-vida varia entre 7 e 8 horas (MONTEIRO et al., 2018a).

Ao ser secretada na bile pelo fígado, uma fração das isoflavonas é eliminada pelas fezes e a outra é canalizada novamente para circulação entero-hepática, após submeter-se a hidrólise bacteriana. A constituição da microbiota intestinal influencia na performance do metabolismo e biodisponibilidade das isoflavonas, assim o intestino saudável, contribui para uma conversão eficiente das formas ativas das isoflavonas (MONTEIRO et al., 2018a).

3.4.2 Isoflavonas e ansiedade e depressão

As isoflavonas da soja são os metabólitos que mais provocam interesse na pesquisa devido ser considerada o principal componente benéfico do grão, e para obter este efeito, o consumo estabelecido é de 20 a 100 mg/dia (SILVA; PRATA; REZENDE, 2013; WALLY-VALLIM et al., 2014; RIZZO; BARONI, 2018). As isoflavonas apresentam efeitos preventivos na osteoporose, doenças cardiovasculares, em alguns cânceres (cólon, mama e próstata), atuam reduzindo sintomas da menopausa (MONTEIRO et al., 2018a), assim como na ansiedade e depressão (LUND; LEPHART, 2001; RODRÍGUEZ-LANDA et al., 2018).

A prevalência de depressão nas mulheres é maior do que nos homens, este dado é relacionado com os hormônios reprodutivos estradiol e progesterona na etiologia da doença

(RANG et al., 2016). As isoflavonas agliconas são as isoflavonas que segundo estudos possuem maior atividade biológica para o tratamento de doenças no período da menopausa (MONTEIRO et al., 2018a).

O consumo regular de isoflavonas, possibilita melhor digestibilidade e biotransformação por parte da microbiota intestinal. As isoflavonas fazem parte da alimentação asiática, fazendo-o consumo em maior quantidade quando comparado com a alimentação ocidental (MONTEIRO et al., 2018b). Foi realizado um estudo transversal por Yu et al. (2015), em uma comunidade rural do nordeste da China com 1.717 residentes com idade mínima de 65 anos, uma população com regular consumo de soja, com frequência média de 4 vezes na semana e obtendo menor chance de vir a ter depressão em comparação com aqueles de consumo médio de 2 a 3 vezes por semana. Em Hong Kong um estudo transversal com 3.999 idosos, observou que a ingestão de isoflavona foi inversamente relacionada ao escore da Escala de Depressão Geriátrica (WOO et al., 2006).

Os hormônios estradiol e progesterona, demonstram interação com o GABA, noradrenalina e serotonina (SASSARINI, 2016). Os efeitos ansiolíticos e antidepressivos das isoflavonas, demonstrou ocorrer através da modulação por seus receptores ERα e ERβ que estão presentes no sistema nervoso central, interagindo com a via serotoninérgica e dopaminérgica (CUI et al., 2020). Os flavonoides também apresentam atuação nos receptores $GABA_A$, antagonizando o receptor (JOHNSTON, 2015; FEDOTOVA et al., 2017). De acordo com o estudo de Hu et al. (2017), o tratamento com genisteína, obteve efeito antidepressivo nos camundongos, aumentando os níveis de serotonina e noradrenalina. Em Cui et al. (2020), foi avaliado a relação do consumo de isoflavonas e sua atividade antidepressiva em homens, demostrando menor prevalência da doença após ingestão diária de isoflavonas, sugerindo atividade através da via serotoninérgica pelos receptores estrogênicos presentes no cérebro.

3.5 KEFIR

A OMS define probióticos como “microrganismos vivos, que quando administrados em quantidades adequadas conferem um benefício à saúde do hospedeiro” (MAHASNEH; MAHASNEH, 2017). Para exercer seus benefícios, os microrganismos precisam ser resistentes ao ambiente gástrico, favorecendo a sobrevivência e colonização intestinal dos microrganismos, e consequentemente inibir o crescimento e fixação de patógenos (BERGMANN et al., 2010; BENGOA et al., 2018a; KRAIMI et al., 2019).

O kefir apresenta uma grande fonte de probióticos (FIORDA et al., 2017). Éle é uma bebida milenar, fermentada, alcoólica com sabor ácido e elaborada através da ação da complexa comunidade microbiana encontrada nos grãos do kefir. Seu nome tem origem turca, no qual a palavra “kef” significa sabor agradável. A bebida possui um tradicional consumo na Europa Ocidental, originando-se do Norte do Cáucaso, e atualmente é encontrada no mundo todo, atraindo atenção devido suas propriedades benéficas à saúde. No Brasil a distribuição do kefir é tradicionalmente praticada através de doações (FRIQUES et al., 2015; TURAN et al., 2015; DIAS et al., 2016; VAN DOAN et al., 2017).

Seus grãos apresentam formas irregulares, consistência gelatinosa, e tamanho variando de 3-3,5 cm de diâmetro (ROSA et al., 2017). Ao se multiplicarem, o kefir também possui a característica de transferir suas propriedades, assim como qualquer outro organismo vivo (YILDIZ-AKGÜL, 2018). A sua qualidade depende da procedência dos grãos, as circustância de armazenamento e seu manuseio, outro fator que dificulta a fabricação industrial é a complexa composição microbiana dos grãos, visto que um kefir de qualidade, depende da obtenção da cultura inicial ótima (YILDIZ-AKGÜL, 2018). Seu cultivo pode ser realizado em açúcar mascavo ou leite, e dependendo do substrato escolhido, a coloração do kefir pode variar, de branco à amarelado em leite e ocre em açúcar mascavo (MOREIRA et al., 2008).

Existem dois tipos de kefir, o de leite e o de água. Cada um deles possui suas características e singularidades no seu preparo. No processo de elaboração os grãos de kefir de leite, possuem como substrato principal a lactose, e em aproximadamente 24 horas ocorre o seu processo fermentativo. Já o kefir de água tem a solução de sacarose como substrato, e ao longo de 2 a 4 dias, em temperatura ambiente, ocorre a fermentação. Ao final, os grãos são separados por peneiramentto, para posterior reutilização onde uma nova biomassa é formada em até 2%, e então é realizado o uso da bebida (LAUREYS; DE VUYST, 2017; ROSA et al., 2017; SOUZA; SILVA, 2017). Assim o resultado da fermentação dos grãos com seu substrato é uma porção sólida rica em microrganismos probióticos e uma porção líquida que está presente os compostos do resultado da atividade microbiana (BRASIL et al., 2018).

O kefir de água apresenta gosto ácido, alcoólico e cor amarelada, enquanto que a de kefir de leite é semelhante ao iogurte, porém mais ácido, este sabor e aroma caracterizante ocorre devido aos compostos gerados pela fermentação, como o ácido lático, etanol e dióxido de carbono (LAUREYS; DE VUYST, 2017; ROSA et al., 2017; SOUZA; SILVA, 2017). A fermentação do kefir ocorre em um recipiente vedado, impossibilitando a entrada de oxigênio, e liberando o dióxido de carbono, deste modo, o processo tem início aeróbico e gradativamente se torna anaeróbico. O oxigênio influência no crescimento, metabolismo e na variedade das espécies microbianas, isto ocorre por este gás ser consumido pelas leveduras e elas produzirem dióxido de carbono (LAUREYS et al., 2018).

Uma alta concentração de nutrientes reflete em uma fermentação mais rápida, altas concentrações de metabólitos, sem reduzir as concentrações totais de carboidratos residuais e valores de pH, diferentemente da baixa concentração de nutrientes. O reflexo disso também atinge o crescimento dos grãos, visto que concentrações insuficientes de nutrientes resultam em uma diminuição lenta e gradual do crescimento de grãos de kefir na água, porém, o excesso de nutrientes, não aumenta o crescimento. Damascos secos,

passas secas e figos secos são as principais fontes de nutrientes utilizadas durante a fermentação (LAUREYS et al., 2018).

A fermentação do kefir com seu substrato, resulta como produtos de conversão, os exopolissacarídeos de dextrano (EPS), etanol, dióxido de carbono, ácido lático, glicerol, ácido acético, manitol e uma variedade de compostos aromáticos (LAUREYS et al., 2018). Determinadas espécies de bactérias ácido lácticas (LABs), como o gênero Lactobacillus, possuem a habilidade de produzir EPS, este produto pode ser isolado de alimentos fermentados, para melhorar a propriedade funcional de alimentos, como a capacidade de proteção das células, ingressando à sua superfície e protegendo contra metais tóxicos, dessecação, ataque de fagos e ainda contribuindo no acolhimento de fatores imunológicos, proteção no trato gastrointestinal, ele também auxilia na agregação bacteriana, através da produção de biofilme, contribuindo com a instalação do microrganismo no intestino e sua consequente interação com a microbiota intestinal (BENGOA et al., 2018b; CHEIRSILP et al., 2018).

Estudos de Fels et al. (2018), demonstrou que os EPS bacterianos estão em grande quantidade no kefir de água, na bebida contém grande quantidade de dextranos e levanos, enquanto que nos grãos apresenta elevada quantidade de glicose ligada na sua estrutura.

3.5.1 Composição

Os grãos apresentam uma composição nutricional de 2,7% de proteína, 0,6% de ácido láctico e menos de 10% de gordura, é fonte de vitaminas, minerais e aminoácidos, além de também apresentar uma complexa constituição microbiana, como lactobacilos, estreptococos lácticos, leuconostocs, leveduras e bactérias de ácido acético (SHARIFI et al., 2017; YILDIZ-AKGÜL, 2018).

As principais bactérias encontradas no kefir de leite são *Lactobacillus paracasei, Lactobacillus kefiri, Lactobacillus parabuchneri* e *Acetobacter lovaniensis*, e de leveduras são *Saccharomyces cerevisiae* e *Kluyveromyces lactis* (SHARIFI et al., 2017), já no kefir de água, os mais eminentes microrganismos são as espécies de bactérias do ácido láctico *Lactobacillus hilgardii, Lactobacillus nagelii* e *Lactobacillus paracasei* e a levedura *Saccharomyces cerevisiae* (LAUREYS et al., 2018). Dependendo da sua procedência e técnica de cultura, a constituição microbiológica dos grãos pode divergir, pois nem sempre um microrganismo presente em uma cultura vai está presente em outra (MOREIRA et al., 2008).

O complexo simbiótico das leveduras e bactérias ácido-láticas e ácido-acéticas, são essenciais para o desenvolvimento dos grãos de kefir, nos quais estão encapsulados por uma matriz contendo proteínas e polissacarídeos, denominada querifano (WESCHENFELDER et al., 2011; PRADO et al., 2015; SOUZA; SILVA, 2017).

O querifano é o polissacarídeo fundamental do kefir, é gerado principalmente pelo *Lactobacillus kefiranofaciens*, uma das suas propriedades mais significantes é o aumento de viscosidade e viscolasticidade de géis de leite ácido, além de aumentar a propriedade reológica (CARVALHO, 2017; SHARIFI et al., 2017).

Pesquisas sobre o kerifano, atestam benefícios à saúde, como diminuição do estresse, através da formação de β-interferon, cortisol e noradrenalina, atividade antimicrobiana, antitumoral, regulação da microbiota intestinal e proteção de patógenos (MOREIRA et al., 2008). A fermentação que ocorre na sua formação, tem como consequência produção de traços sensorial singulares (WESCHENFELDER et al., 2011).

3.5.2 Kefir no controle da ansiedade e depressão

Os microrganismos presentes no TGI, apresentam intensa relação com a imunidade e homeostase (KIM et al., 2018). Os probióticos possuem capacidade de operar no TGI, modificando pH, fornecendo nutrientes, renovando as células intestinais, além de atuar através da regulação da resposta imune do hospedeiro (VAN DOAN et al., 2017; KRAIMI et al., 2019).

A utilização frequente de kefir proporciona benefícios à saúde, ele estimula o sistema imune, possui atividade antimicrobiana contra patógenos e melhora do trânsito intestinal (DIAS et al., 2016). Os microrganismos presentes no kefir competem com os microrganismos patógenos na mucosa intestinal, inibindo-os através da produção de ácidos, bacteriocinas e peróxido de hidrogênio, que este através da sua elevada atividade oxidante atua destruindo o arranjo molecular das proteínas celulares através da peroxidação das membranas lipídicas (LEITE et al., 2013; SANTOS et al., 2013).

No Irã, Fathi et al. (2017), observou mulheres com idades de entre 25 e 45 anos, quanto a relação aos níveis de colesterol total, triglicerídeos, LDL e HDL e consumo de kefir de leite, ao final do estudo demonstrou que o consumo regular de kefir melhorou o perfil lipídico em mulheres com sobrepeso ou obesas, o que supôs que os microrganismos do kefir no intestino poderiam produzir ácidos graxos de cadeia curta e hidrolases de sais biliares.

Foi isolado do kefir de água, o *Lactobacillus mali* APS1 buscando avaliar estenose hepática em ratos (CHEN et al., 2018). Os animais foram divididos em 6 grupos, dieta controlada, dieta gordurosa e tratado com solução salina e com dieta gordurosa tratando com APS1 com gavagem diária por 12 semanas. APS1 obteve como resultado, redução do peso e aumento da atividade antioxidante.

Jeong et al. (2017), desenvolveu uma pesquisa com 16 camundongos divididos em dois grupos o primeiro fazia uso de solução salina e o segundo de solução salina com 2×108 ufc *Lactobacillus kefiranofaciens* por duas semanas, ao final do estudo, demonstrou relação do consumo do *L. kefiranofaciens*, presente no kefir, e a melhora do quadro de constipação em camundongos devido a diminuição da proporção de membros do gênero Clostridium. Assim como também Turan et al. (2015), avaliou a prevenção da constipação em 20 pacientes, divididos entre intestino lento e intestino normal, todos receberam 500 mL da bebida por 4 semanas, e ao final, a utilização do kefir aumentou as evacuações, e diminuiu a necessidade de utilizar laxantes.

Além de diversos estudos que discutem a relação do consumo de probióticos com melhora dos transtornos depressivos e de ansiedade, como um estudo sobre depressão com ratos, constatou que o tratamento com *Bifidobacterium infantis*, houve aumento considerável de triptofano, um precursor serotoninérgico, no plasma sanguíneo, além de contribuir com a diminuição dos marcadores inflamatórios (NADEEM et al., 2019). Em estudo com camundongos livres de germes (GF) sem microbiota intestinal, ao ser exposto ao estresse, a hiperatividade do eixo HPA pôde ser alterada positivamente pela reconstituição com *Bifidobacterium infantis* (WEI et al., 2019).

Em um ensaios clínicos randomizado e controlados com placebo, duplo-cegos e triplo-cegos, realizado com idosos saudáveis, recebendo *Lactobacillus casei* (BENTON; WILLIAMS; BROWN, 2007), estudantes recebendo probióticos multi-espécies (STEENBERGEN et al., 2015), e com adultos, recebendo uma combinação de *Lactobacillus helveticus* R0052 e *Bifidobacterium longum* R0175 (MESSAOUDI et al., 2011), esses estudos obtiveram como resultados melhora de humor no primeiro e segundo ensaios, já no último houve alívio do sentimento depressivo e redução da produção de cortisol.

Além disso, um estudo de Noori et al. (2014) realizado com ratos, buscando analisar atividade do kefir na ansiedade e depressão ocasionada por cessação da nicotina, realizado

com grupos controle negativo e positivo com sertralina, kefir fermentado em leite de gado e kefir fermentado em leite de soja, mostrou que administração de ambas fermentações de kefir obtiveram satisfatórios efeitos antidepressivos e ansiolíticos em ratos, inclusive melhorando aprendizado e memória destes animais.

4 ARTIGO 1

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Study of anxiolytic and antidepressive action of kefir associated with soybean germ in swiss mice

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Abstract

The intestinal microbiota presents thousands of microorganisms that directly influence the bidirectional communication of the brain-intestine axis. Recent research shows that the use of probiotics positively influences the quality of intestinal microorganisms, and the consumption of foods with isoflavones, play a significant role in the mental health of the individual. In this study, the isoflavones present in the fermentation solution of kefir (FSK) and kefir associated with soybean germ (KSG) were quantified, and the effects of this association on anxiety and depression induced by pentylenetetrazole (PTZ) in Swiss mice were evaluated. The daidzein, glycitein and genistein isoflavones analyzed by HPLC-UV, presented a concentration of 9.44, 26.50 and 0.78 µg/ml in the FSK, 6.96, 20.58 and 0.60 µg/ml in KSG on the 1st day of fermentation, 8.57, 28.94 and 0.71 µg/ml, 2nd day of fermentation, 10.07, 31.14 and 0.77 µg/ml, 3rd day of fermentation and 11.74, 40.30 and 0.81 µg/ml on the 4th day of fermentation. KSG administration showed improvement in experimental conditions, demonstrating anxiolytic and antidepressant effect and increased locomotor activity. In this study, improving the content of aglycone isoflavones dependent

on kefir fermentation time was shown, and the results of the various behavioral experiments in mice suggest that treatment for 30 days with KSG presents anxiolytic and antidepressant effect in the experimental conditions used, accompanied by increased locomotor activity.

Keywords: Brain-gut axis. Kefir. Isoflavones. Depression. Anxiety. Behavioral test. Validation. Quantification.

1 Introduction

Anxiety and depression are neuropsychiatric diseases that have a high prevalence, causing changes in behavioral, physical, and physiological responses in the individual [1]. Despite presenting different mechanisms and causes, about 90% of depressed patients have anxiety [1,2]. According to WHO data, about 300 million people are affected by depression, affecting women twice as many [2–4].

Studies conducted in recent years have shown that the intestinal microbiota plays an essential role in the manifestation of pathologies, such as anxiety and depression [5,6]. The brain-intestine axis is a bidirectional link between brain function and host gut by placing the microbiota in the center as the main highlight [7]. It has multiple bonds that connect through the neural, endocrine, and immunological pathways and contribute to the balance of the body [8,9].

