MARIA TATIANA SOARES MARTINS

AÇÃO DA GENISTEÍNA NA PREVENÇÃO DO CÂNCER COLORRETAL E EFEITOS MORFOFISIOLÓGICOS INTESTINAIS DE BEBIDAS À BASE DE SOJA E DO LEITE DE VACA

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Biologia Celular e estrutural, para obtenção do título de *Doctor Scientiae*.

Orientador: Sérgio Luis Pinto da Matta

Coorientadoras: Sirlene Souza Rodrigues Sartori
Reggiani Vilela Gonçalves

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Maria Tatiana Soares Martins
Autor

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Orientador

Ao meu filho Davi.

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“Se eu vi mais longe, foi por estar sobre ombros de gigantes.”
(Isaac Newton)

RESUMO

MARTINS, Maria Tatiana Soares, D.Sc., Universidade Federal de Viçosa, julho de 2024. **Ação da genisteína na prevenção do câncer colorretal e efeitos morfofisiológicos intestinais de bebidas à base de soja e do leite de vaca.** Orientador: Sérgio Luis Pinto da Matta. Coorientadoras: Sirlene Souza Rodrigues Sartori; Reggiani Vilela Gonçalves

O câncer colorretal (CRC) é altamente invasivo, considerado o segundo maior nas causas de mortes por câncer no mundo. A genisteína, principal isoflavona presente na soja e derivados, é amplamente estudada como candidata à prevenção de CRC devido às suas propriedades antioxidantes e anti-inflamatórias. Entretanto, alguns estudos contrariam essas evidências. As bebidas à base de soja (BABS) são alimentos derivados da soja que contêm genisteína. As BABS são amplamente comercializadas e utilizadas em substituição ao leite de vaca. Devido à presença de isoflavonas, como a genisteína, essas bebidas são consideradas anti-inflamatórias e antioxidantes intestinais. Por outro lado, as BABS contêm inibidores de proteases que podem causar efeitos adversos no intestino. Dessa forma, o objetivo desse estudo foi integrar, sistematicamente, estudos pré-clínicos para investigar o potencial da genisteína como alternativa viável para a prevenção e tratamento de CRC, focando na atuação da genisteína em vias de sinalização intracelular intensamente alteradas em condições de câncer. Além disso, objetivou-se avaliar o efeito de duas marcas de BABS comerciais sobre a morfofisiologia intestinal de camundongos Balb/c, comparado ao leite de vaca. A revisão sistemática foi realizada de acordo com o guia para revisões sistemáticas PRISMA, utilizando as plataformas PubMed-Medline, Scopus, Web of Science e Embase. Todos os estudos pré-clínicos que investigaram o efeito da genisteína sobre o CRC até setembro de 2023 foram incluídos. A genisteína possui efeito proliferativo em baixas doses (0.5-2 μM) e antiproliferativo em altas doses (10-100 μM). Os efeitos protetivos da genisteína estão relacionados ao seu potencial de inibição das enzimas tirosina quinase e seu potencial estrogênico parece ser responsável pelo seu efeito proliferativo. De acordo com estudos *in vitro*, genisteína controla a proliferação celular modulando as vias WNT, TGFβ, NFκB, PI3K/Akt/MAPK, vias p53-dependente, respostas oxidantes e inflamatórias e síntese de vitamina D. Os estudos *in vivo* suportam os efeitos quimioprotetivos da genisteína. Para avaliar o efeito das BABS comerciais sobre a morfofisiologia intestinal de camundongos Balb/c, foram utilizados 4 tratamentos: água destilada (controle), BABS

1, BABS 2 e leite de vaca. Foram 6 repetições por tratamento, sendo cada animal uma repetição. Todos os tratamentos foram administrados uma vez ao dia, por gavagem, durante 42 dias. O intestino foi coletado para as análises de enzimas digestivas e histomorfométricas. Em geral ambos, BABS e leite de vaca, não prejudicaram a morfofisiologia intestinal. O leite de vaca aumentou o número de células de Paneth. As BABS diminuíram o número de mitoses e a atividade de tripsina e de lipase. As BABS são alternativas viáveis ao leite de vaca para indivíduos que necessitam de ingestão baixa de calorias. Porém, o efeito de inibição de tripsina e de redução de mitoses levanta alerta sobre o seu consumo, principalmente em indivíduos saudáveis. Sugere-se que a escolha entre o consumo de BABS e de leite de vaca seja acompanhada por profissionais.

Palavras-chave: Revisão sistemática. Soja. Inibidor de tripsina. Inflamação.

ABSTRACT

MARTINS, Maria Tatiana Soares, D.Sc., Universidade Federal de Viçosa, July, 2024. **Action of genistein in the prevention of colorectal cancer and intestinal morphophysiological effects of soy-based beverages and cow's milk.** Adviser: Sérgio Luis Pinto da Matta. Co-adviser: Sirlene Souza Rodrigues Sartori; Reggiani Vilela Gonçalves

Colorectal cancer (CRC) is an invasive tumor, ranking as second in cancer-causing death all around the world. Genistein, the main isoflavone found in soy and its derivatives, is widely studied as a candidate for CRC prevention due to its antioxidant and anti-inflammatory properties. However, some studies contradict this evidence. Soy-based beverages (BABS) are a soy-derived foods that contain genistein. BABS are widely marketed and used as a substitute for cow's milk. Due to the presence of isoflavones, such as genistein, BABS acts as intestinal anti-inflammatory and antioxidant. On the other hand, BABS contain protease inhibitors that may cause adverse effects in the intestine. Thus, the aim of this study was to systematically integrate preclinical studies to investigate the potential of genistein as a viable alternative for the prevention and treatment of CRC, focusing on the role of genistein in intracellular signaling pathways that are intensely altered in cancer conditions. Furthermore, the aim was to evaluate the effect of two commercial BABS brands on the intestinal morphophysiology of Balb/c mice, compared to cow's milk. Was used the PRISMA guidelines from the PubMed-Medline, Scopus, Web of Science, and Embase platforms. All studies that investigated the effect of genistein on CRC in preclinical models up to September 2023 were included. Genistein is a proliferative inductor in low doses (0.5-2 μM) and a chemoprotective in high doses (10-100 μM). The tumor protective abilities of genistein have been linked to their tyrosine kinase inhibitor capacity, and its estrogenic effect is related to cell proliferation. Based on *in vitro* studies, genistein controls CRC by modulating the WNT, TGFβ, NFκB, PI3K/Akt/MAPK, p53-dependent pathways NFκB, the oxidant and inflammatory response, and vitamin D synthesis. The *in vivo* studies support the chemoprotective effect of genistein. To analyze the effect of commercial BABS on the intestinal morphophysiology of Balb/c mice, four treatments were used: distilled water (control), BABS 1, BABS 2 and cow's milk. There were 6 replications per treatment. All treatments were administered once a day, by gavage, for 42 days. The intestine was collected for digestive enzyme and histomorphometric analysis. Generally, both BABS

and cow's milk are not harmful to intestinal health. Cow's milk increased the number of Paneth cells. BABS decreased the number of mitosis and the activity of trypsin and lipase. BABS are a viable alternative to cow's milk for people considering low calorie intake. However, the effect of inhibiting trypsin and reducing mitoses raises a warning about its consumption, especially in healthy individuals. It is suggested that the choice between the consumption of BABS and cow's milk be monitored by professionals.

Keywords: Systematic review. Soybean. Trypsin inhibitor. Inflammation.

LISTA DE SIGLAS E ABREVIATURAS

ABE Altura da Borda Estriada

AE Altura do Epitélio

AKT "Protein Kinase B"

AOM Azoximetano

APC "Adenomatous Polyposis Coli"

ATF3 *"Transcription Factor 3 gene"*

ATM "Ataxia Telangiectasia Mutated"

BABS Bebidas à Base de Soja

BAX "*Bcl-2 Associated X protein"*

BCAC *"β-catenin Deposits Accumulated in Crypt Cells"*

BCL2 "*B-cell lymphoma 2"*

CASP 3 Caspase 3

CASP 8 Caspase 8

CCI Camada Muscular Circular Externa

CCND1 ciclina D

CDKN1A/P21 "Cyclin-Dependent Kinase Inhibitor 1A"

CDKN1B/P27kip1 *"Cyclin-dependent Kinase Inhibitor 1B"*

CDKN2A/P16INK4a "*Cyclin-Dependent Kinase Inhibitor 2A"*

CLE Camada Muscular Longitudinal Externa

COX2 Enzima Ciclooxigenase 2

CRC Câncer Colorretal

CYP Enzima Citocromo P450

CYP24 Enzima 24-hidroxilase

CYP27B1 Enzima 25 Hidroxivitamina D1-α-Hidroxilase

DKK1 "*Dickkopf Protein"*

DMH Dimetilhidrazina

EGF "*Epidermal Growth Factor"*

EGFR "Epidermal Growth Factor Receptor"

ERK ½ *"Extracellular Signal-Regulated Kinases 1 and 2"*

ESR Receptor de estrógeno

FLT4 "*Fms-like Tyrosine Kinase 4"*

FOXO3 "*Forkhead Box O3"*

FRAT 1 “*Frequent Aberration in Colorectal Tumors 1”*

GDF15 “*Nonesteroidal Anti-Inflammatory Drug-Activated Gene (NAG-1)”*

GPER1 Receptor 1 de estrógeno acoplado à proteína G

ID1 “*Inhibitor of DNA Biding/Differetiantion-1”*

IGF1R “Receptor Tyrosine Kinase Insulin-like Growth Factor receptor 1”

KCNK9 Canal de Potássio

MAPK “Mitogen-Activated Protein Kinase”

MMP1 Metaloproteinase de matriz 1

MMP9 Metaloproteinase de matriz 9

MYC c-Myc

NFκB “*Nuclear Factor kappa B”*

PCNA “Proliferating Cell Nuclear Antigen”

PI3K *“Phosphatidylinositol 3-kinase”*

RAS Proteína GTPase

SFRP “*Signal Recognition Particle”*

SGK1” *Serum and Glucocorticoid-regulated Kinase 1”*

Sp-1 *“Specificity Protein 1”*

TCF/LEF “*Transcription Factors T Cell Factor/ Lymphoid Enhancer Factor”*

TGF-β “*Transforming Growth Factor* β”

TIMP1 Inibidor 1 de Metaloproteinase de matriz

TM Túnica Muscular

TNF-α Fator de Necrose Tumoral

TP53 “*P53 Protein”*

VDR Receptor de Vitamina D

WIF1 “*Wnt Inhibitory Factor 1”*

WNT “*Wingless Glycoprotein”*

SUMÁRIO

1 CAPÍTULO 1: INTRODUÇÃO GERAL

1.1 Leite de vaca

O consumo de leite de vaca é recomendado mundialmente pelos guias alimentares (Global Food-Based Dietary Guidelines - FBDGs) por ser fonte de cálcio e por conter múltiplas vitaminas, minerais essenciais e proteínas de alta qualidade nutricional (Comerford *et al*., 2021). Além do papel nutricional, as proteínas do leite contêm peptídeos bioativos (25-130 mg/g de proteína) (Meisel *et al*., 1986) que apresentam importantes propriedades biológicas, como atividade enzimática, bactericida e hormonal, além de possuírem fatores de crescimento (Bos *et al*., 2000). Diversos estudos relatam os benefícios do leite de vaca e de seus compostos bioativos para a saúde intestinal, destacando a estimulação de enzimas digestivas (Hara *et al*., 1992); efeito anti-inflamatório (Ulven *et al*., 2019) e a participação no desenvolvimento da microbiota intestinal em crianças (Guo *et al*., 2024).

Por outro lado, a intolerância à lactose (Obermayer-Pietsch *et al*., 2004) e/ou desenvolvimento de alergias às proteínas (Vojdani *et al*., 2018), e a crescente discussão sobre a sustentabilidade e sobre o consumo de produtos de origem animal (Beacom *et al*., 2021), são algumas limitações ao uso dessa bebida pela população. Além disso, há evidências que o consumo excessivo do leite de vaca, principalmente rico em gordura, está associado ao desenvolvimento de doenças crônicas, como diabetes tipo 2 e doenças cardiovasculares (Drouin-Chartier *et al*., 2016). Essas limitações têm estimulado o aumento da procura por bebidas de origem vegetal em detrimento do leite de vaca, como as bebidas à base de soja (BABS) (Beacom *et al*., 2021).

1.2 Bebidas à base de soja (BABS)

As BABS são amplamente comercializadas e muito utilizadas em substituição ao leite de vaca. Dentre as bebidas de origem vegetal, as BABS são as que mais se assemelham nutricionalmente ao leite de vaca, por conter proporções nutricionais similares de energia, de proteína, de aminoácidos essenciais e não essenciais, e menor proporção de gordura e de carboidratos. Dentre suas proteínas, 65% são as proteínas de reserva glicinina e β-conglicinina. Outras proteínas importantes são a legumina e a vicilina que são parte da família das globulinas. A glicinina é considerada mais importante do ponto de vista nutricional por apresentar maiores concentrações

de aminoácidos sulfurados (metionina e cisteína) (Medic, 2014). Embora a soja contenha menor concentração de cálcio e de algumas vitaminas (B2, B12, D2), esses nutrientes são frequentemente adicionados às BABS. Entretanto, a completa substituição do leite de vaca sem acompanhamento profissional pode gerar deficiências nutricionais a longo prazo (Walther *et al*., 2022).

Apesar das BABS serem consideradas substitutos aceitáveis do leite de vaca, principalmente em casos em que a ingestão do leite de vaca é inviável, algumas características negativas inerentes à soja devem ser consideradas. A soja contém fatores antinutricionais, como os inibidores de proteases (Kong *et al*., 2022) que podem causar efeitos adversos no intestino, especialmente em soja e derivados que não são devidamente aquecidos durante seu processamento. A soja é o vegetal que contém maior concentração desses inibidores, sendo os mais prevalentes o “Kunitz trypsin inhibitor” (KTI) e o “Bowman-Birk inhibitor” (BBI) (Xiao *et al*., 2012). Ainda não existe regulamentação que estabeleça níveis aceitáveis de inibidores ativos de proteases nos alimentos à base de soja, porém, sabe-se que altos níveis (acima de 10%) de atividade dessas moléculas prejudicam a digestibilidade e a biodisponibilidade de proteínas (Muzquiz *et al*., 2012). Além disso, particularmente os inibidores de tripsina, causam hipertrofia das células acinares, aumento do peso do pâncreas e da secreção pancreática em ratos (Xiao *et al*., 2021), o que pode gerar inflamação do intestino (Róka *et al*., 2008). Em humanos, há evidências que grande ingestão de leite de soja resulta em pancreatite (De Souza *et al*., 2021) e até mesmo câncer de pâncreas (Yamagiwa *et al*., 2020). Portanto, novos estudos, principalmente relacionados aos efeitos destas bebidas sobre a morfologia intestinal, são necessários, uma vez que os estudos existentes estão basicamente relacionados aos efeitos da soja sobre a atividade de enzimas digestivas.

Apesar de ainda não existirem estudos que estabeleçam níveis seguros de inibidores de tripsina nas BABS, níveis até 10% parecem ser seguros (Guerrero-Beltrán *et al*., 2009). A manutenção desses níveis (4-10%), além de prevenir a inativação das demais proteínas, o que previne alterações no sabor e no valor nutritivo do alimento (Kwok and Niranjan, 1995), podem atuar contra a inflamação e o câncer intestinal (Losso, 2008; Basson *et al*., 2021). Junto com esses inibidores, outros componentes da soja, como as isoflavonas (Křížová *et al*., 2019), também podem prevenir doenças inflamatórias intestinais, como a colite ulcerativa (Levit *et al*., 2017; Sadeghi *et al*., 2020) e até mesmo o câncer de cólon (Wang *et al*., 2024).

1.3 Câncer colorretal

O câncer colorretal (CRC) é o terceiro mais comum entre os cânceres humanos, representando 10% de todos os diagnósticos de câncer. É o segundo tipo de câncer com maior incidência de morte no mundo. A tendência da doença é aumentar nos próximos anos, devido basicamente ao aumento da expectativa de vida e ao crescimento populacional (Sung *et al*., 2021). O CRC não é facilmente detectável em estágios iniciais, portanto, essa alta incidência ocorre devido ao atraso no diagnóstico, principalmente em países em desenvolvimento, que são privados de técnicas mais avançadas (Issa and Noureddine, 2017). Quimioterapia e cirurgia são as abordagens mais comuns para o tratamento de CRC, mas as taxas de cura ainda são baixas (Kuipers *et al*., 2015). Na quimioterapia, alguns fatores como a resistência das células tumorais e a toxicidade sistêmica explicam a baixa eficiência da abordagem (Bukowski *et al*., 2020). Portanto, estudos sobre novos agentes antitumorais, incluindo aqueles derivados de compostos naturais, são urgentemente necessários.

Em geral, indivíduos a partir de 50 anos de idade são considerados de risco e, portanto, são submetidos a avaliações preventivas. Entretanto, há uma tendência de aumento de CRC em indivíduos mais jovens. Principalmente nesses indivíduos, as principais causas de desenvolvimento de CRC não são por predisposição genética e sim por fatores ambientais como a dieta (Spaander *et al*., 2023). Estudos associam a adoção de hábitos alimentares saudáveis, incluindo grãos, fibras e vegetais com a redução de ocorrência de CRC (Ryan-Harshman and Aldoori, 2007; Veettil *et al*., 2021).

Um dos vegetais conhecido por sua atuação na prevenção de CRC é a soja, devido a presença de fenóis, peptídeos bioativos e grandes quantidades de isoflavonas, dentre elas, genisteína, daidzeína, gliciteína, formononetina, biochanina A e equol. Observa-se relação direta entre o consumo de soja e derivados e menor incidência de CRC em Indivíduos asiáticos, população que mais consome soja no mundo (Yu *et al*., 2016). Para ilustrar, indivíduos asiáticos ingerem 20-50 mg/dia de isoflavonas (Arai *et al*., 2000), enquanto os europeus ingerem apenas 0,437 mg/dia (Grace *et al*., 2004). A recomendação de ingestão segura de isoflavonas pela “Food and Drug Administration” (FDA) é de 50 mg/dia (Krížová *et al*., 2019).

Devido à sua similaridade estrutural aos estrógenos endógenos, as isoflavonas da soja são conhecidas como fitoestrógenos, sendo capazes de exercer efeitos estrogênios ou antiestrogênicos, dependendo da concentração. Além disso, esses compostos possuem propriedades antioxidantes e anti-inflamatórias. As isoflavonas são capazes de inibir enzimas tirosinas quinases, portanto, modificando diversas vias de sinalização intracelular relacionadas à proliferação celular, resultando em parada do ciclo celular, redução de proliferação e migração celular e aumento de apoptose, o que as caracteriza com grande potencial quimioprotetivo (Kim, 2021).

1.4 Genisteína

A genisteína (CHEBI:28088) é a principal isoflavona da soja, com concentração média de 17,89 mg/100g de soja (Sulistyowati *et al*., 2019). É vastamente estudada como candidata à prevenção de CRC em ensaios pré-clínicos *in vitro* (Alorda-Clara *et al*., 2022; Rendón *et al*., 2022; Cheng *et al*., 2023) e mais timidamente em ensaios pré-clínicos *in vivo* (Sekar et at., 2016; Song *et al*., 2018) e clínicos (Pintova *et al*., 2019; Chen te al., 2020). Os benefícios da genisteína estão associados ao seu potencial antioxidante e anti-inflamatório no intestino (Verdrengh *et al*., 2003; Valsecchi *et al*., 2011), atuando na melhoria da barreira intestinal e na modulação das células inflamatórias (Abron *et al*., 2018; He *et al*., 2021). O trato gastrointestinal, além de suas propriedades relacionadas à digestão, apresenta alta atividade imunológica. A mucosa possui uma potente barreira capaz de permitir a entrada nutrientes enquanto restringe a passagem de antígenos (Funk *et al*., 2020; Spencer *et al*., 2023) (fig. 1). Alterações na homeostase desta estrutura afeta a integridade intestinal (fig. 2), levando a doenças inflamatórias intestinais, como doença de Crohn e colite ulcerativa (Martini *et al*., 2017; Ghosh *et al*., 2020). Indivíduos com inflamação crônica intestinal apresentam maior chance de desenvolver CRC (Ahmad *et al*., 2021; Wang *et al*., 2021). Portanto, a genisteína e os derivados de soja poderiam contribuir para a prevenção de CRC por suas atividades anti-inflamatórias.

Os mecanismos de ação da genisteína são devidos à sua similaridade com a molécula de estrógeno (fig. 3), que a permite se ligar aos receptores de estrógenos (ESRs) e gerar efeitos similares aos estrógenos endógenos (Zava and Duwe, 1997, Mukund, 2020). A genisteína possui maior afinidade pelo receptor de estrógeno 2 (ESR2) (Manas *et al*., 2004), que é a isoforma mais abundante nas células do cólon (Maingi *et al*., 2020). Essa molécula também é capaz de inibir a atividade de

enzimas tirosina quinases (Akiyama *et al*., 1987), interferindo assim em diversas vias de sinalização intracelular dependentes de fosforilação. Além disso, ela inibe a enzima DNA Topoisomerase II *in vitro* (Salti *et al*., 2000) e *in vivo* (Baechler *et al*., 2016), prejudicando a manutenção da estrutura do DNA ideal para a correta replicação do DNA. Dessa forma, a genisteína é capaz de inibir o ciclo celular, diminuir a proliferação celular e a metástase e aumentar a apoptose, que a elege como boa candidata à prevenção de CRC. Por outro lado, alguns estudos sugerem que a genisteína induz a proliferação celular (Moore *et al*., 2007; Chen *et al*., 2018). É possível que esses efeitos opostos sejam dose-dependentes. Portanto, considerando as divergências sobre o uso genisteína na prevenção de CRC e a inadequação ainda existente dos tratamentos quimioterápicos, faz-se necessária uma revisão dos estudos existentes para avaliar a eficácia e a segurança da genisteína como alternativa viável para prevenção e tratamento de CRC.

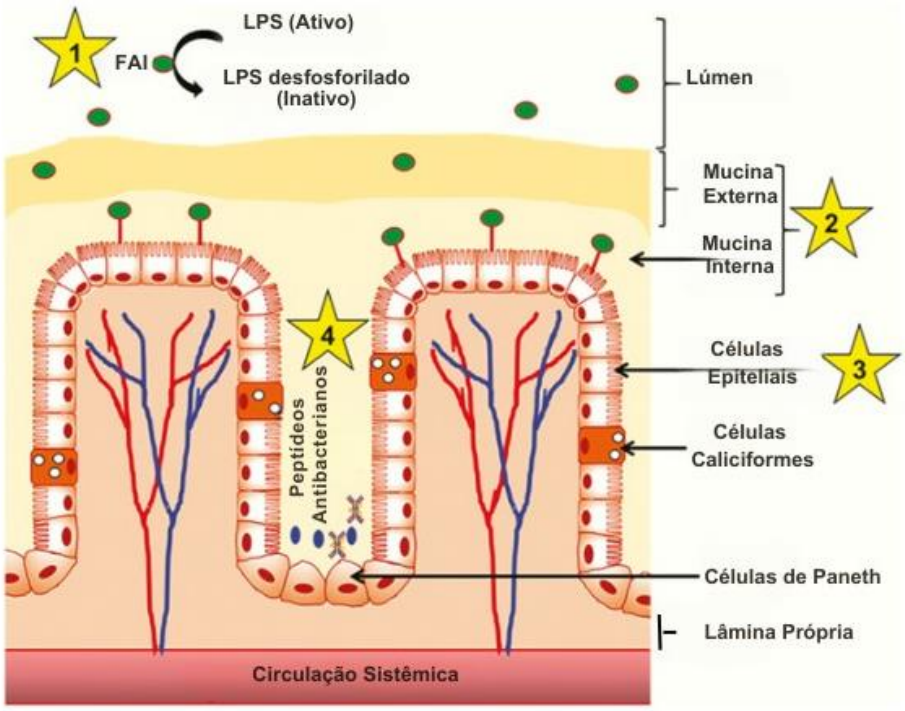

Figura 1 – Representação esquemática dos componentes que formam a barreira intestinal. Em **1**, a fosfatase alcalina intestinal (FAI), secretada pelos enterócitos, neutraliza lipopolissacarídeos de membrana de bactérias (LPS). Em **2**, mucinas secretadas pelas células caliciformes formam uma barreira física que limita o contato de patógenos com as células epiteliais. Em **3**, as células epiteliais são fortemente unidas umas às outras por proteínas que formam as junções de oclusão, formando uma barreira física entre o meio externo e interno. Dentre estas células, encontram-se as células caliciformes

que secretam mucinas responsáveis pela formação da camada observada em 2 e também as células de Paneth. Estas células (**4**) sintetizam e secretam peptídeos antimicrobianos. Todos esses mecanismos modulam a ativação do sistema imune, prevenindo o desencadeamento de respostas inflamatórias. Fonte: (Ghosh *et al*., 2020).

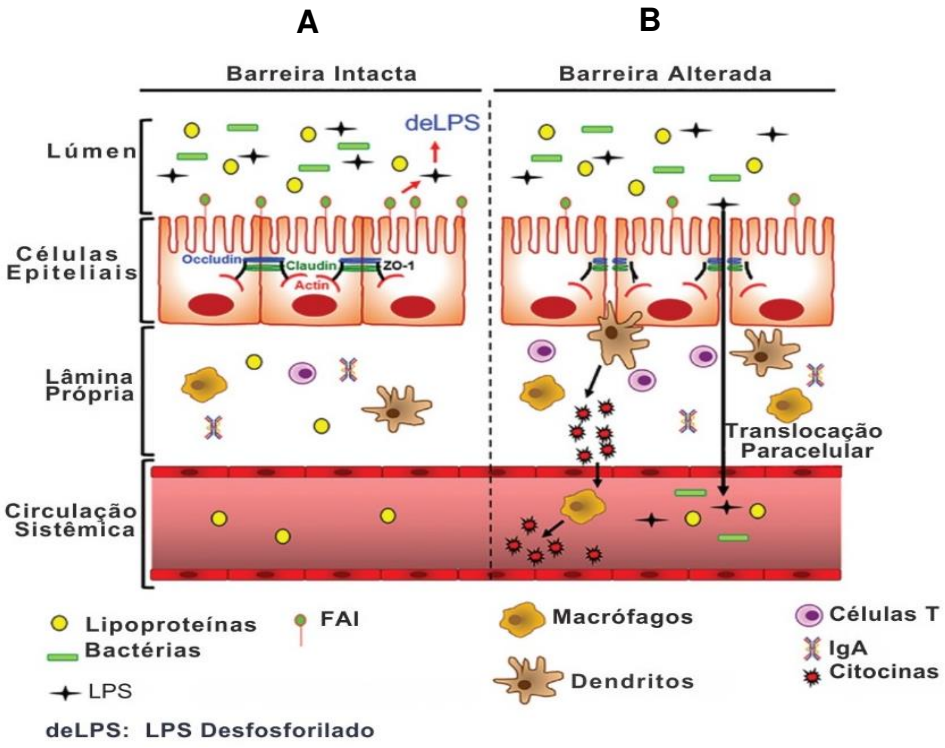

Figura 2 – Representação esquemática da barreira intestinal em condição basal (**A**) e alterada (**B**). Em condições normais, a barreira está ativa e, desta forma, os produtos da digestão são absorvidos pelos enterócitos. Na presença de um estressor, ex. patógeno, essa barreira de defesa é alterada (**B**), permitindo a passagem de antígenos em direção à lâmina própria e ao sangue. Nestas condições, células de defesa liberam citocinas inflamatórias, que podem induzir a inflamação sistêmica. Fonte: (Ghosh *et al*., 2020). LPS, lipossacarídeo de membrana bacteriana; FAI, fosfatase alcalina intestinal; IGa, imunoglobulina A

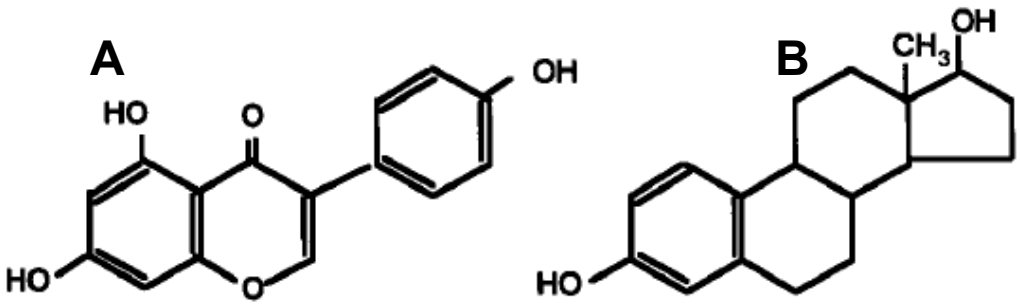

Figura 3 – Estrutura da molécula de genisteína (A) e de estrógeno (B). Fonte: Ishimi *et al*., 1999.

1.5 Objetivos

1.5.1 Objetivo Geral

Realizar uma revisão sistemática de estudos pré-clínicos para avaliar a eficácia e segurança da genisteína como opção ao tratamento de CRC. Também, comparar o efeito de bebidas à base de soja e de leite de vaca sobre a morfometria intestinal e atividade de enzimas digestivas, de camundongos Balb/c.

1.5.2 Objetivos específicos

- Investigar as vias de sinalização intracelular afetadas pela genisteína;
- Determinar a melhor dose de genisteína para utilizar na prevenção/tratamento de CRC;
- Investigar qual é a melhor opção de consumo entre as bebidas analisadas, de acordo com as necessidades e particularidades humanas;
- Orientar sobre o consumo humano de bebidas à base de soja e de leite vaca.

Referências

ABRON, J. D.; SINGH, N. P.; PRICE, R. L.; NAGARKATTI, M.; NAGARKATTI, P. S.; SINGH, U. P. Genistein induces macrophage polarization and systemic cytokine to ameliorate experimental colitis. **PLoS One**, v. 13, n. 7, p. e0199631, 2018.

AHMAD KENDONG, S. M.; RAJA ALI, R. A.; NAWAWI, K. N. M.; AHMAD, H. F.; MOKHTAR, N. M. Gut dysbiosis and intestinal barrier dysfunction: potential explanation for early-onset colorectal cancer. **Frontiers in Cellular and Infection Microbiology**, v. 11, p. 1244, 2021.

AKIYAMA, T.; ISHIDA, J.; NAKAGAWA, S.; OGAWARA, H.; WATANABE, S. I.; ITOH, N.; SHIBUYA, M.; FUKAMI, Y. Genistein, a specific inhibitor of tyrosine-specific protein kinases. **Journal of Biological Chemistry**, v. 262, n. 12, p. 5592-5595, 1987. DOI 10.1016/S0021-9258(18)45614-1.

ALORDA-CLARA, M.; TORRENS-MAS, M.; MORLA-BARCELO, P. C.; SASTRE-SERRA, J.; PONS, D. G. High Concentrations of Genistein Decrease Cell Viability Depending on Oxidative Stress and Inflammation in Colon Cancer Cell Lines**. International Journal of Molecular Sciences,** v. 23, n.14, p. 7526, 2022.

