POLIANA GUIOMAR DE ALMEIDA BRASIEL

EFEITO DO CONSUMO MATERNO DE KEFIR NA LACTAÇÃO E NA PUBERDADE SOBRE A MICROBIOTA INTESTINAL, PARÂMETROS INFLAMATÓRIOS E SUSCEPTIBILIDADE À CARCINOGÊNESE COLORRETAL NA PROGÊNIE DE RATOS WISTAR PROGRAMADOS PELA SUPERALIMENTAÇÃO NEONATAL

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Ciência da Nutrição, para obtenção do título de *Magister Scientiae*.

Orientadora: Maria do Carmo Gouveia Peluzio

VIÇOSA - MINAS GERAIS

2020

Ficha catalográfica preparada pela Biblioteca Central da Universidade Federal de Viçosa - Câmpus Viçosa

T
B823e
2020

Brasiel, Poliana Guiomar de Almeida, 1992-
Efeito do consumo materno de kefir na lactação e na puberdade sobre a microbiota intestinal, parâmetros inflamatórios e susceptibilidade a carcinogênese colorretal na progênie de ratos Wistar programados pela superalimentação neonatal / Poliana Guiomar de Almeida Brasiel. – Viçosa, MG, 2020.
119f. : il. ; 29 cm.

Inclui anexo.
Orientador: Maria do Carmo Gouveia Peluzio.
Dissertação (mestrado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Colón (Anatomia) - Câncer. 2. Kefir. 3. Microbioma Gastrointestinal. 4. Superalimentação. 5. Lactação.
6. Desenvolvimento infantil. 7. Puberdade. I. Universidade Federal de Viçosa. Departamento de Nutrição e Saúde. Programa de Pós-Graduação em Ciência da Nutrição. II. Título.

CDD 22 ed. 616.99435

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APROVADA: 18 de fevereiro de 2020.

Assentimento:

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À minha vó Guiomar, que sempre acreditou no poder do trabalho e do estudo.

DEDICO.

AGRADECIMENTOS

A Deus, por guiar o meu caminho.

A toda minha família, pelo apoio. Sobretudo minha mãe Ana Lucia, meus irmãos, Willie, Abraão, Ariel e Eduardo, e a minha tia Deila querida.

À professora Sheila Cristina Potente Dutra Luquetti, pela amizade, ensinamentos, parcerias, conselhos, boas ideias, por sempre confiar no meu trabalho. Seguimos!

À minha orientadora professora Maria do Carmo Gouveia Peluzio, pela oportunidade de crescimento acadêmico e troca de experiências.

À técnica do Laboratório de Nutrição Experimental (LABNE), Kácia Mateus, pelo apoio e solicitude de sempre. Mais uma ariana desse time!

A toda equipe do LABNE, Maíra, Thaís, Nara, Lucas, Gabriela, Bernardo, Marlon, Érika e Marina. Esse projeto foi para os fortes!

Ao técnico da UFJF, Silvioney, pela disposição incondicional em ajudar, deixando claro que os recursos não eram seus, nem do laboratório, nem da Universidade, mas os recursos são públicos. As universidades precisam de mais profissionais como você!

A todos do Laboratório de Bioquímica Nutricional, Letícia, Bruna, Anny, Iasmim, Felipe, Mariana(s), Rayssa, Andressa, Sandra, Lisiane e Toninho.

Aos amigos de sempre, em especial, Thamara Oliveira e Matheus Ferreira. E aos amigos que tive a oportunidade de conhecer durante minha estada em Viçosa.

Ao G7, Ana Paula, Bruninha, Daiane, Claudia, Elisânia e Ju, eu sempre tive a certeza de como fazer parte desse grupo fez minha formação melhor.

A todos os brilhantes professores que fizeram parte da minha formação e são uma inspiração.

À Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), pelo auxílio financeiro (CDS - APQ-01332-16).

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001.

A todos que, direta ou indiretamente, ajudaram na realização desse trabalho.

‘’Ich habe fleißig seyn müssen; wer eben so fleißig ist, der wird es eben so weit bringen können.’’

Johann Sebastian Bach

RESUMO

BRASIEL, Poliana Guiomar de Almeida, M.Sc., Universidade Federal de Viçosa, fevereiro de 2020. **Efeito do consumo materno de kefir na lactação e na puberdade sobre a microbiota intestinal, parâmetros inflamatórios e susceptibilidade à carcinogênese colorretal na progênie de ratos Wistar programados pela superalimentação neonatal**. Orientadora: Maria do Carmo Gouveia Peluzio.

Alterações nutricionais durante períodos críticos de desenvolvimento, como a lactação e a puberdade, têm impacto no risco de desenvolver doenças na vida adulta. Nesse sentido, o modelo da superalimentação neonatal pode resultar em programação alterada, levando ao aumento da suscetibilidade à obesidade, inflamação e complicações relacionadas. O kefir, um leite fermentado, originado a partir da ação da microbiota natural presente em seus grãos, apresenta uma mistura complexa e específica de bactérias ácido-láticas, ácido-acéticas e leveduras em uma matriz de proteínas e polissacarídeos. Com características probióticas, está associado à atividade antimicrobiana e de imunomodulação. Neste estudo investigamos os efeitos da programação pelo kefir/ superalimentação durante o período de lactação e puberdade da prole na idade adulta induzida à carcinogênese de cólon por 1,2 dimetilhidrazina (DMH), sobre a adiposidade, inflamação, microbiota intestinal e o desenvolvimento da carcinogênese colorretal. Ratas Wistar em lactação foram divididas em quatro grupos: Controle (C, n = 7 filhotes); Controle Kefir (CK, n = 8 filhotes); Superalimentado (S, n = 7 filhotes); Superalimentado Kefir (SK, n = 7 filhotes). As mães dos grupos C e S receberam 1 ml de água destilada por gavagem, uma vez ao dia. Para os outros grupos de teste, os animais receberam 1 ml de kefir de leite (10^8 UFC/ml) por gavagem uma vez ao dia durante os 21 dias de lactação. Após o desmame, todos os filhotes continuaram recebendo o mesmo tratamento materno (água ou kefir) até os 60 dias de idade. Na idade adulta (24 semanas após a última aplicação do DMH), o grupo S apresentou maior somatório de tecido adiposo em comparação ao C (+53,83%; p <0,001), CK (+48,85%; p <0,001) e SK (+20,04 %; p <0,01) grupos. O kefir suprimiu significativamente o número de tumores, mesmo no grupo superalimentado (SK: -71,43%; p <0,01). Houve aumento de citocinas pró-inflamatórias (IL-1β, IL-6 e TNF-α) no tecido do cólon do grupo S. Para a produção de óxido nítrico foi observado um aumento nos animais S, mas que foi reduzido pelo kefir

(grupo SK) (-69,9%, p <0,001). Investigamos pela primeira vez os efeitos do consumo de kefir durante períodos críticos de desenvolvimento e identificamos sua capacidade de reduzir tumores do cólon, danos histológicos e citocinas pró-inflamatórias, bem como diminuir a adiposidade e modular a microbiota intestinal da prole adulta.