The intake of probiotics and adequate foods show, an essential role in the organization of the composition of the intestinal microbiota, these foods and beverages contribute to neuroendocrine molecules and precursors that affect both host physiology, viability and microbial function [10–12].

According to the definition of WHO, probiotics are living microorganisms that, when administered in appropriate quantities, confer benefits to host health [13]. Kefir is a probiotic with a high nutritional value [14]. Its grains are developed by a symbiotic complex of yeasts with acid-lactic and acid-acetic bacteria, fundamental for kefir fermentation. These microorganisms are encapsulated by a protein matrix and polysaccharides, called kefiran [15–17].

The frequent use of kefir provides health benefits because it stimulates the immune system, has antimicrobial activity against pathogens, and improvement of intestinal transit [18]. The microorganisms present in kefir compete with pathogen microorganisms in the intestinal mucosa, inhibiting them through the production of acids, bacteriocins, and hydrogen peroxide, which through their high oxidizing activity acts by destroying the cellular arrangement proteins through the peroxidation of lipid membranes [19,20].

Soybean grain (*Glycine max* L. Meer.) has a high protein content, higher than any other vegetable, saccharides, water-soluble lipids, as well as has a significant source of minerals, vitamins, and various nutritional and functional components, however, isoflavones are mainly responsible for their beneficial health effects [21,22]. Isoflavones belong to the phytoestrogen class, their structure and performance compare with 17β-estradiol, and due to this similarity, isoflavones can act in estrogenic receptors, mainly β (ER β), presenting affinity of 1/3 times higher than in α-type receptors (ER α) [23,24].

Studies report that isoflavones have preventive effects on some diseases, as occurs in osteoporosis, cardiovascular diseases, in some cancers (colon, breast, and prostate), and also act by decreasing menopausal symptoms [25] and anxiety and depression [26,27].

Aglycone isoflavones are the chemical form that has the main health benefits, besides being absorbed more easily by the body among the other types of isoflavones [28]. β-glucosidase, enzymes that are present in human cells and microorganisms of the intestinal microbiota [29], are an enzyme responsible for catalyzing the hydrolysis of glycosidic isoflavones in aglycones [28,30].

Recent studies have shown that the development of soy-based beverages and foods fermented with probiotics increases the bioavailability of aglycone isoflavones as fermentation days go through fermentation days [29]. Thus, the glycosidic isoflavones present in the kefir associated with soybean germ were quantified, and the anxiolytic and antidepressant activities of kefir associated with soybean germ in Swiss mice were evaluated.

2 Material and methods

2.1 Soybean germ

Herborisa Industrial Co., located in the city of Londrina, Paraná, Brazil, supplied the soybean germ. It was obtained from non-transgenic soybeans, and according to the analysis, the report described the presence of proteins, fats, fibers, minerals, and isoflavone.

2.2 Obtaining kefir and kefir associated with soybean germ (KSG)

Kefir grains were obtained from the standard collection stored in the Drugs Research Laboratory, Department of Biological Sciences and Health, Federal University of Amapá, Amapá, Brazil.

For the means of kefir and KSG crops, 40 g/L concentrations of brown sugar, 10 g/L of soybean germ, and 60 g/L of kefir were used, fermented at a temperature of 25°C according to the method described by Oliveira[31]. The cultivation medium containing distilled water and brown sugar was homogenized in an Erlenmeyer and taken to autoclave (Prismatec, Melissa, São Paulo, Brazil) at a temperature of 200°C for a time of 15 minutes. After cooling, at room temperature, the kefir grains were added. In KSG, the pulverized soybean germ has been added. The glasswork was sealed and kept at room temperature in a dry place under the light. On the 4th day of fermentation, the grains and germ were sifted and filtered with filter paper (Whatman®) with micropores 8 µm in diameter and discarded. The fermentative solution of kefir and KSG obtained were used for analyses and animal experimentation.

2.3 Physicochemical analysis of kefir and kefir fermentative solution associated with soybean germ (KSG)

2.3.1 Determination of pH of fermentation solution of kefir (FSK) and kefir associated with soybean germ (KSG)

The pH of the fermentation solution of kefir was evaluated in a digital pHmetro (Edutec, Curitiba, Brazil) on the 1st, 2nd, 3rd, and 4th day of fermentation.

2.3.2 Chromatographic analysis of fermentation solution of kefir (FSK) and kefir associated with soybean germ (KSG)

The extraction of isoflavones present in the KSG was carried out according to the method described by Carrão-Panizzi; Favoni; Kikuchi[32]. Thus, 100 ml of each sample of the fermentation solution of kefir and KSG and filtered in filter paper (Whatman®) with micropores of 8 µm in diameter were removed. The aglycone isoflavones were extracted from the soybean germ following the method described by Lante *et al.*[33], modified. 4 g of soybean germ was crushed and added 100 ml of ultrapure water and submitted to ultrasound (Cristófoli, Campo Mourão, Paraná, Brazil) for 380 seconds. Both solutions were then taken for centrifuge (Nova Técnica, Piracicaba, São Paulo, Brazil) for 10 min at 4000 rpm at 25°C and, the supernatant was filtered through PVDF membrane (Millex® HV Durapore®, Carrighwohill, Co, Cork, Ireland) of 0.45 µm Pore.

The analytical patterns used of isoflavones were daidzein 98%, glycitein 98%, and 98% genistein (Sigma-Aldrich®, St. Louis, MO, USA). The patterns were dissolved in methanol: water solution (80:20 v/v). For chromatographic analysis, HPLC grade methanol reagents (Sigma-Aldrich), HPLC grade acetic acid (Sigma-Aldrich), and HPLC grade acetonitrile (Sigma-Aldrich) were used.

The chromatographic conditions used for separation and quantification of aglycone isoflavones were according to the modified Sun *et al.*[34] method. High-efficiency liquid chromatography (Shimadzu®, Kioto, Japan) was used, with a system equipped with a solvent pumping unit (LC-20AT), Proeminence automatic injector (SIL-20AC), column oven (CTO-10AS/VP). The detection was performed using a UV-VIS detector (SPC-20A), interconnected by a system controller (CBM-20A), and the data were processed using LC-solution software. For the separation of the analytes, the Kinetex C18 column (250 × 4.6 mm) (Phenomenex Inc. was used, CA, USA) with particle size of 5 µm and precolumn C18 2.6 µm, and aliquots of 15 µL were injected into flow of 0.75 mL/min, temperature of 30 °C and wavelength 260 nm, using solvent A: 0.1% acetic acid in water and solvent B: acetonitrile. The linear gradient was started with 15% of solvent B up to the 10-minute time, following to 35% in the time of 25 minutes and to 45% up to 45 minutes, then 65% until 50 minutes and 95% until 65 minutes, decreasing to 80% in 80 minutes and finishing 50% in 65:01 minutes. Calibration was performed with the standards of daidzein, glycitein, and genistein.

2.4 Validation of the method of analysis by HPLC-UV

The method used was validated by the guidelines established by the International Conference on Harmonization [35,36]. The parameters used to validate the technique developed were: Selectivity, linearity, accuracy (intraday, interday), accuracy, sensitivity (detection limit, quantification limit), and robustness.

2.4.1 Selectivity

In the evaluation and determination of selectivity, standard solutions of genistein, glycitein, and daidzein were prepared in methanol: water (80:20 v/v), obtaining the concentration of 333 µg/mL for genistein and 250 µg/mL for glycitein and daidzein. Using the chromatographic method described above, the chromatograms of the patterns, soybean germ sample, the sample of fortified soybean germ with the patterns, and the Sample of the

KSG fortified with the patterns were obtained. The retention time of isoflavone patterns was then compared to the peaks resulting from chromatograms obtained from soybean germ and kefir samples associated with soybean germ.

2.4.2 Linearity

Linearity was evaluated in triplicate with five concentrations (5, 10, 25, 50, and 75 µg/mL) of the standard solution daidzein, glycitein, and genistein. Obtain the calibration curve; it was related to the peak area generated in the chromatogram with the respective concentration of each pattern. From the linear regression method, in which the equation of the straight ((a) was obtained corresponding to the angular coefficient and (b) to the linear coefficient) and the calculation of the linear correlation coefficient (r), with the minimum acceptable parameter of r = 0.990, the statistical analysis of the data was performed.

2.4.3 Detection limits and Quantification Limit

The detection (DL) and quantification (QL) limits of the method were evaluated according to the following equations:

$$DL = \frac{3.3 \times \sigma}{S} \qquad QL = \frac{10 \times \sigma}{S}$$

σ= Standard deviation of the linear coefficient of the equation.
S = Slope or the angular coefficient of the analytical curve

2.4.4 Accuracy (intraday, interday)

In the definition of the accuracy of the method, the triplet analysis of the standard solution of the daidzein, glycitein, and genistein isoflavones was performed at the high, medium and low concentrations present in the calibration curve (5, 25 and 75 µg/mL). The repeatability of the method was achieved by repeating the same procedure three times on the same day for intra-day accuracy. The intermediate efficiency (interday) of the process was analyzed by performing the same method on different days under the same experimental conditions. The results were statistically evaluated in terms of the percentage of the relative standard deviation.

2.4.5 Accuracy

Accuracy was determined by the addition of the standard reference solution of daidzein, glycitein, and genistein isoflavones in the fermentation solution of kefir and KSG samples. Recovery was performed in three different concentrations (5, 25, and 75 µg/mL) present in the linearity range of the method, and each concentration was added, the standard solution at 80%, 100%, and 120%, injected into triplicate. The result of the recovery calculation was expressed as a percentage.

2.4.6 Robustness

Robustness was evaluated under the effect of small, deliberate variations in chromatographic conditions. Among them the flow rate (changed at ± 0.2 mL/min), the column temperature (adjusted at ± 2 °C) and the pH of the sample (changed at ± 0.1). The analyses were performed in triplicate, and the concentrations of the isoflavones were evaluated.

2.5 Evaluation of anxiolytic and antidepressant activities

2.5.1 Animals

Male mice from the Swiss lineage, three months old, acquired from CEMIB UNICAMP - São Paulo, were used. The animals were housed separately in plastic cages (41.4 x 34.4 x 17.8 cm), under standard temperature of 21 ± 2 °C with light/dark cycle of 12h, lights on 7:30 a.m. and off at 6:30 p.m. and with access to food and water until two hours before the experiments, and were adapted to laboratory conditions for two weeks before the start of the tests.

The experimental protocols were by the legislation for animal experimentation established by the National Council for Animal Experimentation of Brazil (CONCEA). The project was approved by the Ethics Committee on the Use of Animals (CEUA) of the Federal University of Amapá-UNIFAP under protocol number 007/2019.

2.5.2 Design experimental

The mice were randomized and divided into seven groups: Group I, II and III received 200, 400 and 600 mg/kg of KSG orally, respectively, Group IV, was administered with FSK (dose 10 µl/kg), Group V, as positive control, was treated with diazepam (DZP - dose of 2 mg/kg i.p.), Group VI, as negative control, received saline solution (dose 10 µl/kg), Group VII, normal, only water.

Groups G1, G2, G3, and G4 were treated for 30 days, from the 31st to the 34th day, standard behavioral methods were performed such as RotaRod, Open Field Test, High Cross Labyrinth, and Forced Swimming Test, to analyze the level of anxiety and depression of all groups. On the 35th to 39th day, three doses of PTZ (dose of 20 mg/kg, i.p.), were injected with 48-hour intervals, with the continuous treatment of KSG, FSK, DZP and saline solution in its corresponding groups. On the 40th day to the 43rd day, all groups were submitted again to behavioral tests (Figure 1).

2.5.2.1 Rotarod Test

This assay was based on the method described by Oliveira *et al.*[37], which consisted of positioning the animals on a platform with a rotational axis, divided by circular plates into four compartments (Rotarod, Hugo Basile, Italy). In the time of 24 hours before the experiment, the animals were selected, rejecting those who could not remain 300 seconds on the rotarod at 32 rpm, without falling in 5 attempts. Soon after a 1-hour interval of treatment, the animals were taken to the appliance and evaluated for the number of falls and the length of stay in the revolving bar. The experiment was recorded using a video camera (Nikon D5100). The maximum number of falls allowed was three, and, after the third, the animal was no longer redirected to the equipment. The maximum length of stay allowed on the rotarod was 1 minute.

2.5.2.2 Open field test

The method described by Carola *et al.* [38], modified was used. The animals were placed 1 hour after treatment in the open field, which is an environment consisting of a box (100 cm x 100 cm x 50 cm) coated with white color and illuminated with a fluorescent lamp 50 cm above the center box. The animals were analyzed for 5 minutes and filmed with a video camera (Nikon D5100). To minimize interference, the box was cleaned with a 70%

hydroethanolic solution after the completion of each animal. The parameters evaluated were: number of central and peripheral quadrants traveled and the length of stay in the central and peripheral quadrants.

2.5.2.3 Elevated plus maze for mice test

This assay occurred as described by Pellow *et al.*[39], the animals were placed in an apparatus composed of four crossed arms, forming a cross, 50 cm long and 10 cm wide, two of them open and two closed with side walls 40 cm high, and the entire structure was raised to a height of 45 cm above the ground. Individually each animal was placed in the central square of the cross maze, facing the open arm. At the same time, its behavior was observed and recorded with a video camera (Nikon D5100) for 5 min. Soon after each attempt, the labyrinth was thoroughly cleaned to erase any trace of previous animals that could interfere with the exploitation by the tested animal. The length of stay and the number of entrances in the open and closed arms were evaluated; the result was expressed as a percentage.

2.5.2.4 Forced swimming test

In this experiment, the procedure proposed by Porsolt; Le Pichon; Jalfre[40] was used. The test took place in a cylindrical glass aquarium 40 cm high and 18 cm in diameter, containing 15 cm of water at 25 °C. The technique consisted of two sections of swimming, the first section of events, the mice were immense in the aquarium individually for 15 minutes (pre-test). After 24 hours, the test was performed, the animals were placed on swimming for 5 minutes, during which they had their behavior recorded in video camera (Nikon D5100), for further analysis. The responses performed were analyzed, including swimming, climbing, and immobility.

2.4 Statistical analysis

The analytical curves were constructed through linear regression analysis. The statistical analysis of the results obtained was used the GraphPad Prism Software version 5.03. The results of the behavioral evaluation trials were expressed as mean ± SEM. Data analysis was performed by variance analysis (ANOVA) followed by the Turkey test. The results with $p < 0.05$ were considered statistically significant.

3 Results

3.1 Validation

3.1.1 Selectivity

The selectivity of the chromatographic analytical method is shown in Figure 2. The isoflavone daidzein, glycitein, and genistein presented the retention time of 27.04 min., 27.95 min. and 32.52 min.. The reference standards were: (A), 27.03 min, 27.89 min, and 32.36 min., for FSK (B), and 27.12 min, 27.96 min, and 32.45 min., for KSG (C).

3.1.2 Linearity

Figure 3 shows the analytical curves of the isoflavones daidzein, glycitein, and genistein (A, B, and C). Linear regression presented correlation coefficients of 0.9952, 0.9941 and 0.9922, and equation of the straight y=77977x-188993, y=9715.9x+11280 and y=139097x+55020, respectively.

3.1.3 Detection and quantification limit

From the data obtained from the analytical curve, the detection limit and the quantification limit of 0.0019 and 0.0058 µg/mL for daidzein, 0.1324, and 0.4012 µg/mL for glycitein and 0.0271 and 0.0822 µg/mL for genistein were calculated, respectively.

3.1.4 Accuracy and robustness

The calculated values of the standard deviation in the evaluation of intraday and interday accuracy were below 2%. For efficiency, the mean percentage of recovery was 99.41 to 100.10% for daidzein, 98.81 to 100.20 for glycitein, and 99.15 to 100.30% for genistein, being within the limits of 100 ± 2%. The method was robust with variations in column temperature, mobile phase flow, and sample pH.

3.2 Application of the validated method in the analysis of the content of isoflavones present in the FSK and KSG

According to the previous results, determined from the validation of the method, the daidzein, glycitein and genistein isoflavones analyzed by HPLC-UV, presented a concentration of 9.44, 26.50 and 0.78 µg/ml in the FSK, 6.96, 20.58 and 0.60 µg/ml in KSG on the 1st day of fermentation, 8.57, 28.94 and 0.71 µg/ml, 2nd day of fermentation, 10.07, 31.14 and 0.77 µg/ml, 3rd day of fermentation and 11.74, 40.30 and 0.81 µg/ml on the 4th day of fermentation. The mean pH was 3.6 on the 1st day of fermentation, 3.2 on the 2nd day of fermentation, 3.07 on the 3rd day of fermentation, and 2.9 on the 4th day of fermentation (Figure 4B).

3.3 Behavioral Analysis

3.3.1 Rotarod

In the first phase of the Rotarod test, animals treated with different doses of KSG remained longer in the appliance compared to the other groups (Figure 5A, $p < 0.01$). In the second phase (Figure 5B, $p < 0.001$), after the administration of pentylenetetrazole, treatment with 400 and 600 mg/kg KSG, did not alter the motor activity of the animals. On the other hand, treatment with kefir showed improvement in locomotor activity in the first phase when compared to the diazepam group and control, however, reduced in the second phase, with a result close to the group treated with saline solution.

3.3.4 Open field test

In the first phase of the open field test, the groups treated with different doses of KSG went through a number of central quadrants near the control group and saline solution (Figure 6A, $p < 0.001$). The group treated with Diazepam traveled a higher number of central and peripheral quadrants (Figure 6B, $p < 0.01$), however, the group treated with 400 mg/kg remained longer in the center of the open field when compared to the other groups (Figure 6C, $p < 0.001$). The group treated with FSK traveled a smaller number of central and peripheral quadrants, remaining longer in outer quadrants (Figure 6D, $p < 0.01$).