ARAI, Y.; UEHARA, M.; SATO, Y.; KIMIRA, M.; EBOSHIDA, A.; ADLERCREUTZ, H.; WATANABE, S. Comparison of isoflavones among dietary intake, plasma concentration and urinary excretion for accurate estimation of phytoestrogen intake. **Journal of Epidemiology**, v. 10, n. 2, p. 127-135, 2000. DOI 10.2188/jea.10.127.

BAECHLER, S. A.; SOUKUP, S. T.; MOLZBERGER, A. F.; KULLING, S. E.; DIEL, P.; MARKO, D. Topoisomerase poisoning by genistein in the intestine of rats. **Toxicology Letters**, v. 243, p. 88-97, 2016.

BASSON, A. R.; AHMED, S.; ALMUTAIRI, R.; SEO, B.; COMINELLI, F. Regulation of intestinal inflammation by soybean and soy-derived compounds. **Foods**, v. 10, n. 4, p. 774, 2021. DOI 10.3390/foods10040774.

BEACOM, E.; BOGUE, J.; REPAR, L. Market-oriented development of plant-based food and beverage products: a usage segmentation approach. **Journal of Food Products Marketing**, v. 27, p. 204–22, 2021. DOI 10.1080/10454446.2021.1955799.

BOS, C.; GAUDICHON, C.; TOMÉ, D. Nutritional and physiological criteria in the assessment of milk protein quality for humans. **Journal of the American College of Nutrition**, v. 19(sup2), p. 191S-205S, 2000.

BUKOWSKI, K.; KCIUK, M.; KONTEK, R. Mechanisms of multidrug resistance in cancer chemotherapy. **International Journal of Molecular Sciences**, v. 21, n. 9, p. 3233, 2020. DOI 10.3390/ijms21093233.

CHEN, H. H.; CHEN, S. P.; ZHENG, Q. L.; NIE, S. P.; LI, W. J.; HU, X. J.; XIE, M. Y. Genistein promotes proliferation of human cervical cancer cells through estrogen receptor-mediated PI3K/Akt-NF-κB pathway. **Journal of Cancer**, v. 9, n. 2, p. 288, 2018. DOI 10.7150/jca.20499.

CHEN, X.; GU, J.; WU, Y.; LIANG, P.; SHEN, M.; XI, J.; QIN, J. Clinical characteristics of colorectal cancer patients and anti-neoplasm activity of genistein. **Biomedicine & Pharmacotherapy**, v. 124, p. 109835, 2020.

CHENG, Y.; TANG, Y.; TAN, Y.; LI, J.; ZHANG, X. KCNK9 mediates the inhibitory effects of genistein on hepatic metastasis from colon cancer. **Clinics**, v. 78, p. 100141, 2023.

COMERFORD, K. B.; MILLER, G. D.; BOILEAU, A. C.; SCHUETTE, S.N. M.; GIDDENS, J. C.; BROWN, K. A. Global review of dairy recommendations in food-based dietary guidelines. **Frontiers in Nutrition**, v. 8, p. 671999, 2021. DOI 10.3389/fnut.2021.671999.

DE SOUZA, I. S.; LIPSITT, A. The soymilk diet: A previously unknown etiology of acute pancreatitis. **The American Journal of Emergency Medicine**, v. 46, p. 798.e5-798.e6, 2021. DOI 10.1016/j.ajem.2021.01.055.

DROUIN-CHARTIER, J. P.; BRASSARD, D.; TESSIER-GRENIER, M.; CÔTÉ, J. A.; LABONTÉ, M. E.; DESROCHES, S.; COUTURE, P.; LAMARCHE, B. Systematic review of the association between dairy product consumption and risk of cardiovascular-related clinical outcomes. **Advances in Nutrition**, v. 7, p. 1026–40, 2016. DOI 10.3945/an.115.011403

FUNK, M. C.; ZHOU, J.; BOUTROS, M. Ageing, metabolism and the intestine. **EMBO Reports**, v. 21, n. 7, p. e50047, 2020.

GHOSH, S. S.; WANG, J.; YANNIE, P. J.; GHOSH, S. Intestinal barrier dysfunction, LPS translocation, and disease development. **Journal of the Endocrine Society**, v. 4, n. 2, p. bvz039, 2020. DOI 10.1210/jendso/bvz039.

GRACE, P. B.; TAYLOR, J. I.; LOW, Y. L.; LUBEN, R. N.; MULLIGAN, A. A.; BOTTING, N. P. Phytoestrogen concentrations in serum and spot urine as biomarkers for dietary phytoestrogen intake and their relation to breast cancer risk in European prospective investigation of cancer and nutrition-norfolk. **Cancer Epidemiology Biomarkers & Prevention**, v. 13, n. 5, p. 698-708, 2004.

GUERRERO-BELTRÁN, J. A.; ESTRADA-GIRÓN, Y.; SWANSON, B. G.; BARBOSA-CÁNOVAS, G. V. Pressure and temperature combination for inactivation of soymilk trypsin inhibitors. **Food Chemistry**, v. 116, n. 3, p. 676-679, 2009. DOI 10.1016/j.foodchem.2009.03.001.

GUO, W.; LIU, S.; KHAN, M. Z.; WANG, J.; CHEN, T.; ALUGONGO, G. M.; LI, S.; CAO, Z. Bovine milk microbiota: Key players, origins, and potential contributions to early-life gut development. **Journal of Advanced Research**, v. 59, p. 49-64, 2024. DOI 10.1016/j.jare.2023.06.016.

HARA, H.; FUJIBAYASHI, A.; KIRIYAMA, S. Pancreatic protease secretion profiles after spontaneous feeding of casein or soybean protein diet in unrestrained conscious rats. **Journal of Nutritional Biochemistry**, v. 3, p. 176–181,1992.

HE, Y.; AYANSOLA, H.; HOU, Q.; LIAO, C.; LEI, J.; LAI, Y.; JIANG, Q.; MASATOSHI, H.; ZHANG, B. Genistein inhibits colonic goblet cell loss and colorectal

inflammation induced by *Salmonella Typhimurium* infection. **Molecular Nutrition & Food Research**, v. 65, n. 16, p. e2100209, 2021. DOI 10.1002/mnfr.202100209.

ISHIMI, Y.; MIYAURA, C.; OHMURA, M.; ONOE, Y.; SATO, T.; UCHIYAMA, Y.; ITO, M.; WANG, X.; SUDA, T.; IKEGAMI, S. Selective effects of genistein, a soybean isoflavone, on B-lymphopoiesis and bone loss caused by estrogen deficiency. **Endocrinology**, v. 140, n. 4, p. 1893-1900, 1999.

ISSA, I. A.; NOUREDDINE, M. Colorectal cancer screening: An updated review of the available options. **World Journal of Gastroenterology**, v. 23, n. 28, p. 5086, 2017.

KONG, X.; LI, Y.; LIU, X. A review of thermosensitive antinutritional factors in plant-based foods. **Journal of Food Biochemistry**, v.46, n. 9, p. e14199, 2022. DOI 10.1111/jfbc.14199.

KŘÍŽOVÁ, L.; DADÁKOVÁ, K.; KAŠPAROVSKÁ, J.; KAŠPAROVSKÝ, T. Isoflavones. **Molecules**, v. 24, n. 6, p. 1076, 2019. DOI 10.3390/molecules24061076.

KUIPERS, E. J.; GRADY, W. M.; LIEBERMAN, D.; SEUFFERLEIN, T.; SUNG, J. J.; BOELENS, P. G.; VAN DE VELDE, C. J. H.; WATANABE, T. Colorectal cancer. **Nature Reviews Disease Primers**, v. 1, p. 15065, 2015. DOI 10.1038/nrdp.2015.65.

KWOK, K. C.; NIRANJAN, K. Effect of thermal processing on soymilk. **International journal of food science & technology**, v. 30, n. 3, p. 263-295, 1995.

LEVIT, R.; DE GIORI, G. S.; DE MORENO DE LEBLANC, A.; LEBLANC, J. G. Evaluation of the effect of soymilk fermented by a riboflavin producing *Lactobacillus plantarum* strain in a murine model of colitis. **Beneficial Microbes**, v. 8, p. 65–72, 2017.

LOSSO, J. N. The biochemical and functional food properties of the Bowman-Birk inhibitor. **Critical Reviews in Food Science and Nutrition**, v. 48, n.1, p. 94-118, 2008.

MAINGI, J. W.; TANG, S.; LIU, S.; NGENYA, W.; BAO, E. Targeting estrogen receptors in colorectal cancer. **Molecular Biology Reports**, v. 47, p. 4087-4091, 2020. DOI 10.1007/s11033-020-05414-6.

MANAS, E. S.; XU, Z. B.; UNWALLA, R. J.; SOMERS, W. S. Understanding the selectivity of genistein for human estrogen receptor-β using X-ray crystallography and computational methods. **Structure**, v. 12, n. 12, p. 2197-2207, 2004.

MARTINI, E.; KRUG, S. M.; SIEGMUND, B.; NEURATH, M. F.; BECKER, C. Mend your fences: the epithelial barrier and its relationship with mucosal immunity in inflammatory bowel disease. **Cellular and Molecular Gastroenterology and Hepatology**, v. 4, n. 1, p. 33-46, 2017. DOI 10.1016/j.jcmgh.2017.03.007.

MEDIC, J.; ATKINSON, C.; HURBURGH, C. R. Current knowledge in soybean composition. **Journal of the American oil chemists' society**, v.91, p. 363-384, 2014.

MEISEL, H. Chemical characterization and opioid activity of an exorphin isolated from *in vivo* digest of casein. **FEBS Letters**, v. 196, p. 223–227, 1986. DOI 10.1016/0014-5793(86)80251-4.

MOORE, A. B.; CASTRO, L.; YU, L.; ZHENG, X.; DI, X.; SIFRE, M. I.; KISSLING, G. E.; NEWBOLD, R. R.; BORTNER, C. D.; DIXON, D. Stimulatory and inhibitory effects of genistein on human uterine leiomyoma cell proliferation are influenced by the concentration. **Human Reproduction**, *v.* 22, n. 10, p. 2623-2631, 2007. DOI 10.1093/humrep/dem185.

MUKUND, V. Genistein: its role in breast cancer growth and metastasis. **Current Drug Metabolism**, v. 21, n. 1, p. 6-10, 2020.

MUZQUIZ, M.; VARELA, A.; BURBANO, C.; CUADRADO, C.; GUILLAMÓN, E.; PEDROSA, M. M. Bioactive compounds in legumes: Pronutritive and antinutritive actions. Implications for nutrition and health. **Phytochemistry Reviews**, v. 11, p. 227–244, 2012.

OBERMAYER-PIETSCH, B. M.; BONELLI, C. M.; WALTER, D. E.; KUHN, R. J.; FAHRLEITNER-PAMMER, A.; BERGHOLD, A.; GOESSLER, W.; STEPAN, V.; DOBNING, L. G; RENNER, W. Genetic predisposition for adult lactose intolerance and relation to diet, bone density, and bone fractures. **Journal of Bone and Mineral Research**, v. 19, p. 42–7, 2004. DOI 10.1359/jbmr.0301207.

PINTOVA, S.; DHARMUPARI, S.; MOSHIER, E.; ZUBIZARRETA, N.; ANG, C.; HOLCOMBE, R. F. Genistein combined with FOLFOX or FOLFOX–Bevacizumab for the treatment of metastatic colorectal cancer: Phase I/II pilot study. **Cancer Chemotherapy and Pharmacology**, p. 84, p. 591-598, 2019.

RENDÓN, J. P.; CAÑAS, A. I.; CORREA, E.; BEDOYA-BETANCUR, V.; OSORIO, M.; CASTRO, C.; NARANJO, T. W. Evaluation of the effects of genistein *in vitro* as a chemopreventive agent for colorectal cancer—strategy to improve its efficiency when administered orally. **Molecules**, v. 27, n. 20, p. 7042, 2022.

RÓKA, R.; WITTMANN, T.; BUENO, L. Altered protease signaling in the gut: A novel pathophysiological factor in irritable bowel syndrome. **Neurogastroenterology & Motility**, v. 20, p. 853–856, 2008.

RYAN-HARSHMAN, M.; ALDOORI, W. Diet and colorectal cancer: Review of the evidence. **Canadian Family Physician**, v. 53, n. 11, p. 1913-1920, 2007.

SADEGHI, O.; MILAJERDI, A.; SIADAT, S. D.; KESHAVARZ, S. A.; SIMA, A. R.; VAHEDI, H.; ADIBI, P.; ESMAILLZADEH, A. Effects of soy milk consumption on gut microbiota, inflammatory markers, and disease severity in patients with ulcerative colitis: a study protocol for a randomized clinical trial. **Trials**, v. 21, p. 565, 2020. DOI 10.1186/s13063-020-04523-8.

SALTI, G. I.; GREWAL, S.; MEHTA, R. R.; GUPTA, T. D.; BODDIE JR, A. W.; CONSTANTINOU, A. I. Genistein induces apoptosis and topoisomerase II-mediated DNA breakage in colon cancer cells. **European Journal of Cancer**, v. 36, n. 6, p. 796-802, 2000.

SEKAR, V.; ANANDASADAGOPAN, S. K.; GANAPASAM, S. Genistein regulates tumor microenvironment and exhibits anticancer effect in dimethyl hydrazine-induced experimental colon carcinogenesis. **Biofactors**, v. 42, n. 6, p. 623-637, 2016.

SONG, S.; CHENG, D.; WEI, S.; WANG, X.; NIU, Y.; QI, W.; WANG, C. Preventive effect of genistein on AOM/DSS-induced colonic neoplasm by modulating the PI3K/AKT/FOXO3 signaling pathway in mice fed a high-fat diet. **Journal of Functional Foods**, v. 46, p. 237-242, 2018.

SPAANDER, M. C.; ZAUBER, A. G.; SYNGAL, S.; BLASER, M. J.; SUNG, J. J.; YOU, Y. N.; KUIPERS, E. J. Young-onset colorectal cancer. **Nature Reviews Disease Primers**, v. 9, n. 1, p. 21, 2023. DOI 10.1038/s41572-023-00432-7.

SPENCER, J.; BEMARK, M. Human intestinal B cells in inflammatory diseases. **Nature Reviews Gastroenterology & Hepatology**, v. 20, n. 4, p. 254-265, 2023.

SULISTYOWATI, E.; MARTONO, S.; RIYANTO, S.; LUKITANINGSIH, E. Development and validation for free aglycones daidzein and genistein in soybeans (*Glycine max* (L.) Merr.) using RP HPLC method. **International Journal of Applied Pharmaceutics**, v. 11, n. 2, p. 138-142, 2019.

SUNG, H.; FERLAY, J.; SIEGEL, R. L.; LAVERSANNE, M.; SOERJOMATARAM, I.; JEMAL, A.; BRAY, F. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. **Cancer Journal for Clinicians**, v. 71, n. 209–49, 2021. DOI 10.3322/caac.21660.

ULVEN, S. M.; HOLVEN, K. B.; GIL, A.; RANGEL-HUERTA, O. D. Milk and dairy product consumption and inflammatory biomarkers: an updated systematic review of randomized clinical trials. **Advances in Nutrition**, v. 10, p. S239-S250, 2019.

VALSECCHI, A. E.; FRANCHI, S.; PANERAI, A. E.; ROSSI, A.; SACERDOTE, P.; COLLEONI, M. The soy isoflavone genistein reverses oxidative and inflammatory state, neuropathic pain, neurotrophic and vasculature deficits in diabetes mouse model. **European Journal of Pharmacology**, v. 650, n. 2-3, p. 694-702, 2011. DOI 10.1007/s00432-020-03496-0.

VEETTIL, S. K.; WONG, T. Y.; LOO, Y. S.; PLAYDON, M. C.; LAI, N. M.; GIOVANNUCCI, E. L.; CHAIYAKUNAPRUK, N. Role of diet in colorectal cancer incidence: umbrella review of meta-analyses of prospective observational studies. **JAMA Network Open**, v. 4, n. 2, p. e2037341-e2037341, 2021. DOI 10.1001/jamanetworkopen.2020.37341.

VERDRENGH, M.; JONSSON, I. M.; HOLMDAHL, R.; TARKOWSKI, A. Genistein as an anti-inflammatory agent. **Inflammation Research**, v. 52, p. 341-346, 2003.

VOJDANI, A.; TURNPAUGH, C.; VOJDANI, E. Immune reactivity against a variety of mammalian milks and plant-based milk substitutes. **Journal of Dairy Research**, v. 85, n. 358–65, 2018. DOI 10.1017/S0022029918000523.

WALTHER, B.; GUGGISBERG, D.; BADERTSCHER, R.; EGGER, L.; PORTMANN, R.; DUBOIS, S.; HALDIMANN, M.; KOPF-BOLANZ, K.; RHYN, P.; ZOLLER, O.; VERAGUTH, R.; REZZI, S. Comparison of nutritional composition between plant-

based drinks and cow's milk. **Frontiers in Nutrition**, v. 9, p. 988707, 2022. DOI 10.3389/fnut.2022.988707.

WAN, Q.; ZHAO, R.; XIA, L.; WU, Y.; ZHOU, Y.; WANG, Y.; CUI, Y.; SHEN, X.; WU, X. T. Inflammatory bowel disease and risk of gastric, small bowel and colorectal cancer: a meta-analysis of 26 observational studies. **Journal of Cancer Research and Clinical Oncology**, v. 147, p. 1077-1087, 2021.

WANG, C., DING, K.; XIE, X.; ZHOU, J.; LIU, P.; WANG, S.; FANG, T.; XU, G.; HONG, H. Soy Product Consumption and the Risk of Cancer: A Systematic Review and Meta-Analysis of Observational Studies. **Nutrients**, v. 16, n. 7, p. 986, 2024.

XIAO, C. W.; WOOD, C. M.; ROBERTSON, P.; GILANI, G. S. Protease inhibitor activities and isoflavone content in commercial soymilks and soy-based infant formulas sold in Ottawa, Canada. **Journal of Food Composition and Analysis**, v. 25, n. 2, p. 130-136, 2012.

XIAO, C. W.; WOOD, C.; CUNNINGHAM, L. A.; LALANDE, M.; RIDING, M. Effects of dietary active soybean trypsin inhibitors on pancreatic weights, histology and expression of STAT3 and receptors for androgen and estrogen in different tissues of rats. **Molecular Biology Reports**, v.48, p. 4591–4600, 2021.

YAMAGIWA, Y.; SAWADA, N.; SHIMAZU, T.; YAMAJI, T.; GOTO, A.; TAKACHI, R.; TSUGANE, S. Soy Food Intake and Pancreatic Cancer Risk: The Japan Public Health Center–based Prospective Study. **Cancer Epidemiology, Biomarkers & Prevention**, v. 29, n. 6, p. 1214-1221, 2020.

YU, Y.; JING, X.; LI, H.; ZHAO, X.; WANG, D. Soy isoflavone consumption and colorectal cancer risk: a systematic review and meta-analysis. **Scientific Reports**, v. 6, n. 1, p. 25939, 2016.

ZAVA, D. T.; DUWE, G. Estrogenic and antiproliferative properties of genistein and other flavonoids in human breast cancer cells *in vitro*. **Nutrition and Cancer**, v. 27, n. 1, p. 31-40, 1997.

2 CAPÍTULO 2: Dose dependent effects of genistein in preclinical studies of colorectal cancer: A systematic review

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Dose dependent effects of genistein in preclinical studies of colorectal cancer: A systematic review

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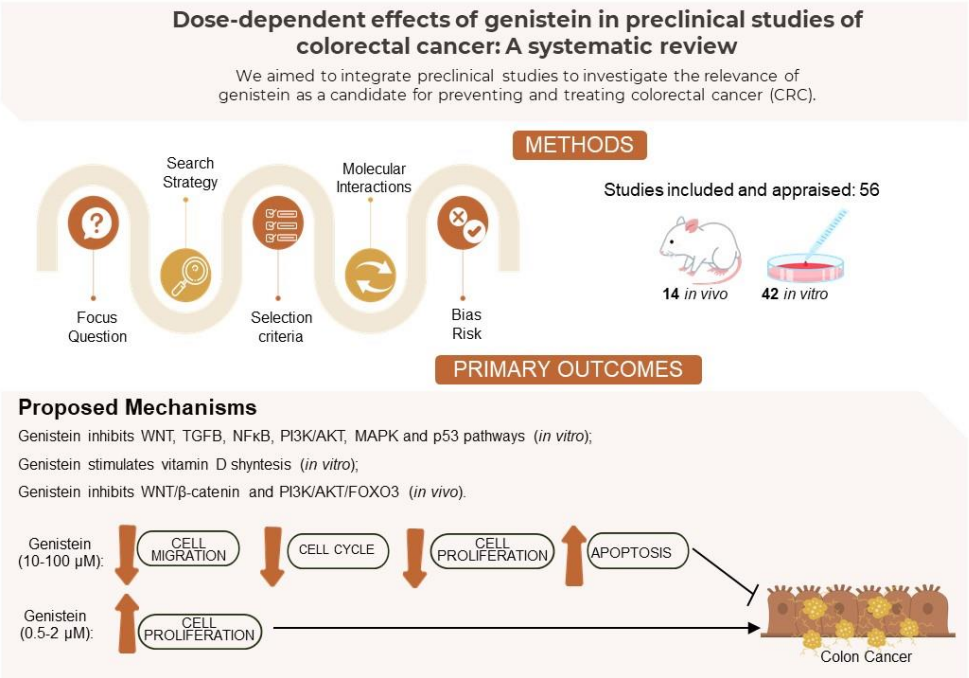
Abstract

Genistein interacts with estrogen receptors and inhibits kinase protein activity, modulating pathways related to cancer development. We integrated preclinical studies to investigate the relevance of genistein for preventing colorectal cancer (CRC). We focused on the signaling pathways in which genistein is involved in the CRC cells. We used the PRISMA guidelines from the PubMed-Medline, Scopus, Web of Science, and Embase platforms. All studies that investigated the effect of genistein on CRC in preclinical models up to September 2023 were included. Bias analysis and the methodological quality of the *in vivo* studies were assessed by SYRCLE's RoB tool. Genistein acts as a proliferative inductor in low doses (0.5-2 μM) and a chemoprotective in high doses (10-100 μM). The tumor protective abilities of genistein have been linked to their tyrosine kinase inhibitor capacity, and its estrogenic effect is related to cell proliferation. Genistein controls CRC by modulating the WNT, TGFβ, NFκB, PI3K/AKT/MAPK, p53-dependent pathways NFκB, the oxidant and inflammatory response, and vitamin D synthesis. According to NFκB-related outcomes, genistein acts as an antioxidant and anti-inflammatory in early-stage CRC cells and as a pro-oxidant and pro-inflammatory in later-stage CRC cells. The *in vivo* studies support the chemoprotective effect of genistein found *in vitro*.

Keywords: Gut; Isoflavone; Signaling pathways; Cancer; Preclinical studies.

Graphical Abstract



2.1 Introduction

Colorectal cancer (CRC) is a highly invasive tumor, considered the third most common cancer in humans, and its incidence is expected to increase in the coming years. It ranks as the second cancer-causing death all around the world. In 2020, 1.93 million new patients were diagnosed with CRC (1). Diagnosis is delayed because the initial symptoms are hidden (2). As for treatment, surgery and chemotherapy are the most common; however, cure and survival rates are still low (3). As for current chemotherapy, lack of selectivity for tumor cells, insufficient drug concentrations in tumor tissues, drug-resistant tumor cell emergence, and systemic toxicity are the main constraints to treatment success (4). Hence, research on novel anti-CRC agents, including those derived from natural compounds, is a strong need.

Although genetic factors play an important role in CRC development, most cases are associated with environmental factors such as diet (4). There is a direct correlation between adopting healthy eating behaviors, which involve consuming fiber, grains, and vegetables, and a reduced occurrence of CRC (5; 6). Soybean consumption and its derivatives are associated with decreased CRC risk, mainly in Asian populations (7). Asian and Western diets are distinct in several aspects, for example, the traditional Asian diet contains large amounts of isoflavones, components

found mainly in soy and its derivatives, which could explain the protective effect against CRC for these populations. To illustrate, the average daily intake of isoflavones in Asia is 20-50 mg/day (8); in contrast, the average in Europe is 0.437 mg/day (9).

Genistein (CHEBI:28088) is an isoflavone mostly present in soybeans, with an average concentration of 17.89 mg per 100 g of soybean (10). It is also commercially available as a nutraceutical synthetic supplement containing about 30 mg per capsule. Genistein is a phytoestrogen because it can bind to estrogen receptors (ESRs) and exert similar effects to endogenous estrogen by modulating ESRs dependent signaling pathways (11; 12). Genistein binds to both estrogen receptor isoforms (ESR1 and ESR2) with a modestly selective affinity for the ESR2 isoform (13), which is relatively more abundant in colon cells than ESR1 (14). Also, it interacts with G protein-coupled estrogen receptor 1 (GPER1) (15; 16). Both ESRs' are expressed in the intestines of women and men, localized to the plasma membrane (GPER1) and intracellularly (ESR1 and ESR2) of the villous and crypt cells (17; 18; 19). Also, genistein is known for its activity as a tyrosine kinase inhibitor (20), thus potentially modulating several kinase-dependent signaling pathways. Genistein may be a promising bioactive compound for aiding in CRC treatments, by promoting cell cycle arrest, apoptosis, and decreasing cell proliferation and metastasis, as observed in several clinical studies (21; 22; 23). On the other hand, evidence suggests that genistein can also induce cell proliferation (24; 25), in which these contrasting effects are dose-dependent. Given the ongoing discussion surrounding genistein and the inadequacy of current anti-CRC medication for chemotherapy, the goal of this systematic review is to synthesize preclinical research and examine genistein's potential as a viable option for the prevention and treatment of CRC. Our primary objective was to investigate and describe the signaling pathways and mediators through which genistein plays a role in CRC cell lines, pointing out new potential molecular targets of genistein that could be used to guide more precise therapies.

2.2 Material and Methods

2.2.1 Focus Questions

What is the effect of genistein on CRC in a preclinical model? What are the signaling pathways affected by genistein? What is the best dose of genistein used in the prevention/treatment of CRC?

2.2.2 Search Strategy

This study design followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (26) (Fig. 1). The protocol for this systematic review was registered on PROSPERO - International Prospective Register of Systematic Reviews (CRD42023394681) and is available in full at https://www.crd.york.ac.uk/PROSPERO/display_record.php?RecordID=394681.

Four online literature databases were explored: Medline/PubMed (https://www.ncbi.nlm.nih.gov/pubmed), Scopus (https://www.scopus.com/home.uri), Web of Science (https://www.webofscience.com/wos/) and Embase (https://www.embase.com/search/quick). The search was completed on September 12, 2023. For all databases, the search filters were based on two complementary levels: (i) genistein and (ii) intestinal cancer, which were combined by the Boolean operator [AND]. Search filters were initially developed for PubMed. The search algorithms [MeSH Terms] and [TIAB] were applied to identify indexed records and those recently published in an indexing process, respectively. The terms used to search PubMed were adapted for Scopus, Web of Science and Embase. The search strategy is detailed in the supplementary materials (Table S1).

2.2.3 Selection Criteria

After record identification through both databases, the duplicate studies were identified and removed. The articles were first screened based on their titles and abstracts. In this selection, we included all preclinical studies that evaluated the effects of genistein on CRC in preclinical *in vitro* and *in vivo* models up to September 2023. At this stage, we excluded reviews, papers not written in English, *in situ*, *ex vivo*, *and in silico* studies, small intestine studies, and all articles that did not fulfill the inclusion criteria. We performed a second screening to select articles for full-text analysis. After reading the selected articles in full, the data were extracted following these sequences:

i) Publication characteristics: author, year, and country;
ii) Characteristics of the animal model: species, gender, age, and weight;
iii) Characteristics of the *in vitro* studies: cell line, time of the exposition, dose;
iv) Intervention: control groups, administered doses and routes, frequency and study duration;
v) Primary outcomes after treatment;
vi) Conversion of the dose administered to animals into its human equivalent dose (HED), whenever possible (27).

Following the methodology of PRISMA, two independent authors (MTSM and LL) analyzed the titles, abstracts, and full-text articles, and another author (MMS) resolved any disagreements over the extracted data. The kappa test (28) was performed for data selection (kappa = 0.979).

2.2.4 Summary of Signaling Pathways and Relevant Molecular Interactions

All the results regarding molecular interactions and altered signaling pathways leading to anti-cancer effects or colon cancer outcomes in CRC cell lines were manually curated and summarized in Table S2. The information was then translated into Figure S1, adapting the adverse outcome pathway (AOP) network framework (29), defining molecular key events on the progression of the biological disturbance, from hypothetical molecular initiating events, as not all the targets for genistein were described in the included papers or are known. The potential key events were organized and described in a sequential and mechanistic way, leading to the adverse outcome (i.e., CRC) or the potential anti-cancer effects. The figure was built using the Path Visio software (30), annotating the references in the network and allowing for the traceability of the information. A machine-readable version of the network in GPML is available in the Supplementary Materials.

2.2.5 Risk of Bias Assessment

The quality of the studies was assessed by the criteria described in the SYRCLE's Risk of Bias (RoB) tool (Systematic Review Centre for Laboratory Animal Experimentation) designed specifically for animal studies (31). This tool is based on the Cochrane Collaboration's guidelines for assessing the risk of bias in randomized trials and is adjusted for conditions of bias specific to animal studies. The following methodological domains based on RoB were evaluated, considering the following: 1)

selection bias (random sequence generation, baseline characteristics, allocation concealment); 2) performance bias (random housing, blinding of caregivers and/or investigators); 3) detection bias (random outcome assessment, blinding of outcome assessment); 4) attrition bias (incomplete outcome data); 5) reporting bias (selective outcome reporting); and other biases (we analyzed whether the studies presented control groups, whether they presented the origin and composition of the basal diet, whether the animals were subjected to a quarantine period; whether the statistical methods used were reported; and whether the ethics committee of the use of experimental animals approved the study). We summarized these analyses in two figures using the Review Manager 5.3 software (Cochrane Collaboration - RoB 2.0) to illustrate the risk of bias across all included studies (Fig. 2). The items in the RoB tool were scored with "yes" (low risk of bias); "no" (high risk of bias); or "unclear" (indicating that the item was not adequately reported and, therefore, the risk of bias was unknown).

2.3 Results

2.3.1 PRISMA Guideline

The search resulted in 314 articles (MEDLINE/PubMed: 139; Scopus: 131; Web of Science: 25; and 19 Embase). One hundred thirty-four duplicated articles were removed, and the remaining 180 articles were evaluated. By screening the title and abstract, 123 articles were removed for being outside the scope of this review; 57 articles were selected for complete analysis, and 1 article was excluded, following the eligibility criteria. Therefore, 56 articles (14 *in vivo* and 42 *in vitro*) were included in this systematic review. The flowchart and each step performed in the selection process to retrieve relevant information are shown in Figure 1.

2.3.2 Characteristics of the Included Studies

The studies were published between 1994 and 2023. Most of the studies (32.14%), were conducted in the USA, followed by China (19.64%) and Japan (10.71%). Four studies (7.14%, each) were performed in Italy and Austria, and two (3.57%, each) in the United Kingdom and Colombia. Only one study was performed in the following countries: Spain, Turkey, Greece, Israel, Brazil, Korea, Iran, England, and India (1.79%, each).