Palavras-chave: Câncer colorretal. Kefir. Microbiota intestinal. Programação. Superalimentação.

ABSTRACT

BRASIEL, Poliana Guiomar de Almeida, M.Sc., Universidade Federal de Viçosa, February, 2020. **Effect of maternal kefir consumption on lactation and puberty on intestinal microbiota, inflammatory parameters and susceptibility to colorectal carcinogenesis in progeny of Wistar rats programmed by neonatal overfeeding**. Advisor: Maria do Carmo Gouveia Peluzio.

Nutritional changes during critical periods of development, such as lactation and puberty, affect the risk of developing a disease later in life. In this sense, the neonatal overfeeding model may result in altered programming, leading to increased susceptibility to obesity, inflammation, and related complications. Kefir, a fermented milk product originated from the action of natural microbiota present in its grains, presents a complex and specific mixture of lactic acid, acetic acid bacteria, and yeast in a matrix of proteins and polysaccharides. With probiotic characteristics, it is associated with antimicrobial and immunomodulation activity. In this study, we investigated the effects of programming by kefir/overfeeding during lactation and puberty in 1,2-dimethylhydrazine (DMH)-induced colon cancer, on adiposity, inflammation, intestinal microbiota, and the development of colorectal carcinogenesis. Lactating Wistar rats were divided into four groups: Control (C, n = 7 pups); Kefir control (CK, n = 8 pups); Overfeeding (S, n = 7 pups); Overfeeding Kefir (SK, n = 7 pups). The dams of groups C and S received 1 ml of distilled water by gavage once a day. For the other test groups, the animals received 1 ml milk kefir (10^8 cfu/ml) by gavage once a day during the 21 days of lactation. After weaning, all pups continued to receive the same maternal treatment (water or kefir) until 60 days of age. In adulthood (24 weeks after the last application of DMH), the S group presented a higher sum of adipose tissue compared to the C (+53.83%; $p < 0.001$), CK (+48.85%; $p < 0.001$) and SK (+20.04%; $p < 0.01$) groups. Kefir significantly suppressed the number of tumors, even in the overfeeding group (SK: -71.43%; $p < 0.01$). There was an increase in proinflammatory cytokines (IL-1β, IL-6, and TNF-α) in the SL group colon tissue. For nitric oxide production, an increase was observed in SL animals but was reduced by kefir (SK group) (-69.9%, $p < 0.001$). We investigated for the first time the effects of kefir consumption during critical developmental periods and identified its ability to

reduce colon tumors, histological damage, and proinflammatory cytokines as well as its potential to decrease adiposity and modulate the gut microbiota of adult offspring.

Keywords: Colorectal cancer. Kefir. Gut microbiota. Programming. Overnutrition.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

AGCC	Ácidos graxos de cadeia curta
ANVISA	Agência Nacional de Vigilância Sanitária
AOM	Azoximetano
BAL	Bactérias ácido-láticas
BDA	Meio batata, dextrose, ágar
CCR	Câncer colorretal
CEA	Coeficiente de eficácia alimentar
CIMP	Fenótipo metilador das ilhotas CpG
DCNT	Doenças crônicas não transmissíveis
DMH	1,2 dimetilhidrazina
DOHaD	Origens Desenvolvimentistas da Saúde e da Doença
ELISA	*Enzyme-Linked Immunosorbent Assay*
EPI	Equipamento de proteção individual
FCA	Focos de criptas aberrantes
H&E	Hematoxilina e eosina
IDH	Índice de Desenvolvimento Humano
IFN	Interferon
IL	Interleucina
INCA	Instituto Nacional de Câncer
LPS	Lipopolissacarídeo
MC	Massa corporal
MRS	Meio de Man, Rogosa e Sharpe
NF-kB	Fator de transcrição nuclear kappa B
OTU	Unidade taxonômica operacional
STAT3	S*ignal transducer and activating factor of transcription 3*
TLR4	Receptores do tipo Toll 4
TNF	Fator de necrose tumoral
UFC	Unidades formadoras de colônias

SUMÁRIO

1. INTRODUÇÃO

A prevalência de doenças crônicas não transmissíveis (DCNT) está aumentando em todo mundo, com destaque para a obesidade e o câncer, que apresentam grande impacto na morbimortalidade, sendo considerados importantes problemas de saúde pública. Desta forma, é essencial a compreensão dos fatores envolvidos na gênese e proteção dessas enfermidades, que são de origem multifatorial, mas que apresentam uma associação entre si (DEKKER et al., 2019).

Segundo dados do Instituto Nacional de Câncer (INCA, 2019), estima-se para os próximos anos a ocorrência de 625 mil novos casos de câncer no Brasil, sendo os cânceres de maior incidência, com exceção do de pele não melanoma, os de próstata, colón e reto e pulmão nos homens e mama, colón e reto e colo de útero nas mulheres. Para o câncer colorretal, a estimativa para cada ano do triênio de 2020-2022, é de 20.520 novos casos em homens e 20.470 em mulheres, evidenciando-se assim, um aumento de casos deste tipo de câncer.

O desenvolvimento do câncer colorretal é resultado de uma interação complexa de fatores ambientais e nutricionais e fatores internos de natureza somática ou hereditária. Para o câncer colorretal, apenas 20% dos casos são de origem hereditária. Os casos mais frequentes são resultantes da exposição à carcinógenos ou fatores ambientais de risco (INCA, 2019).