In the second phase of the open field test, the groups treated with different doses of KSG, went through more central quadrants, compared to groups treated with FSK, saline

solution and control (Figure 7A, $p < 0.05$). The results also showed that treatment with different doses of KSG and FSK decreased peripheral locomotion compared to the group treated with saline solution (Figure 7B, $p < 0.001$). Although the Diazepam group had traveled more central and peripheral quadrants, the length of stay in the center of the field was approximated to the groups treated with KSG (Figure 7C, $p > 0.05$). The group treated with FSK remained longer in the peripheral area (Figure 7D, $p < 0.05$).

3.3.3 Elevated plus maze for mice test

It is observed that in the results of the first phase of the experiment, treatment with 200 mg/kg of KSG increased the length of stay in the open arms compared to the other groups (Figure 8A, $p < 0.001$), as well as the treatment with diazepam, increased the entrances into the open arms (Figure 8B, $p < 0.01$) and reduced the entries into the closed arms (Figure 8C, $p < 0.01$). Treatment with 400 and 600 mg/kg of KSG presented length of stay and numbers of inputs in the approximate open arms, compared to the results of the saline solution and control. The group treated with FSK increased the time significantly in the closed arms (Figure 8D, $p < 0.001$).

In the analysis of the results of the second phase, the animals treated with KSG remained longer in the open arms (Figure 9A). Regarding the entrances into the open arms (Figure 9B) and closed (Figure 9C), the animals treated with KSG presented a percentage similar to the group treated with Diazepam. Treatment with FSK presented a shorter time in the closed arms, only compared to the group treated with saline solution (Figure 9D). However, the data showed no significant difference.

3.3.2 Forced swimming test

In the first phase of the forced swimming test, the animals treated with KSG and FSK had shorter immobility time (Figure 10A, $p < 0.01$) and longer climbing time (Figure 10B, $p < 0.05$), when compared to the control groups.

In the second phase of the experiment, after treatment with pentylenetetrazole, the animals treated with 400 and 600 mg/kg with KSG, remained less immobile time compared to the control groups (Figure 11A, $p < 0.05$) and with longer climbing time (Figure 11B, $p < 0.01$). Treatment with diazepam increased animal swimming time (Figure 11C).

4 Discussion

The validation of an analytical method should ensure that it meets the requirements of analytical applications, ensuring the reliability of the results and their credibility during routine use, and validation studies must be led so that the variation of the concentration range and the types of samples are adequate [41,42]. Thus, the validation of this method provides the declaration of its competence for the identification and quantification of isoflavones daidzein, genistein, and glycitein, offering safety and analytical reliability.

The selectivity of the method presents the ability to identify the analysis substance because of other compounds present in the sample[41,43]. In the present study, selectivity was evaluated by comparing the retention time of aglycone isoflavones. According to the chromatograms obtained from the reference patterns, FSK and KSG, demonstrated the selectivity of the method, showing that there is no significant interference in the presence of other compounds or impurities, using the wavelength of 260 nm.

Linearity is the ability of the analytical methodology to demonstrate that the results obtained are directly proportional to the concentration of the analyte in the sample, within a specified interval, which should contain the concentrations expected for the analysis of Samples [44]. The results obtained by the elaboration of analytical curves presented a correlation coefficient above 0.990, which is the minimum acceptable criterion, ensuring linearity to the method [42]. From the analytical curve, the detection and quantification limits were calculated, in which they are parameters that provide that the proposed approach presents security to detect and quantify a large amount of analyte [45].

Precision is a parameter that assesses the agreement of the results obtained through a series of analyses of the same sample [46]. Accuracy, in turn, refers to the degree of approval of the results obtained by the method understudy to the real value [45]. Robustness refers to the ability of an analytical approach to resist small and deliberate variations in analytical parameters [47]. From the results obtained for these parameters, they ensure that the method is accurate and robust.

In both soybean and non-fermented soy-based foods, glycosidic isoflavones are the main isoflavones present, being biologically inactive [28]. In the study of Rekha, Vijayalakshmi[48], glycosidic isoflavones were present in greater quantity in unfermented soy milk, with only 10% aglycones. The concentration of aglycone isoflavones present in the fermentation process of KSG increased significantly compared to the amount present in the FSK, besides presenting a growing increasingly dependent on fermentation time. This result demonstrates that microorganisms present in kefir culture produced β-glucosidase,

hydrolyzing glycosidic isoflavones, and resulting in increased availability of aglycone isoflavones with fermentation time in the culture medium [28,49]. This result is of considerable significance since this form is rapidly absorbed, and in greater quantity by the human intestine, besides providing improvement in the physiological functions of the organism [29,50].

Similar results showed that the fermentation process of different microorganisms influenced the final content of aglycone isoflavones in fermented beverages, as in the study conducted by Baú; Garcia; Ida[28], in which it showed that kefir crop presented high hydrolysis efficiency of glycosidic isoflavones in aglycones in soy milk, presenting bioconversion of 100% glycitin and daidzein into glycitein and daidzein and 89% genistin into genistein. In the study by Marazza *et al.*[51], fermentation with Lactobacillus rhamnosus in soy milk influenced the gradual increase in the content of the genistein and daidzein isoflavone, occurring total bioconversion after 12 h of incubation.

In the study by Delgado *et al.*[29], commercial soybean beverages fermented with lactobacilli and bifidobacteria strains were used for the study of growth and release of aglycone isoflavones, presenting bioconversion of glycosidic isoflavones in aglycones in 24 and 48 hours of incubation.

The fermentation process of microorganisms with soy-based foods causes the production of secondary metabolites and enzymes, which provide better performance of antioxidant activity, preparation of organic acids, proteins, hydrolyzed acids, and isoflavones aglycones, making it an essential process for the food industry, however, specific parameters such as temperature, pH, moisture content, aeration and microorganisms present in the culture medium, are directly linked with the speed and quality of conversion of isoflavones [48,50].

In the present study, from the fermentation time, a reduction in the pH of the culture medium was observed, this occurs as a result of the production of dairy and acetic acids, as well as the decrease in bacterial growth and reduced glycolic flow, which causes modification in cellular viability [28,29].

The function of the gut-brain axis has as main emphasis the intestinal microbiota; its dysfunction contributes to the onset of diseases, such as anxiety and depression [52]. Studies have shown that the use of probiotics [53,54] and isoflavones [27,55] produced anxiolytic and antidepressant activities.

The microbiota can be affected by stress due to the lifestyle, feeding, and vices of the host, thus reaching the hypothalamus-pituitary-adrenal axis, the hypothalamus is activated by releasing the corticotropin hormone. As a result, the pituitary gland produces the adrenocorticotrophic hormone, and then the adrenal glands produce cortisol.

Intestinal dysbiosis influences increased intestinal permeability, with the consequent release of endotoxins, and improve immune response leading to increased concentration of pro-inflammatory cytokines, activate the metabolic pathways of the tryptophan-kynurenine, and contribute to decreased serotonin production and increased tryptophan catabolite, affecting the activation of the hypothalamus-pituitary-adrenal axis, since both glutamate and serotonin interfere with CRH release in the hypothalamus by harming the host with symptoms of anxiety and depression [4,56,57].

Several pathways of neurotransmission are part of the mediation of anxiety, among them, the gabaergic and serotoninergic systems as well as dopaminergic, neuropeptidícs, in addition to activation of the hypothalamus-pituitary-adrenal axis [58,59]. GABA (gamma-aminobutyric acid) is an inhibitory neurotransmitter of the CNS, which has three receptors. However, $GABA_A$ is the one that is present in greater quantity, through it the main anxiolytic drugs, including barbiturates, z compound, and Benzodiazepines [60,61].

In sub convulsive doses, pentylenetetrazole is a drug that produces an anxiogenic effect on animals [62], it acts as an antagonist of the $GABA_A$ receptor, supplying the action of the inhibitory synapse, by blocking the inflow of Cl^-, causing neuron depolarization, which promotes the intensification of neuronal activity [63,64]. Several studies used pentylenetetrazole as a tool in behavioral experiments to evaluate the anxiolytic activity of new drugs [65,66]. The use of benzodiazepines, such as diazepam, blocks the effect of pentylenetetrazole, potentiating the action of the GABA receptor [61,66].

To evaluate the response of the animals regarding the anxiolytic and antidepressant effects of KSG, this study divided the behavioral experiments into two phases, the first only with treatment for 30 days with KSG and in the second after administration of the pentylenetetrazole.

Rotarod testing is a validated method for assessing locomotor activity and the balance of animals [67]. The results obtained showed that, in the first phase, all animals treated with KSG obtained a significant improvement in locomotor activity in all doses when compared to the control group (Figure 5). In the second phase, after the administration of pentylenetetrazole, the animals treated at doses 200 and 600 mg/kg had a longer length of stay in the stems of the appliance compared to the other groups. According to Lund, Lephart[27], due to steroid action, phytoestrogens can reduce anxiety by increasing exploratory locomotor behavior. These data suggest that 30-day treatment with KSG has enhanced the motor function of the animals.

The open-field test allows the evaluation of the animal's anxious behavior, as well as its exploratory and locomotor activity [68,69]. The aversion of animals caused by the central

area presents a parameter of anxiety [70]. According to the results obtained, although treatment with KSG showed a reduction in the central and peripheral quadrants covered in both phases when compared to the diazepam and control groups, however, the animals remained longer in the central quadrants when crossing them (Figures 6 and 7). Thus, this result demonstrated the anxiolytic action of KSG in both phases of treatment. A similar finding occurred in the study by Wei *et al.*[71], which showed that the administration of *Lactobacillus paracasei* PS23 resulted in an anxiolytic effect in the open field test after administration for 21 days on anxiety and depression induced by corticosterone.

The elevated plus maze is a behavioral experiment carried out to evaluate rodent anxiety, in which there is a confrontation between aversion to the elevated open area and exploratory interest [72,73]. In this study, it was observed that treatment with the dose of 200 mg/kg of KSG produced an anxiolytic effect on animals, demonstrated by the stay time of the animals in the open arms, as well as diazepam, used positive control (Figure 8). In the second phase, even after the administration of pentylenetetrazole, the dose of 200 mg/kg improved the performance of the treated animals, remaining longer in the arms open compared to the animals of the positive and negative control groups (Figure 9).

Performance improvement in the elevated plus-maze was also observed in Tillmann; Wegener[74], in animals treated with probiotics, with the reduction of anxious behavior. Lund; Lephart[27] demonstrated that animals that were fed a diet rich in phytoestrogens remained longer in their arms open and, with more inputs than animals that had a diet deficient in phytoestrogens. Like Rodríguez-Landa et al.[26], they observed that a group of animals treated with genistein and 17β–estradiol remained longer and presented more entries in open arms.

The forced swimming test is a model of animal behavior used to analyze the effect of antidepressant drugs, in which the animal is placed in a situation of despair, and its reaction is evaluated [75]. In this study, we suggest that in the first phase of treatment for 30 days with KSG presented antidepressant effect, since, in the treated group, shorter immobilization time was recorded, longer climbing time, showing better results compared to treated groups with Diazepam and the control group (Figure 10). In the second phase of the experiment, after the administration of pentylenetetrazole, only doses of 400 mg/kg and 600 mg/kg presented a result suggestive of antidepressant effect (Figure 11).

In the study by Li *et al.*[76], after stress induction for four weeks, treatment with three probiotic strains (*L. helveticus*, *L. plantarum* and B. longum), improved the behavior in the forced swimming test, however, in animals without stress, probiotics showed moderate effects when compared to fluoxetine. In the study by Xu *et al.* [77], immobility time was shorter

after treatment with probiotics in mice with intestinal constipation, with depressive behaviors. Liang *et al.*[78] observed that mice with intestinal microbiota disorder early in life showed depressive behavior, in the forced swimming assay, in adulthood, treatment with probiotics, reduced depressive behaviors in these animals.

Different mechanisms may be related to the anxiolytic effect, such as flavonoids, these natural compounds can bind to $GABA_A$ receptors of benzodiazepines and studies have reported that semi-synthetic flavone-derived substances have a higher potency than Diazepam[79]. In other contrast, bacterial strains present in probiotics can produce anxiety-related neurotransmitters such as gamma-aminobutyric acid (GABA), serotonin, catecholamines, and acetylcholine [74]. In addition to reducing intestinal permeability, which consequently mitigates intestinal inflammation, decreases the stress response by the hypothalamus-pituitary-adrenal axis and normalize stemming from the brain-derived neurotrophic factor [80].

5 Conclusion

In this study, increasing the content of aglycone isoflavones dependent on kefir fermentation time was demonstrated, and the results of the various behavioral experiments in mice suggest that treatment for 30 days with KSG presents anxiolytic and antidepressant effect in the experimental conditions used, accompanied by increased locomotor activity. This study supports the studies described for the anxiolytic and antidepressant activity that act through $GABA_A$ receptor action.

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Contributions of the authors

E.L.M, A.M.P, C.L.B.B., K.R.T.P., D.E.S.G., L.C.C.M.V. and J.M.C, realized the analytics experiments and pharmacological assays. H.O.C. supervised the pharmacologic tests. H.R.S. and L.G.S.S supervised the analytics experiments. H.X., reviewed the results and final text and J.C.T.C., Study General Coordinator.

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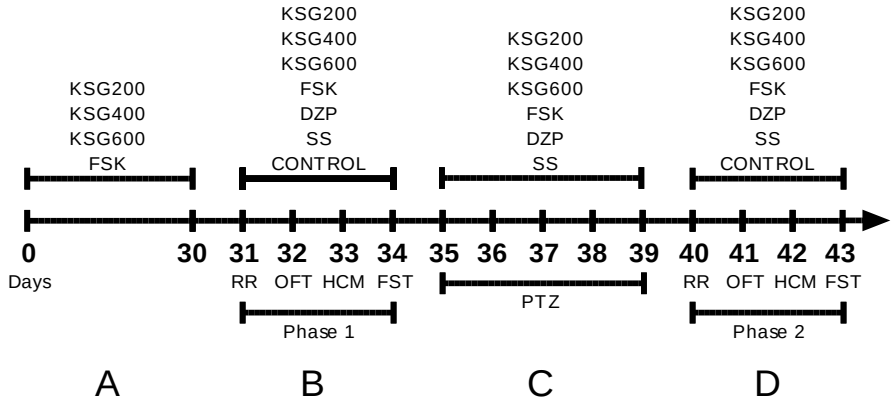

Figure 1. Representation of the experimental design of mice. Timeline: (A) oral treatment with KSG and FSK for 30 days. (B) First phase of rotarod experiment, open field test, elevated plus maze and forced swimming, in groups treated with KSG, FSK, diazepam, saline solution and control. (C) Administration of penthylenetetrazole with a 48-hour interval and continuous treatment with KSG, FSK, diazepam and saline solution. (D) Second phase of rotarod experiment, open field test, elevated plus maze and forced swimming, in groups treated with KSG, FSK, diazepam, saline solution and control, after administration of penthynotetrazole.

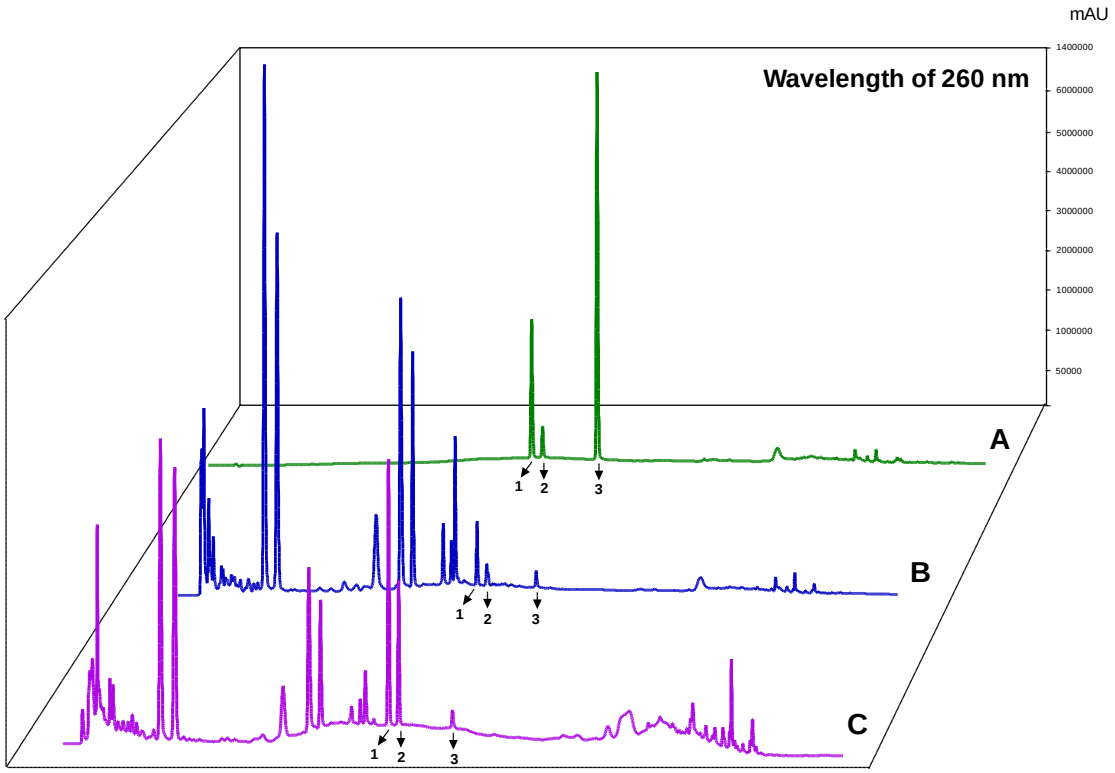

Figure 2. Chromatograms obtained by HPLC-UV with wavelength of 260 nm. (A) Co-injection of analytical patterns of aglycone isoflavones, concentration of 25 µg/mL. Chromatographic peaks: (1) daidzein, (2) glycitein and (3) genistein. (B) Extraction of fermentation solution of kefir (FSK): (1) daidzein, (2) glycitein and (3) genistein. (C) Kefir associated with soybean germ (KSG): (1) daidzein, (2) glycitein and (3) genistein.