The murine models were rats (78.57%) and mice (21.43%). Among the rats, the strain F344 was the most used (45.45%), followed by Wistar and Sprague-Dawley (27.27%, each). Among the mice, the studies were performed with the strain C57BL/6, multiple intestinal neoplasia (Min/+), and Kunming (33.33%, each). As for gender, 78.57% was performed with males, only one study (7.14%) with females, and two studies (14.29%) did not report animal gender. Many studies also fail to report the animals' weight (78.57%) and age (28.57%). For those who reported weight (21.43%) and age (71.43%), the age ranged from 2 to 6 weeks, and the weight ranged from 82 to 200g.

All *in vitro* studies were conducted with CRC cell lines, and often, more than one cell line was used in the same study. 92.86% of the studies were conducted with derived human colorectal carcinoma cell lines (SW837, SW620, SW116, SW480, HT29, HCT8, HCT116, Colo320, LoVo, DLD-1, Caco-2, COGA-1, HCT-15, C22-20, and RKO. Only two studies (4.76%) were conducted with derived murine colon cancer MC-26, and one study (2.38%) was conducted with derived rabbit colon cancer cells. Complete information on the included studies and experimental animals is reported in Table S2 (*in vitro*) and Table S3 (*in vivo*).

2.3.3 In vitro studies

In the *in vitro* studies the minimum dose of genistein administered was 0.01µM and the maximum was 740 µM. The experimental period was different according to each protocol, ranging from 24h hours to 14 days. All studies presented a control group. DMSO was the most used substance as a control (40.48%), followed by untreated cells (29.19%), ethanol (9.52%), and PBS (2.38%). In 5 studies (11.9%) it was only reported that there was a control, but the authors did not clarify what it was. Likewise, one study, reported that the control was a solvent, but the authors did not describe which one. Besides, there was one study in which a positive and negative control were used and there are two studies in which two negative controls were used: untreated cells and DMSO (Table S2).

2.3.4 In vivo studies

Most *in vivo* studies (71.43%) used a soy-free diet, with casein being the main compound used to replace soy. On the other hand, in 28.57% of the studies, it needs

to be clarified whether the control diet offered to the animals during the experiment was soy-free (Table S3). Genistein was administered orally in most studies (92.86%), from which 84.62% were administered in the diet and 18.38% by gavage. Genistein was administered subcutaneously in only one study (7.14%) (Table S4).

As shown in Table S4, the dose of genistein administered ranged from 0.15 to 900 mg/kg of diet. The experimental period ranged from 24 hours to 6 months. Non-isoflavone diet (71.43%), H_2O/5% ethanol, and olive oil (7.14% each) were used as controls. In one study, it was shown that the control was a "normal diet," but the authors did not show the diet description. Besides, in one study only, it was mentioned that there was a control group, but the authors did not clarify more details. Overall, there was no study without a control group.

Only one study (7.14%) did not challenge the animals before or after treatment with genistein. The azoxymethane (AOM) compound was the most used (64.29%), followed by 1,2-dimethylhydrazine (DMH) (31.43%) and ovariectomy (7.14%).

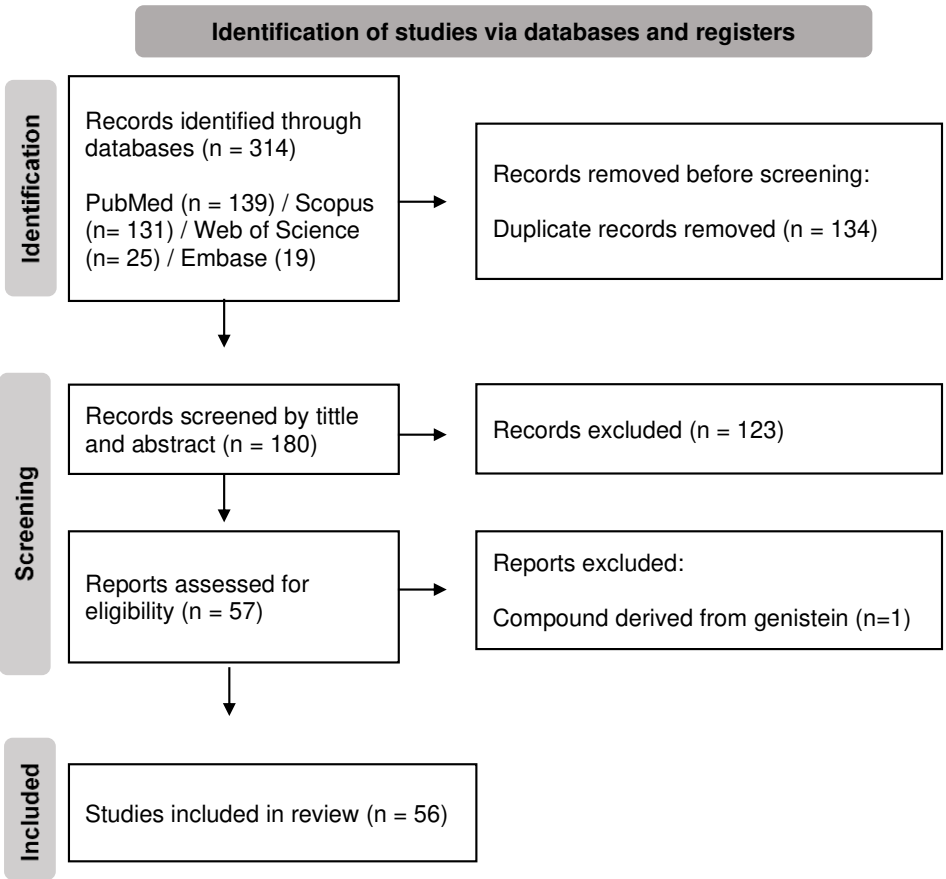


Figure 1: PRISMA workflow. MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, *et al*. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021;372:n71. DOI: 10.1136/bmj.n71 (http://www.prisma-statement.org/).

2.3.5 Primary Outcomes

2.3.6 Genistein Administration on Experimental Colon Cancer, in vitro

In general, genistein mitigated the effects caused by cancer in the murine colon and in the colon cancer cell lines. The figure S1 presents an AOP network framework based on the primary outcomes of the *in vitro* included studies. At high doses (10-100 μM), genistein shows potential for cancer prevention by modulating the WNT, TGFβ, NFκB, PI3K/AKT/MAPK and p53 dependent pathways, the oxidant and inflammatory response, and vitamin D synthesis. As a result, it induces CRC cell cycle disruption, apoptosis and decreases cell proliferation and cell invasion. At low doses (0.5-2 μM), genistein increases CRC cell proliferation, by increasing aromatase activity and changing glycosaminoglycans/proteoglycans ratio, which could lead to cancer induction.

Genistein showed good insights in the 39 (92.86%) (32-70) studies. These studies reported and added evidence that genistein, at 10-100 μM, acts as a chemopreventive substance in the CRC cell lines studied by disrupting the cell cycle, reducing cell invasion and proliferation, and inducing apoptosis of the cancer cells (Fig. S1; Table S2). Only three studies (7.14%) reported negative results induced by genistein: It enhanced aromatase activity and gene expression in HCT8 cells at 1 μM and enhanced cell proliferation at 0.5-2μM (71-73) (Fig. S1; Table S2).

2.3.7 Genistein and WNT/β-catenin pathway

Genistein reduced methylation at the promoter region of the WNT inhibitory factor-1 (WIF1) gene, enhanced histone H3 acetylation at the Dickkopf (DKK1) promoter region, and enhanced the gene and protein expression of these genes in moderate to later stage HT29 and SW480 cell lines (54; 64). WIF1 and DKK1 genes are inhibitors of the signaling WNT pathway, possibly leading to a reduction of the β-catenin gene expression (57). Downstream, genistein reduced levels of pro-oncogenes WNT/β-catenin pathway-associated: c-myc (MYC) and cyclin D (CCND1). Genistein also increased gene and protein expression of invasion and migration-associated factors, including E-cadherin/N-cadherin ratio, matrix metalloproteinase inhibitor protein 1 (TIMP1), and decreased matrix metalloproteinases 1 and 9 (MMP1 and MMP9). The potassium channel (KCNK9) expression inhibition by genistein was also associated with reducing the WNT/β-catenin signaling pathway (70). By these mechanisms, genistein reduced cell invasion, and cell migration, and induced cell cycle

arrest in moderate to late-stage CRC cell lines (54; 57; 64; 70). On the other hand, genistein demethylated the promoter region of WNT5A and increased its expression in early stage SW1116 and DLD-1 cell lines (51). Overall, genistein reduced cell proliferation when WNT gene expression was enhanced or decreased, indicating cooperation of other different regulatory pathways (not explored by these studies) in controlling cell proliferation (Fig. S1; Table S2).

2.3.8 Genistein and Cell Cycle Control

Genistein led to the activation of the tumor suppressor genes as p53 (TP53), p21 (CDKN1A), and p27, resulting in cell cycle arrest and apoptosis (53). Genistein activated the kinase protein Ataxia Telangiectasia Mutated (ATM)/p53-p21 pathway (55), and Nonsteroidal Anti-inflammatory Drug-activated Gene - NAG-1 (GDF15)/p53 pathway (40). In addition, genistein induced cell cycle arrest and apoptosis by directly activating p21 (CDKN1A), a p53-inducible gene (37; 42). Some authors also observed a positive relationship between p53 activation by genistein and the reduction of cell proliferation and induction of apoptosis (65; 69). Besides, genistein inhibits the EGF/FOXO3 cell proliferation pathway (53). The authors observed that genistein-induced FOXO3 interaction with p53 (mut), leads to the expression of p27kip1 (CDKN1B), thereby promoting cell cycle arrest and inhibiting cell proliferation. They speculate that genistein inhibits EGF-induced FOXO3 inactivation by PI3K/AKT pathway (decreased AKT phosphorylation). Genistein did not affect the interaction between TP53 wild type and FOXO3 (Fig S1; Table S2).

Genistein also controlled CRC cell proliferation by modulating estrogen receptors' ESR1 and ESR2 expression. It enhanced gene expression of ESR1 and ESR2, and demethylated the cyclo-inhibitory protein p16INK4a (CDKN2A) promoter (50). As a result, the proliferation of CRC cells was reduced. Genistein affects the extrarenal metabolism of vitamin D by continuing to stimulate estrogen receptors. The inhibition of ESRs resulted in a reduction in the activation of the vitamin D receptor (VDR) by genistein (46). The researchers additionally noted that genistein induced an elevation in intracellular free calcium levels, triggered the phosphorylation of ERK1/2 (MAPK3 and MAPK1), upregulated the transcription factor Sp-1, and induced the expression of both the VDR gene and its associated proteins. The authors propose that genistein enhances intracellular calcium levels, leading to a signaling cascade that activates ERK. This activation then modulates the activity of Sp-1, resulting in an

increased expression of VDR. In this way, genistein increased the expression of VDR through the MAPK/ESR/SP-1 pathway. These findings are corroborated by studies 39 and 47, which demonstrated that genistein increased the activity and mRNA expression of cytochrome P450 (CYP) enzymes, specifically the 25-Hydroxyvitamin D 1-alpha-hydroxylase (CYP27B1) enzyme, while reducing the activity of the 24-hydroxylase enzyme (CYP24) (Fig. 2; Fig. S1; Table S2).

Genistein also inhibited CRC cell proliferation by mechanisms depending on the oxidative status and inflammation in CRC cell lines, as evidenced by decreased cyclooxygenase-2 (COX2) activity. In addition, Genistein enhanced cytokines IL1B, IL2, IL4, IL5, IL6, IL10, IL13, IL17A, IL18, IL27, and GM-CSF (CSF2), and reactive oxygen species (ROS), including H_2O_2 production; and modulated the expression of antioxidant enzymes.

Finally, genistein inhibited CRC cell proliferation by alternative mechanisms, such as DNA topoisomerase II inhibition and DNA strand breakage (DNA damage) (55; 65; 72); changes in the cell membrane characteristics (34); altered polyamine metabolism (43); modulated glycosaminoglycans (GAG)/proteoglycans (PG) content (73); enhanced low-density lipoprotein receptor (LDLR) gene expression and decreased the 3-hydroxy-methylglutarylcoenzymeA (HMGCoA) reductase (49). Furthermore, several other studies corroborate the notion that genistein effectively suppresses cell proliferation and invasion, induces cell cycle arrest, and promotes apoptosis in CRC cell lines that were examined (35; 38, 41; 48; 62) (Fig. S1; Table S2).

2.3.9 Genistein and Apoptosis

Genistein enhanced BAX/BCL2 ratio, caspase 8 (CASP 8) and caspase 3 (CASP 3) gene expression, leading to the activation of the apoptosis mitochondrial pathway. Genistein also inhibited the FLT4 (Vascular Endothelial Growth Factor/ VEGF family of receptors) gene and protein expression together with the matrix metalloproteinases 2 (MMP2), that implicate in CRC control by decreasing metastatic mechanisms (cell invasion and migration) (59). Along with these studies, others propose that genistein inhibits the protein activity of kinases, related to cell proliferation, resulting in the suppression of CRC through a reduction in the expression of the c-myc (MYC) gene (32), and by cell proliferation inhibition and apoptosis induction (33) (Fig. S1; Table S2).

In protein kinase activity regulation, the control and prevention of CRC through genistein requires the downregulation of the long noncoding RNA (lncRNA) TTTY18/AKT signaling pathway, confirmed when genistein's administration reduced Transforming Growth Factor-β (TGF-β) levels and TTTY18 expression (66). This reduction was followed by a decrease in the expression of AKT, Mitogen-activated protein kinases p38 MAPK (MAPK14), and serum and glucocorticoid-regulated kinase 1 (SGK1). Consequently, the later-stage SW480 cell line exhibited decreased cell proliferation and migration while cell apoptosis was increased. Comparable results were observed by other study (61) (Fig. 2; Table S2).

Different from the findings reported by the study cited above (66), there was a positive correlation between elevated levels of TGF-β1 (TGFB1) expression and the control of CRC. Genistein increased the levels of TGF-β1 mRNA, caused the formation of Smad-DNA complexes, and induced phosphorylation of Smad2/3 (SMAD2 and SMAD3) in MC-26 cell lines, indicating that genistein affects the TGF-β1/Smad signaling pathway (45). Therefore, the administration of genistein led to an increase in the expression of TGF-β1/Smad downstream genes, specifically the activating transcription factor 3 gene (ATF3), while simultaneously decreasing the expression of the inhibitor of DNA binding/differentiation-1 (ID1) gene. Consequently, there was a decrease in cell proliferation within the HCT-116 cell line (52) (Fig. S1; Table S2).

By inhibiting the Notch-1 (NOTCH1) protein expression and the expression of both NF-κB p50 (NFKB1) and NF-κB p65 (RELA), genistein modulated the Notch-1/NF-κB/slug (SNAI2)/E-cadherin pathway (63). As a result, genistein suppressed the early-stage CRC cell migration by decreasing the expression of invasion-related genes: slug (SNAI2), ZEB1, ZEB2, foxc-1 (FOXC1), foxc-2 (FOXC2) and TWIST1, leading to the reverse of the Epithelial Mesenchymal Transition (EMT): enhanced expression of the E-cadherin and decreased expression of the N-cadherin. These observations were supported by others, who reported decreasing in NF-κB DNA binding activity, induced dephosphorylation and upregulation of IκB-α (NFKBIA), inhibition of cell proliferation, and induction of apoptosis by genistein in early-stage CRC cells (58). On the other hand, there was a positive CRC cell control by genistein when NF-kB translocation into the nucleus was enhanced, in later-stage SW620 cell lines (67) (Fig. S1; Table S2).

2.3.10 Primary Outcomes of Genistein using In vivo models

Only three studies (21.43%) documented parameters associated with the adverse effects of genistein in murine models. In combined experiments with genistein and cancer-inducing chemicals in rats, genistein enhanced the number of noninvasive and total adenocarcinoma cells in the colon (74) and it enhanced the number and size of β-catenin deposits accumulated in crypt cells (BCAC) and proliferating cell nuclear antigen (PCNA) in BCAC (75; 76). On the other hands, in most of the studies (n=11, 78.57%), genistein did not changes tumor incidence (77; 78) or reduced aberrant crypts (79; 80; 81; 82; 83), reduced the incidence of peritoneal metastasis in intestinal carcinoma, and lymphatic vessel invasion (84), prevented colon shrinkage and architecture damage, and enhanced mucin content (83; 85). The protective effects of genistein have been attributed to its modulation of the WNT/β-catenin pathway, PI3K/AKT/FOXO3 signaling pathway, antioxidant action, and vitamin D synthesis.

Genistein restored baseline levels of WNT signaling genes WNT5A, SFRP1, SFRP2, SFRP5, reduced nuclear β-catenin levels, and WNT target genes cyclin D1 (CCND1) and c-Myc (MYC), in rat colon challenged with azoxymethane (AOM). Besides, the reduction of WNT/β-catenin signaling was correlated with the decrease in aberrant crypt numbers (82). Later, this research group showed that the reduced expression of SFRP2, SFRP5 and WNT5A genes by genistein, in rat colon challenged with AOM, was due to epigenetic mechanisms (DNA methylation and histone modifications) (86). In addition, genistein reduced β-catenin expression, reduced the stem cell protein markers CD133 (PROM1), CD44, the cell proliferation markers argyrophilic nucleolar organizer region (AgNOR), and proliferating cell nuclear antigen (PCNA) (83). Besides, genistein enhanced enzymatic and non-enzymatic antioxidants, and the nuclear factor-erythroid 2 related factor 2 (NFE2L2) associated with detoxification and its downstream target heme oxygenase-1 (HMOX1) expression.

In azoxymethane/dextran sulfate sodium (AOM/DSS) induced colon cancer in high-fat fed mice, genistein decreased PI3K, AKT, COX2, tumor necrosis factor α - TNF-α (TNF) and FRAT1 expression, and enhanced phosphatase and tensin homolog (PTEN) and Forkhead box O3 (FOXO3) expression, and BAX/BCL2 ratio. The authors suggest that genistein inhibited tumor occurrence by modulating the PI3K/AKT/FOXO3 signaling pathway, which led to upregulation of BAX and downregulation of BCL2 (85).

Additionally, genistein exhibited modulation of enzymes associated with the synthesis of the antimitotic hormone 1,25-dihydroxyvitamin D3 (vitamin D), as well as

enhanced expression of the cytochrome P450 enzyme CYP27B1 and decreased expression of CYP24. The authors have linked the preventive effect of genistein on CRC to the synthesis of vitamin D, which is regulated by cytochrome P450 enzymes (87) (Table S4).

2.3.11 Bias Report From In Vivo Studies

Detailed results for bias analysis are shown in Fig. 2. Referring to the criteria described in Fig. 2A, only selective reporting items were evaluated with a low risk of bias (100%, n=14). This result was possible because all studies showed consistency between the methodology and the results presented. The unclear risk of bias was predominant in the following items: baseline characteristics (86%, n=12), incomplete outcome data (50%, n=7), and other biases (64%, n= 9). These studies failed to describe all the parameters related to the characteristics of the animals, mainly the weight, and they did not report the number of animals used in each assay. Many studies exhibited bias by failing to provide information regarding the approval of ethics committees for the use of experimental animals, the composition of the basal diet, the absence of isoflavones, and the quarantine period for the animals. The studies presented a risk of bias (100%, n=12) when evaluating random grouping, blinding of participants and personnel, and blinding on outcome assessment items. The studies were considered unsatisfactory based on their failure to provide information regarding the random distribution of animals in the animal experimentation facilities and the lack of reporting on participant blinding during the execution of the experiment and assays. The items of allocation concealment (71%, n=10) and random outcome assessment (79%, n=11) were primarily assessed with a high risk of bias due to the absence of information regarding the randomization of animal distribution in the experimental groups and the randomization of animals during outcomes assessment in the studies. Using this tool, it was possible to observe that the current evidence has serious limitations because the risk of bias summary showed the predominance of unclear and High risk of bias. However, the number of papers *in vivo* included in this review is very small, compromising the conclusion about the risk of bias.

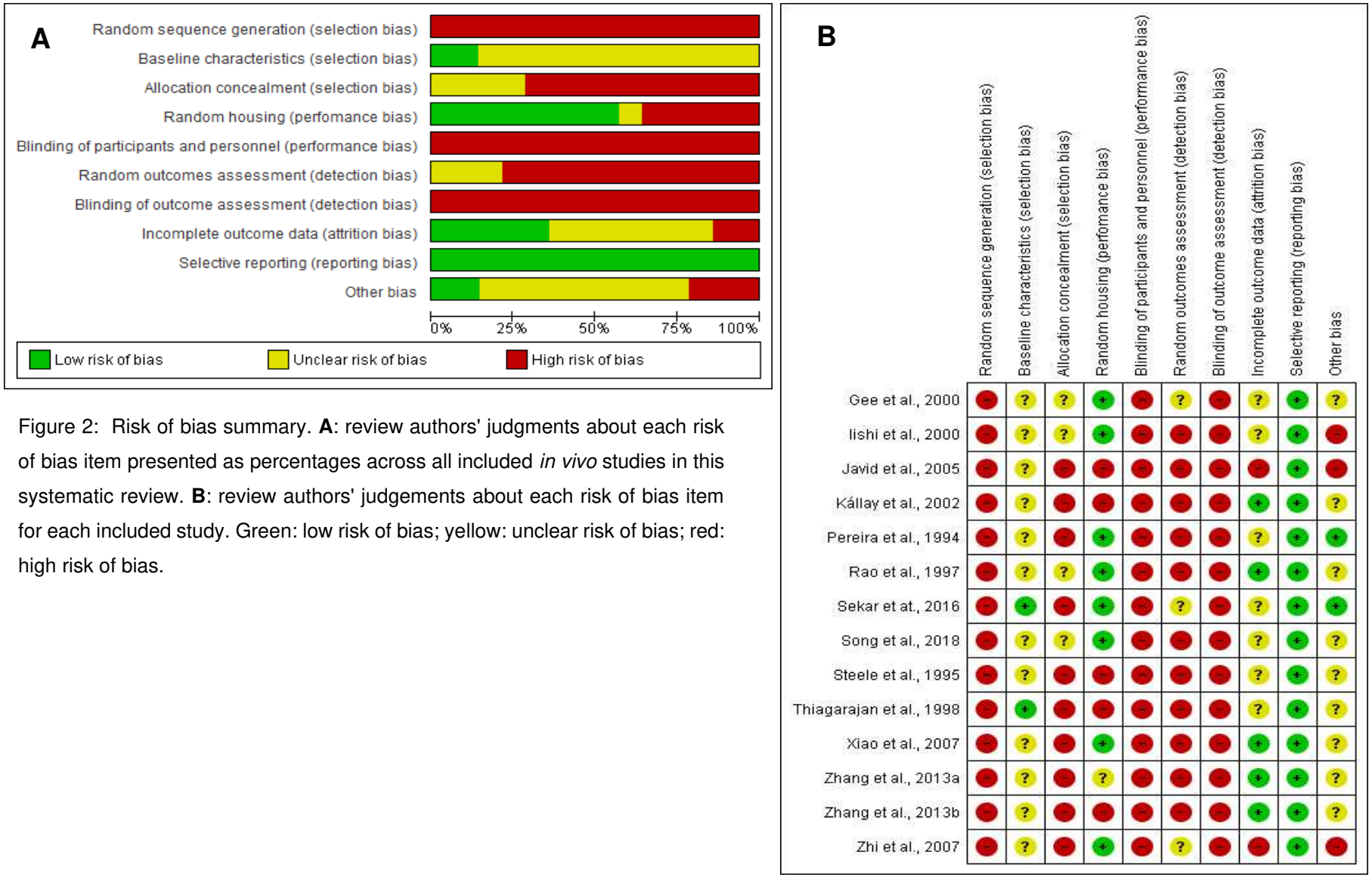


Figure 2: Risk of bias summary. **A**: review authors' judgments about each risk of bias item presented as percentages across all included *in vivo* studies in this systematic review. **B**: review authors' judgements about each risk of bias item for each included study. Green: low risk of bias; yellow: unclear risk of bias; red: high risk of bias.

2.4 Discussion

This systematic review focused on assessing the potential of genistein to prevent and treat CRC. The main finding was that genistein acts dose-dependently, being a chemoprotective agent in high doses (10-100 μM) and proliferative inductor in low doses (0.5-2 μM). Its chemoprotective capacity is due to its potential inhibitory effect on tyrosine kinase enzymes and, its proliferative effect is related to its estrogenic effect. Based on preclinical studies, genistein can potentially be used in CRC prevention and treatment therapies.

2.4.1 Characteristics of the Publications, Animal Models, and Experimental Design

The effects of genistein on CRC have been investigated in preclinical models (*in vivo* and *in vitro*) since 1994. Most of the studies were conducted in the USA, which can be explained by the greater availability of financial support for research in developed countries (88). There are also many studies conducted in Asian countries, which can be explained by the greater interest of this population in the subject since they are the major consumers of soy and derivatives (89).

The most commonly used route of administration in the included studies was the oral route. Although the oral route better mimics human exposure to genistein, it is essential to consider that genistein orally undergoes metabolism by intestinal cells, and very little aglycone genistein reaches the bloodstream. On the other hand, significant quantities of aglycone genistein can be found in the bloodstream following intramuscular or intravenous administration (90). This may explain why the dose in the subcutaneous route was generally lower than the dose provided in the diet.

According to the conversion human equivalent dose (HED) (27), the minimal amount that led to relevant positive outcomes in murine model (2.5 mg/Kg b.w.), by subcutaneous injection, reaches 24 mg/day for a 60 kg human. Considering that isoflavone consumption in countries that most consume soy is 20-50 mg/day (8), ingesting this experimental dose by humans seems to be acceptable. This dose could also be achieved by consuming synthetic genistein. Clinical studies investigating genistein (previously commercialized under the name of Bonistein®), a product containing synthetic genistein, proved its safety and tolerance in maximum oral doses of 120 mg for 14 days (91), or 300 mg administered in a single dose (92).

In vivo studies were performed using rats and mice, with rats predominating over mice. Murine models are frequently used in various research domains due to their anatomical, physiological, and genetic similarities to humans. However, the translatability of outcomes might still be challenging, especially since we are trying to understand the effect of the new formulation due to multiple factors like duration, exposure, and individual variability. The preference for rats over mice may be attributed to the superior efficiency of the carcinogen-induced rat model compared to the mouse model (93). In addition, there is a direct hormonal influence (94) and maybe it can justify the predominance of male animals in the studies of this review (78.57%). However, studies related to human diseases must be conclusive for both genders, accounting for their singularities in physiology avoiding conclusions that comprise half of the population (94). Regarding the animal's weight, 78.57% failed to report this information. This information plays a crucial role in various experimental decision-making processes, including determining the appropriate dosage of treatment and documenting the animal's developmental progress in response to the treatment.

The bias analysis identified an ambiguous risk of bias in the baseline characteristics item, thus corroborating these limitations. In addition, it revealed other limitations, such as the absence of information about randomization in the distribution of animals to treatment groups, the lack of blinding of researchers during the experiments and data analysis, and the absence of information regarding the number of animals in each analysis. Numerous studies also exhibited shortcomings in adequately describing the basal diet and explicitly stating its absence of isoflavones. These details are crucial for ensuring the study's reliability and replicability, as well as validating the statistical analysis.

2.4.2 Primary Outcomes

2.4.3 Primary Outcomes of Genistein on Experimental Colon Cancer, in vitro

Estrogens are known for their role in both inductive and protective effects in CRC. These contradictory effects could be related to different estrogen receptors. For example, the nuclear receptors 1 and 2 (ESR1 and ESR2) are related to different pathways encoded by genes that present different expression patterns. In general, ERS2 is the most prominent among estrogen receptors in colon cells and acts as a tumor suppressor in CRC. ESR1 plays a crucial role in tumor progression in CRC (95). Different of the nuclear receptors that are involved in the genomic response of

estrogen, signaling by GPER initiates a rapid non-genomic reaction of estrogen, mediated by secondary messengers, that modulates several signaling pathways, such as NfκB, RAS/ERK, and AKT (96). The role of GPER-mediated signaling in colon cancer is still controversial. Still, studies provide evidence of its proliferative effect on cancer cells, for example, by activating the tyrosine kinase insulin-like growth factor receptor 1 (IGF1R) and epidermal growth factor receptor (EGFR), which in turn modulates effector proteins (e.g., AKT) and extracellular signal-regulated kinase (ERK) (97).

Because genistein has a similar molecular structure to estrogen (98), it can interact with ESRs (13), triggering downstream signaling cascades, promoting the transcription of target genes directly, and binding to the G protein-coupled receptor GPER1 (16), and finally activating different signaling pathways by modulating kinase protein activity (99). Additionally, genistein has been found to have a significant inhibitory impact on the activity of tyrosine kinase (20) and DNA topoisomerase II (100). As a result, it can disrupt various intracellular signaling pathways and DNA replication processes. Because genistein is more hydrophobic than its related isoflavones (101), it can interact with the cell membrane, preventing the proliferation of cancer cells by changing membrane characteristics (34). Besides, this enhances the ability to interact with membrane lipids and induces protein modifications in signal transduction pathways, creating a molecular network that causes alterations in the expression of multiple genes responsible for regulating cell growth. In this review, genistein modified signal transduction in various signaling pathways that regulate cell proliferation, decreasing cell growth (102). Regarding the extrarenal metabolism of vitamin D, genistein suppresses cell proliferation at low doses (0,01-1 μM) and high doses (10-100 μM). These mechanisms are reported (39; 46; 47), providing evidence that genistein enhances the synthesis of vitamin D, which leads to reduced CRC proliferation, as also reported by other studies (103). Genistein regulates VDR synthesis through ER-dependent pathways since inhibiting ERS2 decreases VDR activation by genistein. We also observed that most of the studies (n=39) presented the chemoprotective effect of genistein in CRC cell lines at high doses. Similar to estrogen effects, genistein, in high doses, could inhibit tyrosine kinase receptors, such as IGF-IR and EGFR, modulating different signaling pathways related to cell proliferation, resulting in proliferation and migration impairment. Genistein modulated p53, TGFβ1, NFκB, PI3/AKT, MAPK and WNT pathways, inducing cell cycle arrest,

apoptosis, reduced cell proliferation, invasion, and migration by change in oxidative status and inflammatory response.

Only three studies have demonstrated the induction of cell proliferation in CRC cells by genistein (71; 72; 73). These occurrences were observed at low concentrations of genistein (0.5-2 μM). The aforementioned studies demonstrate that genistein effectively induces DNA strand breakage, disrupts cell cycle, induces apoptosis, and suppresses cell proliferation when present in high concentrations ranging from 60 to 100 μM. The dose-dependent (biphasic) effect of genistein can account for this phenomenon. At lower concentrations, genistein functions as a phytoestrogen by acting as an estrogen receptor agonist, thereby promoting cellular proliferation. However, when present in higher concentrations, the proliferative action of genistein is overshadowed by its inhibitory effect on tyrosine kinase activity. A schematic diagram is presented in Figure 3 summarizing the main signaling pathways targeted by genistein in CRC cell lines.