O padrão alimentar inadequado, tal como alto consumo de calorias, gorduras, carnes vermelhas, ácidos graxos *trans* e baixo consumo de frutas e hortaliças, associado ao excesso de peso, contribuem para o desencadeamento de uma série de alterações endócrino-metabólicas e no sistema imune, que contribuem para a geração de inflamação crônica de baixo grau (subclínica), resistência à insulina, estresse oxidativo e desequilíbrio na microbiota intestinal (CONCEIÇÃO et al., 2013; MASSODI et al., 2015). Este último favorece a proliferação de bactérias oportunistas, com consequente degradação de ácidos biliares e produção de agentes carcinógenos (LIEBERMAN, 2003; STAMP, 2002; KHAN; AFAQ; MUKHTAR, 2010), e também a maior permeabilidade intestinal, que pode gerar translocação de lipolissacarídeos (LPS), contribuindo para perpetuação da inflamação (SANZ & MOYA-PEREZ, 2014), e criação de um microambiente favorável ao desenvolvimento neoplásico, invasão, metástase e angiogênese (HOOPER et al, 2012; LIU et al, 2014; URONIS et al, 2009).

Paralelamente a estes achados, estudos têm associado o desenvolvimento da obesidade e outras DCNT, com a ocorrência de insultos ou alterações nutricionais e endócrino-metabólicas em períodos críticos do desenvolvimento, como gestação e lactação. Esta relação tem sido denominada de “programação” (DUTRA et al., 2007; DUTRA et al., 2011; PASSOS et al., 2007; PASSOS et al., 2009; RODRIGUES et al., 2007; SAMUELSSON et al., 2008; VIEIRA et al., 2018; XIÃO et al., 2007). Tem sido proposto que as condições ambientais experimentadas no início da vida podem influenciar profundamente a biologia humana e a saúde a longo prazo. A janela da plasticidade do desenvolvimento se estende da pré-concepção à primeira infância e envolve respostas epigenéticas às mudanças ambientais, que exercem seus efeitos durante as transições das diferentes fases da vida (BARKER, 1993; FERNANDEZ-TWINN & OZANNE, 2010; SOMINSKY et al., 2018).

Embora diversos estudos relacionem o processo de carcinogênese colorretal com a exposição a fatores nutricionais, que podem atuar como moduladores de risco e prevenção no seu desenvolvimento (BUTT & SULTAN, 2009; KIM & KWON, 2009; MARSHALL, 2008), poucos trabalhos relacionam esta exposição durante períodos críticos do desenvolvimento, com a carcinogênese colorretal (LOPES, 2014; XIÃO et al., 2007). Desta forma, a relação entre programação e câncer colorretal ainda demanda mais estudos, em especial os que relacionam a obesidade neonatal com o desenvolvimento desta neoplasia.

Assim, um modelo experimental de programação neonatal por redução da ninhada, foi proposto como uma forma de simular o cenário nutricional atual e o desenvolvimento de doenças. Neste modelo, os animais desenvolvem aumento da adiposidade corporal, hiperinsulinemia e estresse oxidativo. Acreditamos que estes animais também apresentam maior ativação de vias inflamatórias e alteração da microbiota intestinal. Todos esses fatores associados poderiam favorecer o desenvolvimento da carcinogênese colorretal. Desta forma, este modelo é interessante para se estudar fatores envolvidos na relação entre obesidade e câncer colorretal. Por outro lado, não identificamos estudos que tenham avaliado parâmetros inflamatórios e a microbiota intestinal nesse modelo, até a presente data.

Considerando a importância de fatores nutricionais na carcinogênese colorretal, estudos apontam que os probióticos parecem atuar como preventivos, uma vez que apresentam potencial na modulação da imunidade intestinal, induzindo a maturação de células dendríticas e subsequente ativação das células T presentes no

intestino. Seu consumo também tem sido correlacionado a maior produção de IL-10 e defensinas (AZCÁRATE-PERIL et al., 2011; CHAN et al., 2009; KHOURY et al., 2014).

Nesta perspectiva, os probióticos vêm sendo estudados e inseridos na alimentação humana. Entretanto, há diferenças em seus efeitos em relação ao tipo de cepa que é consumida. Várias cepas de bactérias, incluindo *Lactobacillus acidophilus* e *Eubacterium aerofaciens* estão associados ao baixo risco para o desenvolvimento de câncer colorretal, enquanto *Streptococus bovis* e *Escherichia coli*, foram demonstradas com maior risco (ARTHUR et al., 2012; REDDY et al., 1985). Desta forma, ainda não há um consenso quanto ao seu papel nesta neoplasia, e muito se deve as diferenças entre as cepas utilizadas, ao modo de preparo do probiótico e as doses administradas.

O kefir é um leite fermentado, originado a partir da ação da microbiota natural presente em seus grãos ou grumos. Apresenta uma mistura complexa e específica de bactérias ácido-láticas, ácido-acéticas e leveduras em uma matriz de polissacarídeos e proteína. Sua composição bioquímica e microbiológica o classifica como um probiótico, sendo associado à atividade antimicrobiana e de imunomodulação (FARNWORTH, 2005; SARKAR, 2008). O baixo custo e a facilidade no preparo impulsionam sua utilização, e exigem mais pesquisas que comprovem a segurança de seu consumo e benefícios a saúde (HONG et al., 2009; RATTRAY & O'CONNEL, 2011; SILVA et al., 2009).

Existem poucos estudos avaliando o efeito do consumo materno de kefir ou outro probiótico em períodos críticos do desenvolvimento, como a lactação, sobre a saúde da progênie. Entretanto, é conhecido que a colonização intestinal do recém-nascido é essencial para a maturação, estabelecimento e manutenção da barreira da mucosa intestinal. Tem-se evidencias que a colonização microbiana inicial exerce forte impacto sobre a saúde do lactente e do indivíduo adulto. (EDWARDS, 2017; MOHAJERI et al., 2018; SJÖGREN et al., 2009).

Desta forma, considerando que a prole pode adaptar seu desenvolvimento em resposta a modificações no início da vida, e tendo em vista a ausência de estudos que relacionem os possíveis efeitos da superalimentação neonatal e/ou do consumo de kefir sobre a suscetibilidade ao câncer colorretal nos descendentes adultos, torna-se relevante avaliar as consequências dessa exposição precoce sobre o desenvolvimento deste tipo de neoplasia, bem como as alterações em biomarcadores

inflamatórios e na microbiota intestinal, na progênie adulta programada pela superalimentação neonatal.