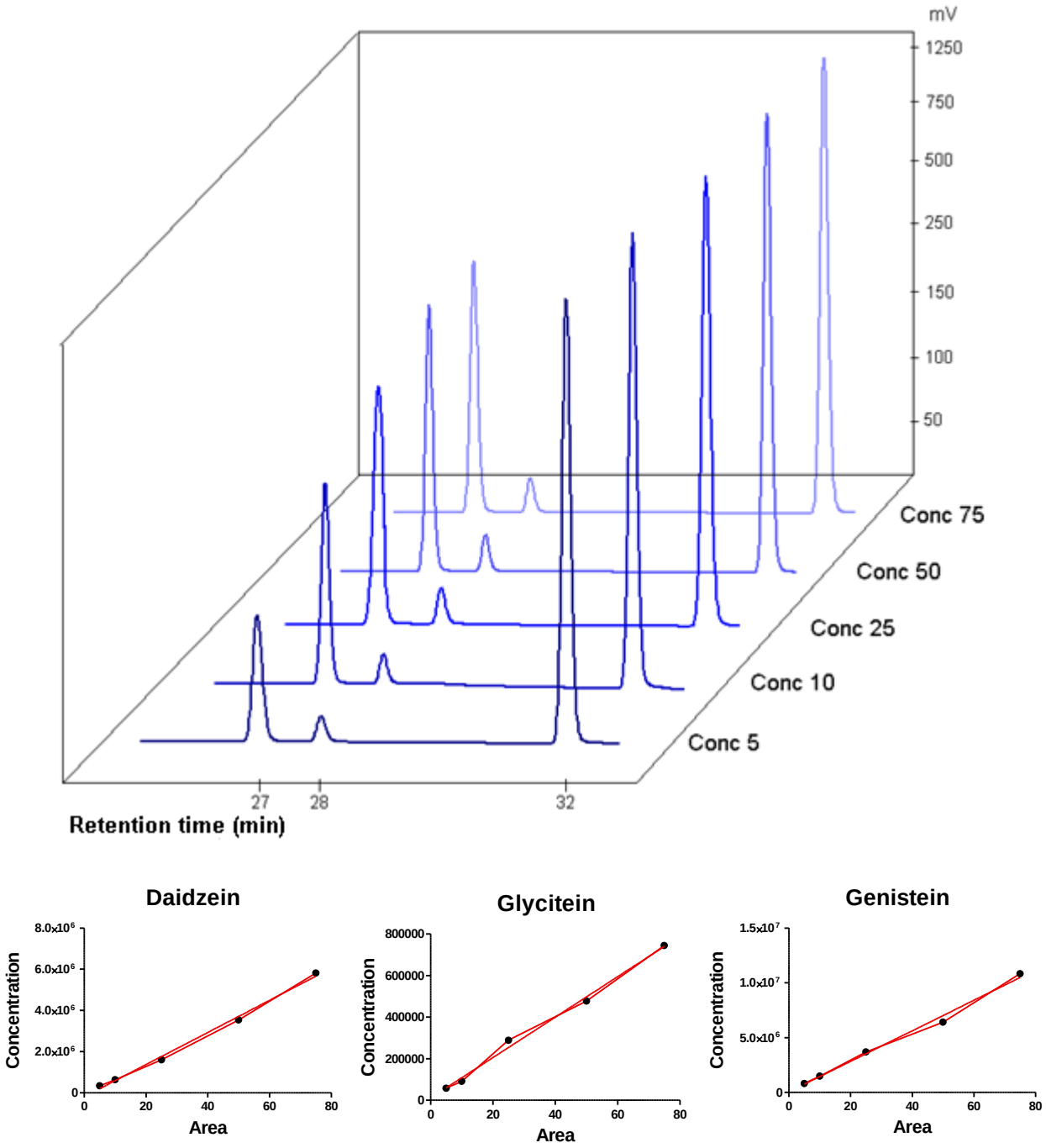

Figure 3. Analytical curves obtained by HPLC-UV for (A) Daidzein, concentration range between 5 and 75 µg/mL. Straight equation: y=77977x-188993, r^2 = 0.9952. (B) Glycitein, concentration ranges between 5 and 75 µg/mL. Straight equation: y=9715.9x+11280, r^2 = 0.9941. (C) Genistein, concentration ranges between 5 and 75 µg/mL. Straight equation: y=139097x+55020, r^2 = 0.9922. Overlap of the co-injection chromatograms of the analytical patterns of isoflavones.

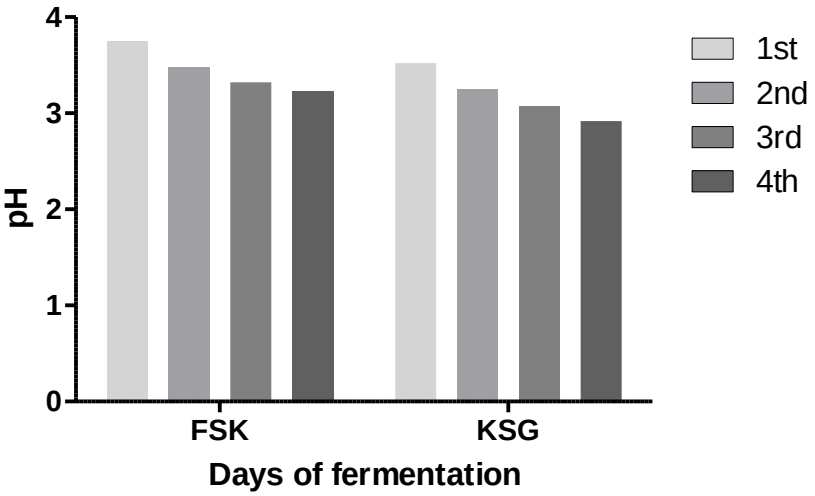

Figure 4. Graphical representation of kefir pH associated with soybean germ (KSG) and fermentation solution of kefir (FSK), according to fermentation days.

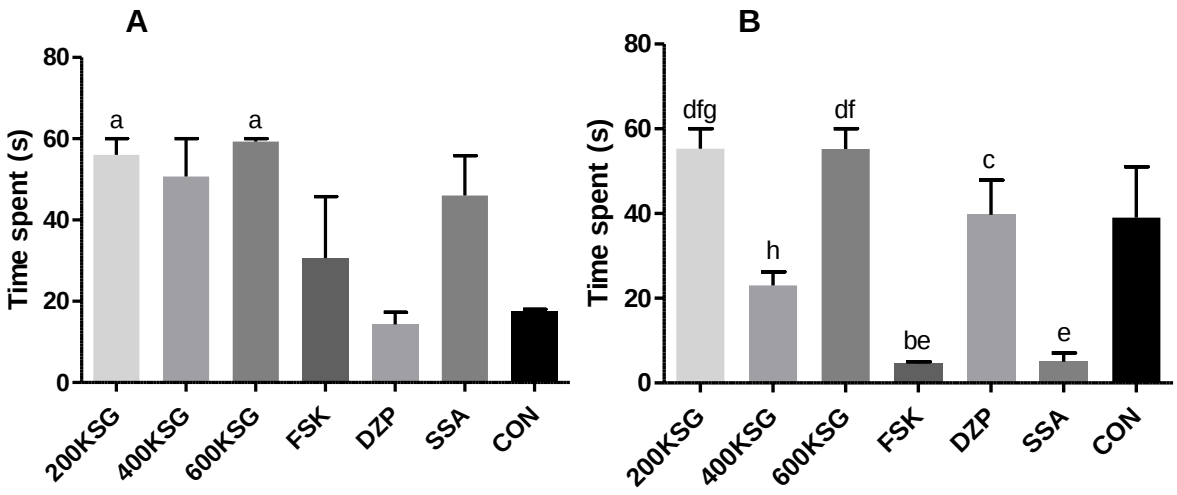

Figure 5. Effect of kefir administration associated with soybean germ (KSG) and fermentation solution of kefir (FSK) in mice in rotarod test (time spent in seconds). (A) First phase of the experiment, KSG (200 mg/kg, 400 mg/kg and 600 mg/kg, vo); FSK (10 µl/kg, vo); saline solution (SSA, 10 µl/kg, vo); negative control (CON); diazepam (DZP, 2 mg/kg, ip). (B) Second phase of the experiment, after intraperitoneal administration of Penthylenetetrazole (15 mg/kg); KSG (200 mg/kg, 400 mg/kg and 600 mg/kg, vo); FSK (10 µl/kg, vo); saline solution (SSA, 10 µl/kg, vo); negative control (CON); diazepam (DZP, 2 mg/kg, ip). The columns represent the mean ± SEM (n = 5/group), [a] $p < 0.05$ vs. Diazepam; [b] $p < 0.01$ vs. Diazepam. [c] $p < 0.01$ vs. Saline solution; [d] $p < 0.001$ vs. Saline solution. [e] $p < 0.05$ vs. Control; [f] $p < 0.001$ vs. Kefir. [g] $p < 0.05$ vs. 400KSG. [h] $p < 0.01$ vs. 600KSG. A análise estatística foi realizada pelo teste one-way ANOVA seguido pelo teste de Tukey.

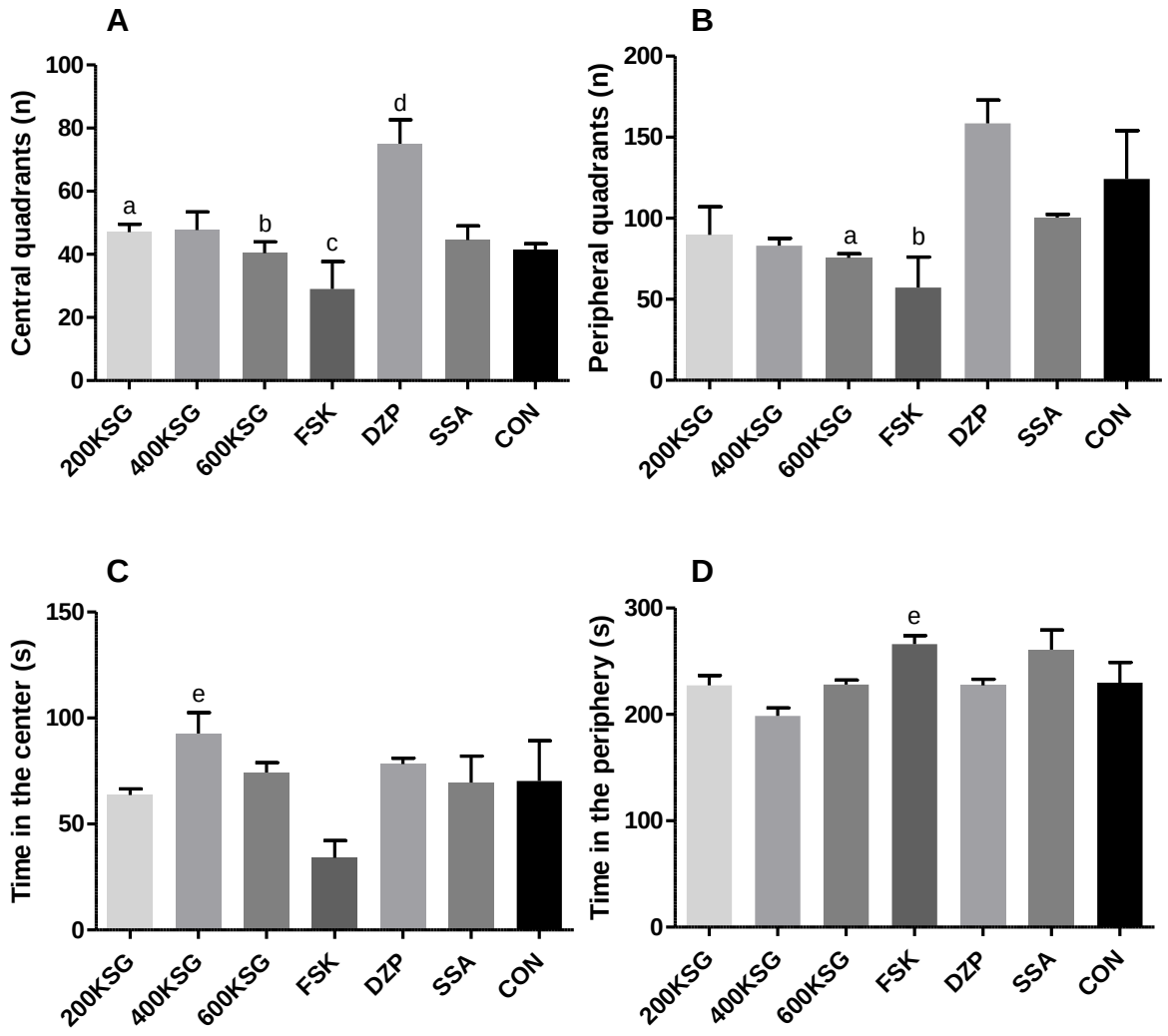

Figure 6. Effect of kefir administration associated with soybean germ (KSG) and fermentation solution of kefir (FSK) in mice in the open field test (number of central and peripheral quadrants, time in the center of the field and in the periphery). First phase of the experiment, KSG (200 mg/kg, 400 mg/kg and 600 mg/kg, vo); FSK (10 µl/kg, vo); saline solution (SSA, 10 µl/kg, vo); negative control (CON); diazepam (DZP, 2 mg/kg, ip). The columns represent the mean ± SEM (n = 5/group), [a] $p < 0.05$ vs. Diazepam; [b] $p < 0.01$ vs. Diazepam; [c] $p < 0.001$ vs. Diazepam. [d] $p < 0.01$ vs. Control. [e] $p < 0.05$ vs. Kefir. Statistical analysis was performed by the ANOVA one-way test followed by the Tukey test.

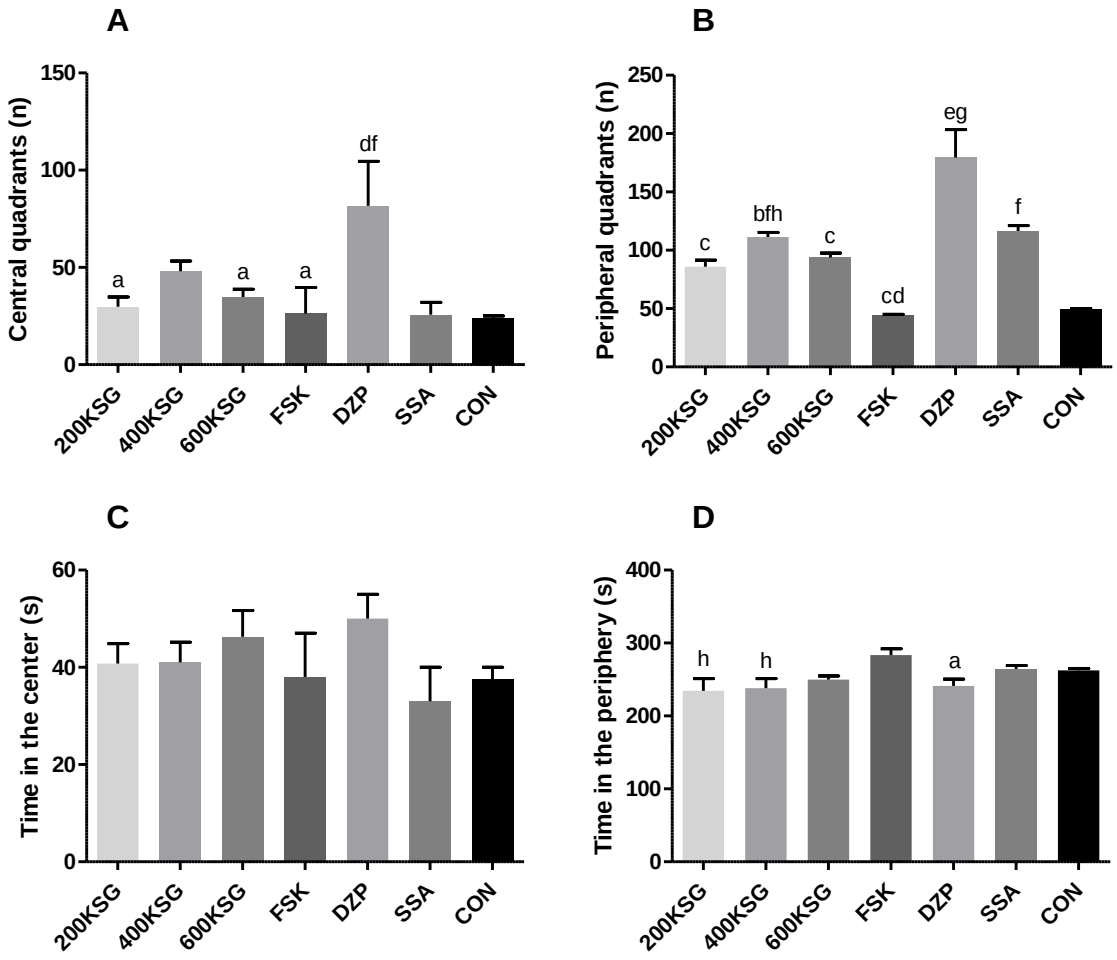

Figure 7. Effect of kefir administration associated with soybean germ (KSG) and fermentation solution of kefir (FSK) in mice in the open field test (number of central and peripheral quadrants, time in the center of the field and in the periphery). Second phase of the experiment, after intraperitoneal administration of Penthylenetetrazole (15 mg/kg); KSG (200 mg/kg, 400 mg/kg and 600 mg/kg, vo); FSK (10 µl/kg, vo); saline solution (SSA, 10 µl/kg, vo); negative control (CON); diazepam (DZP, 2 mg/kg, ip). The columns represent the mean ± SEM (n = 5/group), [a] $p < 0.05$ vs. Diazepam; [b] $p < 0.01$ vs. Diazepam; [c] $p < 0.001$ vs. Diazepam. [d] $p < 0.05$ vs. Saline solution; [e] $p < 0.01$ vs. Saline solution. [f] $p < 0.05$ vs. Control; [g] $p < 0.001$ vs. Control. [h] $p < 0.05$ vs. Kefir. Statistical analysis was performed by the ANOVA one-way test followed by the Tukey test.