2.4.4 Genistein controls the WNT, TGFβ, NF-κB, PI3K/AKT/MAPK, P53 Pathways

The signaling WNT pathway is fundamental for self-renewing intestines in adult mammals (104). This pathway occurs in different (canonical and non-canonical) versions. In the canonical pathway, signaling by WNT glycoprotein leads to the stabilization of β-catenin in the cytoplasm, thus, β-catenin translocates to the nucleus, where it interacts with the transcription factors T cell factor/lymphoid enhancer factor (TCF/LEF), increasing proliferation, invasion, and migration of the cells. The non-canonical versions are diverse and does not directly depend on β-catenin stabilization and nucleus translocation; instead, WNT ligands activate different intracellular signaling cascades, such as, WNT planer cell polarity (WNT/PCP) and the WNT/calcium pathways, which result in the regulation of several effector proteins. The canonical WNT pathway can be modulated by non-canonical WNT signaling. The WNT pathway is related mainly to organizing the orientation and migration of the cells (105; 106; 107). Mutations in the WNT pathway cause disturbances in intestinal homeostasis, leading to pathological conditions such as CRC. This pathway is altered in over 90% of human CRC (108). In CRC, several intermediaries of this pathway are mutated, such as mutations in protein members of the multiprotein complex of β-catenin, the adenomatous polyposis coli (APC) and axin, and mutations in the degradation-inducing phosphorylation sites in β-catenin, which in turn decrease β-

catenin degradation. As a result, intense interaction with the TCF/LEF transcription factors in the nucleus leads to increased proliferation, invasion, and migration of the cells, by the canonical WNT signaling pathway (109). In human colorectal cancer, there can also occur inactivation of WNT antagonists, such as WIF1 and DKK1, leading to intense activation of the WNT signaling (110; 111). Therefore, WNT pathways were one of the most discussed pathways in our review. In this review, genistein reduced methylation at the promoter region of WIF1, enhanced histone H3 acetylation at the DKK1 promoter region and enhanced the gene and protein expression of these genes in moderate to later-stage (54; 64). The WIF1 and DKK1 genes inhibit the WNT gene, which reduces the expression of β-catenin (110; 111). Genistein also inhibited KCNK9 expression, which was associated with the reduction of the WNT/ β-catenin signaling pathway (70).

On the other hand, genistein enhanced the expression of the WNT5A gene in early-stage CRC cells (51). Interestingly, genistein reduced proliferation in these cells. WNT5A is a member of the WNT family with oncogenic or tumor-suppressive actions, regulating mainly non-canonical signaling pathways. By this pathway, degradation of the β-catenin is independent of the multiprotein complex (APC- GSK-3β- axin). WNT5A effects are conditioned by the isoform type (WNT5A-S / WNT5A-L), cell/tissue and receptor effects. However, in CRC, WNT5A seems to act as a tumor suppressor (107). In our revision, genistein did not change the transcript levels of the APC, but it reduced β-catenin transcript levels, suggesting that genistein decreased the cytoplasmic β-catenin levels independently of the degradation of β-catenin by the multiprotein complex (APC- GSK-3β- axin) (57). In addition to this, β-catenin has another role beyond signaling, which is cell adhesion on epithelial cells through its interaction with cadherins (112), and WNT5A is involved in this process (113). Thus, genistein may also have reduced the transcriptional levels of β-catenin by stimulating WNT5A, which led to the stabilization of β-catenin in the β-catenin/E-cadherin complex. This prevented its migration to the nucleus and the transcription of target genes, as newly synthesized β-catenin is first directed to the β-catenin/E-cadherin complex (109). Furthermore, through this pathway, genistein contributed to the increase in cell adhesion, which in turn, prevented cell migration.

Results of the studies included in this review show that genistein reduced levels of c-myc (MYC) and cyclin D (CCND1), increased the E-cadherin/N-cadherin ratio, increased matrix metalloproteinase inhibitor protein 1 (TIMP1), and decreased

matrix metalloproteinases 1 and 9 (MMP1/MMP9). Also, we believe that the reduction of the metastatic FLT4 levels by genistein (59) is related to WNT signaling, as observed in the literature (114). So, it seems that genistein reduces cell invasion and migration modulating several intermediates of the WNT signaling pathway.

By modulating different signaling pathways related to p53, such as the ATM/p53-p21 pathway, the NAG-1 (GDF15)/p53 pathway, and the EGF/FOXO3/p53 pathway, genistein activated the tumor suppressor p53 genes, resulting in cell cycle arrest, apoptosis, and thus, antiproliferative effect. Genistein also directly activates p53 and/or p21, avoiding the progression of S to the G2 phases in the cellular cycle. The P53 gene is considered the guardian of the genome, being activated in several stress responses, such as DNA damage or oncogene activation, which in turn, lead to several downstream responses, including cell cycle arrest and apoptosis. In CRC, the role of P53 in suppressing tumorigenesis is relevant because it is the most frequently mutated gene in human tumors, contributing to tumor progression (115). The ATM/p53-p21waf/cip1 pathway activation by genistein in CRC cells (56), could result in DNA strand breakage by genistein (65; 72). ATM phosphorylates P53, which activates p21 in response to DNA damage (116).

Another protein kinase-dependent pathway modulated by genistein is TGF-β1/Smad/MAPK. Transforming growth factor-β (TGFβ) is a multifunctional cytokine that presents pleiotropic functions in cancer development. TGFβ acts as a tumor suppressor in premalignant cells by inhibiting cell proliferation and activating apoptosis. However, tumoral cells become insensitive to the suppressive effects of TGFβ and begin to use it to their advantage to trigger tumor progression and metastasis. In non-cancerous or premalignant cells, TGFβ acts mainly by its canonical pathway, binding to the receptor and initiating intracellular signaling by phosphorylating protein SMADs that modulate genes related to cell proliferation. In cancer progression, TGFβ promotes tumoral growth through non-canonical pathways as p38 mitogen-activated protein kinase (MAPK), AKT, nuclear factor κ-light-chain enhancer of activated B cells (NF-κB) pathways (117; 118). In this review, genistein induced the formation of Smad–DNA complexes and phosphorylation of Smad2/3 (45); increased the transcription factor ATF3 and inhibited the transcription factor inhibitor of DNA binding (ID1) (52), showing its role in the canonical TGFβ pathway. IDI is a protein that regulates the maintenance of cell renewal in normal cells. IDI proteins are highly expressed in cancer, and their deregulation has a direct role in cancer development (119). Genistein also modulates

the non-canonical TGFβ pathways, by downregulating the proteins Mitogen-activated protein kinases (p38 MAPK) SGK1, and AKT (66). Interestingly, genistein showed antiproliferative effects by increasing TGFβ1 levels in the mouse CRC cell line MC-26 (45), and decreasing them in the metastatic SW480 cell line (66).

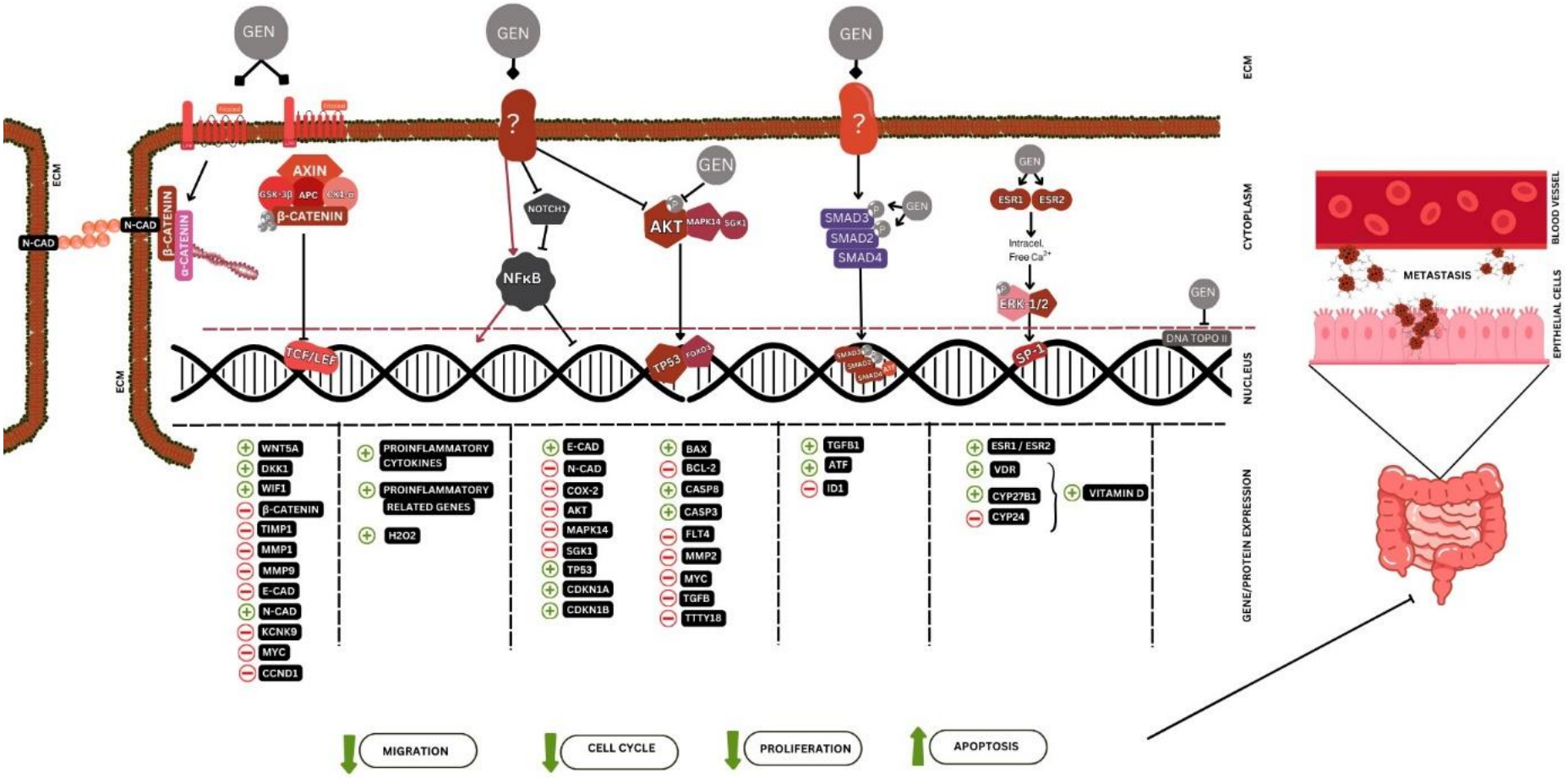
According to the tumoral stage, genistein presents a dual effect on the NF-κB pathway. It inhibited the notch1/NF-κB pathway in LoVo and colon primary cancer HT-29 cells, reducing cell proliferation, migration and induction of apoptosis (58; 63). Also, genistein inhibited the pro-inflammatory enzyme COX-2 activity in DLD1 CRC cells decreasing the chronic inflammation process (36). However, in metastatic CRC SW620 cells, genistein increased the translocation of NF-κB to the nucleus, leading to decreased cell viability, cell cycle changes, and apoptosis (67). This same study showed that genistein increased H_2O_2 production and enhanced the expression of antioxidant enzymes and inflammation-related genes. Besides, genistein increased the production of reactive oxygen species (ROS), and increased the pro-inflammatory cytokines in metastatic SW620 and SW480 cells (69). So, genistein seems to stimulate the NF-κB pathway through its prooxidant effect (120). Indeed, genistein acts as an antioxidant (121) or pro-oxidant (122) in cell proliferation control, depending on the cell stage.

2.4.5 Primary Outcomes of Genistein on Experimental Colon Cancer, in vivo

Overall, our findings indicate that genistein effectively alleviates intestinal disorders induced by experimental CRC in murine models, modulating mainly the WNT/β-catenin and PI3K/AKT/FOXO3 signaling pathways, changing oxidative and inflammatory properties and enhancing vitamin D synthesis. Genistein demonstrated favorable outcomes in 11 studies, while only 3 exhibited adverse effects. Experimental data demonstrate an increase in colon tumors through the administration of genistein in rats subjected to azoxymethane or dimethylhydrazine (74; 75; 76). The authors document a rise in the number of noninvasive and total adenocarcinoma cases and increase of the abnormal crypts (ACF) or crypts accumulated with β-catenin (BCAC), in the colon. However, these studies did not demonstrate any changes in the progression of the invasive tumor, crypt cell proliferation, or apoptosis. Furthermore, these studies' outcomes indicate no observed alterations in animal performance across all groups treated with genistein (74; 75). Meanwhile, the additional studies incorporated in this revision demonstrate encouraging outcomes of genistein in

alleviating CRC-related symptoms. Therefore, these few adverse effects do not invalidate studies on the impact of genistein in the prevention and control of CRC. However, other studies addressing the limitations described here would be needed to confirm and validate the positive outcomes.

Overall, the signaling pathways mentioned here are responsible for maintaining intestinal homeostasis and are highly activated in CRC. Genistein was able to reverse this. According to our molecular network proposed using CRC cell lines (Fig. S1), genistein decreases cell invasion and proliferation, disrupts the cell cycle, and induces apoptosis by activating or inhibiting WNT/β-catenin pathway, modulating the different signaling pathways, which in turn leads to activation of the tumor suppressor P53*,* and modulating signaling pathways AKT, MAPK, and TGF-β1 dependents. Thus, genistein, a natural compound, seems a good candidate for therapeutic strategies to prevent or aid in treating CRC. Crosstalk commonly occurs among the signaling pathways, resulting in synergistic or antagonistic effects, including in CRC conditions (117; 118). Thus, detailing the complexity of this signaling pathway crosstalk is a limitation of this study, and more studies are necessary to investigate more specific gene targets for genistein in CRC cell lines. While this review indicates that genistein's effect on CRC depends on the dosage, only three studies have reported its effect at low doses. Thus, additional research employing lower doses of genistein is required to substantiate its potential. In preclinical studies, a dose range of 10-100 μM genistein appears promising for treating CRC.


GEN
ECM
CYTOPLASM
NUCLEUS
GENE/PROTEIN EXPRESSION
N-CAD
β-CATENIN
α-CATENIN
AXIN
GSK-3β
APC
CK1-α
β-CATENIN
NOTCH1
NFκB
AKT
MAPK14
SGK1
SMAD3
SMAD2
SMAD4
ESR1
ESR2
Intracel. Free Ca2+
ERK1/2
DNA TOPO II
TCF/LEF
TP53
FOXO3
SP-1
WNT5A
DKK1
WIF1
β-CATENIN
TIMP1
MMP1
MMP9
E-CAD
N-CAD
KCNK9
MYC
CCND1
PROINFLAMMATORY CYTOKINES
PROINFLAMMATORY RELATED GENES
H2O2
E-CAD
N-CAD
COX-2
AKT
MAPK14
SGK1
TP53
CDKN1A
CDKN1B
BAX
BCL-2
CASP8
CASP3
FLT4
MMP2
MYC
TGFB
TTTY18
TGFB1
ATF
ID1
ESR1 / ESR2
VDR
CYP27B1
CYP24
VITAMIN D
MIGRATION
CELL CYCLE
PROLIFERATION
APOPTOSIS
BLOOD VESSEL
METASTASIS
EPITHELIAL CELLS

1

Figure 3: Schematic diagram summarizing molecular targets for genistein (10-100 μM) based on *in vitro* primary outcomes. According to these outcomes we hypothesize that genistein modulates several intermediates of the WNT, TGFβ, NFκB, PI3K/AKT/MAPK, vitamin D and p53-dependent pathways, resulting in decrease of proliferation, migration, cell cycle arrest and increase of apoptosis. This scenario disfavors metastasis, which is desirable for the prevention and treatment of CRC. GEN, Genistein; WNT5A, Wingless-type family member 5A, DKK1, Dickkopf-1; WIF1, WNT inhibitory factor-1; TIMP1, Matrix metalloproteinase inhibitor protein 1; MMP1, Matrix metalloproteinase 1; MMP2, Matrix metalloproteinase 2; MMP9, Matrix metalloproteinase 9; E-CAD, E-cadherin; N-CAD, N-cadherin; KCNK9, Potassium channel; MYC, c-MYC; CCND1, Cyclin D1; COX-2, Cyclooxygenase-2; CDKN1A, Cyclin Dependent Kinase Inhibitor 1A; CDKN1B, Cyclin Dependent Kinase Inhibitor 1B; CASP8, Caspase 8; CASP3, Caspase 3; FLT4, VEGF family of receptors; TGFB, Transforming Growth Factor B; TTY18, long noncoding RNA (lncRNA); ATF3, Activating transcription factor 3 gene; ID1, Inhibitor of DNA binding/differentiation-1 (ID1) gene; ESR; Estrogen receptor; VDR, Vitamin D receptor; CYP27B1, 25-Hydroxyvitamin D 1-alpha-hydroxylase; CYP24, 24-hydroxylase enzyme.

2.5 Conclusion

Based on *in vitro* investigations, it has been observed that genistein exhibits proliferative effects at low concentrations (0.5-2 μM) and chemoprotective effects at higher concentrations (10-100 μM). *In vitro*, genistein's cancer-protective properties are attributed to its ability to inhibit tyrosine kinase, while its estrogenic effect is associated with cell proliferation, except for the vitamin D synthesis. Genistein regulates CRC by influencing the activity of WNT, TGFβ, NFκB, PI3K/AKT/MAPK, the oxidant, and inflammatory responses and promoting vitamin D synthesis in the models analyzed. Based on NFκB -related outcomes, genistein exhibits antioxidant and anti-inflammatory properties in early-stage colorectal cancer cells while demonstrating pro-oxidant and pro-inflammatory effects in later-stage CRC cells. The chemoprotective effect of genistein is supported by *in vivo* studies, which primarily inhibit the activation of the WNT/β-catenin and PI3K/AKT/FOXO3 signaling pathways.

2.6 Supplementary materials

All supplementary tables and figures are available at Soares Martins, M. T., Ladeira, L., Matias Sarandy, M., Mattosinhos, P., Neves, C., Matta, S., & Vilela Goncalves, R. (2024). Supplementary Materials for Martins *et al*. - Genistein and Colorectal Cancer - systematic review (1.0). Zenodo. https://doi.org/10.5281/zenodo.12534221, under the License Creative Commons Attribution 4.0 International (CC BY 4.0) License https://creativecommons.org/licenses/by/4.0/).

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References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soeriomataram I, Jemal A, *et al*. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians* **2021**.
2. Issa IA, Noureddine M. Colorectal cancer screening: An updated review of the available options. *World Journal of Gastroenterology* **2017**;23(28):5086.
3. Kuipers EJ, Grady WM, Lieberman D, Seufferlein T, Sung JJ, Boenlens PG, *et al*. Colorectal cancer. *Nature Reviews Disease Primers* **2015**;1:15065.
4. Bukowski K, Kciuk M, Kontek R. Mechanisms of multidrug resistance in cancer chemotherapy. *International Journal of Molecular Sciences **2020**;*21(9):3233.
5. Ryan-harshman, M.; Aldoori, W. Diet and colorectal cancer: Review of the evidence. *Canadian Family Physician* **2007**;53(11):1913-1920.
6. Veettil SK, Wong TY, Loo YS, Playdon MC, Lai NM, Giovannucci EL, Chaiyakunapruk N. Role of diet in colorectal cancer incidence: umbrella review of meta-analyses of prospective observational studies. *JAMA Network Open* **2021**;4(2):e2037341-e2037341.
7. Yu Y, Jing X, Li H, Zhao X, Wang D. Soy isoflavone consumption and colorectal cancer risk: a systematic review and meta-analysis. *Scientific Reports* **2016**; 6(1):25939.
8. Arai Y, Uehara M, Sato Y, Kimira M, Eboshida A, Adlercreutz H, Watanabe S. Comparison of isoflavones among dietary intake, plasma concentration and urinary excretion for accurate estimation of phytoestrogen intake. *Journal of Epidemiology* **2000**;*10*(2):127-135.
9. Grace PB, Taylor JI, Low YL, Luben RN, Mulligan AA, Botting NP. Phytoestrogen concentrations in serum and spot urine as biomarkers for dietary phytoestrogen intake and their relation to breast cancer risk in European prospective investigation of cancer and nutrition-norfolk. *Cancer Epidemiology Biomarkers & Prevention* **2004**;13(5):698-708.
10. Sulistyowati E, Martono S, Riyanto S, Lukitaningsih E. Development and validation for free aglycones daidzein and genistein in soybeans (*Glycine max* (L.) Merr.) using RP HPLC method. *International Journal of Applied Pharmaceutics* **2019**;11(2):138-142.

11. Zava DT, Duwe G. Estrogenic and antiproliferative properties of genistein and other flavonoids in human breast cancer cells *in vitro*. *Nutrition and cancer* **1997**;27(1):31-40.
12. Mukund V. Genistein: its role in breast cancer growth and metastasis. *Current drug Metabolism* **2020**;21(1):6-10.
13. Manas ES, Xu ZB, Unwalla RJ, Somers WS. Understanding the selectivity of genistein for human estrogen receptor-β using X-ray crystallography and computational methods. *Structure* **2004**;12(12):2197-2207.
14. Maingi JW, Tang S, Liu S, Ngenya W, Bao E. Targeting estrogen receptors in colorectal cancer. *Molecular Biology Reports* **2020**;47:4087-4091.
15. Thomas P, Dong J. Binding and activation of the seven-transmembrane estrogen receptor GPR30 by environmental estrogens: a potential novel mechanism of endocrine disruption. *The Journal of Steroid Biochemistry and Molecular Biology* **2006**;102(1-5):175-179.
16. Du ZR, Feng XQ, Li N, Qu JX, Feng L, Chen L, *et al*. G protein-coupled estrogen receptor is involved in the anti-inflammatory effects of genistein in microglia. *Phytomedicine* **2018**;43:11-20.
17. Konstantinopoulos PA, Kominea A, Vandoros G, Sykiotis GP, Andricopoulos P, Varakis I, *et al.* Oestrogen receptor beta (ERβ) is abundantly expressed in normal colonic mucosa, but declines in colon adenocarcinoma paralleling the tumour's dedifferentiation. *European Journal of Cancer* **2003**;39(9):1251-1258.
18. Tuo B, Wen G, Wei J, Liu X, Wang X, Zhang Y*, et al*. Estrogen regulation of duodenal bicarbonate secretion and sex-specific protection of human duodenum. *Gastroenterology* **2011**;141(3):854-863.
19. Yang X, Guo Y, He J, Zhang F, Sun X, Yang S, Dong H. Estrogen and estrogen receptors in the modulation of gastrointestinal epithelial secretion. *Oncotarget* **2017**;8(57):97683.
20. Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe SI, Itoh N, *et al.* Genistein, a specific inhibitor of tyrosine-specific protein kinases. *Journal of Biological Chemistry* **1987**;262(12):5592-5595.
21. Ko KP, Yeo Y, Yoon JH, Kim CS, Tokudome S, Koriyama C, *et al*. Plasma phytoestrogens concentration and risk of colorectal cancer in two different Asian populations. *Clinical Nutrition* **2018**;37(5):1675-1682.

22. Chen X, Gu J, Wu Y, Liang P, Shen M, Xi J, *et al*. Clinical characteristics of colorectal cancer patients and anti-neoplasm activity of genistein. *Biomedicine & Pharmacotherapy* **2020**;124:0753-3322.
23. Naeem H, Momal U, Imran M, Shahbaz M, Hussain M, Alsagaby SA, *et al*. Anticancer perspectives of genistein: a comprehensive review. *International Journal of Food Properties* **2023**;26(2):3305-3341.
24. Moore AB, Castro L, Yu L, Zheng X, Di X, Sifre MI, *et al*. Stimulatory and inhibitory effects of genistein on human uterine leiomyoma cell proliferation are influenced by the concentration. *Human Reproduction* **2007**;22(10):2623-2631.
25. Chen HH, Chen SP, Zheng QL, Nie SP, Li WJ, Hu XJ, *et al*. Genistein promotes proliferation of human cervical cancer cells through estrogen receptor-mediated PI3K/AKT-NF-κB pathway. *Journal of Cancer* **2018**;9(2):288.
26. Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, *et al*. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. *BMJ* **2021**;372:71.
27. Nair AB, Jacob SA. simple practice guide for dose conversion between animals and human. *Journal of Basic and Clinical Pharmacy* **2016**;7(2):27.
28. Viera AJ, Garrett JM.Understanding interobserver agreement: the kappa statistic. *Family Medicine* **2005**;37(5):360-363.
29. Vinken M, Knapen D, Vergauwen L, Hengstler JG, Angrish M, Whelan M. Adverse outcome pathways: a concise introduction for toxicologists. *Archives of Toxicology* **2017**;91:3697-3707.
30. Kutmon M, Van Iersel MP, Bohler A, Kelder T, Nunes N, Pico AR, *et al*. PathVisio 3: an extendable pathway analysis toolbox. *PLoS Computational Biology* **2015**;*11*(2):e1004085.
31. Hooijmans CR, Rovers MM, De Vries RB, Leenaars M, Ritskes-Hoitinga, M, Langendam MW. SYRCLE's risk of bias tool for animal studies. *BMC Medical Research Methodology* **2014**;14:1-9.
32. Heruth DP, Wetmore LA, Leyva A, Rothberg PG. Influence of protein tyrosine phosphorylation on the expression of the c-myc oncogene in cancer of the large bowel. *Journal of Cellular Biochemistry* **1995**;*58*(1):83-94.
33. Booth C, Hargreaves DF, Hadfield JA, McGown AT, Potten CS. Isoflavones inhibit intestinal epithelial cell proliferation and induce apoptosis *in vitro*. *British Journal of Cancer* **1999**;*80*(10):1550-1557.

34. Yu J, Cheng Y, Zhang R. (1999). Effects of genistein and daidzein on membrane characteristics of HCT cells. *Nutrition and Cancer* **1999**;33(1):100-4.
35. Arai N, Ström A, Rafter J. J, Gustafsson JÅ. Estrogen receptor β mRNA in colon cancer cells: growth effects of estrogen and genistein. *Biochemical and Biophysical Research Communications* **2000**;270(2):425-431.
36. Mutoh M, Takahashi M, Fukuda K, Matsushima-Hibiya Y, Mutoh H, Sugimura T, Wakabayashi K. Suppression of cyclooxygenase-2 promoter-dependent transcriptional activity in colon cancer cells by chemopreventive agents with a resorcin-type structure. *Carcinogenesis* **2000**;*21*(5):959-963.
37. Park JH, Oh EJ, Choi YH, Kang CD, Kang HS, Kim DK*, et al*. Synergistic effects of dexamethasone and genistein on the expression of Cdk inhibitor $p21^{WAF1/CIP1}$ in human hepatocellular and colorectal carcinoma cells. *International Journal of Oncology* **2001**;*18*(5):997-1002.
38. Plewa MJ, Berhow MA, Vaughn SF, Woods EJ, Rundell M, Naschansky K, *et al.* Isolating antigenotoxic components and cancer cell growth suppressors from agricultural by-products. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **2001**;*480*:109-120.
39. Lechner D, Cross HS. Phytoestrogens and 17β-Estradiol Influence Vitamin D Metabolism and Receptor Expression—Relevance for Colon Cancer Prevention. *In Vitamin D Analogs in Cancer Prevention and Therapy*. Berlin, Heidelberg: Springer Berlin Heidelberg **2003**; 379-391.
40. Wilson LC, Baek SJ, Call A, Eling TE. Nonsteroidal anti-inflammatory drug-activated gene (NAG-1) is induced by genistein through the expression of p53 in colorectal cancer cells. *International Journal of Cancer* **2003**;*105*(6):747-753.
41. Bayazit V. Cytotoxic effects of some animal and vegetable extracts and some chemicals on liver and colon carcinoma and myosarcoma. *Saudi Medical Jornal* **2004**;*25*(2):156-163.
42. Yu Z, Li W, Liu F. Inhibition of proliferation and induction of apoptosis by genistein in colon cancer HT-29 cells. *Cancer Letters* **2004**;*215*(2):159-166.
43. Linsalata M, Russo F, Notarnicola M, Guerra V, Cavallini A, Clemente C, *et al.* Effects of genistein on the polyamine metabolism and cell growth in DLD-1 human colon cancer cells. *Nutrition and Cancer* **2005**;*52*(1):84-93.
44. Prete SP, Rossi L, Correale PP, Turriziani M, Baier S, Tamburrelli G, *et al*. Combined effects of protein kinase inhibitors and 5-fluorouracil on CEA

expression in human colon cancer cells. *Pharmacological Research* **2005**;*52*(2):167-173.

45. Yu Z, Tang Y, Hu D, Li J. Inhibitory effect of genistein on mouse colon cancer MC-26 cells involved TGF-β1/Smad pathway. *Biochemical and Biophysical Research Communications* **2005**;*333*(3):827-832.
46. Gilad LA, Tirosh O, Schwartz B. Phytoestrogens regulate transcription and translation of vitamin D receptor in colon cancer cells. *Journal of Endocrinology* **2006**;*191*(2):387-398.
47. Lechner D, Bajna E, Adlercreutz H, Cross HS. Genistein and 17β-estradiol, but not equol, regulate vitamin D synthesis in human colon and breast cancer cells. *Anticancer Research* **2006**;*26*(4A):2597-2603.
48. Ogasawara M, Matsunaga T, Suzuki H. Differential effects of antioxidants on the *in vitro* invasion, growth and lung metastasis of murine colon cancer cells. *Biological and Pharmaceutical Bulletin* **2007**;*30*(1):200-204.
49. Caruso MG, Messa C, Orlando A, D'Attoma B, Notarnicola M. Early induction of LDL receptor gene expression by genistein in DLD-1 colon cancer cell line. *Fitoterapia* **2008**;*79*(7-8):524-528.
50. Berner C, Aumüller E, Gnauck A, Nestelberger M, Just A, Haslberger AG. Epigenetic control of estrogen receptor expression and tumor suppressor genes is modulated by bioactive food compounds. *Annals of Nutrition and Metabolism* **2010**;*57*(3-4):183-189.
51. Wang Z, Chen H. Genistein increases gene expression by demethylation of WNT5a promoter in colon cancer cell line SW1116. *Anticancer Research* **2010**;*30*(11):4537-4545.
52. Bottone Jr FG, Alston-Mills B. The dietary compounds resveratrol and genistein induce activating transcription factor 3 while suppressing inhibitor of DNA binding/differentiation-1. *Journal of Medicinal Food* **2011**;*14*(6):584-593.
53. Qi W, Weber CR, Wasland K, Savkovic SD. Genistein inhibits proliferation of colon cancer cells by attenuating a negative effect of epidermal growth factor on tumor suppressor FOXO3 activity. *BMC Cancer* **2011**;(11):1-9.
54. Wang H, Li Q, Chen H. Genistein affects histone modifications on Dickkopf-related protein 1 (DKK1) gene in SW480 human colon cancer cell line. *PloS One* **2012**;*7*(7):e40955.