2. JUSTIFICATIVA

Esse trabalho possibilitará definir os mecanismos da programação e a suscetibilidade ao desenvolvimento do câncer colorretal, abrindo novos caminhos nesta área, e direcionar o desenvolvimento de estudos de intervenção para prevenir doenças adquiridas na idade adulta como a obesidade e o câncer colorretal, que representam um importante problema de saúde pública em nosso país e no mundo. Da mesma forma, determinar os efeitos do consumo do kefir em fases críticas do desenvolvimento, e o seu impacto na prevenção de alterações inflamatórias e da microbiota intestinal, que possam ter impacto na suscetibilidade ao desenvolvimento da carcinogênese colorretal na idade adulta.

3. REVISÃO BIBLIOGRÁFICA

3.1 Câncer Colorretal

As doenças crônicas não transmissíveis (DCNT) representam as principais causas de morbimortalidade no mundo, com destaque para as doenças cardiovasculares e o câncer, representando 48% e 21% das DCNT, respectivamente (WHO, 2013).

No mundo, os tipos de câncer mais incidentes foram o de pulmão (1,8 milhão), mama (1,7 milhão), intestino (1,4 milhão) e próstata (1,1 milhão). Nos homens, os mais frequentes foram pulmão (16,7%), próstata (15,0%), intestino (10,0%), estômago (8,5%) e fígado (7,5%). Em mulheres, as maiores frequências foram encontradas na mama (25,2%), intestino (9,2%), pulmão (8,7%), colo do útero (7,9%) e estômago (4,8%) (FERLAY et al., 2013).

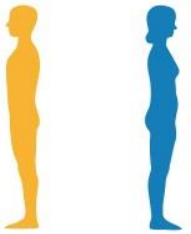
Estima-se, para o Brasil, no triênio de 2020-2022, a ocorrência de 625 mil casos novos de câncer, para cada ano. Com exceção do câncer de pele não melanoma, ocorrerão 450 mil casos novos de câncer (INCA, 2019). Considerando o cálculo global corrigido para o sub-registro, tem-se a ocorrência de 685 mil casos novos (MATHERS et al., 2003). Essas estimativas refletem o perfil de um país que possui os cânceres de próstata, pulmão, mama feminina e cólon e reto entre os mais incidentes, entretanto ainda apresenta altas taxas para os cânceres do colo do útero, estômago e esôfago (INCA, 2017).

A distribuição da incidência por região geográfica mostra que a Região Sudeste concentra mais de 60% da incidência, seguida pelas Regiões Nordeste (27,8%) e Sul (23,4%). Existe, entretanto, grande variação na magnitude e nos tipos de câncer entre as diferentes regiões do Brasil. Nas Regiões Sul e Sudeste, o padrão da incidência mostra que predominam os cânceres de próstata e de mama feminina, bem como os cânceres de pulmão e de intestino (INCA, 2019).

Considerando o câncer de cólon e reto, para o Brasil, estimam-se 20.520 casos novos em homens e 20.470 em mulheres para cada ano do triênio 2020-2022. Esses valores correspondem a um risco estimado de 19,63 casos novos a cada 100 mil homens e 19,03 para cada 100 mil mulheres. Representando a segunda neoplasia mais frequente em homens e mulheres (Figura 1) (INCA, 2019).

Distribuição proporcional dos dez tipos de câncer mais incidentes estimados para 2020 por sexo, exceto pele não melanoma*

Localização Primária	Casos	%
Próstata	65.840	29,2%
Cólon e reto	20.520	9,1%
Traqueia, brônquio e pulmão	17.760	7,9%
Estômago	13.360	5,9%
Cavidade oral	11.180	5,0%
Esôfago	8.690	3,9%
Bexiga	7.590	3,4%
Linfoma não Hodgkin	6.580	2,9%
Laringe	6.470	2,9%
Leucemias	5.920	2,6%



Localização Primária	Casos	%
Mama feminina	66.280	29,7%
Cólon e reto	20.470	9,2%
Colo do útero	16.590	7,4%
Traqueia,brônquio e pulmão	12.440	5,6%
Glândula tireoide	11.950	5,4%
Estômago	7.870	3,5%
Ovário	6.650	3,0%
Corpo do útero	6.540	2,9%
Linfoma não Hodgkin	5.450	2,4%
Sistema nervoso central	5.220	2,3%

*Números arredondados para múltiplos de 10.

Figura 1. Distribuição proporcional dos dez tipos de câncer mais incidentes estimados para 2020 (INCA, 2019).

O câncer de cólon e reto possui relevância epidemiológica mundial, uma vez que é a terceira neoplasia maligna mais comumente diagnosticada e a quarta principal causa de morte por câncer, representando 1,4 milhão de casos novos e quase 700 mil óbitos no ano de 2012. O padrão da incidência difere entre os sexos, com taxas de 20,6/100 mil para os homens e de 14,3/100 mil para as mulheres (FERLAY et al., 2013). Uma grande variação geográfica tem sido observada, com taxas elevadas nos países mais desenvolvidos comparados aos menos desenvolvidos (CENTER; JEMAL; WARD, 2009; FERLAY et al., 2013, 2015).

As taxas de incidência e de mortalidade por câncer colorretal apresentam grande variação no mundo segundo o Índice de Desenvolvimento Humano (IDH), sendo identificados três padrões de distribuição da doença: elevação de ambas as taxas nas mais recentes décadas em países que passaram por uma rápida transição econômica, entre eles o Brasil; aumento da incidência e diminuição da mortalidade em países com alto IDH, incluindo Canadá, Reino Unido, Singapura e Dinamarca; e diminuição de ambas as taxas nos países com IDH muito elevado, como Estados Unidos, Japão e França (ARNOLD et al., 2016).

O câncer colorretal é uma doença multifatorial influenciada por fatores genéticos, ambientais e relacionados ao estilo de vida. Os fatores hereditários, como o histórico familiar de câncer de cólon e reto e as doenças inflamatórias intestinais, representam apenas uma pequena proporção da variação observada na carga global da doença. Nesse sentido, as diferenças geográficas observadas na incidência possivelmente refletem a adoção de hábitos de vida ocidentais (ARNOLD et al., 2016).

É evidente a ocorrência de uma transição nutricional, em todo o mundo, que afeta principalmente os países em desenvolvimento. Assim, os fatores de risco ligados ao estilo de vida são modificáveis e incluem: o consumo de bebidas alcoólicas, a baixa ingestão de frutas e vegetais, o alto consumo de carnes vermelhas e de alimentos processados, a obesidade, o tabagismo e a inatividade física (BOUVARD et al., 2015; FEDIRKO et al., 2011; HARRISS et al., 2009; WALTER, 2014; WORLD CANCER RESEARCH FUNDATION, 2012).