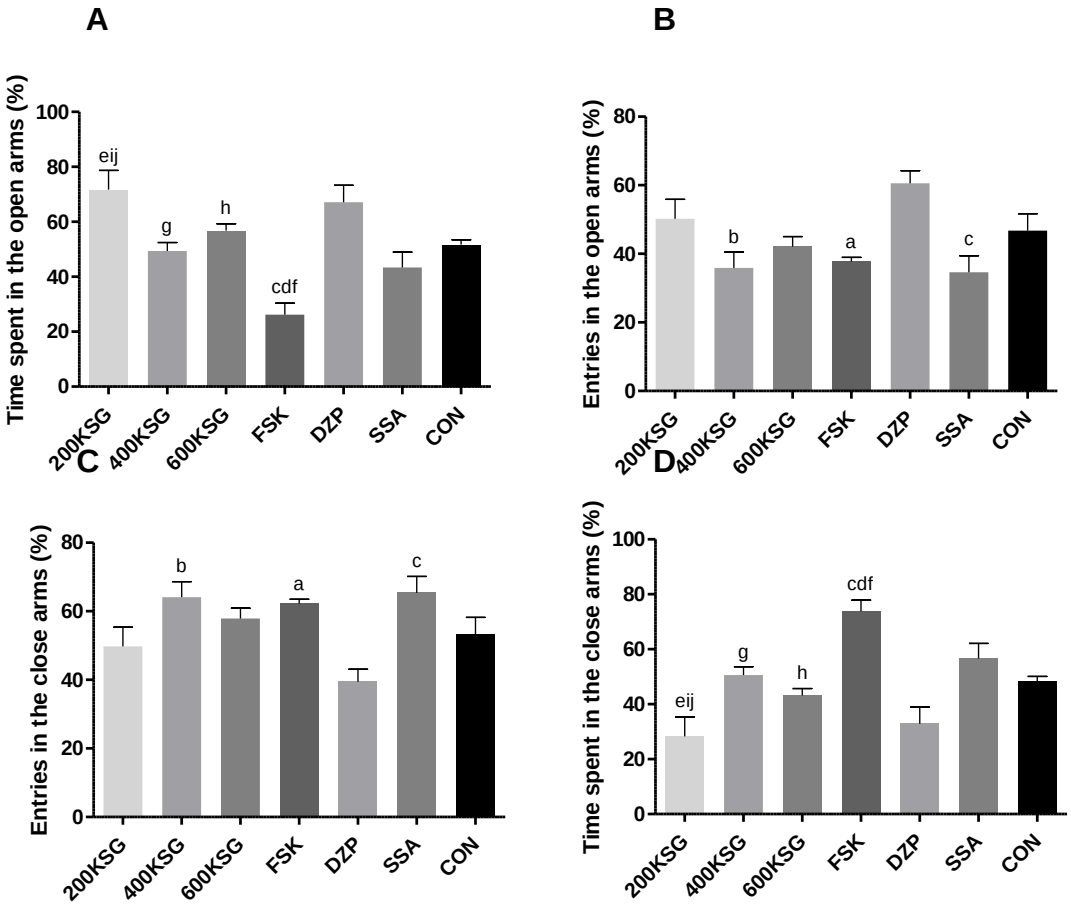

Figure 8. Effect of kefir administration associated with soybean germ (KSG) and fermentation solution of kefir (FSK) in mice in elevated plus maze test (percentage of time spent in the open and close arms, percentage of entries in the open and close arms). First phase of the experiment, KSG (200 mg/kg, 400 mg/kg and 600 mg/kg, vo); FSK (10 µl/kg, vo); saline solution (SSA, 10 µl/kg, vo); negative control (CON); diazepam (DZP, 2 mg/kg, ip). The columns represent the mean ± SEM (n = 5/group), [a] $p < 0.05$ vs. Diazepam; [b] $p < 0.01$ vs. Diazepam; [c] $p < 0.001$ vs. Diazepam. [d] $p < 0.05$ vs. Saline solution; [e] $p < 0.01$ vs. Saline solution. [f] $p < 0.05$ vs. Control; [g] $p < 0.05$ vs. FSK; [h] $p < 0.01$ vs. Controle; [i] $p < 0.001$ vs. Control. [j] $p < 0.001$ vs. 400KSG. Statistical analysis was performed by the ANOVA one-way test followed by the Tukey test.

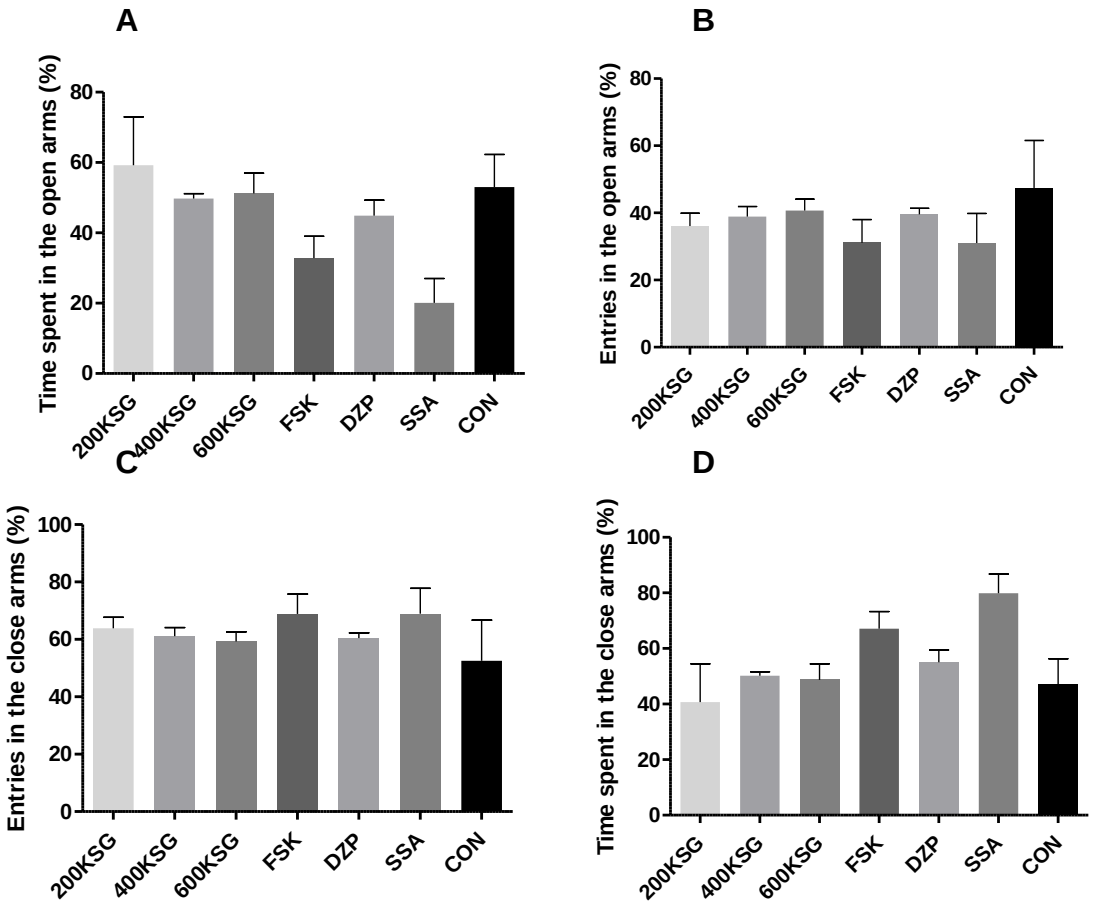

Figure 9. Effect of kefir administration associated with soybean germ (KSG) and fermentation solution of kefir (FSK) in mice in elevated plus maze test (percentage of time spent in the open and close arms, percentage of entries in the open and close arms). Second phase of the experiment, after intraperitoneal administration of Penthylenetetrazole (15 mg/kg); KSG (200 mg/kg, 400 mg/kg and 600 mg/kg, vo); FSK (10 µl/kg, vo); saline solution (SSA, 10 µl/kg, vo); negative control (CON); diazepam (DZP, 2 mg/kg, ip). The columns represent the mean ± SEM (n = 5/group). Statistical analysis was performed by the ANOVA one-way test followed by the Tukey test.

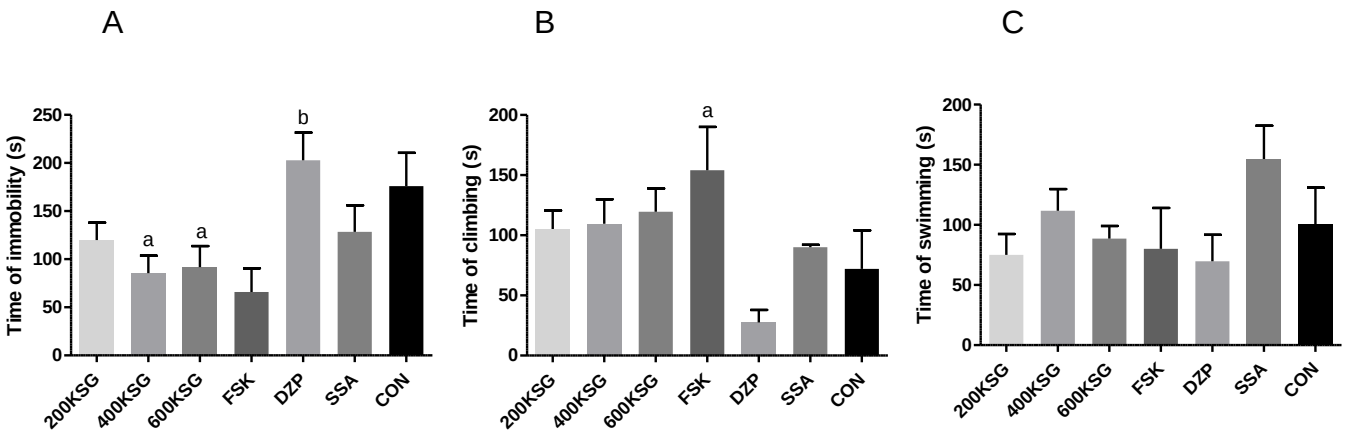

Figure 10. Effect of kefir administration associated with soybean germ (KSG) and fermentation solution of kefir (FSK) in mice in the forced swimming test (time in seconds of swimming, immobility and climbing). First phase of the experiment, KSG (200 mg/kg, 400 mg/kg and 600 mg/kg, vo); FSK (10 µl/kg, vo); saline solution (SSA, 10 µl/kg, vo); negative control (CON); diazepam (DZP, 2 mg/kg, ip). The columns represent the mean ± SEM (n = 5/group), [a] $p < 0.05$ vs. Diazepam; [b] $p < 0.01$ vs. Diazepam. Statistical analysis was performed by the ANOVA one-way test followed by the Tukey test.

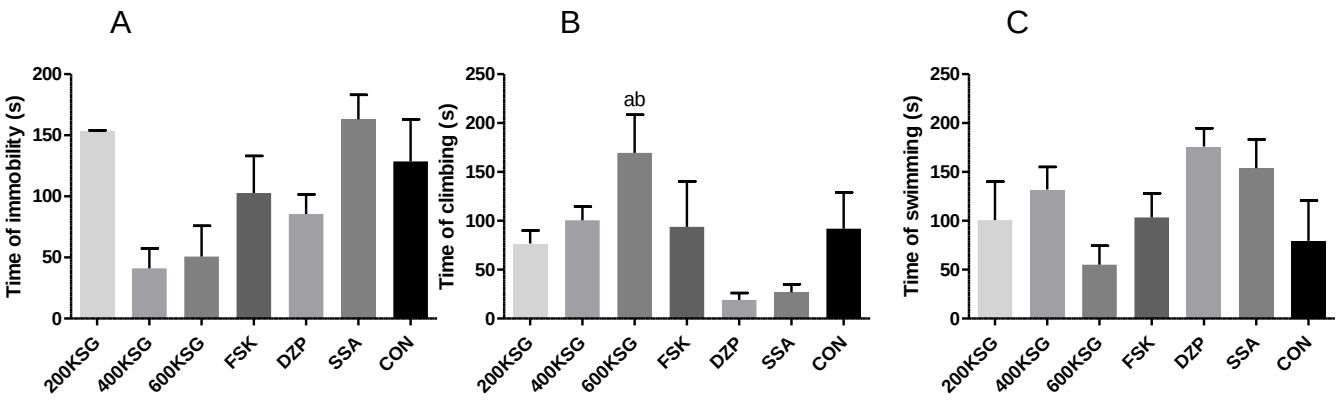

Figure 11. Effect of kefir administration associated with soybean germ (KSG) and fermentation solution of kefir (FSK) in mice in the forced swimming test (time in seconds of swimming, immobility and climbing). Second phase of the experiment, after intraperitoneal administration of Penthylenetetrazole (15 mg/kg); KSG (200 mg/kg, 400 mg/kg and 600 mg/kg, vo); FSK (10 µl/kg, vo); saline solution (SSA, 10 µl/kg, vo)); negative control (CON); diazepam (DZP, 2 mg/kg, ip). The columns represent the mean ± SEM (n = 5/group), [a] $p < 0.01$ vs. Diazepam; [b] $p < 0.05$ vs. Saline solution. Statistical analysis was performed by the ANOVA one-way test followed by the Tukey test.

5 ARTIGO 2

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Evaluation of the in vitro release of isoflavones from soybean germ associated with kefir culture in the gastrointestinal tract and anxiolytic and anti-depressive actions in zebrafish (*Danio rerio*)

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Abstract

The gastrointestinal tract has thousands of microorganisms present in the mucosa, derived from several different species, and these microorganisms have a direct relationship with immunity, homeostasis, and certain systemic diseases. Kefir interacts with the gastrointestinal, immune system and also with the intestinal microbiota. Isoflavones are the most commonly consumed phytoestrogens, with aglycone and glycoside forms being the main ones. Therefore, in this study, the content of aglycone isoflavones released from kefir associated with soybean germ (KSG) was evaluated in a gastrointestinal digestion model in vitro, and the anxiolytic and antidepressant activity of KSG and fermentation solution of kefir (FSK) were evaluated in models of anxiety and depression in zebrafish (*Danio rerio*). It has been demonstrated an increase in the release of the isoflavone content in the gastric phase and a reduction in the intestinal and colon phases with KSG, and the data set of the

behavioral patterns obtained in the different experiments demonstrated that KSG and FSK, present anxiolytic and antidepressant effects.

Keywords: Brain-intestine axis. Kefir. Isoflavones. Depression. Anxiety. Gastrointestinal tract in vitro.

1 Introduction

The gastrointestinal tract has thousands of microorganisms present in the mucosa, derived from several different species; these microorganisms have a direct relationship with immunity, homeostasis, and certain systemic diseases (Kim, Jeong, Kim, & Seo, 2018). Probiotics act directly on the gastrointestinal microbiota, regulate the immune response of the host that works by interacting with epithelial cells, increasing the secretion of anti-inflammatory cytokines (Van Doan, Hoseinifar, Tapingkae, & Khamtavee, 2017). They also carry out preventive activities in gastrointestinal diseases, with positive results in gastric injury models, such as anti-inflammatory and antioxidant, alluring to the symptoms of constipation, as well as immunological and metabolic diseases (Barboza et al., 2018; Chiu, Lin, Tsai, & Lin, 2014; Turan, Dedeli, Bor, & Ilter, 2014).

Kefir grains are composed of extractive exopolysaccharides (EPS), which is a conversion product with their substrates, such as ethanol, carbon dioxide, lactic acid, glycerol, acetic acid, mannitol and a variety of aromatic compounds (Laureys, Aerts, Vandamme, & De Vuyst, 2018). EPS have the protective capacity of cells, as well as in the protection of the gastrointestinal tract, such as low pH, bile salts, pancreatic and gastric enzymes, and still plays a function in bacterial aggregation, through biofilm production, interacting with cells of the intestinal epithelium, assisting in the permanence of the microorganism in the intestine, as well as its interaction with the gastrointestinal, immune system and also with the intestinal microbiota (Bengoa et al., 2018; Cheirsilp, Suksawang, Yeesang, & Boonsawang, 2018).

Isoflavones are part of the flavonoid group, in which they are present in the secondary metabolites of vegetables (Simões et al., 2007). Soy isoflavones present four different chemical forms, showing three types of isoflavones, each: aglycones (genistein, daidzein, and glycitein), glycosidic (daidzein, genistin, and glycitin), acetyl-glucoside (acetyl-daidzein, acetyl-genistin, acetyl-glycitin) and malonyl-glucoside (malonyl-daidzein, malonyl-genistin, malonyl-glycitin) (Dong, Xu, Sikes, & Wu, 2012). They are the phytoestrogens most

commonly researched and consumed, with the forms aglycones and glycosides being the main ones (Carbonel et al., 2011; Dong et al., 2012).

The specific action and high molecular weight of glycosidic isoflavones provide low absorption in the small intestine, unlike aglycone isoflavones that are absorbed efficiently. Glycosidic isoflavones are only absorbed after their hydrolysis, which promotes the removal of the glucose molecule and exerts their metabolic action by binding on the estrogen receptor (Larkin, Price, & Astheimer, 2008; Monteiro, Queirós, Lopes, Pedro, & Macedo, 2018).