55. Mizushina Y, Shiomi K, Kuriyama I, Takahashi Y, Yoshida H. Inhibitory effects of a major soy isoflavone, genistein, on human DNA topoisomerase II activity and cancer cell proliferation. *International Journal of Oncology* **2013**;*43*(4):1117-1124.
56. Zhang Z, Wang CZ, Du GJ, Qi LW, Calway T, He TC, *et al*. Genistein induces G2/M cell cycle arrest and apoptosis via ATM/p53-dependent pathway in human colon cancer cells. *International Journal of Oncology* **2013**;*43*(1):289-296.
57. Lepri SR, Zanelatto LC, Da Silva PBG, Sartori D, Ribeiro LR, Mantovani MS.The effects of genistein and daidzein on cell proliferation kinetics in HT29 colon cancer cells: the expression of CTNNBIP1 (β-catenin), APC (adenomatous polyposis coli) and BIRC5 (survivin). *Human Cell* **2014**;*27*:78-84.
58. Luo Y, Wang SX, Zhou ZQ, Wang Z, Zhang YG, Zhang Y, *et al.* Apoptotic effect of genistein on human colon cancer cells via inhibiting the nuclear factor-kappa B (NF-κB) pathway. *Tumor Biology* **2014**;*35*(11):11483-11488.
59. Xiao X, Liu Z, Wang R, Wang J, Zhang S, Cai X, *et al.* Genistein suppresses FLT4 and inhibits human colorectal cancer metastasis. *Oncotarget* **2015**;*6*(5):3225.
60. Qin J, Teng J, Zhu Z, Chen J, Huang WJ. Genistein induces activation of the mitochondrial apoptosis pathway by inhibiting phosphorylation of Akt in colorectal cancer cells. *Pharmaceutical Biology* **2016**;*54*(1):74-79.
61. Shafiee G, Saidijam M, Tavilani H, Ghasemkhani N, Khodadadi I. Genistein induces apoptosis and inhibits proliferation of HT29 colon cancer cells. *International Journal of Molecular and Cellular Medicine* **2016**;*5*(3):178.
62. Pintova S, Planutis K, Planutiene M, Holcombe RF. ME-143 is superior to genistein in suppression of WNT signaling in colon cancer cells. *Anticancer Research* **2017**;*37*(4):1647-1653.
63. Zhou P, Wang C, Hu Z, Chen W, Qi W, Li A. Genistein induces apoptosis of colon cancer cells by reversal of epithelial-to-mesenchymal via a Notch1/NF-κB/slug/E-cadherin pathway. *BMC cancer* **2017**;*17*:1-10.
64. Zhu J, Ren J, Tang L. Genistein inhibits invasion and migration of colon cancer cells by recovering WIF1 expression. *Molecular Medicine Reports* **2018**;*17*(5):7265-7273.

65. Schroeter A, Aichinger G, Stornig K, Marko D. Impact of oxidative metabolism on the cytotoxic and genotoxic potential of genistein in human colon cancer cells. *Molecular Nutrition & Food Research* **2019**;*63*(2):1800635.
66. Chen X, Wu Y, Gu J, Liang P, Shen M, Xi J, Qin J. Anti-invasive effect and pharmacological mechanism of genistein against colorectal cancer. *Biofactors* **2020**;*46*(4):620-628.
67. Alorda-Clara M, Torrens-Mas M, Morla-Barcelo PM, Roca P, Sastre-Serra J, Pons D G, *et al.* High Concentrations of Genistein Decrease Cell Viability Depending on Oxidative Stress and Inflammation in Colon Cancer Cell Lines. *International Journal of Molecular Sciences* **2022**;*23*(14):7526.
68. Castaño M, Martínez E, Osorio M, Castro C. Development of genistein drug delivery systems based on bacterial nanocellulose for potential colorectal cancer chemoprevention: effect of nanocellulose surface modification on genistein adsorption. *Molecules* **2022**;*27*(21):7201.
69. Rendón JP, Cañas AI, Correa E, Bedoya-Betancur V, Osorio M, Castro C, Naranjo TW. Evaluation of the effects of genistein in vitro as a chemopreventive agent for colorectal cancer—strategy to improve its efficiency when administered orally. *Molecules* **2022**;*27*(20):7042.
70. Cheng Y, Tang Y, Tan Y, Li J, Zhang X. KCNK9 mediates the inhibitory effects of genistein on hepatic metastasis from colon cancer. *Clinics* **2023**;*78*:100141.
71. Fiorelli G, Picariello L, Martineti V, Tonelli F, Brandi ML. Estrogen synthesis in human colon cancer epithelial cells. *The Journal of Steroid Biochemistry and Molecular Biology* **1999**;*71*(5-6):223-230.
72. Salti GI, Grewal S, Mehta RR, Gupta TD, Boddie Jr AW, Constantinou AI. Genistein induces apoptosis and topoisomerase II-mediated DNA breakage in colon cancer cells. *European Journal of Cancer* **2000**;*36*(6):796-802.
73. Chatzinikolaou G, Nikitovic D, Stathopoulos EN, Velegrakis GA, Karamanos NK, Tzanakakis GN. Protein tyrosine kinase and estrogen receptor-dependent pathways regulate the synthesis and distribution of glycosaminoglycans/proteoglycans produced by two human colon cancer cell lines. *Anticancer Research* **2007**;*27*(6B): 4101-4106.
74. Rao CV, Wang CX, Simi B, Lubet R, Kelloff G, Steele V, Reddy BS. Enhancement of experimental colon cancer by genistein. *Cancer research* **1997**;57(17):3717-3722.

75. Gee JM, Noteborn HPJM, Polley ACJ, Johnson IT. Increased induction of aberrant crypt foci by 1, 2-dimethylhydrazine in rats fed diets containing purified genistein or genistein-rich soya protein. *Carcinogenesis* **2000**;21(12):2255-2259.
76. Zhi H, Yamada Y, Hirose Y, Kato K, Sheng H, Zheng Q, Mori H. Effect of 2-(carboxyphenyl) retinamide and genistein on the formation of early lesions in 1, 2-dimethylhydrazine-induced colon carcinogenesis in rats. *Asian Pacific Journal of Cancer Prevention* **2007**;8(1):33-38.
77. Javid SH, Moran AE, Carothers AM, Redston M, Bertagnolli MM. Modulation of tumor formation and intestinal cell migration by estrogens in the Apc Min/+ mouse model of colorectal cancer. *Carcinogenesis* **2005**;26(3):587-595.
78. Xiao R, Hennings LJ, Badger TM, Simmen FA. Fetal programing of colon cancer in adult rats: correlations with altered neonatal growth trajectory, circulating IGF-I and IGF binding proteins, and testosterone. *Journal of Endocrinology* **2007**; 195(1):79-87.
79. Pereira MA, Barnes LH, Rassman VL, Kelloff GV, Steele VE. Use of azoxymethane-induced foci of aberrant crypts in rat colon to identify potential cancer chemopreventive agents. *Carcinogenesis* **1994**;15(5):1049-1054.
80. Steele VE, Pereira MA, Sigman CC, Kelloff GJ. Cancer chemoprevention agent development strategies for genistein. *The Journal of nutrition* **1995**;125:713S-716S.
81. Thiagarajan DG, Bennink MR, Bourquin LD, Kavas FA. Prevention of precancerous colonic lesions in rats by soy flakes, soy flour, genistein, and calcium. *The American journal of Clinical Nutrition* **1998**;68(6):1394S-1399S.
82. Zhang Y, Li Q, Zhou D, Chen H. Genistein, a soya isoflavone, prevents azoxymethane-induced up-regulation of WNT/β-catenin signalling and reduces colon pre-neoplasia in rats. *British Journal of Nutrition* **2013**;109(1):33-42.
83. Sekar V, Anandasadagopan SK, Ganapasam S. Genistein regulates tumor microenvironment and exhibits anticancer effect in dimethyl hydrazine-induced experimental colon carcinogenesis. *Biofactors* **2016**;42(6):623-637.
84. Iishi H, Tatsuta M, Baba M, Yano H, Sakai N, Akedo H. Genistein attenuates peritoneal metastasis of azoxymethane-induced intestinal adenocarcinomas in Wistar rats. *International Journal of Cancer* **2000**;86(3):416-420.
85. Song S, Cheng D, Wei S, Wang X, Niu Y, Qi W, Wang C. Preventive effect of genistein on AOM/DSS-induced colonic neoplasm by modulating the

PI3K/AKT/FOXO3 signaling pathway in mice fed a high-fat diet. *Journal of Functional Foods* **2018**;46:237-242.

86. Zhang Y, Li Q, Chen H. DNA methylation and histone modifications of Wnt genes by genistein during colon cancer development. *Carcinogenesis* **2013**;34(8): 1756-1763.
87. Kállay E, Farhan H, Lechner D, Bajna E, Cross HS, Adlercreutz H, Campbell M. Phytoestrogens regulate vitamin D metabolism in the mouse colon: relevance for colon tumor prevention and therapy. *The Journal of Nutrition* **2002**;132(11):3490S-3493S.
88. Salager-Meyer F. Scientific publishing in developing countries: Challenges for the future. *Journal of English for Academic Purposes* **2008**;7(2):121-132.
89. He FJ, Chen JQ. Consumption of soybean, soy foods, soy isoflavones and breast cancer incidence: Differences between Chinese women and women in Western countries and possible mechanisms. *Food Science and Human Wellness* **2013**; 2(3-4):146-161.
90. Barnes S, Prasain J, D'Alessandro T, Arabshahi A, Botting N, Lila MA, *et al*. The metabolism and analysis of isoflavones and other dietary polyphenols in foods and biological systems. *Food & Function* **2011**;2(5):235-244.
91. Ullmann U, Oberwittle H, Grossmann M, Riegger C. Repeated Oral Once Daily Intake of Increasing Doses of the Novel Synthetic Genistein Product Bonistein™ in Healthy Volunteers. *Planta Medica* **2005**;71(10):891-896.
92. Ullmann U, Metzner J, Frank T, Cohn W, Riegger C. Safety, tolerability, and pharmacokinetics of single ascending doses of synthetic genistein (Bonistein™) in healthy volunteers. Advances in Therapy **2005**;22:65-78.
93. Corpet DE, Pierre F. How good are rodent models of carcinogenesis in predicting efficacy in humans? A systematic review and meta-analysis of colon chemoprevention in rats, mice and men. *European Journal of Cancer* **2005**;41(13): 1911-1922.
94. Plevkova J, Brozmanova M, Harsanyiova J, Sterusky M, Honetschlager J, Buday T. Various aspects of sex and gender bias in biomedical research. *Physiological Research* **2020**;69(Suppl 3):S367.
95. Caiazza F, Ryan EJ, Doherty G, Winter DC, Sheahan K. Estrogen receptors and their implications in colorectal carcinogenesis. *Frontiers in oncology* **2015**;*5*:19.

96. Das PK, Saha J, Pillai S, Lam AKY, Gopalan V, Islam F. Implications of estrogen and its receptors in colorectal carcinoma. *Cancer Medicine* **2023**;12(4): 4367-4379.
97. Pisano A, Santolla MF, De Francesco EM, De Marco P, Rigiracciolo DC, Perri MG*, et al*. GPER, IGF-IR, and EGFR transduction signaling are involved in stimulatory effects of zinc in breast cancer cells and cancer-associated fibroblasts. *Molecular Carcinogenesis* **2017**;56(2):580-593.
98. Kuiper GG, Lemmen JG, Carlsson BO, Corton JC, Safe SH, Van Der Saag PT, *et al*. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. *Endocrinology* **1998**;139(10):4252-4263.
99. Li J, Li J., Yue Y, Hu Y, Cheng W, Liu R, *et al*. Genistein suppresses tumor necrosis factor α-induced inflammation via modulating reactive oxygen species/AKT/nuclear factor κB and adenosine monophosphate-activated protein kinase signal pathways in human synoviocyte MH7A cells. *Drug Design, Development and Therapy* **2014**;17(8):315-323.
100. Baechler SA, Soukup ST, Molzberger AF, Kulling SE, Diel P, Marko D. Topoisomerase poisoning by genistein in the intestine of rats. *Toxicology Letters* **2016**;243:88-97.
101. Whaley WL, Rummel JD, Kastrapeli N. Interactions of genistein and related isoflavones with lipid micelles. *Langmuir* **2006**;22(17):7175-7184.
102. Chen AC, Donovan SM. Genistein at a concentration present in soy infant formula inhibits Caco-2BBe cell proliferation by causing G2/M cell cycle arrest. *The Journal of Nutrition* **2004**;134(6):1303-1308.
103. Ng K. Vitamin D for prevention and treatment of colorectal cancer: what is the evidence? *Current colorectal Cancer Reports* **2014**;10:339-345.
104. Pinto D, Clevers H. Wnt control of stem cells and differentiation in the intestinal epithelium. *Experimental Cell Research* **2005**;*306*(2):357-363.
105. McDonald SL, Silver A. The opposing roles of Wnt-5a in cancer. *British Journal of Cancer* **2009**;101(2):209-214.
106. De A. WNT/Ca^{2+} signaling pathway: a brief overview. *Acta Biochimica et Biophysica Sinica* **2011**;43:745–756.
107. Bueno MLP, Saad STO, Roversi FM. WNT5A in tumor development and progression: A comprehensive review. *Biomedicine & Pharmacotherapy* **2022**;155: 113599.

108. The Cancer Genome Atlas Network. 2012. Comprehensive molecular characterization of human colon and rectal cancer. *Nature* **2012**;487:330-337.
109. Clevers H. WNT/β-catenin signaling in development and disease. *Cell* **2006**;127(3):469-480.
110. Taniguchi H, Yamamoto H, Hirata T, Miyamoto N, Oki M, Nosho K, *et al*. Frequent epigenetic inactivation of WNT inhibitory factor-1 in human gastrointestinal cancers. *Oncogene* **2005**;24(53):7946-7952.
111. Aguilera O, Fraga MF, Ballestar E, Paz, MF, Herranz M, Espada J, *et al*. Epigenetic inactivation of the WNT antagonist DICKKOPF-1 (DKK-1) gene in human colorectal cancer. *Oncogene* **2006**;25(29):4116-4121.
112. Peifer M, McCrea PD, Green KJ, Wieschaus E, Gumbiner BM. The vertebrate adhesive junction proteins beta-catenin and plakoglobin and the Drosophila segment polarity gene armadillo form a multigene family with similar properties. *The Journal of Cell Biology* **1992**;118(3):681-691.
113. Medrek C, Landberg G, Andersson T, Leandersson K. WNT-5a-CKIα signaling promotes β-catenin/E-cadherin complex formation and intercellular adhesion in human breast epithelial cells. *Journal of Biological Chemistry* **2009**;284(16):10968–10979.
114. Wang Y, Sang A, Zhu M, Zhang G, Guan H, Ji M, Chen H. Tissue factor induces VEGF expression via activation of the WNT/β-catenin signaling pathway in ARPE-19 cells. *Molecular Vision* **2016**;22:886.
115. Liebl MC, Hofmann TG. The role of p53 signaling in colorectal cancer. *Cancers* **2021**;13(9):2125.
116. Al Bitar S, Gali-Muhtasib H. The role of the cyclin dependent kinase inhibitor p21cip1/waf1 in targeting cancer: Molecular mechanisms and novel therapeutics. *Cancers* **2019**;11(10): 1475.
117. Cheruku HR, Mohamedali A, Cantor DI, Tan SH, Nice EC, Baker MS.Transforming growth factor-β, MAPK and WNT signaling interactions in colorectal cancer*. EuPA Open Proteomics* **2015;**8:104-115.
118. Liu S, Ren J, Ten Dijke P. Targeting TGFβ signal transduction for cancer therapy. *Signal Transduction and Targeted Therapy* **2021**;6(1):8.
119. Lasorella A, Benezra R, Iavarone A. The ID proteins: master regulators of cancer stem cells and tumour aggressiveness. *Nature Reviews Cancer* **2014**; 14(2):77-91.

120. Flohé L, Brigelius-Flohé R, Saliou C, Traber MG, Packer L. Redox regulation of NF-kappa B activation. *Free Radical Biology and Medicine* **1997**;22(6):1115-1126.
121. Ganai AA, Farooqi H. Bioactivity of genistein: A review of in vitro and in vivo studies. *Biomedicine & Pharmacotherapy* **2015**;76:30-38.
122. Sánchez Y, Amrán D, Fernández C, de Blas E, Aller P. Genistein selectively potentiates arsenic trioxide-induced apoptosis in human leukemia cells via reactive oxygen species generation and activation of reactive oxygen species-inducible protein kinases (p38-MAPK, AMPK). *International Journal of Cancer* **2008**;123(5):1205-1214.

Appendix A - Supplementary Tables

Table S1 - Search filters used in PubMed, Scopus, Web of Science and Embase databases

Database	Descriptores	Found items	Date	Time
Pubmed	Genistein: #1 (“Genistein"[Mesh Terms] OR "Genistin" [Supplementary Concept] OR Genistein [Tiab] OR Genistin [Tiab])	13,151	09/12/23	11:16
Pubmed	Cancer: #2 (“Intestinal neoplasm*” [MeSH Terms] OR “aberrant crypt foci” [MeSH Terms] OR “cecal neoplasm*” [MeSH Terms] OR “colorectal neoplasm*” [MeSH Terms] OR “adenomatous polyposis coli” [MeSH Terms] OR “colonic neoplasm*” [MeSH Terms] OR “colorectal neoplasm*” [MeSH Terms] OR “rectal neoplasm*” [MeSH Terms] OR “duodenal neoplasm*” [MeSH Terms] OR “ileal neoplasm*” [MeSH Terms] OR “jejunal neoplasm*” [MeSH Terms] OR (“Intestinal neoplasm*” [Tiab] OR “intestinal cancer*” [Tiab] OR “aberrant crypt foci” [Tiab] OR “cecal neoplasm*” [Tiab] OR “colorectal neoplasm*” [Tiab] OR “adenomatous polyposis coli” [Tiab] OR “colonic neoplasm*” [Tiab] OR “colorectal neoplasm*” [Tiab] OR “rectal neoplasm*” OR “ileal neoplasm*” [Tiab] OR “jejunal neoplasm*” [Tiab])	271,287	09/12/2023	11:22
Combined	#1 AND #2	139	09/12/23	11:24
Scopus	Genistein (Genistein OR Genistin)	22,888	09/12/23	11:39
Scopus	Cancer: #2 (“Intestinal neoplasm*” OR “cancer, intestinal*” OR “aberrant crypt foci” OR “cecal neoplasm*” OR “colorectal neoplasm*” OR “adenomatous polyposis coli” OR “colonic neoplasm*” OR “colorectal neoplasm*” OR “rectal neoplasm*” OR “duodenal neoplasm*” OR “ileal neoplasm*” OR “jejunal neoplasm*”)	235,893	09/12/23	11:40
Combined	#1 AND #2	131	09/12/23	11:44
Web of Science	Genistein (Genistein OR Genistin)	17,022	09/12/23	11:59
Web of Science	Cancer: #2 (“Intestinal neoplasm*” OR “cancer, intestinal*” OR “aberrant crypt foci” OR “cecal neoplasm*” OR “colorectal neoplasm*” OR “adenomatous polyposis coli” OR “colonic neoplasm*” OR “colorectal	16,013	09/12/23	12:00

	neoplasm*” OR “rectal neoplasm*” OR “duodenal neoplasm*” OR “ileal neoplasm*” OR “jejunal neoplasm*”)			
Combined	#1 AND #2	25	09/12/23	12:02
Embase	Genistein (Genistein OR Genistin)	21,734		12:06
Embase	Cancer: #2 (“Intestinal neoplasm*” OR “cancer, intestinal*” OR “aberrant crypt foci” OR “cecal neoplasm*” OR “colorectal neoplasm*” OR “adenomatous polyposis coli” OR “colonic neoplasm*” OR “colorectal neoplasm*” OR “rectal neoplasm*” OR “duodenal neoplasm*” OR “ileal neoplasm*” OR “jejunal neoplasm*”)	29,481		12:07
Combined	#1 AND #2	19		12:10

Table S2 - Description of the main experimental characteristics of the studies that evaluated the effect of genistein on colorectal cancer, *in vitro*

Author	Country	Cell line	Dose (genistein)	Control	Time of the exposition	Outcomes
Herut *et al*., 1995	USA	HCT8, SW837	74, 148, 222, 296, 370, 444 μM	Untreated cells	3 days	Genistein decreased the proto-oncogene c-myc expression and inhibited cell proliferation.
Booth *et al*., 1999	United Kingdom	SW620, HT29	92 μM	Untreated cells	72h	Genistein inhibited cell proliferation, via apoptosis.
Fiorelli *et al*., 1999	Italy	HCT8, HCT116	1 μM	Untreated cells	24h	Genistein enhanced aromatase activity and gene expression in HCT8 cells.
Yu *et al*., 1999	China	HCT	10, 20, 30, 40, 50 μM	DMSO	24h and 48h	Genistein reduced the fluidity of the membrane, the density of cell surface charge, and the coil proportion of the cell membrane protein. It also inhibited the HCT cell growth, but it did not alter the growth of the normal cell IBRS2.
Arai *et al*., 2000	Japan	HT-29, Colo320, Lovo, SW480, HCT116	1,10 μM	Ethanol	96h	Genistein inhibited growth of HT-29, Colo320 and Lovo cells, at 10 μM. The estrogen receptor 2 (ESR2), but not estrogen receptor 1 (ESR1), was expressed in all of the colon cancer cell lines.
Mutoh *et al*., 2000	Japan	DLD-1	40 μM	Untreated cells	48h	Genistein suppressed cyclooxigenase-2 (COX-2) activity.
Salti *et al*., 2000	USA	HT-29	2-200 μM	Untreated cells	1h-4 days	Genistein induced DNA strand breakage and arrested G2/M phase (100 μM), induced apoptosis and inhibited cell growth (60 μM). Also, it enhanced cell proliferation at 1-2 μM.
Park *et al*., 2001	Korea	Colo320	10, 30, 60 μM	Untreated cells	48h	Genistein decreased cell growth, arrested G2/M phase and induced p21 expression.
Plewa *et al*., 2001	USA	HT-29	7-740 μM	Untreated cells	72 h	Genistein induced DNA damage, at740 μM and suppressed the cell growth at 42.5 μM.
Lechner *et al*., 2003	Austria	Caco-2, COGA-1	10 - 100 μM	DMSO	96 h	Genistein downregulated the 24-hydroxylase enzyme (CYP24) expression and induced the vitamin D receptor (VDR) expression, at 100 μM.
Wilson *et al*., 2003	USA	HCT-116, HCT-15	25, 50, 100 μM	DMSO	48h	Genistein induced p53, p21, and the Nonsteroidal Anti-inflammatory Drug-activated Gene (NAG-1) expression. It

						also inhibited the cell growth and induced apoptosis.
Bayazit, 2004	Turkey	Rabbit's colon cancer cells	1,000-3,000 µM	Untreated cells	24-48h	Genistein induced apoptosis in cells in G_2, M and G_o phases.
Yu *et al*., 2004	China	HT-29	15, 30, 60, 120 µM	Ethanol	72 h	Genistein decreased Bcl-2 expression and increased Bax, $p21^{WAF1}$ expression. It also reduced the cell number, G2/M cell cycle arrest, and induced apoptosis.
Linsalata *et al*., 2005	Italy	DLD-1	0.01, 0.1, 1, 10, 20, 30, 100 µM	Untreated cells and DMSO	24 h	Genistein reduced the ornithine decarboxylase (ODC) activity and the polyamine contents. It also decreased the cell proliferation and increased apoptosis, at from 10 µM. DLD-1 cells expressed only ERβ subtype.
Prete *et al*., 2005	Italy	C22-20	2, 10, 50 µM	Untreated cells	3 days	Genistein did not change the carcinoembryonic antigen (CEA) expression.
Yu *et al*., 2005	China	Mouse colon cancer MC-26	20,40,60 µM	Ethanol	24h	Genistein enhanced TGF-β1 expression and induced formation of smad-DNA complexes and phosphorylation of smad2/3.
Gilad *et al*., 2006	Israel	HT29	0.01, 10 µM	DMSO	3 and 6 days	Genistein enhanced intracellular free calcium, activated ERK ½ phosphorylation; upregulated transcription factor Sp-1 and vitamin D receptor (VDR) expression. It also inhibited cell proliferation.
Lechner *et al*., 2006	Austria	Caco-2	1 µM	Ethanol	8h	Genistein enhanced 25-Hydroxyvitamin D 1-alpha-hydroxylase (CYP27B1) expression and reduced CYP24 expression.
Chatzinikolaou *et al.,* 2007	Greece	HT-29, SW-1116	1.85, 37, 111 µM	Untreated cells	48 h	Genistein enhanced synthesis of heparan sulfate (HS), at 37 µM, in HT-29. At 111 µM, it suppressed synthesis of HS and increased of galactosaminoglycans (GalAG). In SW-116 cell line, genistein suppressed the HA, HS and GalAG. Genistein increased cell proliferation at 1.85 µM and suppressed at 111 µM, in HT-29. In SW-116, genistein suppressed it in both concentrations.
Ogasawara *et al*., 2007	Japan	26-L5 murine	50 µM	DMSO	48h	Genistein decreased cell invasion and cell proliferation.
Caruso *et al*., 2008	Italy	DLD-1	0.01, 1, 10, 50 µM	Untreated and DMSO	24 h	Genistein up regulated LDL receptor expression and downregulated the 3-hydroxy methylglutarylcoenzymeA (HMGCoA) reductase.

Berner *et al*., 2010	Austria	Caco-2	200, 500 µM	Untreated cells	48 h	Genistein hypermethylated the ESR1 promoter, enhanced expression of ESR1 and ESR2; demethylated the cyclo-inhibitory protein p16 ^{INK4a} promoter (CDKN2A). It also inhibited cell growth.
Wang and Chen, 2010	USA	DLD-1, SW480, SW1116	75 µM	DMSO	1-4 days	Genistein reduced WNT5A promoter methylation in SW1116 and enhanced WNT5A expression in DLD-1 and in SW1116. It also inhibited cell proliferation.
Bottone *et al*., 2011	USA	HCT-116	10-100 µM	DMSO	24 h	Genistein induced activating of the transcription factor 3 (ATF3) and suppressed the inhibitor of DNA binding/ differentiation-1 (Id1). Genistein inhibited cell proliferation.
Qi *et al*., 2011	USA	HT-29, HCT116	10, 50,100, 150 µM	Untreated cells	48 h	Genistein inhibited EGF-induced proliferation, FOXO3 phosphorylation and stabilized it in the nucleus and decreased the Akt phosphorylation during EGF treatment. Genistein also increased p27kip1 (CDKN1B) expression and inhibited EGF-induced FOXO3 disassociation from promoter of CDKN1B promoter. Besides, it increased expression of p53(mut) and induced FOXO3 interaction with p53(mut). Genistein also induced cell cycle arrest and inhibited cell proliferation.
Wang *et al*., 2012	USA	SW480	0, 1, 5, 15, 25, 50, 75 µM	DMSO	2 to 4 days	Genistein enhanced histone H3 acetylation at the DKK1 promoter region and enhanced expression of this gene. Genistein also decreased cyclin D1 (CCND1) gene expression, inhibited cell cycle: decreased G1/S and increased in G2/M phase and, inhibited cell proliferation.
Mizushina *et al*., 2013	Japan	HCT116	50,100,150, 200 µM	DMSO	24 h	Genistein inhibited DNA topoisomerase II (topo II), biding it directly, because it was not observed effect of the genistein on the thermal transition of dsDNA. It also disrupted cell cycle and suppressed cell growth in dose-dependent manner, with a 50 % lethal dose (LD_{50}) of 94.0 µM.
Zhang *et al*., 2013c	USA	HCT116, SW480	2.5, 5, 10, 25, 50, 100 µM	DMSO	24, 48, 72 h	Genistein enhanced ATM/p53-p21 expression, modulated cell cycle arrest relates genes: enhanced p53, p21(CDKN1A), BRCA1 and E2F4 expression and decreased Bcl2, CDK6 and CUL3 expression. It also induced cell cycle arrest in a p53-dependent way, inhibited cell proliferation and induced apoptosis.

Lepri *et al*., 2014	Brazil	HT29	10, 25, 50, 100 µM	DMSO	24, 48, 72, 96 h	Genistein reduced β-catenin expression but it did not change the expression of the adenomatous polyposis coli (APC) and the surviving gene (BIR5). At 96 h and 100 µM of genistein, there was reduction of the cell proliferation in 97%.
Luo *et al*., 2014	China	LoVo, HT29	0-200 µM	Untreated cells	24 h	Genistein decreased NF-κB DNA binding activity, induced dephosphorylation and upregulation of IκB-α, and downregulated Bcl-2 and upregulated Bax expression. It also inhibited cell population growth and induced apoptosis.
Xiao *et al*., 2014	China	HCT116, SW620, HT 29	0, 10, 25, 50 µM	DMSO	Up to 5 days	Genistein decreased Fms-Related Tyrosine Kinase (FLT4) and matrix metalloproteinase 2 (MMP2) expression. It also inhibited cell growth at 25 µM and 50 µM and inhibited cell invasion and migration at 10 µM.
Qin *et al*., 2016	China	HCT116, LoVo	0, 25, 50, 100 µM	Untreated cells	24, 48, 72 h	Genistein upregulated Bax expression and downregulated Akt phosphorylation. It also inhibited cell growth and induced apoptosis.
Shafiee *et al*., 2016	Iran	HT29	10, 30, 50, 70, 90 µM	DMSO	12, 24, 48, 72 h	Enhanced caspase-3 expression and its enzymatic activity, inhibited p38 MAPK (MAPK14), and reduced metalloproteinase enzyme activity (MMP2). It also enhanced apoptosis and reduced cell migration and proliferation. The 50 µM of genistein was found as IC50 of genistein for HT29 cells.
Pintova *et al*., 2017	USA	RKO, DLD1	25, 50, 75, 100 µM	DMSO	72 h	Genistein inhibited proliferation Rko and DLD1 cells, with IC_{50} of 50 µM and 75 µM, respectively.
Zhou *et al*., 2017	China	HT29	25-400 µM	Untreated cells	48 h	Genistein downregulated expression of both NF-κB p50 and NF-κB p65, inhibited notch-1 expression and induced expression of Bax/Bcl-2, Caspase-8 and caspase-3. It also enhanced the expression of the E-cadherin and decreased of the N-cadherin, decreased the expression of invasion -related genes: slug, zeb1, zeb2, foxc-1, foxc-2 and twist1 and reversed the Epithelial Mesenchymal Transition (EMT) of the cells. Genistein inhibited cell proliferation and invasion ability, disrupted cell cycle and induced apoptosis. Genistein inhibited cell migration at 200 µM.