As doenças inflamatórias intestinais, incluindo a Retocolite ulcerativa e a doença de Crohn, também estão associadas a um risco aumentado de desenvolvimento do câncer colorretal. Várias vias de sinalização imunológica associada à colite parecem ligadas ao câncer. Modelos de inflamação intestinal crônica foram determinados para apoiar a iniciação do tumor através de mutações induzidas por estresse oxidativo. Um microambiente pró-inflamatório que se desenvolve possivelmente como resultado da modificação da função de barreira intestinal e interações hospedeiro-microbiota, parecem contribuir para a promoção do tumor. Diversas vias moleculares, incluindo TNF/NF-kB ou IL-6/STAT3 (*signal transducer and activating factor of transcription 3*), foram identificadas como importantes contribuintes para o desenvolvimento do câncer colorretal associado a colite, sendo alvos terapêuticos promissores para a prevenção e tratamento dessa neoplasia (WALDNER; NEURATH, 2015).

A carcinogênese é um processo complexo, envolvendo uma série de mudanças genéticas e epigenéticas que ocorrem em níveis morfológicos, celulares e moleculares podendo ser dividida em três estágios principais: iniciação, promoção e progressão (PITOT, 2001, 2007; VICENT & GATENBY, 2008).

No caso da carcinogênese colorretal, a transformação neoplásica da mucosa colônica normal em um adenoma e, posteriormente em um adenocarcinoma, envolve uma série de alterações genéticas e eventos progressivos conhecidos como sequência adenoma-adenocarcinoma (FEARON & VOLGESTEIN, 1990; YANG et al., 2018). O desequilíbrio fisiológico e cíclico da renovação epitelial (proliferação e morte celular) resulta nas neoplasias no epitélio intestinal onde o aumento na proliferação celular é considerado o evento celular mais precoce da carcinogênese de cólon (CAMPLEJOHN et al., 2003; FEARON, 2011).

Conforme a teoria da sequência adenoma-carcinoma, a maior parte dos casos de câncer colorretal é de origem multifatorial, incluindo fatores intrínsecos (idade,

obesidade, polipose adenomatosa familiar, e doença inflamatória intestinal) e extrínsecos (fumo, álcool, e alto teor de gordura na dieta), grande parte se desenvolve a partir de pólipos de adenoma pré-formados. O potencial maligno do pólipo adenomatoso está associado ao seu tamanho, grau de displasia e gravidade de atipia. Alterações moleculares, genéticas e imunológicas parecem estar envolvidas nesta sequência (Figura 2) (CUI et al., 2017; BARKER et al., 2009).

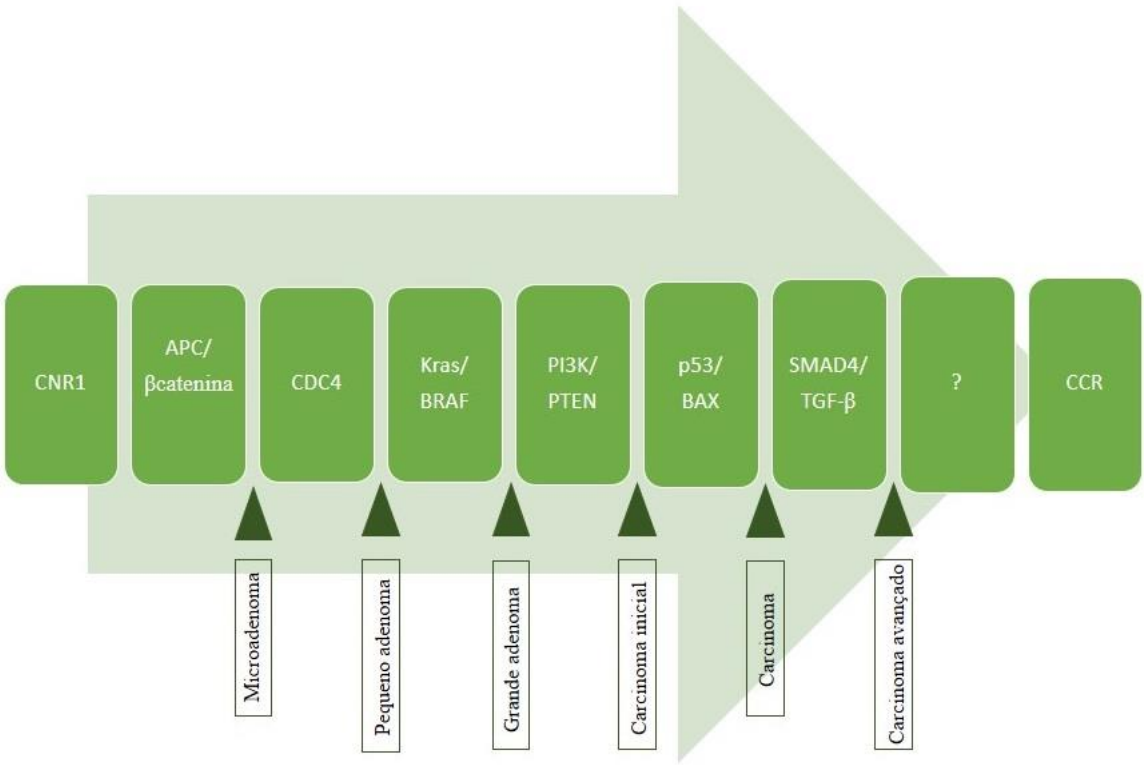


Figura 2. Diferentes estágios durante a progressão do câncer colorretal (Adaptado de Donovan et al., 2017).

Três vias moleculares do câncer colorretal foram identificadas, e incluem: instabilidade cromossômica, caracterizada por cariótipos anormais, aneuploidia e perda de heterozigose; instabilidade de microssatélites, com silenciamento de mecanismos de reparo do DNA; e fenótipo metilador das ilhotas CpG (CIMP), associado a hipermetilação e silenciamento de genes supressores de tumor (MÁRMOL et al., 2017; MUNDADE et al., 2014).

Do ponto de vista clínico, evolutivo e comportamental, as neoplasias são divididas em duas categorias: benignas e malignas. As neoplasias benignas em geral, têm suas células bem diferenciadas, as atipias celulares e arquiteturais são discretas,

possuem baixo índice mitótico, o crescimento tende a ser lento e expansivo e o tumor é bem delimitado. As neoplasias malignas têm células mais indiferenciadas, caracterizadas por expressiva atipias celulares, alto índice mitótico e geralmente provocam metástase (INCA, 2011).