Zebrafish (*Danio rerio*) has become an important organism used to evaluate neuropsychiatric diseases, showing advantages because it is an economic model of simple creation and with a rapid reproductive cycle (Kalueff, Stewart, & Gerlai, 2014; Nguyen, Stewart, & Kalueff, 2014). In several studies, this model was used to evaluate the anxiolytic and antidepressant activity of new compounds (Costa de Melo et al., 2019; Dos Santos Sampaio et al., 2018). However, we did not find studies where the anxiolytic and antidepressant effect was evaluated, and the evaluation of isoflavone release in an in vitro digestion model. A model that is useful tools with low cost and use little time to simulation and evaluate structural changes and bioavailability of components (Dong et al., 2012; Rui et al., 2016; Singh & Vij, 2018).

Therefore, in this study, the in vitro gastrointestinal digestion model was applied, in which the content of aglycone isoflavones released from the kefir associated with soybean germ was applied in each phase of digestion and, evaluating anxiolytic and antidepressant using zebrafish as an animal model.

2 Material and methods

2.1 Soybean germ

Herborisa Industrial Co., located in the city of Londrina, Paraná, Brazil, supplied the soybean germ. It was obtained from non-transgenic soybeans, and according to the analysis, the report described the presence of proteins, fats, fibers, minerals, and isoflavone.

2.2 Obtaining kefir and kefir associated with soybean germ (KSG)

Kefir grains were obtained from the standard collection stored in the Drugs Research Laboratory, Department of Biological Sciences and Health, Federal University of Amapá, Amapá, Brazil.

For the means of kefir and KSG crops, 40 g/L concentrations of brown sugar, 10 g/L of soybean germ and 60 g/L of kefir were used, fermented at a temperature of 25°C according to the method described by Oliveira et al., (2017). The cultivation medium containing distilled water and brown sugar was homogenized in an Erlenmeyer and taken to autoclave (Prismatec, Melissa, São Paulo, Brazil) at a temperature of 200°C for a time of 15 minutes. After cooling, at room temperature, the kefir grains were added. In KSG, the pulverized soybean germ has been added. The glasswork was sealed and kept at room temperature in a dry place under the light. On the 4th day of fermentation, the grains and germ were sifted and filtered with filter paper (Whatman®) with micropores eight μm in diameter and discarded. The fermentative solution of kefir and KSG obtained were used for analyses and animal experimentation.

2.3 Evaluation of in vitro release of KSG isoflavones in the gastrointestinal tract

The evaluation of the release was based on the method described by Breynaert et al. (2015) modified, which was divided into the gastric, intestinal, and colon phases.

2.3.1 Gastric phase

The gastric phase was performed in a dissolution device in vats (Nova Ética, Vargem Grande Paulista, São Paulo, Brazil). Forty-seven g of the sample was added in pH adjusted to 2.0 utilizing 6 mol/L of HCl (Alphatec), and pH evaluated in digital pHmetro (Edutec, Curitiba, Brazil). Then added 3 ml of the pepsin solution (16% pepsin (m/v), Sigma Aldrich, St. Louis, MO, USA, dissolved in 0.1 mmol/L of HCl), the samples were incubated during one hour in a water bath with agitation at 37° C (120 rotations/min). After gastric digestion, 3 ml aliquots were collected taken for centrifuge (Nova Técnica, Piracicaba, São Paulo, Brazil) for 10 min at 4000 rpm at 25°C and the supernatants filtered through PVDF membrane (Millex® HV Durapore®, Carrighwohill, Co, Cork, Ireland) of 0.45 μm of pore, and analyzed in HPLC-UV (Shimadzu®, Kioto, Japão).

2.3.2 Intestinal phase

The simulated intestinal phase occurred in the dissolution (Nova Ética, Vargem Grande Paulista, São Paulo, Brazil). The content of gastric digestion was transferred to a vat and added 100 ml of deionized water, followed by the addition of a dialysis bag containing NaHCO3 1mol/L. Next, 0.5 mol/L of NaOH (Alphatec) was titled, necessary for gastrointestinal digestion, to present a pH of 7.5 (pHmetro Edutec, Curitiba, Brazil). After 30 min, 15 ml of biliary pancreatin (pH 7.5) was added, prepared in a pancreatin solution (0.4% pancreatin (m/v), Sigma Aldrich, St. Louis, MO, USA) and bile (0.76% bile (m/v), Sigma Aldrich, St. Louis, MO, USA) at 0.1 mmol/L from NaHCO3 (Sigma Aldrich, St. Louis, MO, USA). The sample was incubated for one hour, with a temperature of 37°C. After 1 hour, the fluid was analyzed by HPLC-UV (Shimadzu®, Kioto, Japan), removing rates of 3 ml and centrifuges in centrifuge (Nova Técnica, Piracicaba, São Paulo, Brazil) for 10 min at 4000 rpm at 25°C and supernatants filtered through PVDF membrane (Millex® HV Durapore®, Carrighwohill, Co, Cork, Ireland) of 0.45 µm pore.

2.3.3 Colon phase

For the colon phase, a 10% human fecal suspension (p:v) was prepared, homogenized with sterile phosphate buffer solution (0.1 mmol/L, pH 7.0), and was stored at -80 °C before use. The samples retained from the intestinal phase were the substrate for the colon microbiota. A 50 mL of the suspension of fecal bacteria was added, and digestion pH was maintained at ± 5.8 (1 mmol/L of HCl (Alphatec)). Dialysis was performed for 1 hour, and the final product was analyzed by HPLC-UV (Shimadzu®, Kioto, Japan), in which 3 ml aliquots were removed and taken to centrifuge (Nova Técnica, Piracicaba, São Paulo, Brazil) for 10 min at 4000 rpm at 25°C and the supernatants filtered through PVDF membrane (Millex® HV Durapore®, Carrighwohill, Co, Cork, Ireland) of 0.45 µm pore.

2.4 Analysis of KSG isoflavones by high performance liquid chromatography

The analytical patterns used of isoflavones were daidzein 98%, glycitein 98%, and 98% genistein (Sigma-Aldrich®, St. Louis, MO, USA). The patterns were dissolved in methanol: water solution (80:20 v/v). For chromatographic analysis, HPLC grade methanol reagents (Sigma-Aldrich), HPLC grade acetic acid (Sigma-Aldrich), and HPLC grade acetonitrile (Sigma-Aldrich) were used.

The chromatographic conditions used for separation and quantification of aglycone isoflavones were according to the modified Sun et al. (2011) method. High-efficiency liquid chromatography (Shimadzu®, Kioto, Japan) was used, with a system equipped with a solvent pumping unit (LC-20AT), Proeminence automatic injector (SIL-20AC), column oven (CTO-10AS/VP). The detection was performed using a UV-VIS detector (SPC-20A), interconnected by a system controller (CBM-20A), and the data were processed using LC-solution software. For the separation of the analytes, the Kinetex C18 column (250 × 4.6 mm) (Phenomenex Inc. was used, CA, USA) with particle size of 5 µm and precolumn C18 2.6 µm, and aliquots of 15 µL were injected into flow of 0.75 mL/min, temperature of 30 °C and wavelength 260 nm, using solvent A: 0.1% acetic acid in water and solvent B: acetonitrile. The linear gradient was started with 15% of solvent B up to the 10-minute time, following to 35% in the time of 25 minutes and to 45% up to 45 minutes, then 65% until 50 minutes and 95% until 65 minutes, decreasing to 80% in 80 minutes and finishing 50% in 65:01 minutes.

2.5 Evaluation of the anxiolytic and anti-depressive activities in zebrafish of KSG

The experimental protocols were by the legislation for animal experimentation established by the National Council for Animal Experimentation of Brazil (CONCEA). The project was approved by the Ethics Committee on the Use of Animals (CEUA) of the Federal University of Amapá-UNIFAP under protocol number 007/2019.

The animals (Zebrafish, *Danio rerio*) of both sexes (six-month-old AB wild strain, 3.5–4.0 cm long, weighing around 200–1000 mg, were obtained from São Caetano Animal Trade Eireli, located in Santo André, São Paulo, Brazil.

The animals were kept on the Zebrafish Platform of the Pharmaceutical Research Laboratory, Department of Biological and Health Sciences, Federal University of Amapá, Amapa, Brazil, at a temperature of 26 ± 2ºC with a 12 h light/dark cycle. The animals were kept in tanks, in which the water conditions were controlled for 2 months before the tests, as described by Carvalho et al. (2017).

The experiments were carried out in accordance with the rules established for the care of animals and, the feeding was carried out twice a day with *Artemia salina* and/or specific food for the species (with morning food from 8 to 9 am and with *Artemia salina* in the afternoon at 16 pm).

2.5.1 Experimental design

The oral administration of the drugs tests occurred according to the method described by Dos Santos Sampaio et al. (2018). The animals were divided into six groups (n=6/group): Group I and II received 200 and 400 mg/kg of KSG respectively, and Group III received 2 µl/kg of FSK for 30 days. After this treatment period, Group I was treated with 200 mg/kg of KSG + 100 mg/kg of caffeine (anhydrous, Jilin Shulan Co., batch CA201609033, China), Group II was treated with 400 mg/kg of KSG + 100 mg/kg of caffeine, Group III was treated with 2 µl/kg of FSK + 100 mg/kg of caffeine, and Group IV was treated with 25 mg/kg of buspirone (Libbs Farmacêutica Ltda., Lote 18I0127, São Paulo) + 100 mg/kg of caffeine, in these groups, caffeine was administered 30 minutes after receiving the initial substance. Group V was treated with 100 mg/kg of caffeine and Group VI, naive (Figure 1). After 60 minutes, the animals were submitted to the test.

2.5.2 Scototaxis Test (Light-Dark Test)

The method described by Dos Santos Sampaio et al. (2018) was used. The animals were filmed with a video camera (Nikon D5100) positioned 56 cm above the test tank. Individualized behavioral analysis was performed using the ToxTrac software (software-v2.61).

After oral treatment, the animals were individually placed in the central compartment of the aquarium for 3 minutes, and then the hatches from the central compartment were removed. The behavior of the animals was recorded for 15 minutes. Animals that did not cross the midline in the 15-minute session were discarded to avoid false positives in the preference measures (Noakes & Baylis, 1990).

The following reactions of behaviors were evaluated: length of stay in the white compartment(s), latency (s, time to enter the white compartment), alternation (n, the total number of times that the central point of the animal crossed the center of the tank that divided the black and white compartments), duration of trigotaxis (s, the proportion of the time of remained in the white compartment used in prolonged swimming at a distance of up to 2 cm from the nearest wall), and risk assessment (n), (Figure 2A) (Dos Santos Sampaio et al., 2018).

2.6 Evaluation of anti-depressive activity in zebrafish

2.6.1 Experimental design

For the evaluation of antidepressant activity, the treatment occurred orally according to the method described by Dos Santos Sampaio et al. (2018). The animals were divided into six groups (n=6/group): Group I and II received 200 and 400 mg/kg of KSG, respectively, and Group III received 2 µl/kg of FSK for 30 days. After this treatment period, Group I was treated with 200 mg/kg ksg, Group II was treated with 400 mg/kg kSG, Group III was treated with 2 µl/kg of FSK, Group IV was treated with 20 mg/kg of fluoxetine (Medquímica Pharmaceutical Industry Ltda., Lote 86829S, Minas Gerais), Group V was treated with 1% ethanol (Solven Solvents and Chemicals Co., lot 536/16, São Paulo) and Group VI, navei (Figure 3). After 60 minutes of treatment, the animals were submitted to the trial.

2.6.2 New Tank Diving Test (NTDT)

The method described by Dos Santos Sampaio et al. (2018) was used using a standard aquarium for this experiment. At a distance of 35 cm from the aquarium, a video camera (Nikon D3300 model) was placed for filming and subsequent analysis of the behavioral reactions. The ToxTrac software (software-v2.61) was used for behavioral analysis.

After 60 minutes of treatment of the animals with their respective test substances, the animals were transferred to the central compartment of the aquarium test; without acclimatization period, the animal's behavior was recorded for 6 min (Cachat et al., 2010). The variables analyzed were: length of stay at the top (s); number of erratic swimming events (n) (multiple occurrences of accelerated swimming and with rapid succession of change of motion direction); freezing time (s) (time in which the animal remained immobile, except for opercula and eyes), distance traveled (mm), quadrants crossed (n) and latency (s) (Figure 2B) (Dos Santos Sampaio et al., 2018).

2.7 Statistical analyze

GraphPad Prism software (version 5.0) was used for statistical analysis of the results obtained. For the behavioral evaluation of the animals, the ANOVA test (one-way) was used, followed by Tukey tests to compare the test and control groups. The results were expressed

as the mean ± standard deviation of the mean. Values with $p < 0.05$ were considered significant.

3 Results

3.1 Identification and quantification of isoflavones present in KSG after the in vitro digestion process

In the chromatograms presented in Figure 4, the aglycone isoflavones of the 4th day of fermentation of KSG, before and after the gastric, intestinal, colon and analytical patterns phases are demonstrated, presenting retention time of 27.04, 27.12, 27.15, 27.25 and 27.06 min., respectively, for daidzein, 27.95, 27.96, 27.89, 27.82 and 27.52 min., respectively, for glycitein and 32.52, 32.45, 32.16, 32.18 and 32.22 for genistein. The ratio of the peak area of the patterns of the isoflavones daidzein, glycitein, and genistein for the evaluation of concentration was established through linear regression of the standard curves, in which it is shown in Figure 5. The levels of the content of the aglycone isoflavones before and after in vitro digestion are shown in Table 1.

3.2 Scototaxis Test (Light-Dark Test)

Treatment with KSG (400 mg/kg) and FSK showed a significant increase in the number of alternating in comparison with the other treatments. In contrast, the group treated with KSG (200 mg/kg) presented a number of alternations similar to the group treated with buspirone (Figure 6A, $p < 0.001$).

Regarding risk assessment, the groups treated with KSG, KSG (200 and 400 mg/kg) and FSK (2 µl/kg), significantly reduced the risks, comparing with the buspirone, caffeine and control groups (Figure 6B, $p < 0.01$).

Treatment with KSG (200 mg/kg) and FSK (2 µl/kg) reduced the time of trigotaxis when compared to the negative group. However, only KSG (200 mg/kg) showed time approaching the buspirone group. The group, KSG (400 mg/kg), presented longer trigotaxis time (Figure 6C, $p < 0.01$).

In determining latency time, it was observed that the groups treated with KSG (400 mg/kg) and FSK (2 µl/kg) showed a significant reduction in time when compared to the other groups. Unlike treatment with KSG (200 mg/kg), which reduced only compared to the control group (Figure 7A, $p < 0.01$).

Treatment with KSG (400 mg/kg) and FSK (2 µl/kg), prolonged the permanence of animals in the white compartment compared to the other groups. Treatment with KSG (200 mg/kg) showed a result close to buspirone treatment (Figure 7B, $p < 0.001$).

3.4 New Tank Diving Test (NTDT)

The data obtained from the length of stay at the top of the aquarium show that the animals treated with FSK (2 µl/kg), presented more time at the top compared to the other groups. However, the KSG groups (200 and 400 mg/kg) demonstrated a similar time to the fluoxetine group. (Figure 8A, $p < 0.01$).

Treatment with KSG (200 and 400 mg/kg) and FSK (2 µl/kg) reduced the latency time of the animals, compared with the results obtained by the fluoxetine, alcohol, and control groups, as observed in Figure 8B ($p < 0.05$).

In the evaluation of the number of cross quadrants, the results show that the groups treated with KSG (200 and 400 mg/kg), FSK (2 µl/kg) and fluoxetine crossed more quadrants when compared to the alcohol and control groups (Figure 8C, $p < 0.001$).

Treatment with KSG (200 mg/kg) and FSK (2 µl/kg) increased the distance traveled of animals compared to the other groups. The group treated with KSG (400 mg/kg) presented greater distance than alcohol (Figure 9A, $p < 0.05$).

Regarding freezing time, the groups treated with KSG (200 and 400 mg/kg), FSK (2 µl/kg) and fluoxetine had significantly shorter time compared to groups treated with alcohol and control (Figure 9B, $p < 0.001$).

The animals treated with KSG (200 and 400 mg/kg), and FSK (2 µl/kg), showed an increase in erratic swimming compared to fluoxetine and control groups. However, KSG (400 mg/kg) showed a reduction in the amount to the alcohol group (Figure 9C), $p < 0.01$).

4 Discussion

In vitro digestion models are techniques widely used in the reproduction of biochemical development and in the set of conditions that occur in the gastrointestinal tract (Berthelsen, Klitgaard, Rades, & Müllertz, 2019; Lucas-González, Viuda-Martos, Pérez-Alvarez, & Fernández-López, 2018). As animal and human digestion models, they present high price, technical difficulties, and legal limitations, the in vitro model offers excellent cost-benefit, speed, and efficiency (Lucas-González et al., 2018).

In this study, an in vitro gastrointestinal digestion, an increase in the release of aglycone isoflavones on the 4th day of fermentation of KSG was demonstrated after the gastric phase of digestion. However, there was a decrease in the concentration of aglycone isoflavones in the intestinal phase and, consequently, in the colon phase. This result suggests that enzymatic activity and acid pH may be related to increased aglycone isoflavones, releasing from the food matrix. At the same time, the reduction in the following phases may be associated with enzyme activity bacterial β-glycosidase, by contributing to the conversion of glycosidic isoflavones into aglycones and subsequently absorbed and metabolized by the microbiota present in the large intestine (Da Silva Fernandes et al., 2017; Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso, 2013; Spínola, Llorent-Martínez, & Castilho, 2018).

Da Silva Fernandes et al. (2017), in the evaluation of fermented soy milk with kefir, demonstrated that after the simulation of the digestive system in vitro, the release of isoflavones aglycones was higher in fermented soybean milk compared to unfermented soy milk. Rodríguez-Roque et al. (2013) demonstrated an increase in the release of isoflavones and phenolic compounds of soy milk after the simulation of gastric digestion in vitro, being significantly reduced in the intestinal fraction. Peñalvo (2004) found that under acidic conditions, acetyl and malonyl conjugate separate dwell from isoflavones, allowing the release of 7- O- glucosides and free aglycones.