Zhu *et al*., 2018	China	HT29	0, 5, 10, 20, 40, 60 µM	Untreated cells	12, 24, 48, 72 h	Genistein reduced methylation at the promoter region of WNT inhibitory factor-1 (WIF1) gene and enhanced expression of this gene. It also enhanced expression of E-cadherin and metalloproteinase inhibitor protein 1 (TIMP1) and decreased matrix metalloproteinases 1 and 9 (MMP1/MMP9) and β-catenin expression. Genistein also reduced the pro-oncogenes WNT/β-catenin pathway-associated: cMyc, cyclin D. It also suppressed cell viability, invasion and migration.
Schroeter *et al*., 2019	Austria	HT29	0.1 - 250 µM	DMSO	Different for each protocol	Genistein inhibited Human Topoisomerase II and caused DNA Strand Breaking (DNA damage), at 250 µM, activated p53, increased ROS generation and reduced mitochondrial activity (cytotoxicity), at 200 µM. It also induced apoptosis, at 100 µM.
Chen *et al*., 2020	China	SW480	0, 25, 50, 100 µM	Solvent	48h/ 14 days	Genistein reduced TGF-β1 content, SGK1, Akt^{Ser473}, p38 $MAPK^{Tyr323}$ expressions and lncRNA TTTY18 expression. It also decreased cell proliferation and migration, and enhanced apoptosis.
Alorda-Clara *et al*., 2022	Spain	HT29, SW620	1, 5, 50, 100 µM	DMSO	48h	Genistein enhanced the NF-*k*B translocation into the nucleus, enhanced inflammation-related genes, interleukins and their receptors, in HT29 cells: It enhanced TNF, IL1B, CXCR2, HPSE, and IL10, and decreased CXCL8 expression. In SW620, genistein enhanced TNF, CXCL8, CXCR2, HSPE, IL1B, and decreased PPARG expression. Genistein enhanced H_2O_2 production, SOD, GPX expression, and decreased CAT expression. Genistein enhanced stress fibers and filopodia number (actin cytoskeleton remodeling). Genistein modulated mitochondrial regulatory genes: In HT29 cells, it decreased PPARGC1a and ESRRA, enhanced TFAM and SSBP1, expression. In SW620 cells, genistein enhanced ESRRA, TFAM, and SSBP1 expression. Genistein decreased mitochondrial DNA expression. Genistein also induced cell cycle arrest and apoptosis. It decreased cell viability only at 50 and 100 µM, more pronounced in SW620. Overall, genistein showed a more pronounced oxidative and pro-

						inflammatory effect on the metastatic cell (SW620).
Castaño *et al.*, 2022	Colombia	SW480, SW620	7 different doses starting at 150 μM	Untreated cells	24 and 48h	The inhibitory concentration (IC50) for genistein was 134.67 μM for SW480 and 94.1 μM for SW 620, at 48h.
Rendón *et al.*, 2022	Colombia	SW480, SW620	3.7–740 μM	DMSO	2 to 6 days	Genistein enhanced production of reactive oxygen species (ROS), caspase 3, p53, Cytochrome c and cleaved PARP proteins. It also stimulated cytokines IL-1B, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17A, IL-18, IL-27, and GM-CSF levels. Genistein reduced cell viability, proliferation and induced apoptosis.
Cheng *et al.*, 2023	China	HT-29, SW480	?	PBS	48h	Genistein reduced KCNK9, β-catenin and cMyc expression. Genistein was more efficient to changes expression of Bax, Bcl-2, caspase-3, cleaved caspase-3, PARP, and cleaved PARP, p53, CyclinD1, CDK6, and CDK4 were altered after silencing of KCNK9. Genistein also was better to inhibit viability and cell invasion, to induce apoptosis, and to cause cell cycle arrest, in KCNK9 knockdown cell lines than in control cell lines SW480.

Note: ?, unreported data

Table S3 - Characteristics of the experimental models and experimental diet of the studies that evaluated genistein on colorectal cancer*, in vivo.*

Study	Country	Animal model	Strain	Sex	Weight (g)	Age (wk)	Dietary strategy	Diet description
Pereira *et al*., 1994	USA	rats	F344	male	?	5	AIN-76A	20% casein, 0.3% DL-methionine, 52% corn starch, 13% dextrose, 5% corn oil, 5% alphacel, 3.5% AIN mineral mixture, 1% AIN vitamin mixture, 0.2% choline biturate
Steele *et al*., 1995	USA	rats	F344	male	?	5	AIN-76A	20% casein, 0.3% DL-methionine, 52% corn starch, 13% dextrose, 5% corn oil, 5% alphacel, 3.5% AIN mineral mixture, 1% AIN vitamin mixture, 0.2% choline biturate
Rao *et al*., 1997	USA	rats	F344	male	?	5	AIN-76A	?
Thiagarajan *et al*., 1998	USA	rats	F344	male	82.0	3	diet contained negligible amounts of isoflavones	?
Gee *et al*., 2000	United Kingdom	rats	Wistar	male	150-180	?	semi-synthetic diet	Casein (200 g/Kg), dextrose (438 g/Kg), corn oil (200 g/Kg), Cellulose (100 g/Kg), mineral mix (40 g/Kg), vitamin mix (20 g/Kg), DL-methionine (2g/Kg)
Iishi *et al*., 2000	Japan	rats	Wistar	male	?	6	regular chow pellets	?
Kállay *et al*., 2002	England	mice	C57BL /6J	male	?	2-3	AIN-76A	?
Javid *et al*., 2005	USA	mice	Min/+	female	?	5	AIN-76A	?
Zhi *et al*., 2007	Japan	rats	F344	male	?	4	AIN-76A	?
Xiao *et al*., 2007	USA	rats	Sprague–Dawley	male	?	?	AIN-93G	?
Zhang *et al*., 2013(a)	USA	rats	Sprague–Dawley	male	?	?	AIN-93G	?
Zhang *et al*., 2013(b)	USA	rats	Sprague–Dawley	male	?	?	AIN-93G	?
Sekar *et al*., 2016	India	rats	Wistar	?	180-200	4	standard rat feed	?

Song *et al*., 2018	China	mice	Kun Ming	?	?	4	normal diet or high-fat diet (HFD)	66.8% common feed, 10% egg yolk, 12% lard, 1% cholesterol, 0.2% bile salt, 10% sucrose (HFD)

Note: ?, unreported data.

Table S4 - Main outcomes of the included studies assessing the effect of genistein on colorectal cancer, *in vivo*

Author	Challenge	Control	Animal dose	Human dose – mg/60 kg human	Administration route/ period	Main outcomes
Pereira *et al*., 1994	Azoxymethane	Soy-free diet (AIN-76A)	75 / 150 mg/Kg diet	NA	Oral (diet), for 5 weeks	Genistein decreased No. Foci / colon.
Steele *et al*., 1995	Azoxymethane	Soy-free diet (AIN-76A)	75 / 150 mg/Kg diet	NA	Oral (diet), for 5 weeks	Genistein decreased Foci aberrant crypt / colon, but it did not change aberrant crypt / foci.
Rao *et al*., 1997	Azoxymethane	Soy-free diet (AIN-76A)	250 mg/Kg diet	NA	Oral (diet), for 54 weeks	Genistein enhanced noninvasive tumor adenocarcinoma total, but it did not change invasive tumor.
Thiagarajan *et al*., 1998	Azoxymethane	Diet contained negligible amounts of isoflavones	0.15 mg/Kg diet	NA	Oral (diet), for 12 weeks	Genistein decreased foci aberrant crypt / colon.
Gee *et al*., 2000	1,2-Dimethyllhydrazine (DMH)	Semi-synthetic diet with casein	250 mg/Kg diet	NA	Oral (diet), for 42 days	Genistein did not changes crypt cell mitosis / apoptosis, but it enhanced aberrant crypts before DMH treatment.
Iishi *et al*., 2000	Azoxymethane	Olive oil	5 / 10 mg/Kg b.w.	48.4 / 96.8	Subcutaneous injections, for 29 weeks	Genistein reduced incidence of peritoneal metastasis on intestinal carcinoma and lymphatic vessel invasion.
Kállay *et al*., 2002	NA	H2O/5% ethanol	0.25 mg/Kg b.w.	1.22	Oral (gavage), single dose	Genistein enhanced 25-Hydroxyvitamin D 1-alpha-hydroxylase enzyme (CYP27B1) expression and it decreased 24-hydroxylase enzyme (CYP24) expression.
Javid *et al*., 2005	Ovariectomy	Soy-free diet (AIN-76A)	1 mg/Kg diet	NA	Oral (diet), for 10 weeks	Genistein did not changes tumor counts.
Zhi *et al*., 2007	1,2-Dimethyllhydrazine (DMH)	Soy-free diet (AIN-76A)	250 mg/Kg diet	NA	Oral (diet), for 9 weeks	Genistein enhanced multiplicity and size of β-catenin accumulated crypts (BCAC) and PCNA indices in BCAC. But it did not change aberrant crypt foci (ACF) and PCNA indices in ACF.
Xiao *et al*., 2007	Azoxymethane	Soy-free diet (AIN-93G)	2.500 mg/Kg diet	NA	Oral (diet), for gestational period (17 days)	Genistein did not changes progeny colon tumor incidence.

Zhang *et al*., 2013a	Azoxymethane	Soy-free diet (AIN-93G)	140 mg/Kg diet	NA	Oral (diet), for gestation to 13 weeks of age	Genistein reduced total aberrant crypts, nuclear β-catenin protein, c-Myc and cyclin D1 expression. It also regulated expression of WNT related genes: Wnt5a, Sfrp1, Sfrp2, Sfrp5.
Zhang *et al*., 2013b	Azoxymethane	Soy-free diet (AIN-93G)	140 mg/Kg diet	NA	Oral (diet), for gestation to 13 weeks of age	Genistein reduced expression of Sfrp2, Sfrp5 and Wnt5a genes by DNA methylation and histone modifications.
Sekar et at., 2016	1,2-Dimethyllhydrazine (DMH)	?	2.5 mg/Kg b.w.	24	Oral, 2 weeks	Genistein reduced aberrant crypt foci, markers argyrophilic nucleolar organizer region (AgNOR), proliferating cell nuclear antigen (PCNA), β-catenin expression, stem cell protein markers CD133 (PROM1) and CD44, nuclear factor-erythroid 2 related factor 2 (NFE2L2) and heme oxygenase-1 (HMOX1) expression. It also enhanced superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase activity, vitamin C, E, A, glutathione levels, improved colonic architecture and mucin content.
Song *et al*., 2018	Azoxymethane/DSS/High fat diet	Normal diet	225/450/900 mg/Kg diet	NA	Oral (diet), for 6 months	Genistein decreased cyclooxygenase-2 (COX-2), TNF-α, FRAT1, P13K, AKT expression. It also enhanced phosphatase and tensin homolog (PTEN), forkhead box O3 (FOXO3) expression and BAX/BCL-2 ratio. Genistein prevented colon shrinkage and architecture damage.

Note: NA, not applicable; ?, unreported data.

3 CAPÍTULO 3: Efeito de bebidas à base de soja e de leite de vaca sobre a morfofisiologia intestinal de camundongos Balb/c

3.1 Introdução

A inclusão do leite de vaca na dieta é fortemente recomendada pelos guias alimentares (Global Food-Based Dietary Guidelines - FBDGs), por ser ótima fonte de cálcio, vitaminas, minerais essenciais e proteínas de alto valor nutricional (Comerford *et al*., 2021). Entretanto, em alguns casos seu consumo é limitado, como em indivíduos que possuem intolerância à lactose (Obermayer-Pietsch *et al*., 2004) e alergias (Vojdani *et al*., 2018). Outra limitação é o aumento da preocupação sobre o consumo de alimentos mais sustentáveis e de origem vegetal (Beacom *et al*., 2021). Além disso, o consumo de leite de vaca, principalmente rico em gorduras, pode contribuir para o desenvolvimento de doenças crônicas, como diabetes tipo 2 e doenças cardiovasculares (Drouin-Chartier *et al*., 2016). Essas limitações têm estimulado a procura por substitutos ao leite de vaca, com as bebidas à base de soja (BABS) (Beacom *et al*., 2021).

As BABS são as bebidas de origem vegetal que mais se assemelham, nutricionalmente, ao leite de vaca. Essas bebidas contêm proporções nutricionais similares de energia, de proteína, e menor proporção de gordura e de carboidratos, comparado ao leite de vaca (Walther *et al.,* 2022). Além disso, é livre de colesterol e de lactose (Walther *et al*., 2022), prejudiciais à saúde. Alguns nutrientes como o cálcio e vitaminas B2, B12 e D2 estão presentes em baixas concentrações nas BABS, porém, são frequentemente adicionados às BABS. Entretanto, a completa substituição de leite de vaca por BABS sem acompanhamento profissional, a longo prazo, pode gerar deficiências nutricionais (Singhal *et al*., 2017; Walther *et al*., 2022).

Além das propriedades nutricionais, as BABS são consideradas anti-inflamatórias e antioxidantes, devido à presença de riboflavina - B2 (Walther *et al*., 2022) e de isoflavonas (12–130 mg/kg) (Krížová *et al*., 2019). Dessa forma, a bebida previne doenças inflamatórias intestinais, como a colite ulcerativa, em camundongos e humanos (Levit *et al*., 2017; Sadeghi *et al*., 2020) e está associada ao menor risco de incidência de câncer de cólon em humanos (Wang *et al*., 2024). Além disso, a ingestão de BABS contribui para a manutenção da microbiota intestinal, reduzindo os organismos patógenos e aumentando a proliferação de gêneros benéficos como

Bifidobacterium e Lactobacillus (Pei *et al*., 2020). Por outro lado, a soja contém fatores antinutricionais, como os inibidores de tripsina, as lectinas, as ureases e as lipoxigenases (Armour *et al*., 1998; Kong *et al*., 2022) que podem causar efeitos adversos no intestino, especialmente em soja e derivados que não sejam devidamente aquecidos durante seu preparo (Giri and Mangaraj, 2012; Xiao *et al*., 2012). Os fatores antinutricionais inibem a ação das enzimas digestivas, prejudicando a digestibilidade e a biodisponibilidade de nutrientes (Muzquiz *et al*., 2012). Além disso, particularmente os inibidores de tripsina, causam hipertrofia das células acinares, aumento do peso do pâncreas e da secreção pancreática em ratos (Xiao *et al*., 2021), o que pode gerar inflamação do intestino (Róka *et al*., 2008). Em humanos, há evidências que grande ingestão de leite de soja resulta em pancreatite (De Souza *et al*., 2021) e até mesmo câncer de pâncreas (Yamagiwa *et al*., 2020). Portanto, baseado no aumento da demanda pelo consumo de BABS e nas incertezas sobre seu efeito sobre a saúde intestinal, objetivamos avaliar o efeito de duas marcas comerciais de BABS sobre a morfofisiologia intestinal de camundongos, comparado ao leite de vaca.

3.2 Material e Métodos

Este estudo foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Federal de Viçosa CEUA/UFV, protocolo nº 01/2022 (Anexo B), e está de acordo com os princípios éticos da experimentação animal, estabelecido pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA) e com a legislação vigente.

3.2.1 Animais, tratamentos e condições experimentais

Foram utilizados 24 camundongos machos Balb/c adultos (32,76 ± 3,15 g; 50 dias de vida), obtidos do Biotério Central do Centro de Ciências Biológicas e da Saúde da Universidade Federal de Viçosa. Os animais foram transportados em veículo com velocidade e temperatura controladas, mantidos em adaptação por uma semana no Biotério Setorial do Departamento de Biologia Geral, onde o experimento foi realizado.

O experimento foi realizado em delineamento inteiramente casualizado, com 4 tratamentos: água destilada (controle); BABS 1 (Ingredientes: água, grãos de soja, minerais - cálcio e zinco, maltodextrina, sal, vitaminas – E, B6, ácido fólico, D, e B12, aromatizante, estabilizantes: citrato de sódio, goma gelana e goma xantana,

emulsificante lecitina de soja e edulcorante sucralose; BABS 2 (Ingredientes: Água, grãos de soja, açúcar, minerais (cálcio e zinco), sal, vitaminas (E, B6, A, ácido fólico, D e B12), aromatizante, estabilizantes: citrato de sódio, goma gelana e goma xantana, emulsificante lecitina de soja e edulcorante sucralose); leite de vaca (Leite pasteurizado integral). Foram 6 repetições por tratamento, sendo cada animal uma repetição, mantidos em gaiolas individuais. Todos os tratamentos foram administrados uma vez ao dia, por gavagem (0,7 mL), durante 42 dias. A dose de 0,7 mL utilizada foi baseada no valor máximo suportado pela administração por gavagem em camundongos adultos (McConnell *et al*., 2008). As BABS 1, BABS 2 e o leite de vaca utilizados foram de produção comercial. As bebidas foram renovadas a cada três dias e mantidas sob refrigeração (-20 °C). Os componentes nutricionais dos produtos utilizados estão representados na Tabela 1.

Os animais foram mantidos sob controle de luminosidade (12h claro/ 12h escuro) e temperatura (21ºC ± 1) e receberam água e ração padrão (Tabela 2) *ad libitum*. O peso dos animais foi mensurado semanalmente, durante o período experimental.

Tabela 1 - Informação nutricional das BABS 1, BABS 2 e do leite de vaca, referente a quantidade de 100 mL

Informação nutricional	**BABS 1**	**BABS 2**	**Leite de vaca**
Valor energético	69 Kcal = 290 kJ	95 Kcal = 399 kJ	126 Kcal = 529 kJ
Carboidratos	1,2%	6 %	5 %
Lactose	0 %	0%	*
Proteínas	3,2 %	2,7 %	3,5 %
Gorduras Totais, dos quais:	1,8 %	1,5 %	3,2 %
Gorduras Saturadas	0,4 %	0,9 %	2,0 %
Gorduras Trans	0 %	0 %	*
Gorduras Monoinsaturadas	0,5 %	*	*
Gorduras Poli-insaturadas	0,9 %	*	*
Colesterol	0 %	*	*
Fibra alimentar	0,0005 %	0,4 %	*
Sódio	0,089 %	0,0475 %	0,06 %
Vitamina A	8,1e-5 %	*	*
Vitamina D	1,4e-6 %	*	*
Vitamina E	0,0014 %	*	*
Vitamina B6	1e-4 %	*	*
Ácido Fólico	1,8e-5 %	*	*

Vitamina B12	6e-7 %	*	*
Cálcio	0,132 %	0,12 %	0,12 %
Zinco	1,1 mg	*	*

*Valores não informados pelo fabricante.
Fonte: rótulo da embalagem.

Tabela 2 - Informação nutricional fornecida pelo fabricante da ração padrão (Presence) utilizada durante o experimento

Informação nutricional	
Umidade	12,5%
Proteína Bruta	23%
Extrato Etéreo	4%
Matéria Mineral	9 %
Fibra Bruta	5 %
Cálcio (min.)	12 g/kg
Cálcio (Máx.)	13 g/kg
Fósforo (Min.)	8,5 g/kg
Sódio (Min.)	0,27 %
Lisina (Min.)	12,5 g/kg
Metionina (Min.)	4 g/kg

Fonte: rótulo da embalagem.

3.2.2 Coleta de amostras

Ao final de 42 dias, os animais foram pesados em balança de precisão 0,01g (AS500, Marte) e anestesiados com 10 mg/Kg xilazina e 75 mg/Kg cetamina, via intraperitoneal e eutanasiados por exsanguinação. Em seguida, foi feita a abertura da cavidade abdominal a partir de incisão na linha alba para dissecação e obtenção do intestino. O intestino de cada animal foi dividido em partes: duodeno, jejuno, íleo e cólon. Para manter consistência amostral, a coleta foi padronizada, de acordo com o tamanho do intestino de camundongos (Stephens *et al.*, 2002; Ruehl-Fehlert *et al*., 2003). Os segmentos foram coletados da seguinte forma: os 4 primeiros centímetros após o estômago, o duodeno; os próximos 14 cm, o jejuno; os 14 cm antes do ceco, o íleo; e a região após o ceco, o cólon. Para a avaliação histológica, foi separado o primeiro cm de cada região coletada e fixado em solução Karnovsky (Karnovsky, 1965). Para avaliação das enzimas digestivas, foram coletados 3 cm de duodeno, 7 cm de jejuno, 7 cm de íleo (a partir do ceco) e todo o restante do cólon. Estas amostras foram armazenadas em freezer -80°C.

3.2.3 Extração de inibidores de tripsina

A avaliação da atividade de inibição de tripsina foi realizada no Laboratório de Enzimologia, Bioquímica de Proteínas e Peptídeos do Instituto de Biotecnologia Aplicada à Agropecuária (BIOAGRO), da UFV. A extração de inibidores de tripsina foi realizada de acordo com Xiao *et al*., 2012, adicionando-se 1 mL das amostras (BABS 1; BABS 2; leite de vaca) em 1 mL de NaOH 0,01 M e submetidos ao agitador Thermo Scientific/modelo MaxQ 4000, a 1000 rpm, a 4°C, por 3 horas. O material foi centrifugado a 14.000xg, à 4°C, por 15 min. O sobrenadante foi coletado para a análise de atividade de inibição de tripsina.

3.2.4 Determinação de inibição de atividade de tripsina

A avaliação de inibição da atividade de tripsina foi realizada utilizando-se substrato N-benzoil-L-arginil-p-nitroanilida (L-BApNA) (5 mM), em tampão Tris-HCl 0,1 M, pH8,2 contendo 20 mM de $CaCl_2$ e tripsina bovina (1mg/mL). Os inibidores de tripsina da soja inibem tripsina bovina de forma similar à tripsina humana (Weder, 1986). A mistura da reação continha 100 µL de substrato, 885 µL solução tampão, 5 µL de tripsina e 10 µL de amostra. Para o controle, utilizaram-se os mesmos reagentes citados, exceto a amostra. A velocidade da reação foi determinada pela formação do produto p-nitroanilida, em espectrofotômetro Hitachi UV-VIS/modelo U-5100, a 410 nm, sendo calculada a partir da diferença da absorbância final e inicial (Δ), no tempo de 2,5 minutos (Erlanger *et al*.,1961).

As porcentagens de inibição sobre a enzima tripsina foram calculadas comparando-se a absorbância da formação de produto no meio reacional. Os valores correspondentes à absorbância da enzima tripsina bovina mais o substrato L-BApNA, forneceram o referencial da atividade máxima da enzima utilizada, tendo sido considerada a atividade da enzima igual a 100% sem as amostras de BABS e de leite de vaca. Dessa forma, as porcentagens de inibição das amostras foram calculadas de acordo com a seguinte equação: % inibição = [(ΔAc-ΔA) / ΔAc] * 100, Em que: ΔAc representa a absorbância do controle, enzimas na ausência das amostras e ΔA representa a absorbância na presença das amostras (Voet *et al*., 2016; Berg *et al*., 2018).

3.2.5 Avaliação das enzimas digestivas

A avaliação da atividade das enzimas digestivas protease total, tripsina, amilase e lipase foi realizada no Laboratório de Enzimologia, Bioquímica de Proteínas e Peptídeos do Instituto de Biotecnologia Aplicada à Agropecuária (BIOAGRO), da UFV. Foram analisadas as atividades das enzimas digestivas de cada região do intestino (duodeno, jejuno, íleo e cólon), em 4 animais por tratamento. O intestino foi macerado com almofariz e pistilo de porcelana em banhos de nitrogênio líquido. Em seguida, foi adicionado HCl 10 mM (4°C) em uma proporção de 500µL por 100 mg de tecido. O homogenato foi centrifugado a 10.000 rpm por 10 minutos a 4°C. O sobrenadante foi coletado para determinar a atividade das enzimas digestivas e a concentração de proteínas totais.

A concentração de proteínas totais foi determinada pelo método de Bradford (1976). Foi utilizado espectrofotômetro de varredura de microplacas (Multiskan GO, Thermo Scientific) no comprimento de onda de 595 nm, com albumina sérica bovina (BSA) como proteína padrão.

A atividade da protease total foi determinada pelo método Tomarelli *et al*., (1949). Foi utilizado espectrofotômetro Hitachi UV-VIS/modelo U-5100, a 440 nm, com azocaseína 2%(p/v) como substrato em tampão Tris-HCl 0,1 M, pH 8,0, 37°C. A mistura da reação continha 50 µL de substrato, 50 µL da solução tampão e 50 µL da amostra, tendo sido incubada por 30 minutos, a 37°C. A reação foi interrompida pela adição de 240 µL de ácido tricloroacético (TCA) 10% (p/v). Após repouso no gelo por 15 minutos, as amostras foram centrifugadas a 10.000 rpm, por 5 minutos, a 25 °C, para remoção da proteína precipitada. Para a realização da leitura foram utilizados 240 µL do sobrenadante, acrescidos de 280 µL NaOH 1M. Os valores da absorbância (abs) foram divididos pela concentração de proteínas totais para obtenção da atividade específica de proteases totais, expressa em abs/mg proteína.

A atividade da tripsina foi determinada apenas no segmento duodenal. Os demais segmentos não apresentaram atividade desta enzima, pelo método descrito por Erlanger *et al*., (1961). Esse método utiliza o substrato L-BApNA 1,2 mM, em tampão Tris-HCl 0,1 M, pH8,2 contendo 20 mM de $CaCl_2$. A mistura da reação continha 300 µL de substrato, 690 µL solução tampão e 10 µL da amostra. A velocidade da reação foi determinada pela formação do produto p-nitroanilida, em espectrofotômetro Hitachi UV-VIS/modelo U-5100, a 410 nm, sendo calculada a partir da diferença da absorbância final e inicial no tempo de 2,5 minutos e dividida pelo

coeficiente de extinção molar 8800 (M^{-1} x cm^{-1}) para o produto. Os valores da velocidade de reação foram divididos pela concentração de proteínas totais para obtenção da atividade específica da tripsina, expressa em $nM.s^{-1}$.ng $proteina^{-1}$.

A atividade da amilase foi determinada pelo kit enzimático colorimétrico BIOCLIN® (Belo Horizonte, Brasil), baseado na metodologia de Caraway (1959). A mistura de reação continha 2 µL da amostra e 100 µL de substrato (amido a 0,4 g L^{-1} em tampão fosfato 100 mM, pH 7,0). O material foi incubado por 7,5 minutos, à 37°C, em banho-maria. A seguir, foram adicionados 0,1 mL do reagente de cor (solução estoque de iodo 50 mM) e 0,8 mL de água destilada. A leitura da absorbância foi realizada a 660 nm, em espectrofotômetro Hitachi UV-VIS/modelo U-5100. A atividade total da amilase foi calculada baseada em instruções do kit. A atividade específica da amilase foi calculada dividindo-se o valor da atividade total pela concentração de proteínas totais, sendo expressa em U. dL^{-1}.mg $proteína^{-1}$.

A atividade da lipase foi determinada pelo kit enzimático colorimétrico BIOCLIN® (Belo Horizonte, Brasil), baseado na metodologia de Cherry & Crandall (1932). A mistura da reação continha 180 µL do tampão Tris-HCl 100 mM pH 8,5; 10 µL da amostra; 10 µL do inibidor enzimático fenilmetil sulfonil fluoreto 8 mM e 20 µL do reagente de cor DTNB (ácido ditionitrobenzóico) 3 mM com acetato de sódio 100 mM. A mistura foi homogeneizada e aquecida em banho-maria, à 37°C, por dois minutos. A seguir, foram adicionados 10 µL do substrato tributirato ditiopropanol 20 mM. A reação foi incubada por 30 minutos, à 37°C, em banho-maria. A reação foi interrompida pela adição de 400 µL de acetona e centrifugada a 3.500 rpm, por 5 minutos, à 25 °C. A leitura da absorbância foi realizada a 410 nm em espectrofotômetro Hitachi UV-VIS/modelo U-5100. A atividade total da lipase foi calculada baseada em instruções do kit. A atividade específica da lipase foi calculada dividindo-se o valor da atividade total pela concentração de proteínas totais, sendo expressa em U. dL^{-1}.mg $proteína^{-1}$.

3.2.6 Processamento do material histológico para microscopia de luz

Fragmentos de cada segmento do intestino (duodeno, jejuno, íleo e cólon), destinados ao estudo em microscopia de luz, foram desidratados em concentrações crescentes de etanol, incluídos em 2-hidroxietil metacrilato (Historesin®, Leica), seccionados em micrótomo rotativo na espessura de 2µm, mantendo-se um intervalo de, pelo menos, 10 cortes entre secções e corados com azul de toluidina-borato de

sódio 1% ou submetidos à testes histoquímicos (Ácido periódico de Schiff – PAS, Alcian blue 2,5 – AB e PAS/AB (Bancroft and Gamble, 2008). As preparações foram montadas com Entellan® (Merck, Frankfurt, Alemanha). Imagens histológicas foram obtidas em microscópio Olympus BX-53, com diferentes aumentos de acordo com o objetivo das análises.

3.2.7 Histomorfometria e contagem de células intestinais

As análises histomorfométricas e de contagem celular foram realizadas em cada segmento intestinal, em 6 animais por tratamento, utilizando-se o *software* Image J. Foram avaliadas a altura da mucosa intestinal (objetiva 10x),dos enterócitos, da borda estriada, da espessura das camadas musculares longitudinal externa (CLE) e circular interna (CCI) e da túnica muscular (CLE + CCI) (objetiva 40x), em cinco diferentes regiões de cada imagem, totalizando 25 medidas por animal e 150 medidas por tratamento. A figura 1 mostra exemplares de cada uma dessas medidas.

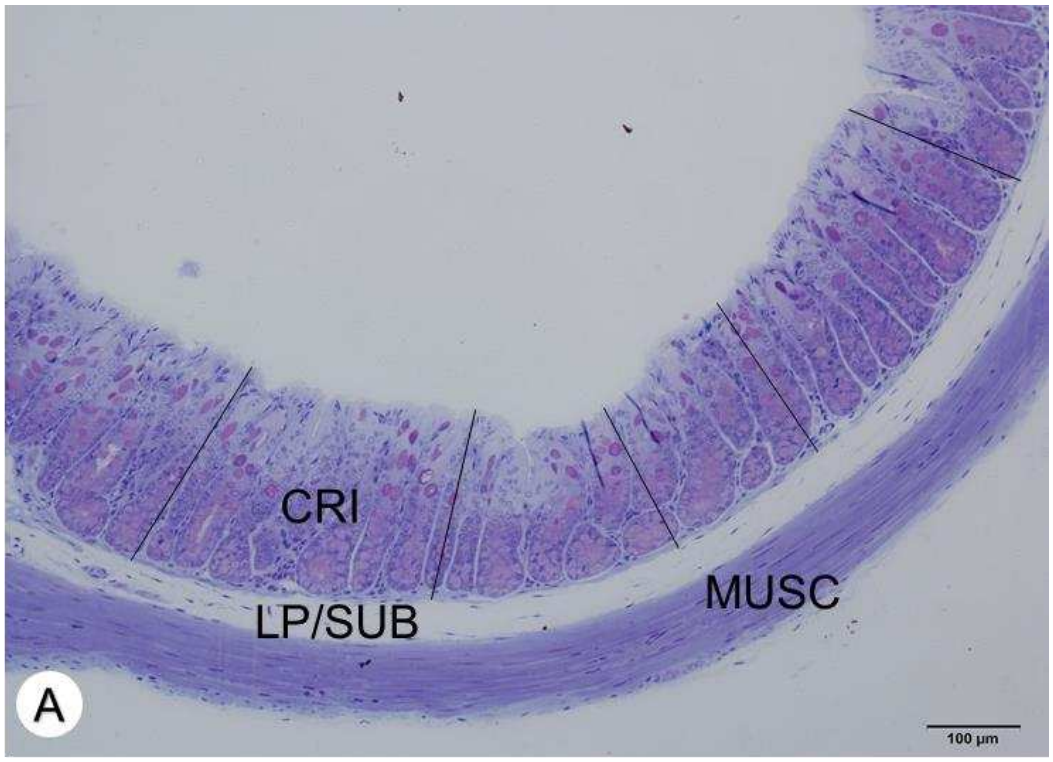
Para as análises de contagem células intestinais foi utilizado um retículo com 336 interseções (pontos) (figura 2. A), no qual o número de pontos por animal variou de acordo com a região do intestino. Foram contadas células caliciformes (figura 2. B, C, D), células inflamatórias: mastócitos, plasmócitos e linfócitos (figura 2. B, D), polimorfonucleares – PMNs (figura 2. D), células de Paneth (figura 2. C) e mitose das células intestinais (figura 2. D), em campos aleatoriamente distribuídos nas preparações histológicas das diferentes regiões intestinais de cada animal. No intestino delgado, a contagem dos pontos foi feita até se esgotar a amostra, partindo da base da cripta até o ápice da vilosidade. Dessa forma, no duodeno e no jejuno, foram contados 5.040 pontos por animal, totalizando 30.240 pontos por tratamento. No íleo, foram contados 1.680 pontos por animal, sendo 10.080 pontos por tratamento. No cólon, contou-se apenas as células caliciformes e as mitoses, em regiões de criptas, utilizando-se a mesma contagem de pontos realizada no íleo.