Considerando a relevância da doença neoplásica, além da necessidade de entender a fisiopatologia do surgimento das lesões precoces, utilizam-se diversos modelos experimentais de carcinogênese colorretal (FEARON & VOGELSTEIN, 1990; BIRD, 1995). O modelo de Bird promove a carcinogênese por 1,2 dimetilhidrazina (DMH) ou azoximetano (AOM) e avalia a formação de criptas aberrantes em mucosa cólica de roedores, sendo amplamente utilizado em pesquisas experimentais. As lesões induzidas por estas drogas ocorrem de modo semelhante ao câncer colorretal em humanos (BIRD, 1987; RONCUCCI et al., 2000; BIRD, 2000).

3.2 Programação Metabólica

Desde o período de desenvolvimento intrauterino pode-se expor o feto ao risco de desenvolver doenças na idade adulta. Nesse aspecto, a hipótese denominada de Origens Desenvolvimentistas da Saúde e da Doença (DOHaD), destaca a relação entre os estímulos em fases iniciais da vida e o posterior desenvolvimento de doenças. Esse modelo investiga as adaptações que ocorrem no feto em resposta a sinais do ambiente intrauterino, que resultam em permanente ajuste de sistemas homeostáticos com a finalidade de ajudar na sobrevida imediata e na melhora do sucesso em um ambiente pós-natal adverso. No entanto, interpretações inadequadas ou mudanças ambientais podem levar a uma incompatibilidade entre as previsões pré-natais e a realidade pós-natal (GLUCKMAN et al., 2008; LAKER et al., 2013, CHANGO & POGRIBNY, 2015).

Logo, essas adaptações conhecidas como respostas adaptativas preditivas, podem ser desvantajosas na vida adulta, conduzindo para um aumento do risco de doenças que podem ser transmitidas as próximas gerações. Nesta perspectiva, tem-se estabelecido que alterações nutricionais e endócrino-metabólicas na mãe e no neonato em fases de desenvolvimento, podem levar a alterações em tecidos e órgãos, que se estendem ao longo da vida; podendo ainda, haver um período de latência e as manifestações ocorrerem somente da vida adulta, originando doenças. Esta relação

tem sido denominada de “programação” (BARKER, 1993; FERNANDEZ-TWINN & OZANNE, 2010; PATEL; SRINIVASAN, 2011; SUTTON et al., 2016).

A capacidade de um genótipo produzir diferentes fenótipos em resposta a ambientes distintos, denominada plasticidade, parece apresentar atividade máxima durante o desenvolvimento. A plasticidade na programação evoluiu para fornecer as melhores chances de sobrevivência e sucesso reprodutivo. Desta forma, as condições ambientais no início da vida podem influenciar profundamente aspectos biológicos humanos, e a saúde em longo prazo. A nutrição e o estresse são algumas das condições que influenciam o risco para o desenvolvimento de doenças metabólicas, diabetes mellitus tipo 2 e doenças cardiovasculares na vida adulta (HOCHBERG et al., 2011).

Sinais de disponibilidade energética podem modular essa plasticidade, tanto de forma intrínseca (interno), como extrínseca (ambiental). Entre os sinais intrínsecos tem-se, a leptina, o eixo hipotálamo-hipófise-adrenal, grelina, hormônios tireoidianos, insulina e cortisol. Enquanto fazem parte dos sinais ambientais, a nutrição pré e pós-natal, estressores e desreguladores endócrinos (HOCHBERG et al., 2011; KAMITAKAHARA et al., 2018).

Propõem-se que mecanismos epigenéticos estão envolvidos na plasticidade fenotípica e na programação adaptativa. A epigenética fornece um mecanismo molecular para programação, ligando genes, ambiente pré-natal, intrauterino, crescimento e suscetibilidade à doenças. A reprogramação representa um exemplo do estado dinâmico epigenético. Essa flexibilidade contrasta com a repressão em longo prazo que é provocada pela metilação do DNA e associada a modificações de histonas, sendo observado em genes cruciais para a pluripotência durante a diferenciação (FEINBERG, 2007; STOVER et al., 2018).

Neste sentido, os nutrientes e intermediários metabólicos relacionados podem atuar como moléculas sinalizadoras que alteram as funções do genoma, permitindo adaptações celulares ao meio ambiente. Os nutrientes e seus metabólitos regulam a expressão gênica através de diversos mecanismos, incluindo a atuação como ligantes para fatores de transcrição de receptores nucleares e influenciando a atividade de microRNA e outros pequenos RNA que regulam a função gênica (NOLTE-‘T HOEN et al., 2015; RABHI et al., 2017).

É importante ressaltar que os nutrientes e metabólitos relacionados podem modificar diretamente elementos da cromatina, incluindo a sequência primária de DNA e as proteínas histonas em locais distintos que determinam a estrutura da cromatina, levando a alterações nos níveis de expressão gênica e estabilidade do genoma. A variação genética humana interindividual pode influenciar a arquitetura epigenética e a capacidade de resposta às mudanças na exposição de nutrientes e atividade metabólica, estando estes mecanismos envolvidos em vários desfechos de saúde, como crescimento, desenvolvimento, risco para DCNT, como o câncer e expectativa de vida (NOLTE-'T HOEN et al., 2015; RABHI et al., 2017; STOVER et al., 2018; TEH et al., 2014).

Em consonância, estudo inicial desenvolvido por Kennedy (1953), onde se alterou o plano de nutrição durante o período de amamentação pela manipulação do tamanho da ninhada, observou que ratos criados em ninhadas pequenas, com pouca concorrência para o leite materno, ganharam mais peso durante a lactação e permaneceram mais gordos e mais pesados ao longo da vida, mesmo quando alimentados com uma dieta padrão. Porém, os ratos criados em ninhadas grandes recebem menos leite e, consequentemente, ganharam menos peso. Estes animais permanecem com menor peso ao longo da vida. Com base nestes resultados foi sugerido que o apetite era determinado durante o período de amamentação e que o hipotálamo tinha um papel importante na mediação desses efeitos. Esses achados foram apoiados por pesquisas posteriores (BOURET; LEVIN; OZANNE, 2015; PATEL; SRINIVASAN, 2011; WIDDOWSON; MCCANCE, 1963).