However, different studies show contradictory results, such as Wyspiańska, Kucharska, Sokół-Łętowska, & Kolniak-Ostek (2019), in which a significant reduction in isotonic beverage release enriched with maltodextrin and inulin was observed after digestion simulated gastric. Piskula (2000) found that after gastric digestion, there was a reduction in the content of isoflavones. The controversial results obtained by different studies indicate that the variation of the food matrix, that isoflavones are inserted, the pH of the medium, as well as the intestinal microbiota of the host can influence the release and absorption of isoflavones aglycones during digestion of the gastrointestinal tract (Da Silva Fernandes et al., 2017; Wyspiańska et al., 2019).

Studies attribute anxiolytic and antidepressant activity to isoflavones (Friedman & Frye, 2011; Khan, Perviz, Sureda, Nabavi, & Tejada, 2018) and probiotics (Jiang et al., 2018; Luna & Foster, 2015). The complex relationship of central nervous system disorders and intestinal microbiota has been demonstrated in several studies using zebrafish, including Parkinson's disease, schizophrenia, autism, anxiety, and depression (De Abreu et al., 2019).

Zebrafish is an analogous animal model for humans (De Abreu et al., 2019). Their digestive tract bears similarities compared to that of mammals, according to their

development, organization, and function, in addition to extensive homology with innate and adaptive immune systems (Borrelli et al., 2016), making it an organism conducive to research related to the connection of the intestinal microbiota and anxiety and depression. In this study, the effects of kefir treatment associated with soybean germ by oral administration in zebrafish for 30 days were demonstrated, and anxiety and depression behavior was evaluated.

In the stereotaxis test, treatment with KSG (400 mg/kg) and FSK (2 µl/kg) increased length of stay in the white compartment, decreased latency to enter the dark area, and increased the number of alternations. The Groups KSG (200 mg/kg) and FSK (2 µl/kg) showed a reduction in trigotaxis time, while KSG (200 and 400 mg/kg) and FSK (2 µl/kg) decreased risk behavior. In this trial, zebrafish presents natural preference for the dark area of the aquarium, however, in studies conducted to evaluate the behavior after treatment of anxiolytic drugs, the choice for the dark area was reduced (Duarte et al., 2019; Facciol, Tran, & Gerlai, 2017; Mezzomo, Silveira, Giuliani, Quadros, & Rosemberg, 2016). Thus, from the results obtained, the treatment of KSG (400 mg/kg) and FSK (2 µl/kg) suggest an anxiolytic action on zebrafish.

In this study, the ansiogenic effect caused by caffeine administration is aligned with the effects caused by neurotransmitter modulation, such as monoamines, acetylcholine, and GABA. Another factor is the increase in intracellular calcium levels, in which it is responsible for the increase in metabolic rate, and nitric oxide levels, resulting in psychiatric disorders (Khurana & Bansal, 2019). In zebrafish, treatment with high concentrations of caffeine causes anxiety-related responses, causing increased freezing and decreased locomotion, and this behavior is related to the antagonism of A1 adenosine receptors (Burbano-L. & Porfiri, 2020). However, buspirone, an anxiolytic drug, partial serotonin receptor agonist, has the binding capacity to 5-HT A1 receptors present in zebrafish (Maaswinkel, Zhu, & Weng, 2012; Maximino, da Silva, Gouveia, & Herculano, 2011).

In the new tank test, the anxious behavior of the zebrafish is evaluated against the animal's instinct in an unknown environment (Mi et al., 2019). In this study, the groups treated with KSG and FSK increased the number of cross quadrants, reduced latency and freezing time, and increased the distance traveled compared to the alcohol and control groups. FSK remained longer at the top, compared with the other groups, and KSG (200 and 400 kg/kg) showed time similar to fluoxetine, while KSG (400 mg/kg) reduced erratic swimming when compared to the negative group. In this experiment, when entering the new environment, zebrafish initially presents an inclination to remain at the bottom of the aquarium, and then gradually explores the top of the aquarium (Mezzomo et al., 2016). Thus,

the behavioral responses obtained by treatment with FSK, KSG (200 and 400 mg/kg), are associated with an antidepressant action.

Ethanol leads to a depressive effect at the cellular level in the CNS. However, it shows an increase in neuronal activity in some regions of CNS, as in the dopaminergic pathway of the mesencephalic. Its mechanism occurs by increased inhibition mediated by glycine and GABA, in which the action of GABA on $GABA_A$ receptors increases, inhibition of Ca^{2+} input and activation of specific K^+ channels, inhibition of glutamate inotropic receptor activity, inhibiting NMDA receptor, and inhibition of adenosine transport in A_1 receptors (Rang, Ritter, Flower, & Henderson, 2016). Fluoxetine is a selective serotonin receptor inhibitor. This drug has high selectivity for 5-HT carriers and an increased amount of serotonin in the synaptic cleft, resulting in the potentiation of the response (Golan, Tashjian, Armstrong, & Armstrong, 2009).

Through different mechanisms, probiotics contribute to the modulation of the host's intestinal microbiota, thus providing to the colonization of beneficial microorganisms in the intestine, and resulting in the prevention and treatment of diseases (Nadeem, Rahman, Ad-Dab'bagh, & Akhtar, 2019; Valles-Colomer et al., 2019). Davies et al. (2016) reported reducing anxiety from treatment with *L. plantarum* in adult zebrafish through protection against dysbiosis caused by stress. Borrelli et al. (2016) demonstrated that the administration of *L. rhamnosus* in zebrafish for 28 days presented an altered expression of the brain-derived neurotrophic factor (BDNF) and serotonin signaling. While Bugel & Tanguay (2018), observed hyperactive locomotor response through the bioassay in Zebrafish LCR using flavonoids, suggesting there was no mediation by $GABA_A$.

5 Conclusion

Based on the results obtained in the models and animal species employed, this study demonstrates that there is a better release of the content of isoflavones in the gastric phase and reduction in the intestinal and colon phases with treatment with KSG. The set of data from behavioral patterns obtained in the various experiments on zebrafish demonstrated that KSG and FSK present anxiolytic and antidepressant effects.

Ethics Statements File

Authors followed the guidelines of the legislation for animal experimentation established by the National Council for Animal Experimentation of Brazil (CONCEA). The

project was approved by the Ethics Committee on the Use of Animals (CEUA) of the Federal University of Amapá-UNIFAP under protocol number 007/2019.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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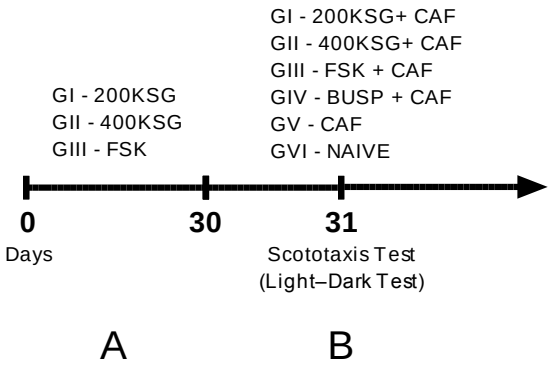

Figure 1. Graphic representation of the experimental design of the Scototaxis test in zebrafish (Light-Dark Test). Timeline: (A) oral treatment of KSG and FSK for 30 days. (B) Scototaxis test in the groups GI (KSG (200 mg/kg) + caffeine (100 mg/kg)), GII (KSG (400 mg/kg) + caffeine (100 mg/kg)), GIII (FSK + caffeine (100 mg/kg)), GIV (buspirone (25 mg/kg) + caffeine (100 mg/kg)), GV (caffeine (100 mg/kg)) and GVI.

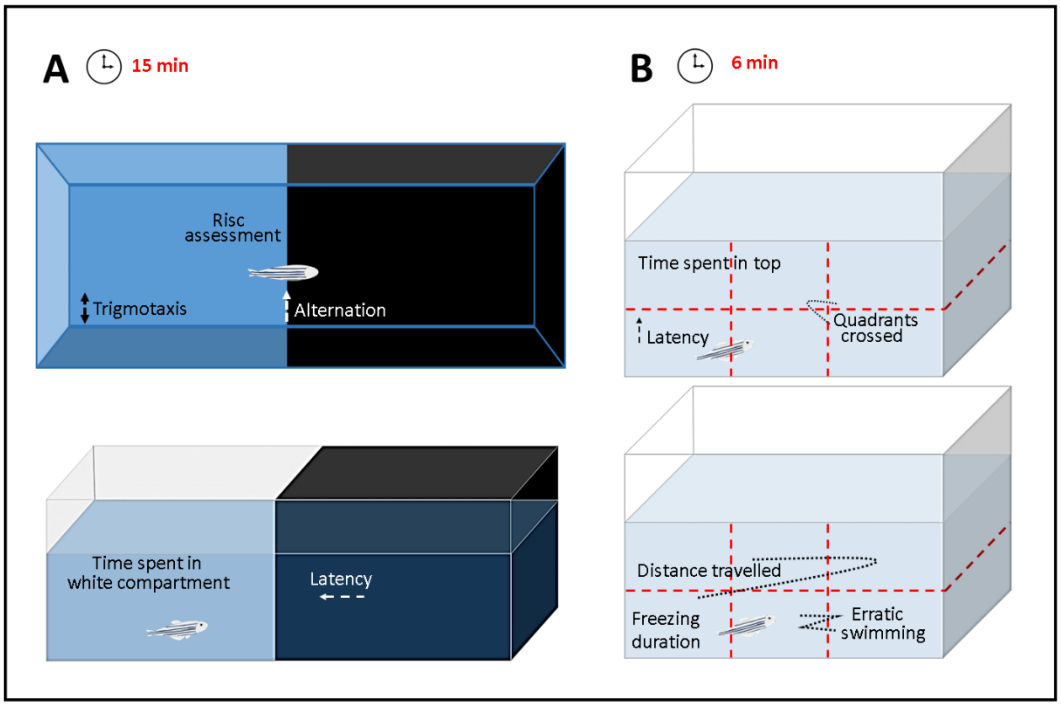

Figure 2. Behavioral tests in Zebrafish: (A) Scototaxis test in zebrafish (Light-Dark Test). (B) New Tank Test.

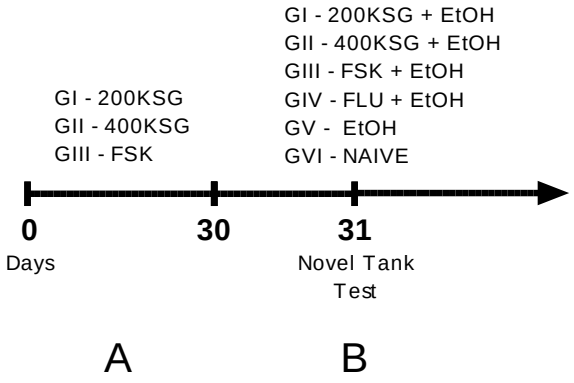

Figure 3. Graphic representation of the experimental design of the New Tank test in zebrafish (Light-Dark Test). Timeline: (A) oral treatment of KSG and FSK for 30 days. (B) New Tank test in the groups GI (KSG (200 mg/kg) + 1% ethanol), GII (KSG (400 mg/kg) + 1% ethanol), GIII (FSK + 1% ethanol), GIV (buspirone (25 mg/kg) + 1% ethanol), GV (1% ethanol) and GVI.

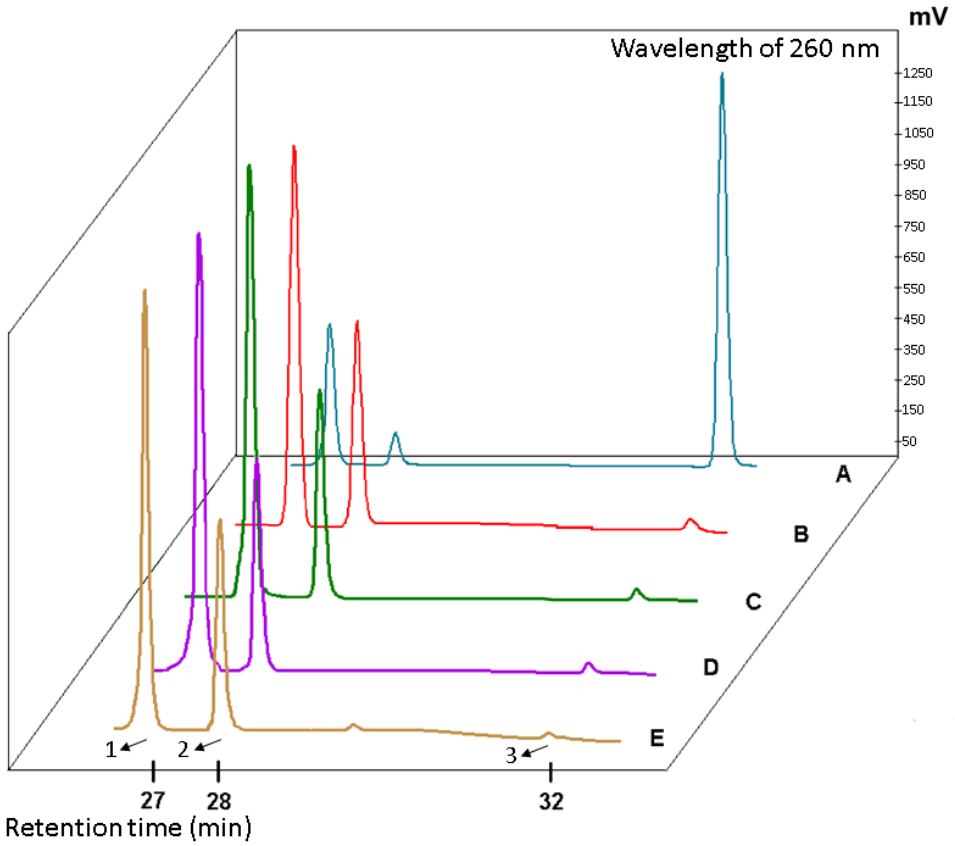

Figure 4. Chromatograms obtained by HPLC-UV with a wavelength of 260 nm. (A) co-injection of the analytical standards of the aglycone isoflavones, concentration of 25 µg/mL. (B) 4th day of fermentation. (C) gastric phase. (D) intestinal phase. (E) colon phase. Chromatographic peaks: (1) daidzein, (2) glycitein and (3) genistein.

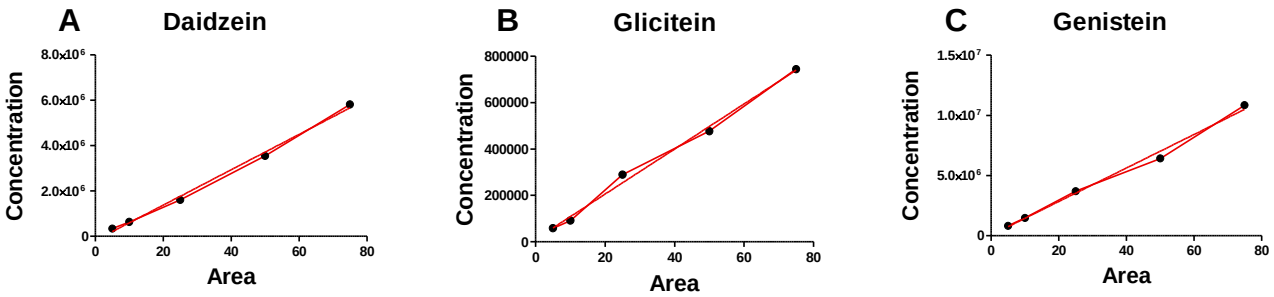

Figure 5. Graphical representation of the analytical curves obtained by HPLC-UV for (A) Daidzeína, concentration range between 5 to 75 µg/mL. Line equation: y = 77977x-188993, r^2 = 0.9952. (B) Glycitein, concentration ranges between 5 to 75 µg/mL. Line equation: y = 9715.9x + 11280, r^2 = 0.9941. (C) Genistein, concentration range between 5 to 75 µg / mL. Line equation: y = 139097x + 55020, r^2 = 0.9922.