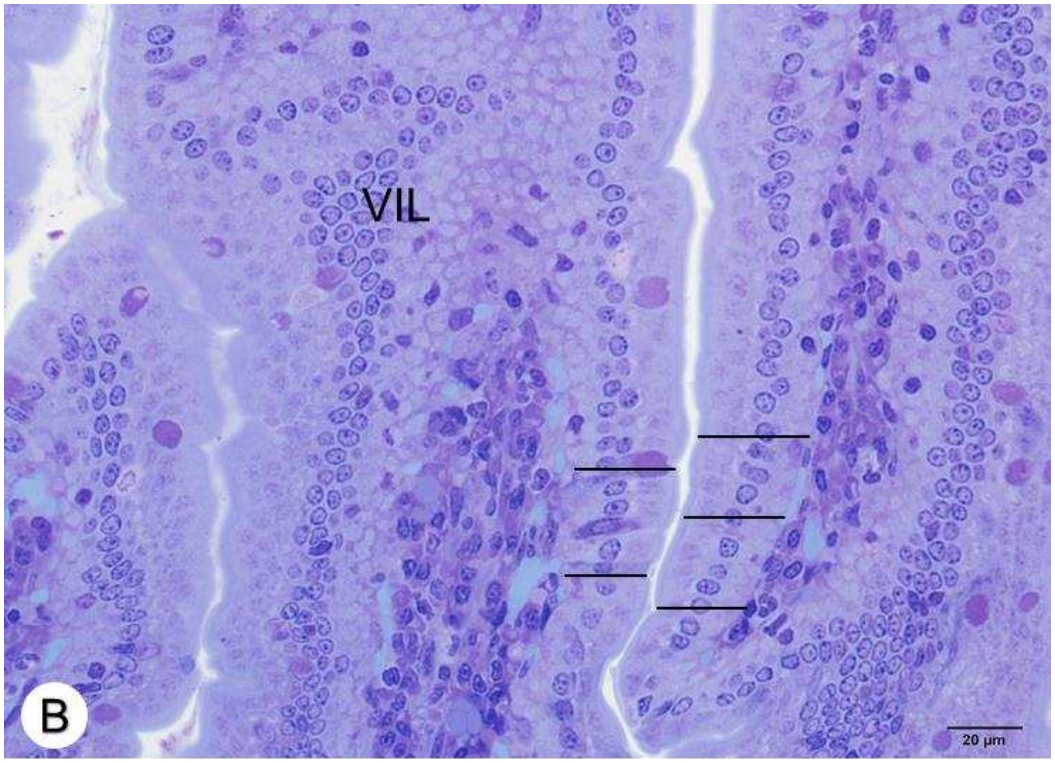
As células inflamatórias foram reconhecidas por suas características morfológicas e/ou tintoriais, nas regiões do intestino delgado (duodeno, jejuno e íleo) corados pelo azul de toluidina (Bloom and Fawcett, 1986). Não houve separação entre a lâmina própria e a submucosa, uma vez que esta última é extremamente fina e de difícil distinção no intestino delgado de camundongos. Portanto, quando referimos à presença de células inflamatórias na região da mucosa do intestino delgado de camundongos, destacamos que poucas células foram observadas na túnica

submucosa. Como representado na figura 2.B, C e D as seguintes características foram consideradas: os mastócitos pela metacromasia de seus grânulos quando corados pelo azul de toluidina; os plasmócitos, principalmente pela presença de núcleo com grande quantidade de cromatina organizada em forma de “roda de carroça”, sendo também consideradas a basofilia e a ocorrência da área negativa de Golgi; células com núcleos menores, arredondados e heterocromáticos, presentes tanto no tecido conjuntivo quanto no epitélio, foram identificadas como linfócitos; células com núcleos polimórficos foram denominadas em conjunto por polimorfonucleares (PMNs), pois não foi possível diferenciá-las com grau razoável de certeza.

3.2.8 Análises estatísticas

Os efeitos dos tratamentos foram avaliados usando análise de variância (ANOVA). Os resíduos foram testados pelos testes de Shapiro-Wilk e Bartlett para confirmar a normalidade da distribuição e homogeneidade das variâncias, respectivamente. Em seguida, as diferenças significativas entre as médias foram determinadas pelo teste de Tukey. Todas as análises foram realizadas utilizando o pacote ExpDes.pt (Ferreira *et al.*, 2021) do *software* R® versão 4.2.1 (R Core Team, 2022) ao nível de significância de 5%.


CRI
LP/SUB
MUSC
A
100 µm


VIL
B
20 µm

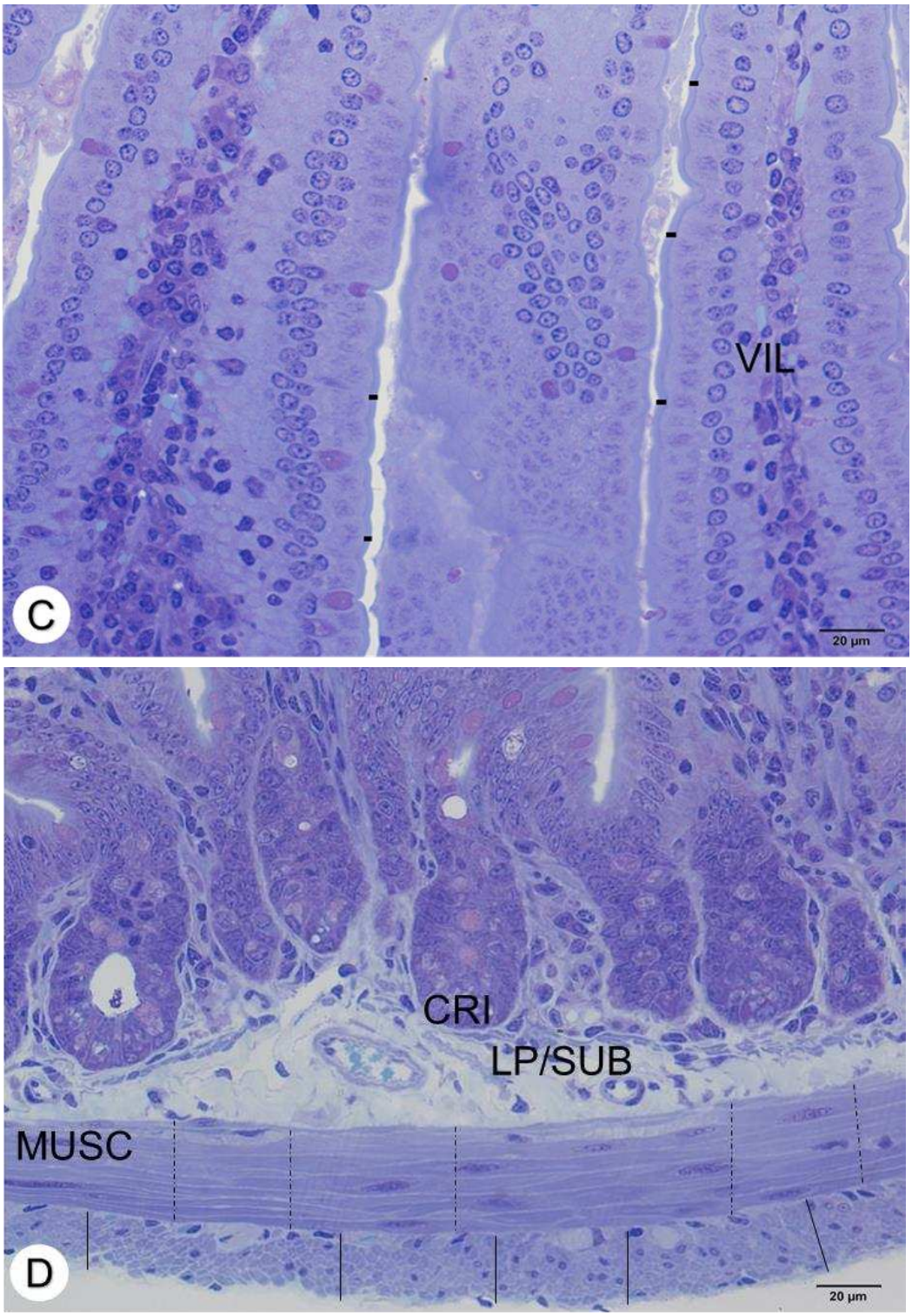
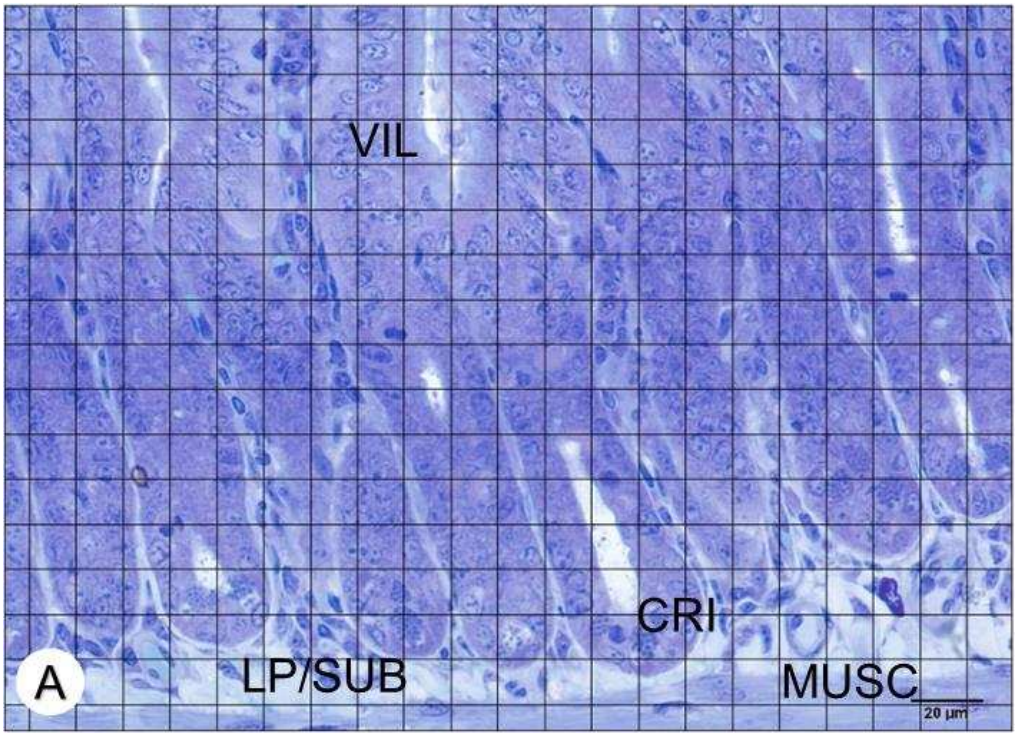
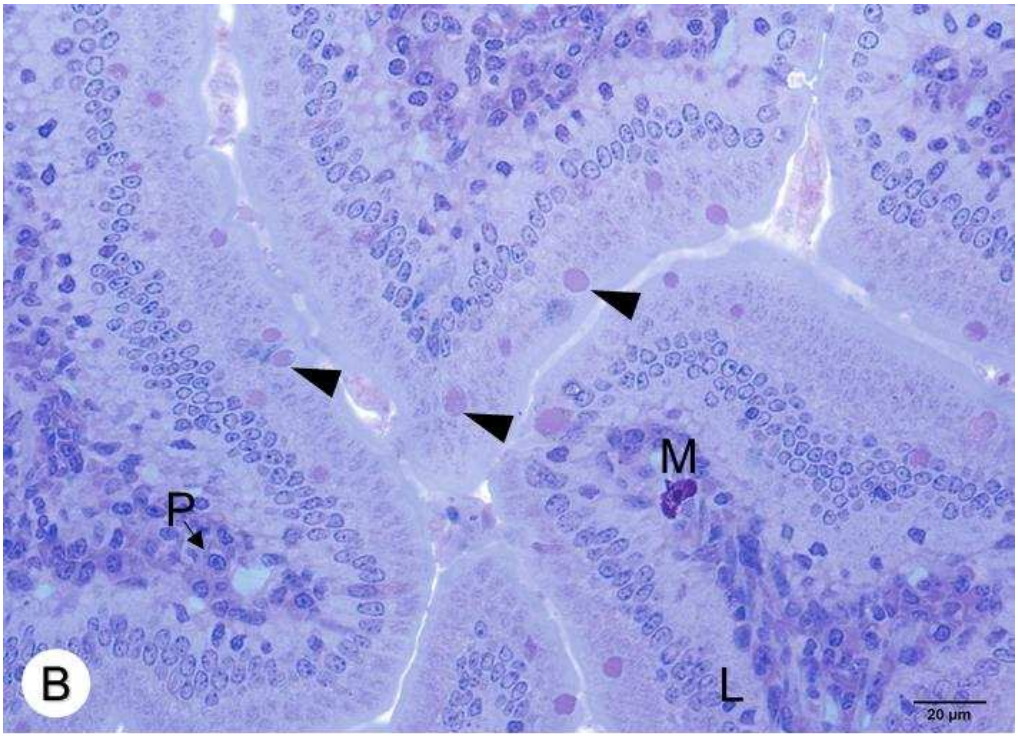


Figura 1 - Fotomicrografia de diferentes segmentos intestinais corados com azul de toluidina. As linhas ilustram as cinco medidas realizadas em cada imagem. **A**, espessura da mucosa; **B**, espessura do epitélio; **C**, espessura da borda estriada; **D**, espessura da camada muscular longitudinal externa (linha contínua) e da camada circular interna (linha pontilhada). VIL, vilosidade; CRI, cripta; LP/SUB, lâmina própria/submucosa; MUSC, túnica muscular.


VIL
CRI
A
LP/SUB
MUSC
20 µm


M
P
B
L
20 µm

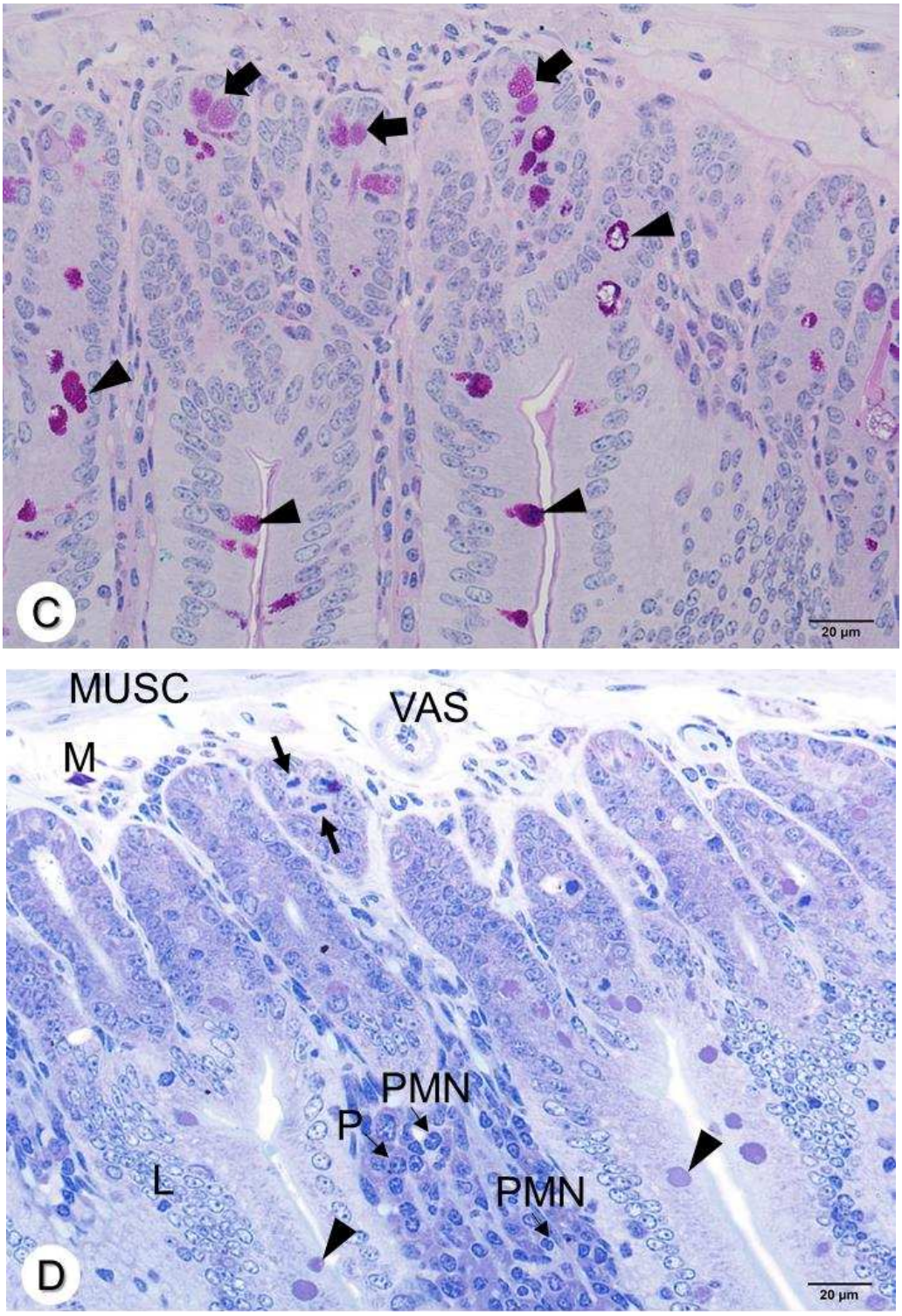


Figura 2 - **A, B, D**- Fotomicrografia da região do jejuno em preparação com azul de toluidina. **C** - Fotomicrografia da região do duodeno em preparação com PAS + Alcian Blue. **A** representa uma imagem do retículo com 336 interseções (pontos), onde foram contadas as estruturas de interesse, presentes nas interseções. Em **B**, **C** e **D**, encontram-se exemplares das estruturas que foram contadas: ponta de seta = células caliciformes; seta delgada = mitose; seta espessa = células de Paneth; P = plasmócito; M = mastócito; L = linfócito; PMN = polimorfonucleares. VIL = vilosidade; CRI = cripta; LP/SUB = lâmina própria/submucosa; MUSC = túnica muscular; VAS = vaso sanguíneo.

3.3 Resultados

3.3.1 Inibição de atividade de tripsina do leite de vaca e das BABS

A atividade de inibição de tripsina foi semelhante entre as BABS (BABS 1= 45,1% e BABS 2 = 39,3%), que foi maior do que nos animais tratados com leite de vaca (1,6%).

3.3.2 Peso Corporal

Os camundongos tratados com BABS 2 apresentaram menor peso corporal do que o controle (P≤0,05), porém não diferiu do peso dos animais dos demais tratamentos. Os animais tratados com BABS 1 e leite de vaca não diferiam do controle (P>0,05) (Tabela 3).

3.3.3 Enzimas digestivas

No duodeno, tanto BABS quanto leite de vaca aumentaram a atividade de amilase, em relação ao controle (P≤0,05). Os animais que receberam BABS 1 apresentaram maior atividade de lipase e de protease total em relação ao grupo controle, mas os demais tratamentos não diferiram do controle e entre si (P>0,05) (Tabela 3). Para a atividade de tripsina, os animais que receberam BABS 2 e leite de vaca apresentaram maior atividade em relação ao controle (P≤0,05), porém, os animais que receberam BABS 2 apresentaram menor atividade desta enzima em relação ao grupo que recebeu leite de vaca (P≤0,05).

No jejuno, a atividade de lipase dos animais tratados não diferiu do grupo controle (P>0,05). Porém, os animais que receberam BABS apresentaram menor atividade desta enzima em relação ao grupo que recebeu leite de vaca (P≤0,05) (Tabela 3). Os tratamentos não influenciaram significativamente a atividade das enzimas amilase e proteases totais nesse segmento intestinal (Tabela 3).

No íleo, os tratamentos não influenciaram significativamente a atividade das enzimas digestivas analisadas (P>0,05) (Tabela 3).

No cólon, os animais que receberam leite de vaca apresentaram menor atividade da lipase do que o controle (P≤0,05), porém, não diferiu dos animais tratados com BABS, que por sua vez, não diferiram do controle (Tabela 3). Os tratamentos não influenciaram significativamente a atividade das demais enzimas digestivas analisadas nesse segmento intestinal.

3.3.4 Morfologia intestinal

Os animais que receberam leite de vaca apresentaram espessura menor da camada muscular longitudinal externa (CLE) e da túnica muscular (TM) duodenal em relação ao controle (P≤0,05), mas não houve diferença dessas variáveis entre as BABS e o leite de vaca. As demais variáveis histológicas avaliadas não foram influenciadas pelos tratamentos (Tabela 4).

No jejuno, os animais que receberam BABS 2 apresentaram maior altura de epitélio (AE) em relação ao controle (P≤0,05), porém, não diferiu da BABS 1 e do leite de vaca. BABS 1 e BABS 2 diminuíram a CLE, em relação ao controle, mas não diferiram do leite de vaca (Tabela 4).

No íleo, a CLE foi menor nos animais que receberam BABS 1 em relação aos que receberam BABS 2, mas diferiram do controle e do leite de vaca. Além disso, os animais que receberam BABS 1 apresentaram menores medidas de camada circular interna (CCI) e de TM, em relação ao controle e ao grupo que recebeu BABS 2, porém não diferiram do leite de vaca. Comparado ao leite de vaca, BABS 2 aumentou a altura da borda estriada (ABE) (Tabela 4).

No cólon, os animais que receberam leite de vaca apresentaram maiores medidas de AE e de ABE em relação ao controle e aos animais que receberam BABS (P≤0,05). Além disso, o leite de vaca e BABS 1 reduziram a CLE, a CCI e a TM, em relação ao controle (Tabela 4).

3.3.5 Células intestinais e mitose

No duodeno, os animais que receberam leite de vaca e BABS 2 obtiveram aumento do número de células caliciformes (CAL) e diminuição do número de linfócitos (LINF) em relação ao controle (P≤0,05), porém, não houve diferença entre as BABS e o leite de vaca (P>0,05). Os animais tratados apresentaram menor número de plasmócitos (PLASM) em relação ao controle, mas não diferiram entre si. Além disso, o leite de vaca aumentou o número de células de Paneth em relação ao controle (P≤0,05), porém, não diferiram entre si (Tabela 5).

No jejuno, os animais que receberam leite de vaca apresentaram maior número de células caliciformes (CAL) em relação aos grupos controle e BABS 2 (P≤0,05), mas não diferiu de BABS 1 (P>0,05). As demais variáveis avaliadas não foram influenciadas significativamente pelos tratamentos (Tabela 5).

No íleo, os animais que receberam BABS 1 apresentaram diminuição significativa no número de células caliciformes (CAL) em relação controle (P≤0,05), mas não diferiu dos demais tratamentos. BABS 1 e leite de vaca diminuíram o número de plasmócitos (PLASM) em relação ao controle, mas também não diferiram entre si e da BABS 2. Além disso, BABS1 e BABS 2 apresentaram menor número de mitoses das células do epitélio intestinal, em relação ao leite de vaca, mas não diferiram do controle (P>0,05). Os tratamentos não influenciaram as variáveis analisadas no cólon (Tabela 5).

Tabela 3 - Valores médios (± desvio padrão) do peso corporal final e da atividade específica de enzimas digestivas de camundongos Balb/c, adultos, expostos às BABS 1, BABS 2 e ao leite de vaca, durante 42 dias.

	Controle	**BABS 1**	**BABS 2**	**Leite de vaca**	**P-valor**
PF (g)	36,55 ± 2,68a	32,70 ± 2,80ab	32,12 ± 3,03b	35,73 ± 2,13ab	0,0126
			Duodeno		
Amilase	67,63 ± 25,04 b	299,71 ± 59,57a	238,61 ± 41,42a	271,7± 67,06 a	0,000138
Lipase	20,05 ± 12,54b	45,62 ± 9,76a	29,74 ± 0,97ab	36, 55 ± 13,79 ab	0,0299
Protease total	0,21 ± 0,04b	0,36 ± 0,08a	0,30 ± 0,08ab	0,34 ± 0,06ab	0,0373
Tripsina	3,83 ± 2,45c	2,12 ± 0,70c	9,48 ± 2,33b	13, 72 ± 1,54a	<0,000
			Jejuno		
Amilase	279,01 ± 79,22	198,15 ± 22,41	193,76 ± 53,08	193, 38 ± 52,60	0,131
Lipase	36,01 ± 7,64ab	26,34 ± 3,18b	24,44 ± 4,55b	42, 72 ± 6,19a	0,00196
Protease total	0,27 ± 0,12	0,28 ±0,03	0,29 ± 0,11	0,16 ± 0,10	0,279
			Íleo		
Amilase	322,9 ± 38,18	232,94 ± 36,20	297,18 ± 94,12	232, 09 ± 40,79	0,105
Lipase	57,68 ± 7,09	55,63 ± 4,18	52,67 ± 11,51	53,60 ± 10,53	0,856
Protease total	0,34 ± 0,15	0,28 ± 0,11	0,26 ± 0,11	0,097 ± 0,095	0,0747
			Cólon		
Amilase	36,47 ± 56,71	285,65 ± 116,68	239,16 ± 98,42	155,92 ± 121,28	0,0708
Lipase	64,11 ± 6,91a	57,54 ± 9,24ab	59,06 ± 3,82ab	46,75 ± 7,52b	0,0306
Protease total	0,17 ± 0,12	0,03 ± 0,013	0,05 ± 0,03	0,05 ± 0,03	0,0954

PF, Peso corporal final; Amilase e lipase: U/dL × mg proteína $^{-1}$; tripsina: nM s^{-1} x ng proteína $^{-1}$; Protease total: abs/mg proteína. Dados são média ± desvio padrão (n=4). Médias, seguidas de diferentes letras, nas linhas, diferem entre si ao nível de 5% de probabilidade, pelo teste de Tukey. n = 4 por tratamento.

Tabela 4 - Valores médios (± desvio padrão) de parâmetros histomorfométricos do intestino de camundongos Balb/c, adultos, expostos às BABS 1, BABS 2 e ao leite de vaca, durante 42 dias.

	Controle	BABS 1	BABS 2	Leite de vaca	P-valor
			Duodeno		
AM (µM)	479 ,03 ± 129,52	355,31 ± 121,37	341,91 ± 137,49	363,91 ± 139,20	0,288
AE (µM)	14,56 ± 0,46	14,73 ± 0,61	14,55 ± 0,73	15,26 ± 0,38	0,126
ABE (µM)	1,08 ± 0,06	1,05 ± 0,05	0,99 ± 0,08	1,01 ± 0,100	0,252
CLE (µM)	11,29 ± 2,83a	9,15 ± 2,61ab	8,99 ± 1,98ab	6,83 ± 0,93b	0,0203
CCI (µM)	16,23 ± 3,75	13,43 ± 3,37	13,25 ± 3,61	11,61 ± 0,81	0,114
TM (µM)	27,52 ± 6,53a	24,14 ± 4,16ab	22,23 ± 5,57ab	18,46 ± 1,66b	0,0328
			Jejuno		
AM (µM)	442,31 ± 109,95	371,11 ± 147,37	515,58 ± 145,91	392,60 ± 160,39	0,328
AE (µM)	12,28 ± 0,60b	12,49 ± 0,42ab	13,64 ± 0,58a	12,46 ± 1,18ab	0,0207
ABE (µM)	1,03 ± 0,08	1,04 ± 0,12	1,20 ± 0,13	1,16 ± 0,24	0,166
CLE (µM)	9,09 ± 1,04a	6,36 ± 1,83b	6,23 ± 1,53b	6,94 ± 1,51ab	0,0127
CCI (µM)	12,53 ± 1,56	10,58 ± 3,50	9,89 ± 0,10	10,57 ± 1,63	0,199
TM (µM)	21,21 ± 2,89	17,70 ± 5,95	15,65 ± 1,77	17,51 ± 3,01	0,107
			Íleo		
AM (µM)	212,41 ± 68,80	156,57 ± 64,02	139,15 ± 47,15	157,31 ± 64,17	0,22
AE (µM)	12,38 ± 0,79	12,50 ± 0,73	12,93 ± 0,87	11,72 ± 1,65	0,303
ABE (µM)	0,95 ± 0,18ab	0,95 ± 0,07ab	1,21 ± 0,23a	0,90 ± 0,11b	0,0154
CLE (µM)	10,86 ± 1,80ab	7,89 ± 2,94b	13,18 ± 2,94a	10,41 ± 1,99ab	0,0131
CCI (µM)	17,55 ± 4,61a	10,32 ± 4,58b	17,12 ± 3,77a	14,88 ± 2,55ab	0,019
TM (µM)	29,29 ± 7,26a	18,21 ±7,44b	30,30 ±6,66a	25,46 ± 4,00ab	0,0174
			Cólon		
AM (µM)	72,73 ± 26,36	94,88 ± 49,98	103,41 ± 60,85	86,36 ± 29,43	0,667
AE (µM)	14,82 ± 1,92b	17,12 ± 2,03b	16,34 ± 2,20b	37,42 ± 1,66a	<0,001
ABE (µM)	0,69 ± 0,09b	0,72 ± 0,06b	0,77 ± 0,08b	1,27 ± 0,09a	<0,001
CLE (µM)	11,43 ± 2,99a	6,61 ± 1,86bc	8,13 ± 3,04ab	3,23 ± 0,81c	<0,001
CCI (µM)	36,98 ± 7,65a	23,04 ± 4,96b	27,70 ± 10,78ab	16,36 ± 4,59b	<0,001
TM (µM)	48,33 ± 10,36a	29,65 ± 6,53b	35,83 ± 13,40ab	20,31 ± 6,93b	<0,001

Dados são média ± desvio padrão (n=6). Médias, seguidas de diferentes letras, nas linhas, diferem entre si ao nível de 5% de probabilidade, pelo teste de Tukey. AM, altura da mucosa; AE, altura do epitélio; ABE, altura da borda estriada; CLE, camada muscular longitudinal externa; CCI, camada muscular circular interna; TM, túnica muscular. n = 6 por tratamento.