Um número crescente de estudos destaca a relevância da nutrição materna, da concepção a lactação, na programação de sistemas e vias homeostáticas da prole (Figura 3). Neste contexto, o sistema imunológico em desenvolvimento pode ser particularmente vulnerável. De fato, exemplos de programação imunológica mediada por nutrição podem ser encontrados na literatura, atuando sobre retardo do crescimento intra-uterino, deficiências de micronutrientes maternos e alimentação infantil. Um mecanismo de programação envolvido é a ativação do eixo hipotálamo-hipófise-adrenal materno em resposta ao estresse nutricional. A exposição fetal ou neonatal a hormônios do estresse elevados está ligada a alterações nas interações neuroendócrino-imunes, com manifestações diversas, como resposta inflamatória atenuada ou resistência reduzida à colonização tumoral (LEE, 2015; SOMINSKY et al., 2018).

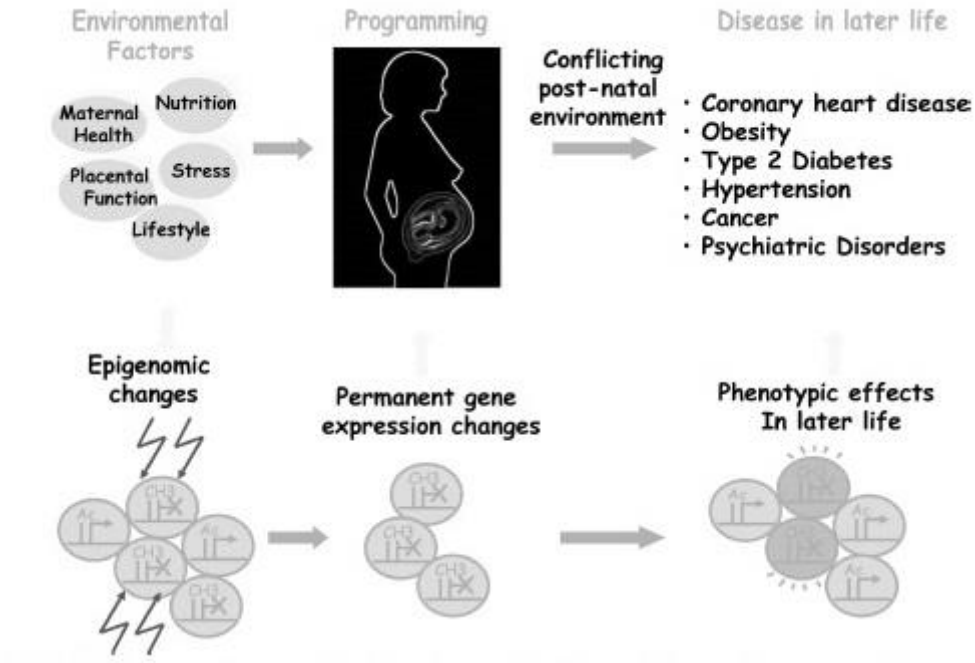


Figure 3. Complexa rede que modula a programação metabólica e o desenvolvimento de doenças (Hochberg et al., 2011).

Modificações epigenéticas induzidas por alterações na nutrição materna podem modificar células T reguladoras em desenvolvimento, e subsequente risco para alergias ou asma; afetar mecanismos de transferência placentária e/ou via leite materno, influenciando na quantidade e qualidade dos fatores transferidos. As implicações para a saúde pública da programação mediada pela nutrição são de particular importância no mundo em desenvolvimento, onde as DCNT e as doenças imunomediadas apresentam grande impacto na morbimortalidade da população (CHADIO et al., 2016; PALMER, 2011).

Os mecanismos envolvidos na programação ainda não foram totalmente elucidados, mas acredita-se que haja uma relação com alterações no desenvolvimento estrutural dos órgãos, ou alteração persistente ao nível celular, sendo postulado de acordo com Koletzko et al. (2011):

- Participação da memória epigenética, com modificação no processo de transcrição;
- Alteração da estrutura dos órgãos na vascularização, inervação e justaposição, como por exemplo, a posição dos hepatócitos, células

endoteliais e células de Kuppfer, que durante a organogênese podem modificar o metabolismo de forma permanente;

- Ocorrência de hiperplasia ou hipertrofia, levando a alterações no número e tamanho de células;
- Crescimento anormal das células de proliferação rápida em condições metabólicas específicas (Seleção Clonal);
- Processo de diferenciação metabólica.

Nota-se que os mecanismos moleculares propostos, incluem as alterações agudas ou crônicas na expressão gênica, através de diversas vias epigenéticas, onde existe uma inter-relação entre determinados genes, exposição a fatores ambientais e eventos biológicos posteriores (HANLEY et al., 2010). Dado que a regulação epigenética durante o desenvolvimento sofre alterações dinâmicas, o epigenoma apresenta uma natureza instável, o que lhe permite responder e adaptar-se às pressões do ambiente, incluindo as modificações nutricionais (VICKERS, 2014).

Ainda assim, há muito que se compreender, embora a epigenética ajude a entender como a exposição aos fatores ambientais, em períodos críticos de desenvolvimento levam a alterações na vida adulta. É necessário desvendar as modificações pós-epigenéticas envolvidas nos diferentes processos que levam ao surgimento das doenças (KOLETZKO et al., 2011).

3.3 Superalimentação

Em modelos experimentais com animais, a modificação no tamanho da ninhada pode ter efeitos em longo prazo na homeostase metabólica, com ninhadas reduzidas promovendo a superalimentação. Sugere-se que os efeitos sejam devidos a mudanças na ingestão alimentar durante a amamentação e/ou maternal (ARGENTE-ARIZÓN et al., 2016; STEFANIDIS; SPENCER, 2012).

Trabalhos que utilizaram ninhadas reduzidas (3 a 4 filhotes/mãe lactante) demonstraram que na idade adulta, a prole apresentou massa corporal aumentada, aumento de adiposidade central e total, hiperfagia, hipertensão arterial, resistência à insulina, hiperleptinemia, aumento do estresse oxidativo e alterações em estruturas

hipotalâmicas de controle alimentar (ARGENTE-ARIZÓN et al., 2018; BEI et al., 2015; CONCEIÇÃO et al., 2013; RODRIGUES et al., 2009; RODRIGUES et al., 2011; VELKOSKA et al., 2005).