Table 1. Concentration of aglycone isoflavones before and after in vitro digestion of kefir associated with soyben germ (KSG)

	DAIDZEIN	GLICITEIN	GENISTEIN
KSG 4th DAY OF FERMENTATION	11,74 µg/ml	40,30 µg/ml	0,81 µg/ml
GASTRIC PHASE	19,17 µg/ml	40,97 µg/ml	0,82 µg/ml
INTESTINAL PHASE	14,49 µg/ml	18,54 µg/ml	0,56 µg/ml
COLON PHASE	3,64 µg/ml	11,73 µg/ml	0,39 µg/ml

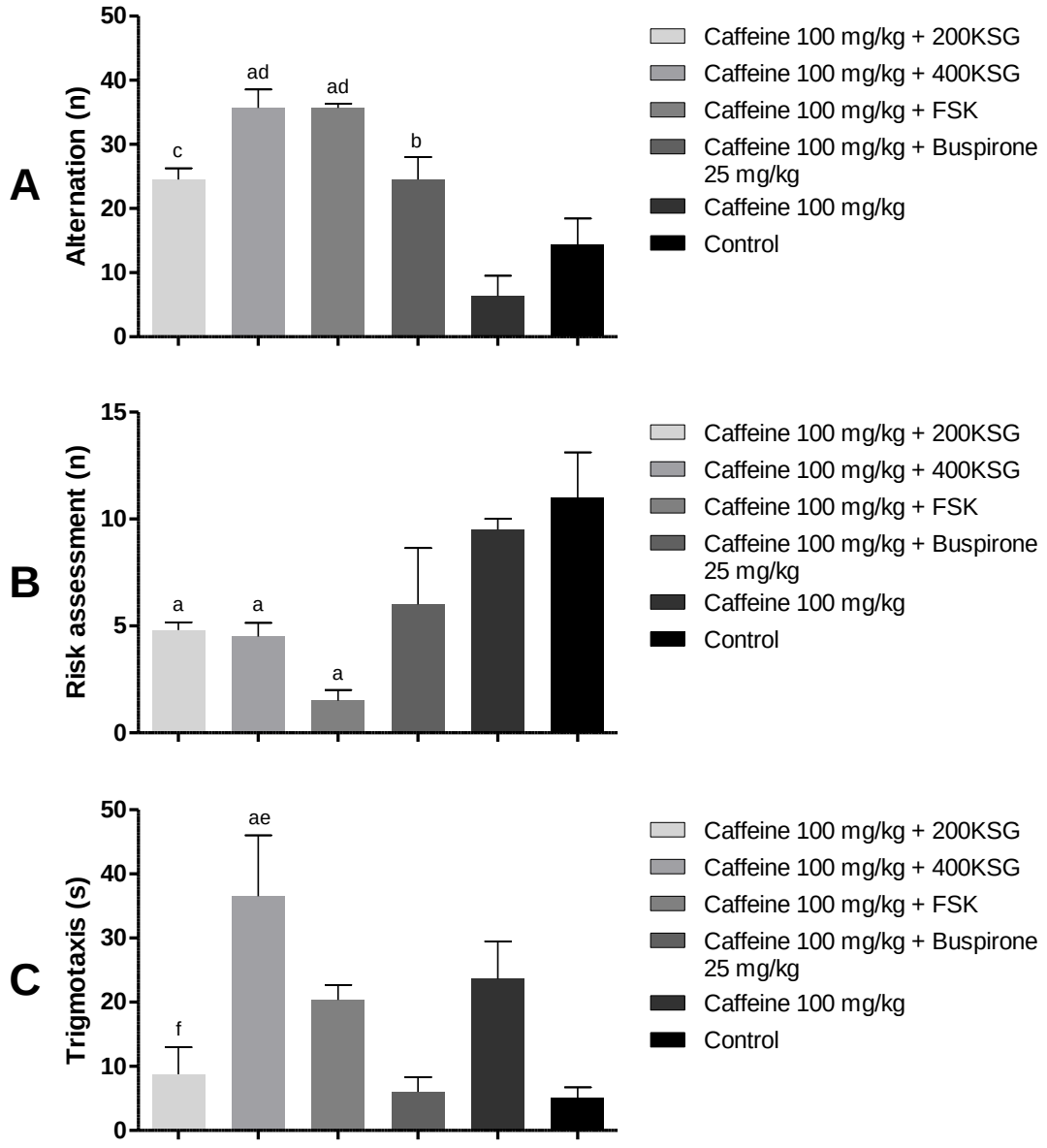

Figure 6. Effect of kefir administration associated with soybean germ (KSG), fermentation solution of kefir (FSK), buspirone and caffeine on zebrafish behavior in the scototaxis test **(A)** Alternation. **(B)** Risk assessment. **(C)** Trigmotaxis. Data are presented as mean ± SEM (n=12/group), [a] $p < 0.01$ vs. Control. [b] $p < 0.05$ vs. Caffeine; [c] $p < 0.01$ vs. Caffeine; [d] $p < 0.001$ vs. Caffeine. [e] $p < 0.01$ vs. Buspirone. [f] $p < 0.05$ vs. 400KSG, statistical analysis was done by one-way ANOVA followed by Tukey test.

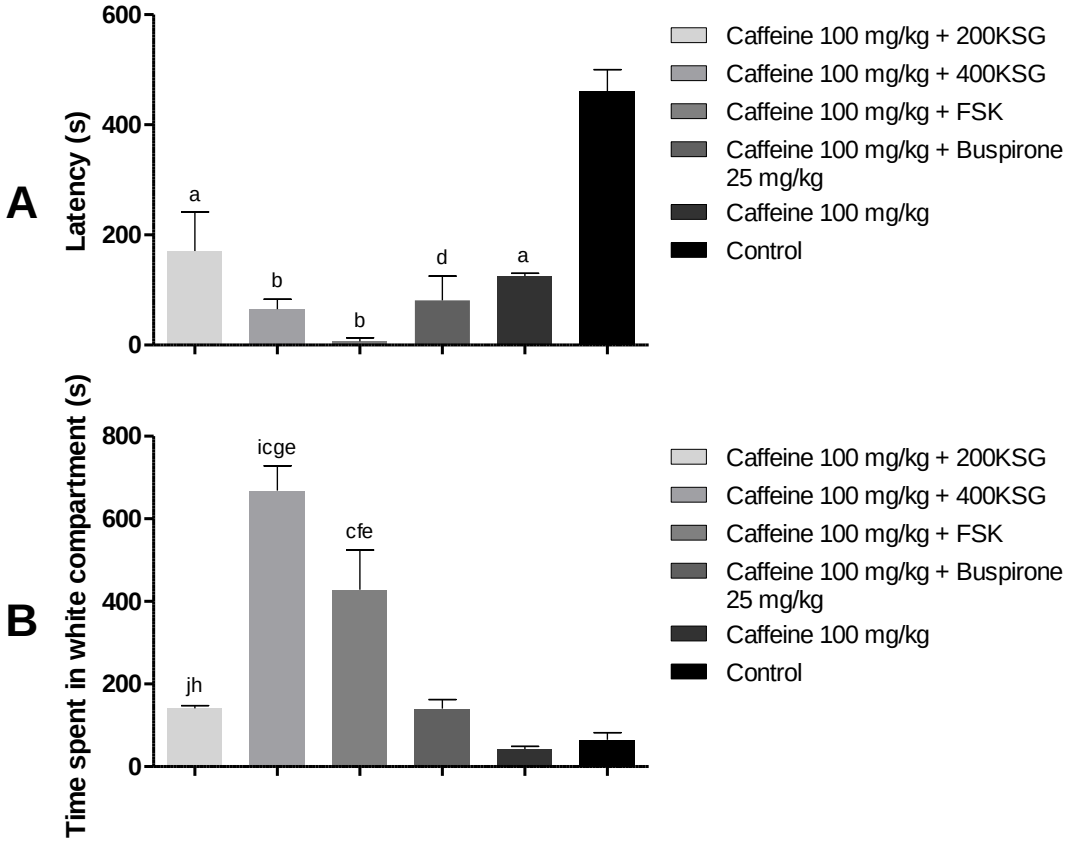

Figure 7. Effect of kefir administration associated with soybean germ (KSG), fermentation solution of kefir (FSK), buspirone and caffeine on zebrafish behavior in the scototaxis test **(A)** Latency. **(B)** Time spent in white compartment. Data are presented as mean ± SEM (n=12/group), [a] $p < 0.05$ vs. Control; [b] $p < 0.01$ vs. Control; [c] $p < 0.001$ vs. Control. [d] $p < 0.01$ vs. Caffeine; [e] $p < 0.001$ vs. Caffeine. [f] $p < 0.01$ vs. Buspirone; [g] $p < 0.001$ vs. Buspirone. [h] $p < 0.001$ vs. 400KSG. [i] $p < 0.05$ vs. FSK; [j] $p < 0.01$ vs. FSK, statistical analysis was done by one-way ANOVA followed by Tukey test.

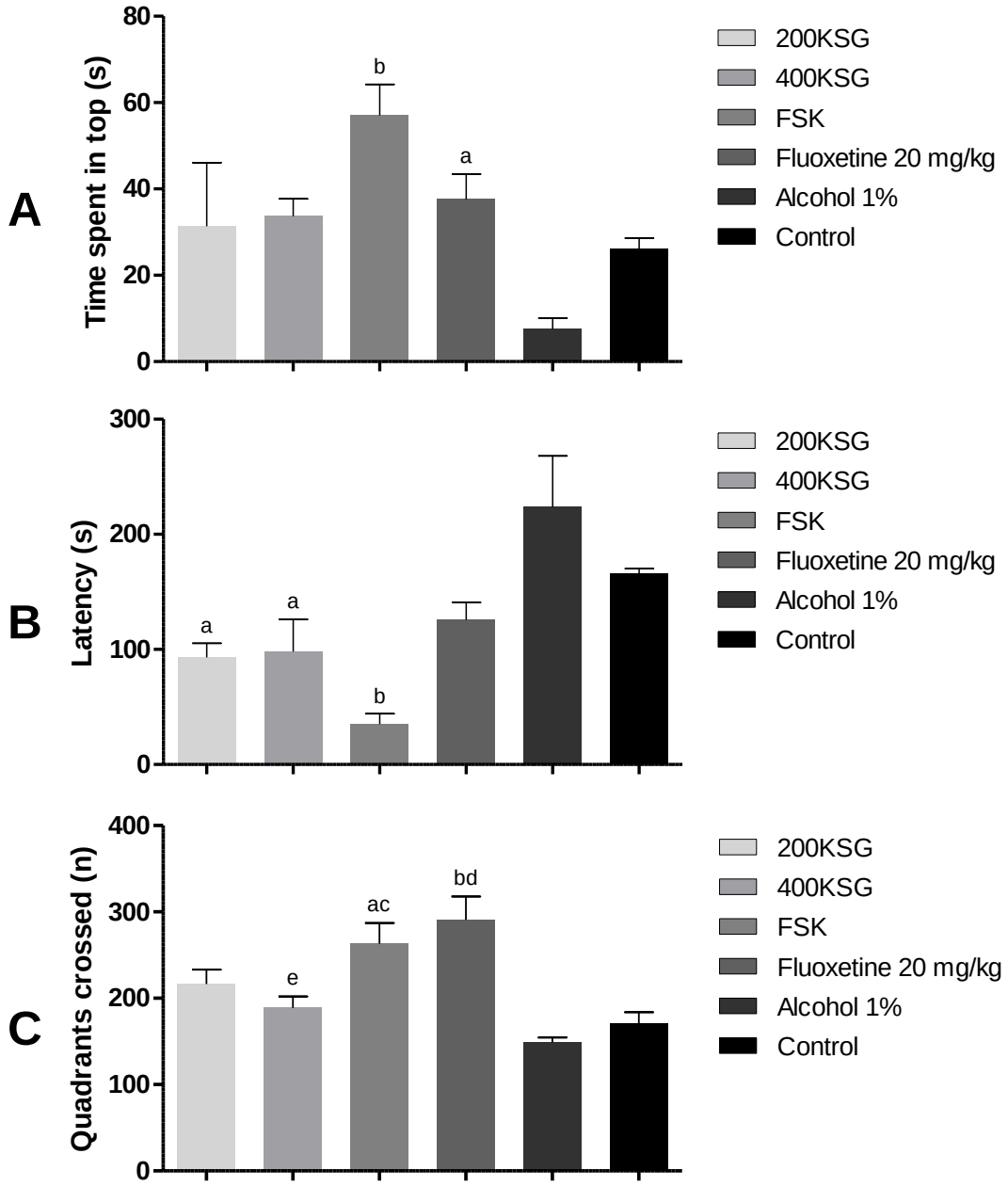

Figure 8. Effect of kefir administration associated with soybean germ (KSG), fermentation solution of kefir (FSK), fluoxetine and alcohol on the induction of depression-like behavior in zebrafish in the novel tank diving test **(A)** Time spent in top. **(B)** Latency. **(C)** Quadrants crossed. Data are presented as mean ± SEM (n=12/group), [a] $p < 0.05$ vs. Alcohol; [b] $p < 0.01$ vs. Alcohol. [c] $p < 0.05$ vs. Control; [d] $p < 0.01$ vs. Control. [e] $p < 0.05$ vs. Fluoxetine, statistical analysis was done by one-way ANOVA followed by Tukey test.

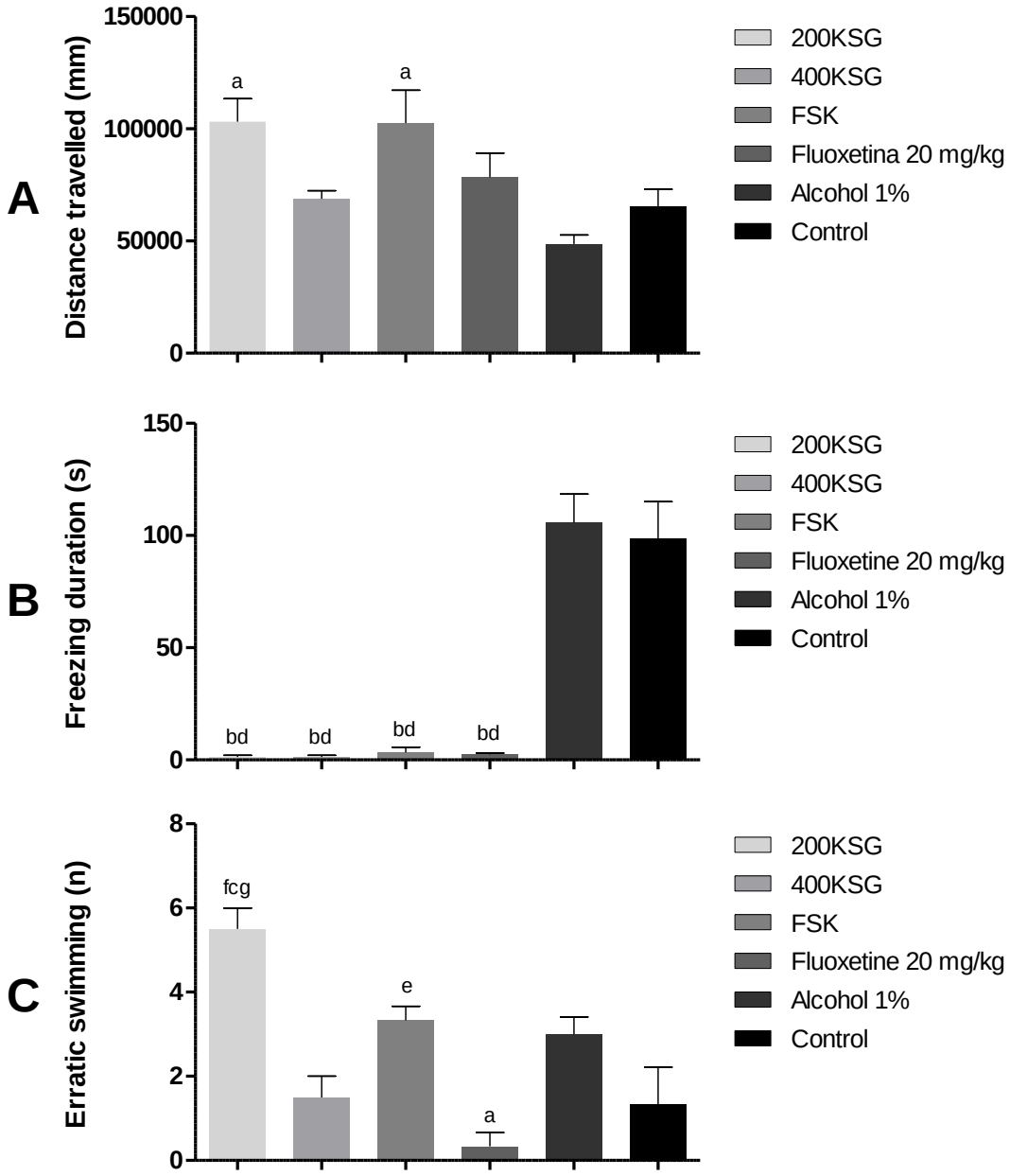

Figure 9. Effect of kefir administration associated with soybean germ (KSG), fermentation solution of kefir (FSK), fluoxetine and alcohol on the induction of depression-like behavior in zebrafish in the novel tank diving test **(A)** Distance traveled. **(B)** Freezing duration. **(C)** Erratic swimming. Data are presented as mean ± SEM (n=12/group), [a] $p < 0.05$ vs. Alcohol; [b] $p < 0.001$ vs. Alcohol. [c] $p < 0.01$ vs. Control; [d] $p < 0.001$ vs. Control. [e] $p < 0.05$ vs. Fluoxetine; [f] $p < 0.001$ vs. Fluoxetine. [g] $p < 0.01$ vs. 400KSG, statistical analysis was done by one-way ANOVA followed by Tukey test.

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ANEXOS E APÊNDICES

Anexo 1 – Parecer do Comitê de Ética

UNIVERSIDADE FEDERAL DO AMAPÁ
PRÓ-REITORIA DE PESQUISA E PÓS- GRADUAÇÃO
COMITE DE ÉTICA NO USO DE ANIMAIS – CEUA – UNIFAP

CERTIFICADO

A Comissão de Ética no Uso de Animais da Universidade Federal do Amapá **APROVOU,** na data de 12 de março de 2019, o parecer referente ao protocolo no. **007/2019** e certifica que o Projeto de Pesquisa intitulado "**ESTUDO DA AÇÃO ANTIDEPRESSIVA E ANSIOLÍTICA DO KEFIR ASSOCIADO AO GÉRMEN DE SOJA EM CAMUNDONGOS SWISS E Danio rerio (ZEBRAFISH)**" coordenado por **Ester Lopes de Melo**, está de acordo com os princípios de ética e bem estar animal.

CERTIFICATE

The Ethics Committee on Animal Use of the Amapá Federal University **APPROVED** at the meeting of 12 March 2019, the final decision about the Protocol **007/2019** and certify that the research project entitled "**ESTUDO DA AÇÃO ANTIDEPRESSIVA E ANSIOLÍTICA DO KEFIR ASSOCIADO AO GÉRMEN DE SOJA EM CAMUNDONGOS SWISS E Danio rerio (ZEBRAFISH)**" coordinated by **Ester Lopes de Melo**, is in accordance with the principles of ethics and animal welfare.

Macapá, 12 de março de 2019

Prof. Tit. José Carlos Tavares Carvalho
Presidente CEUA-UNIFAP
Port. No. 1733/2014

Coordenador(a) do Comitê de Ética no Uso de Animais - CEUAS
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Anexo 2 – Normas de publicação do periódico

Gmail **José Carlos Tavares <jctcarvalho@gmail.com>**

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