Tabela 5 - Valores médios (± desvio padrão) do número das células caliciformes (CAL), de células de Paneth, de plasmócitos (PLASM), de linfócitos (LINF), de mastócitos (MAST), de polimorfonucleares (PMNs) e de mitose, do intestino de camundongos Balb/c, adultos, expostos às BABS 1, BABS 2 e ao leite de vaca, durante 42 dias.

	Controle	**BABS 1**	**BABS 2**	**Leite de vaca**	**P-valor**
			Duodeno		
CAL	4,63 ± 2,20b	8,48 ± 2,37ab	9,56 ± 1,37a	10,57 ± 2,95a	0,00205
Paneth	4,70 ± 1,74b	5,88 ± 2,94ab	5,68 ± 1,75ab	8,90 ± 1,67a	0,0142
PLASM	11,32 ± 1,31a	8,12 ± 2,90b	6,76 ± 1,84b	7,00 ± 0,95b	0,0017
LINF	3,27 ± 1,52a	1,60 ± 0,71ab	0,96 ± 0,38b	1,50 ± 1,11b	0,0103
MAST	0,20 ± 0,25	0,12 ± 0,11	0,12 ± 0,11	0,07 ± 0,10	0,567
PMNs	1,03 ± 0,39	0,80 ± 0,37	0,96 ± 0,26	0,77 ± 0,46	0,605
Mitose	1,03 ± 0,43	0,92 ± 0,18	0,96 ± 0,38	0,56 ± 0,29	0,121
			Jejuno		
CAL	7,53 ± 1,97b	12,60 ± 2,38ab	10,53 ± 2,20b	16,83 ± 5,45a	0,000881
Paneth	5,43 ± 2,14	5,46 ± 1,60	4,33 ± 1,12	4,07 ± 1,24	0,308
PLASM	8,90 ± 2,04	9,16 ± 2,43	7,73 ± 1,38	6,77 ± 2,02	0,174
LINF	1,13 ± 0,43	0,87 ± 0,30	1,23 ± 0,64	1,10 ± 0,83	0,739
MAST	0,00 ± 0,00	0,10 ± 0,17	0,07 ± 0,10	0,03 ± 0,08	0,422
PMNs	0,66 ± 0,53	1,06 ± 0,74	0,43 ± 0,29	1,23 ± 0,46	0,0652
Mitose	0,60 ± 0,38	0,63 ± 0,60	0,73 ± 0,35	1,16 ± 0,50	0,165
			Íleo		
CAL	16,88 ± 8,42a	8,97 ± 2,02b	11,03 ± 1,06ab	13,66 ± 3,51ab	0,0464
Paneth	6,92 ± 3,61ab	5,06 ± 1,70b	7,93 ± 2,54ab	10,36 ± 2,54a	0,021
PLASM	2,70 ± 1,12a	0,87 ± 1,06b	1,43 ± 0,85ab	1,03 ± 0,64b	0,0123
LINF	0,23 ± 0,32	0,06 ± 0,10	0,26 ± 0,27	0,20 ± 0,13	0,457
MAST	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,03 ± 0,018	0,413
PMNs	0,43 ± 0,34	0,16 ± 0,32	0,13 ± 0,10	0,33 ± 0,24	0,209
Mitose	0,90 ± 0,37ab	0,50 ± 0,27b	0,43 ± 0,15b	1,00 ± 0,38a	0,00925
			Cólon		
CAL	56,90 ± 7,47	58,76 ± 7,47	54,53 ± 7,76	53,20 ± 4,60	0,529
Mitose	0,50 ± 0,33	0,33 ± 0,24	0,23 ± 0,26	0,30 ± 0,17	0,348

Dados são média ± desvio padrão (n=6). Médias, seguidas de diferentes letras, nas linhas, diferem entre si ao nível de 5% de probabilidade, pelo teste de Tukey. n = 6 por tratamento.

3.4 Discussão

As bebidas avaliadas influenciaram a atividade de enzimas digestivas principalmente no duodeno, provavelmente por ser local de lançamento das enzimas pancreáticas no intestino e o principal local de digestão dos nutrientes em mamíferos (Guyton and Hall, 2021). A BABS 1 foi a bebida que mais influenciou as enzimas digestivas nesse segmento, com aumento da atividade da amilase, lipase e proteases totais, porém, causou redução na atividade da lipase no jejuno. O aumento na atividade de enzimas digestivas promove maior digestibilidade dos nutrientes e, consequentemente, maior eficiência de utilização dos nutrientes para o crescimento e manutenção da homeostase e da saúde dos animais (Guyton and Hall, 2021). Já a BABS 2 e o leite de vaca causaram aumento na atividade da amilase e da tripsina no duodeno. Portanto, a principal diferença entre os efeitos da BABS 1 em relação a BABS 2 e o leite de vaca são os efeitos sobre a digestão de proteínas. A maior atividade da tripsina nos animais tratados com leite de vaca se deve à ausência de inibidores de tripsina, porém, a maior atividade desta enzima nos animais que receberam BABS 2 em relação ao controle é paradoxal, pois na presença de inibidores de tripsina seria esperado redução da atividade desta enzima (Xiao et al., 2012). As BABS utilizadas neste estudo apresentaram, em média, 42,2%de atividade de inibição de tripsina, o que está coerente com Xiao *et al*., 2012, que encontraram de 9,6% a 45,2% de inibição de atividade de tripsina, em diferentes marcas comerciais de BABS, em relação ao extrato de soja cru. Portanto, mesmo que as BABS sejam submetidas ao aquecimento, que inibe parcialmente esses fatores antinutricionais (Xiao et al., 2012) ainda exibem considerável inibição de tripsina.

A tripsina é uma enzima chave tanto na digestão de proteínas, como na ativação de outras enzimas digestivas no duodeno como a do quimiotripsinogênio, a proelastase, a procarboxipeptidase e algumas prolipases (Guyton and Hall, 2021). Sua forma inativa (tripsinogênio) é produzida e liberada pelas células acinares do pâncreas, tornando-se ativa no duodeno (Logsdon *et al*., 2013). A síntese dessa enzima pelo pâncreas é regulada por mecanismo de feedback negativo. Quando sua atividade é inibida, o pâncreas é estimulado a sintetizar e secretar mais tripsinogênio, o que pode causar superestimulação do órgão (Yuan *et al*., 2008). Altos níveis de inibidores de tripsina causam aumento do peso do pâncreas e dos níveis de tripsinogênio, bem como hipertrofia pancreática (Xiao, 2021), pancreatite (De Souza

et al., 2021) e aumento no risco de desenvolver câncer de pâncreas (Yamagiwa *et al*., 2020), o que resulta em diminuição da digestão e da biodisponibilidade de proteínas.

Ratos que ingeriram dietas contendo níveis elevados de inibidores de tripsina, similares aos encontrados em algumas BABS comercializadas (46,7%) (Xiao *et al*., 2012) apresentaram hipertrofia das células acinares, aumento do peso do pâncreas e aumento da secreção de tripsinogênio, mas essas alterações não são observadas nos animais que ingeriram dietas contendo níveis baixos (6,5%) de inibidores de tripsina (Xiao, *et al*., 2021). A retenção de menores níveis (4-10%) de inibidores de tripsina nas BABS é desejável, uma vez que a completa inativação desses inativa outras proteínas, afetando o valor nutricional e a sabor do alimento (Kwok and Niranjan, 1995). Além disso, a manutenção de níveis baixos desses compostos na bebida pode atuar contra a inflamação e câncer intestinal (Losso, 2008; Basson *et al*., 2021). Embora mais pesquisas sejam necessárias para definir níveis seguros de inibidores de tripsina ativos nos alimentos à base de soja, sugere-se que o valor de 10% de atividade de inibição seja desejável (Guerrero-Beltrán *et al*., 2009). Baseado nisso, a descrição da porcentagem desses inibidores nos rótulos dos alimentos é recomendada para o consumo mais consciente. Dessa forma, presume-se que os níveis de inibição da tripsina encontrados nas BABS utilizadas neste estudo (BABS 1 = 45,1% e BABS = 39,3%) poderiam explicar as leves alterações das variáveis morfofisiológicas analisadas no intestino.

Os camundongos que receberam BABS apresentaram menor atividade de lipase, no jejuno, em relação aos que receberam leite de vaca. Provavelmente, a maior porcentagem de gordura no leite de vaca em relação às BABS, (Tabela 1) pode ter estimulado a maior síntese de lipase nesses animais. A concentração de substrato tem relação direta com a produção de enzimas (Scops, 2002). Esse resultado sugere que a ingestão de BABS resulta em menor digestibilidade dos lipídeos no intestino, proporcionando menor absorção desse nutriente, sendo, portanto, alternativa viável para indivíduos que necessitam reduzir o peso corporal. Já no cólon, os animais tratados com leite de vaca apresentaram menor atividade de lipase em relação ao grupo controle. O leite de vaca pode ter alterado a microbiota dessa região o que pode ter influenciado indiretamente na atividade da lipase (McAllan *et al*., 2014; Boscaini *et al*., 2019).

Em geral, tanto BABS quanto o leite de vaca diminuíram a espessura das camadas musculares (CLE, CCI, TM) e aumentaram a altura do epitélio (AE) e da

borda estriada (ABE) nos diferentes segmentos intestinais. Sendo assim, sugere-se que todos os tratamentos beneficiaram o desenvolvimento da área de digestão e absorção no intestino. Provavelmente, todos os tratamentos também facilitaram o trânsito intestinal (Kapadia *et al*., 1993; Dalziel *et al*., 2017; Miao *et al*., 2018), refletindo na diminuição da espessura das camadas musculares.

Considerando que os tratamentos favoreceram o desenvolvimento do epitélio e da borda estriada, os animais estavam em boas condições para a absorção de nutrientes, o que está coerente com a manutenção do peso corporal entre os tratamentos e também em relação ao controle, exceto pela BABS 2. Em BABS 2, pode ter havido maior influência dos inibidores de proteases (Kong *et al*., 2022). No cólon, o epitélio e a borda estriada se apresentaram mais desenvolvidos nos animais que ingeriram leite de vaca. O leite de vaca, por conter mais gorduras que as BABS (Tabela 1) exige mais tempo para completar a digestão e, dessa forma, pode ter restado nutrientes, principalmente os ácidos graxos de cadeia curta (McNeil *et al*.,1978), para serem absorvidos no intestino grosso, o que estimulou o desenvolvimento dessas estruturas.

Em geral, tanto BABS quanto leite de vaca aumentaram o número de células caliciformes no intestino dos camundongos. As células caliciformes são importantes por formar muco, que previne a entrada de patógenos pela barreira intestinal (Kim and Ho, 2010). Adicionalmente, todos os tratamentos diminuíram o número de células inflamatórias intestinais (linfócitos e plasmócitos), o que pode estar diretamente relacionado com a maior proteção intestinal gerada pelas células caliciformes. Outra hipótese é que tanto BABS (Sadeghi *et al*., 2020) quanto o leite de vaca (Ulven *et al*., 2019) possuem funções anti-inflamatórias e podem ter atuado no combate a possíveis patógenos e toxinas.

No íleo, o leite de vaca aumentou o número de células de Paneth em relação à BABS 1. As células de Paneth estão localizadas principalmente na base das criptas de Lieberkuhn, no intestino delgado. Essas células sintetizam e secretam peptídeos antimicrobianos, como as α-defensinas, as lisozimas, a fosfolipase A2 e a RegIIIA (“regenerating islet-derievd protein IIIA”). Esses peptídeos são mediadores chave na modulação da composição da microbiota intestinal e na proteção inata contra patógenos, estando diretamente relacionadas com prevenção de doenças inflamatórias intestinais (Lueschow and McElroy, 2020). Portanto, o aumento das células de Paneth nos animais que receberam leite de vaca na dieta, sugere que

indivíduos que ingerem leite vaca em vez de BABS, apresentam maior defesa imune intestinal. Além disso, as células de Paneth secretam fatores de crescimento como EGF e WNT3 que modulam o desenvolvimento das células tronco intestinais, demonstrando íntima interação entre essas células na renovação do epitélio (Clevers and Bevins, 2013).

Os animais que ingeriram BABS apresentaram menor número de mitoses no íleo do que os que ingeriram leite de vaca. A diminuição da divisão celular pelas BABS pode estar relacionada com a ação de compostos bioativos presentes na soja, como a genisteína (Sulistyowati et al., 2019). A genisteína é capaz de inibir enzimas tirosina quinases que estão envolvidas em diversas vias de sinalização relacionadas a proliferação celular, com a as WNT/β catenina e AKT/MAPK (Zhu *et al*., 2018; Chen *et al*., 2020), além de inibir a enzima DNA topoisomerase, que é essencial para a manutenção da integridade do DNA durante a replicação (Salti *et al*., 2000). Logo, a inibição destas vias resulta em menor proliferação celular. Esta condição é vantajosa para algumas condições clínicas, como o câncer, mas alterações nestas enzimas em indivíduos saudáveis devem ser vistas com cautela.

3.5 Conclusão

Em geral, tanto as BABS quanto o leite de vaca não são prejudiciais para a morfofisiologia intestinal. BABS é uma alternativa viável ao leite de vaca para indivíduos que necessitam de ingestão baixa de gordura. Entretanto, o aumento do número das células de Paneth nas criptas intestinais dos animais que receberam leite de vaca, sugere que a ingestão dessa bebida possa promover algum benefício para saúde intestinal, se comparado à ingestão de BABS. Além disso, o efeito de inibição de tripsina e de diminuição de mitose levanta alerta sobre o comportamento de BABS sobre a digestão e a renovação do epitélio intestinal. A escolha entre o consumo de BABS e de leite de vaca deve ser realizada de forma individual e com acompanhamento profissional. Sugerem-se novos estudos pré-clínicos e clínicos que comparem os efeitos destas bebidas, a partir de análises complementares às realizadas neste estudo, como a avaliação da expressão gênica de inibidores de proteases e de enzimas digestivas, além da avaliação da concentração de genisteína nas BABS.

Referências

ARMOUR, J. C.; CHANAKA PERERA, R. L.; BUCHAN, W. C.; GRANT, G. Protease inhibitors and lectins in soya beans and effects of aqueous heat-treatment. **Journal of the Science of Food and Agriculture**, v. 78, p. 225–231, 1998.

BANCROFT, J. D.; GAMBLE, M. **Theory and Practice of Histological Techniques**. 2 ed. Churchill Livingstone, 725 p., 2008.

BASSON, A. R.; AHMED, S.; ALMUTAIRI, R.; SEO, B.; COMINELLI, F. Regulation of intestinal inflammation by soybean and soy-derived compounds. **Foods**, v. 10, n. 4, p. 774, 2021. DOI 10.3390/foods10040774.

BEACOM, E.; BOGUE, J.; REPAR, L. Market-oriented development of plant-based food and beverage products: a usage segmentation approach. **Journal of Food Products Marketing**, v. 27, p. 204–22, 2021. DOI 10.1080/10454446.2021.1955799.

BERG, J. M.; TYMOCZKO, J. L.; GATTO JR, G. J.; STRYER, L. **Stryer biochemie**. Heidelberg: Springer Spektrum, 1401 p., 2018.

BLOOM, W.; FAWCETT, D. W. **Text Book of Histology**. 11 ed. W.B. Saunders Company, 1017 p., 1986.

BOSCAINI, S.; CABRERA-RUBIO, R.; SPEAKMAN, J. R.; COTTER, P. D.; CRYAN, J. F.; NILAWEERA, K. N. Dietary α-lactalbumin alters energy balance, gut microbiota composition and intestinal nutrient transporter expression in high-fat diet-fed mice. **British Journal of Nutrition**, v. 121, n. 10, p. 1097-1107, 2019.

BRADFORD, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. **Analytical Biochemistry**, v. 72, p. 248–254, 1976. DOI 10. 1016/ 0003- 2697(76) 90527-3.

CARAWAY, W. T. A stable starch substrate for the determination of amylase in serum and other body fluids. **American Journal of Clinical Pathology**, v. 32, p. 97-99, 1959.

CHEN, X.; WU, Y.; GU, J.; LIANG, P.; SHEN, M.; XI, J.; QIN, J. Anti-invasive effect and pharmacological mechanism of genistein against colorectal cancer. **Biofactors**, v. 46, n. 4, p. 620-628, 2020.

CHERRY, I. S.; CRANDALl JR, L. A. The specificity of pancreatic lipase: its appearance in the blood after pancreatic injury. **American Journal of Physiology-Legacy Content**, v. 100, n. 2, p. 266-273, 1932.

CLEVERS, H. C.; BEVINS, C. L. Paneth cells: maestros of the small intestinal crypts. **Annual Review of Physiology**, v. 75, p. 289-311, 2013.

COMERFORD, K. B.; MILLER, G. D.; BOILEAU, A. C.; MASIELLO SCHUETTE, S. N.; GIDDENS, J. C.; BROWN, K. A. Global review of dairy recommendations in food-based dietary guidelines. **Frontiers in Nutrition**, v. 8, p. 671999, 2021. DOI 10.3389/fnut.2021.671999. eCollection 2021.

DALZIEL, J. E.; YOUNG, W.; MCKENZIE, C. M.; HAGGARTY, N. W.; ROY, N. C. Gastric emptying and gastrointestinal transit compared among native and hydrolyzed whey and casein milk proteins in an aged rat model. **Nutrients**, v. 9, n. 12, p. 1351, 2017.

DE SOUZA, I. S.; LIPSITT, A. The soymilk diet: A previously unknown etiology of acute pancreatitis. **The American Journal of Emergency Medicine**, v. 46, p. 798.e5-798.e6, 2021. DOI 10.1016/j.ajem.2021.01.055.

DROUIN-CHARTIER, J. P.; BRASSARD, D.; TESSIER-GRENIER, M.; COTE, J. A.; LABONTE, M. E.; DESROCHES, S.; COUTURE, P.; LAMARCHE, B. Systematic review of the association between dairy product consumption and risk of cardiovascular-related clinical outcomes. **Advances in Nutrition**, v. 7, p. 1026–40, 2016. DOI 10.3945/an.115.011403.

ERLANGER, B. F.; KOKOWSKY, N.; COHEN, W. The preparation and properties of two new chromogenic substrates of trypsin. **Archives of Biochemistry and Biophysics**, v. 95, p. 271-278, 1961.

FERREIRA, E. B.; CAVALCANTI, P. P.; NOGUEIRA, D. A. ExpDes.pt: pacote experimental designs (portuguese): R package version 1.2.0 [online]. Available from: https://CRAN.R-project.org/package=ExpDes.pt. 2018 [viewed 24 December 2021].

GIRI, S. K.; MANGARAJ, S. Processing influences on composition and quality attributes of soymilk and its powder. **Food Engineering Reviews**, v. 4, n. 3, p. 49-164, 2012. DOI 10.1007/s12393-012-9053-0.

GUERRERO-BELTRÁN, J. A.; ESTRADA-GIRÓN, Y.; SWANSON, B. G.; BARBOSA-CÁNOVAS, G. V. Pressure and temperature combination for inactivation of soymilk trypsin inhibitors. **Food Chemistry**, v.116, n. 3, p. 676-679, 2009.

HALL, J. E.; GUYTON, A. C. **Tratado de Fisiología médica**. 14 ed. Elsevier, 940 p., 2021.

KAPADIA, S. A.; RAIMUNDO, A. H.; SILK, D. B. The effect of a fibre free and fibre supplemented polymeric enteral diet on normal human bowel function. **Clinical Nutrition**, v. 12, n. 5, p. 272-276, 1993. DOI 10.1016/0261-5614(93)90045-6.

KARNOVSKY, M. J. A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. **Journal of Cell Biology**, v. 27:137A, 1965.

KIM, Y. S.; HO, S. B. Intestinal goblet cells and mucins in health and disease: recent insights and progress. **Current Gastroenterology Reports**, v. 12, p. 319-330, 2010.

KONG, X.; LI, Y.; LIU, X. A review of thermosensitive antinutritional factors in plant-based foods. **Journal of Food Biochemistry**, v.46 n. 9, p. e14199, 2022. DOI 10.1111/jfbc.14199.

KŘÍŽOVÁ, L.; DADÁKOVÁ, K.; KAŠPAROVSKÁ, J.; KAŠPAROVSKÝ, T. Isoflavones. **Molecules**, p. 24, n. 6, p. 1076, 2019.

KWOK, K. C.; Niranjan, K. Effect of thermal processing on soymilk. **International Journal of Food Science & Technology**, v. 30, n. 3, p. 263-295, 1995.

LEVIT, R.; DE GIORI, G. S.; DE MORENO DE LEBLANC, A.; LEBLANC, J. G. Evaluation of the effect of soymilk fermented by a riboflavin producing *Lactobacillus plantarum* strain in a murine model of colitis. **Beneficial Microbes**, v. 8, p. 65–72, 2017.

LOGSDON, C. D.; JI, B. The role of protein synthesis and digestive enzymes in acinar cell injury. **Nature Reviews Gastroenterology & Hepatology**, v. 10, p. 362–370, 2013.

LOSSO, J. N. The biochemical and functional food properties of the Bowman-Birk inhibitor. **Critical Reviews in Food Science and Nutrition**, v. 48, n.1, p. 94-118, 2008.

LUESCHOW, S. R.; MCELROY, S. J. The Paneth cell: the curator and defender of the immature small intestine. **Frontiers in Immunology**, v. 11, p. 587, 2020. DOI 10.3389/fimmu.2020.00587.

MCALLAN, L.; SKUSE, P.; COTTER, P. D.; CONNOR, P. O.; CRYAN, J. F.; ROSS, R. P.; FITZGERALD, G.; ROCHE, H. M.; NILAWEERA, K. N. Protein quality and the protein to carbohydrate ratio within a high fat diet influences energy balance and the gut microbiota in C57BL/6J mice. **PLoS One**, v. 9, n. 2, p. e88904, 2014. DOI 10.1371/journal.pone.0088904.

MCCONNELL, E. L.; BASIT, A. W.; MURDAN, S. Measurements of rat and mouse gastrointestinal pH, fluid and lymphoid tissue, and implications for *in-vivo* experiments. **Journal of Pharmacy and Pharmacology**, v. 60, n. 1, p. 63-70, 2008.

MCNEIL, N. I.; CUMMINGS, J. H.; JAMES, W. P. Short chain fatty acid absorption by the human large intestine. **Gut**, v. 19, n. 9, p. 819-822, 1978.

MIAO, S.; ZHAO, C.; ZHU, J.; HU, J.; DONG, X.; SUN, L. Dietary soybean meal affects intestinal homoeostasis by altering the microbiota, morphology and inflammatory cytokine gene expression in northern snakehead. **Scientific Reports**, v. 8, n. 1, p. 113, 2018.

MUZQUIZ, M.; VARELA, A.; BURBANO, C.; CUADRADO, C.; GUILLAMÓN, E.; PEDROSA, M. M. Bioactive compounds in legumes: Pronutritive and antinutritive actions. Implications for nutrition and health. **Phytochemistry Reviews**, v. 11, p. 227–244, 2012.

OBERMAYER-PIETSCH, B. M.; BONELLI, C. M.; WALTER, D.E; KUHN, R.J; FAHRLEITNER-PAMMER, A.; BERGHOLD, A.; GOESSLER, W.; STEPAN, V.; DOBNING, H.; LEB, G.; RENNER, W. Genetic predisposition for adult lactose intolerance and relation to diet, bone density, and bone fractures. **Journal of Bone and Mineral Research,** v. 19, p. 42–7, 2004. DOI 10.1359/jbmr.0301207.

PEI, R.; LIU, X.; BOLLING, B. Flavonoids and gut health. **Current Opinion in Biotechnology**, v. 61, p. 153-159, 2020. DOI 10.1016/j.copbio.2019.12.018.

RÓKA, R.; WITTMANN, T.; BUENO, L. Altered protease signalling in the gut: A novel pathophysiological factor in irritable bowel syndrome. **Journal of Neurogastroenterology and Motility,** v. 20, p. 853–856, 2008.

RUEHL-FEHLERT, C.; KITTEL, B.; MORAWIETZ, G.; DESLEX, P.; KEENAN, C.; MAHRT, C. R.; DESCHL, U. Revised guides for organ sampling and trimming in rats and mice–part 1: A joint publication of the RITA and NACAD groups. **Experimental and Toxicologic Pathology**, v. 55, n. 2-3, p. 91-106, 2003.

SADEGHI, O.; MILAJERDI, A.; SIADAT, S. D.; KESHAVARZ, S. A.; SIMA, A. R.; VAHEDI, H.; ADIBI, P.; ESMAILLZADEH, A. Effects of soy milk consumption on gut microbiota, inflammatory markers, and disease severity in patients with ulcerative colitis: a study protocol for a randomized clinical trial. **Trials**, v. 21, p. 565, 2020. DOI 10.1186/s13063-020-04523-8.

SALTI, G. I.; GREWAL, S.; MEHTA, R. R.; GUPTA, T. D.; BODDIE JR, A. W.; CONSTANTINOU, A. I. Genistein induces apoptosis and topoisomerase II-mediated DNA breakage in colon cancer cells. **European Journal of Cancer**, v.36, n. 6, p. 796-802, 2000.

SCOPES, R. K. Enzyme activity and assays. **Encyclopedia of Life Sciences (eLS),** 2002.

SINGHAL, S.; BAKER, R. D.; BAKER, S. S. A comparison of the nutritional value of cow's milk and nondairy beverages. **Journal of Pediatric Gastroenterology and Nutrition**, v. 64, n. 5, p. 799-805, 2017. DOI 10.1097/MPG.0000000000001380.

STEPHENS, R. H.; TANIANIS-HUGHES, J.; HIGGS, N. B.; HUMPHREY, M.; WARHURST, G. Region-dependent modulation of intestinal permeability by drug efflux transporters: *in vitro* studies in mdr1a (−/−) mouse intestine. **Journal of Pharmacology and Experimental Therapeutics**, v. *303*, n. 3, p. 1095-1101, 2002.

SULISTYOWATI, E.; MARTONO, S.; RIYANTO, S.; LUKITANINGSIH, E. Development and validation for free aglycones daidzein and genistein in soybeans (*Glycine max* (L.) Merr.) using RP HPLC method. **International Journal of Applied Pharmaceutics,** v. 11, n. 2, p. 138-142, 2019.

TOMARELLI, R. M.; CHARNEY, J.; HARDING, M. L. The use of azoalbumin as a substrate in the colorimetric determination of peptic and tryptic activity. **Journal Laboratory Clinical Medical**, v. 34, p. 428-433, 1949.

ULVEN, S. M.; HOLVEN, K. B.; GIL, A.; RANGEL-HUERTA, O. D. Milk and dairy product consumption and inflammatory biomarkers: an updated systematic review of randomized clinical trials. **Advances in Nutrition**, v. 10, p. S239-S250, 2019. DOI 10.1093/advances/nmy072.

VOET, D.; VOET, J. G.; PRATT, C. W. **Fundamentals of Biochemistry: Life at the Molecular Level**. John Wiley & Sons, ed. 5, 1184 p., 2016.

VOJDANI, A.; TURNPAUGH, C.; VOJDANI, E. Immune reactivity against a variety of mammalian milks and plant-based milk substitutes. **Journal of Dairy Research**, v. 85, n.3, p. 358–365, 2018. DOI 10.1017/S0022029918000523.

WALTHER, B.; GUGGISBERG, D.; BADERTSCHER, R.; EGGER, L.; PORTMANN, R.; DUBOIS, S.; REZZI, S. Comparison of nutritional composition between plant-based drinks and cow's milk. **Frontiers in Nutrition**, v. 9, p. 988707, 2022. DOI 10.3389/fnut.2022.988707. eCollection 2022.

WANG, C., DING, K.; XIE, X.; ZHOU, J.; LIU, P.; WANG, S.; FANG, T.; XU, G.; HONG, H. Soy Product Consumption and the Risk of Cancer: A Systematic Review and Meta-Analysis of Observational Studies. **Nutrients**, v. 16, n. 7, p. 986, 2024.

WEDER, J. K. Inhibition of human proteinases by grain legumes. **Advances in Experimental Medicine and Biology,** v. 199, p. 239–279, 1986.

XIAO, C. W.; WOOD, C. M.; ROBERTSON, P.; GILANI, G. S. Protease inhibitor activities and isoflavone content in commercial soymilks and soy-based infant formulas sold in Ottawa, Canada. **Journal of Food Composition and Analysis**, v. 25, n. 2, p. 130-136, 2012.

XIAO, C. W.; WOOD, C.; CUNNINGHAM, L. A.; LALANDE, M.; RIDING, M. Effects of dietary active soybean trypsin inhibitors on pancreatic weights, histology and expression of STAT3 and receptors for androgen and estrogen in different tissues of rats. **Molecular Biology Reports**, v.48, p. 4591–4600, 2021.

YAMAGIWA, Y.; SAWADA, N.; SHIMAZU, T.; YAMAJI, T.; GOTO, A.; TAKACHI, R.; TSUGANE, S. Soy Food Intake and Pancreatic Cancer Risk: The Japan Public Health Center–based Prospective Study. **Cancer Epidemiology, Biomarkers & Prevention**, v. 29, n. 6, p. 1214-1221, 2020.

Yuan, S.; Chang, S. K.; Liu, Z.; Xu, B. Elimination of trypsin inhibitor activity and beany flavor in soy milk by consecutive blanching and ultrahigh-temperature (UHT) processing. **Journal of Agricultural and Food Chemistry**, v. 56, p. 7957–7963, 2008.

ZHU, J.; REN, J.; TANG, L. Genistein inhibits invasion and migration of colon cancer cells by recovering WIF1 expression. **Molecular Medicine Reports**, v. 17, n. 5, p. 7265-7273, 2018.

Anexo A

Certificado de aprovação do projeto pela Comissão de Ética no Uso de Animais da Universidade Federal de Viçosa CEUA/UFV.

CERTIFICADO

A Comissão de Ética no Uso de Animais - CEUA/UFV certifica que o processo nº 01/2022, intitulado **"Ação do leite se soja e leite de vaca sobre o sistema reprodutor masculino de camundongos Balb C adultos",** coordenado pelo professor Sérgio Luis Pinto da Matta do Departamento de Biologia Geral, está de acordo com a Legislação vigente (Lei Nº 11.794, de 08 de outubro de 2008), as Resoluções Normativas editadas pelo CONCEA/MCTIC, a DBCA (Diretriz Brasileira de Prática para o Cuidado e a Utilização de Animais para Fins Científicos e Didáticos) e as Diretrizes da Prática de Eutanásia preconizadas pelo CONCEA/MCTIC, portanto sendo aprovado por esta Comissão em 25/04/2022, com validade de 12 meses.

CERTIFICATE

The Ethic Committee in Animal Use/UFV certify that the process number 01/2022, named **"Action of soy milk and cow's milk on the male reproductive system of adult Balb C mice"**, is in agreement with the a actual Brazilian legislation (Lei Nº 11.794, 2008, Normative Resolutions edited by CONCEA/MCTIC, the DBCA (Brazilian Practice Guideline for the Care and Use of Animals for Scientific and the Guidelines of Practice the Euthanasia recommended by CONCEA/MCTIC therefore being approved by the Committee on April 25, 2022 valid for 12 months.

Prof. ª Mariella Bontempo Duca de Freitas
Coordenadora
Comissão de Ética no Uso de Animais – CEUA/UFV