A hipótese de superalimentação na lactação é sustentada pelo fato de que o animal neonato parece não ter controle da ingestão até o 14º-16º dia de vida pós-natal. Assim, quando há grande oferta de leite, os filhotes ingerem o volume máximo da capacidade gástrica. Esta abundante ingestão pode levar à hiperalimentação, visto que o controle hipotalâmico no início da vida pós-natal ainda não está totalmente estruturado. Portanto, a indução do excesso de alimentação perinatal tem sido relacionada à instalação de excesso de peso e hiperfagia na vida adulta (MCMILLEN; ADAM; MÜHLHÄUSLER, 2005; SEKAR; WANG; CHOW, 2017).

Esse modelo tem sido utilizado para determinar o papel da nutrição neonatal na capacidade inflamatória do tecido adiposo e na disfunção metabólica. O tecido adiposo branco, em particular, contribui para este estado de inflamação metabólica ou "*meta-inflammation*", e sofre modificações consideráveis na composição de leucócitos e produção de citocinas e adipocinas na obesidade. Macrófagos do tecido adiposo são contribuintes centrais para a ‘’*meta-inflammation*’’, onde a obesidade leva ao influxo de macrófagos tipo 1 pró-inflamatórios (M1) que superam a proporção decrescente de macrófagos residentes e anti-inflamatórios do tipo 2 (M2). Os macrófagos M1 recrutados secretam uma série de citocinas e quimiocinas que perpetuam a inflamação e prejudicam a função dos adipócitos (AOUADI et al., 2013; GREGOR; HOTAMISLIGIL, 2011; KAYSER; GORAN; BOURET, 2015).

Diante o exposto, a atual epidemia de obesidade no mundo pode estar não só associada ao padrão de consumo alimentar ocidental, mas ao fato de que as novas gerações estão sendo expostas durante as fases de desenvolvimento, como gestação, lactação e adolescência, a fatores que podem programar para sobrepeso e obesidade na vida adulta, mesmo com a ingestão de uma dieta adequada após esses períodos críticos (ARGENTE-ARIZÓN et al., 2016; BARKER, 2007; COLLDEN et al., 2015; LONG et al., 2015; SPENCER, 2012).

3.4 Kefir

O kefir é um leite fermentado, de fácil preparo e economicamente acessível, originado da ação da microbiota natural presente em seus grãos ou grumos

(MARCHIORI, 2007). Os grãos são descritos como uma associação simbiótica de leveduras, bactérias ácido-láticas e bactérias ácido-acéticas, envolvidas por uma matriz de polissacarídeos denominados kefiram. A composição microbiana dos grãos de kefir apresenta variação dependente da região de origem, tempo de utilização, substrato de proliferação dos grãos e as técnicas utilizadas em sua manipulação (LEITE et al., 2015; SATIR; GUZEL-SEYDIM, 2016; VIEIRA et al., 2015; WESCHENFELDER et al., 2011).

A composição microbiológica do kefir o caracteriza como um alimento complexo, com um grande número de microrganismos simbióticos, dos quais várias bactérias têm sido identificadas como probióticas (Figura 4) (FARNWORTH et al., 2005).

Lactobacilli

Lactobacillus kefir [a,c,j,n,o,p,r]
Lactobacillus kefiranofaciens [l,m,p]
Lactobacillus kefirgranum [n]
Lactobacillus parakefir [n,o]
Lactobacillus brevis [g,h,p,r]
Lactobacillus plantarum [o,p]
Lactobacillus helveticus [a,b,h]
Lactobacillus acidophilus [g,p,r]
Lactobacillus delbrueckii [a,h,p]
Lactobacillus rhamnosus [a,r]
Lactobacillus casei [h]
Lactobacilli paracasei [p]
Lactobacillus fructivorans [k]
Lactobacillus hilgardii [k]
Lactobacillus fermentum [r]
Lactobacillus viridescens [r]

Lactococci

Lactococcus lactis subsp. lactis [a,c,e,f,g,h,k,o,r]
Lactococcus lactis subsp. cremoris [a,e,f]

Streptococci

Streptococcus thermophilus [e,h]

Enterococci

Enterococcus durans [d*,e*]
(reported as *Streptobacterium durans* in ref. d; reported as *Streptococcus durans* in ref. e)

Leuconostocs

Leuconostoc sp.[r]
Leuconostoc mesenteroides [a,h,g*,o]
(reported as *Leuconostoc kefir* in ref. g)

Acetic acid bacteria

Acetobacter sp.[o]
Acetobacter pasteurianus [g*]
(reported as *Acetobacter rancens* in ref. g)
Acetobacter aceti [a,d]

Other bacteria

Bacillus sp.[r]
Bacillus subtilis [g]
Micrococcus sp.[r]
Escherichia coli [r]

Kluyveromyces marxianus [a,b,f*,g,h,i,j,k,m*,n]
(reported as *Saccharomyces lactis* in ref. f; reported as *Kluyveromyces lactis* in ref. m)
Saccharomyces sp.[k]
Saccharomyces cerevisiae [a,d,e,f*,g,j,m,n]
(reported as *Saccharomyces carlbergensis* in ref. f)
Saccharomyces unisporus [c,h,j,m]
Saccharomyces exiguus [l*]
(reported as *Torolopsis holmii* in ref. l)
Saccharomyces turicensis [h]
Saccharomyces delbrueckii [d]
Saccharomyces dairensis [n]
Torulaspora delbrueckii [a,h,m]
Brettanomyces anomalus [h]
Issatchenkia occidentalis [j]
Candida friedrichii [n]
Candida pseudotropicalis [f]
Candida tenuis [f]
Candida inconspicua [g]
Candida maris [g]
Candida lambica [j]
Candida tannotelerans [e]
Candida valida 6 [e]
Candida kefyr [a,j,n]
Candida holmii [j,m]
Pichia fermentans [b,m,n]

Figura 4. Bactérias e leveduras encontradas no kefir e em seus grãos (Farnworth, 2005).

Os grãos são adicionados ao leite em recipiente de vidro, esterilizado, o qual fermenta em temperatura ambiente (± 25 °C) por aproximadamente 24 horas. Após a

fermentação, os grãos são coados, e o líquido resultante é o kefir, que pode ser consumido fresco ou maturado. A maturação consiste em fermentação secundária por 24 horas ou mais a temperatura de aproximadamente 10°C, para promover o crescimento de leveduras e conferir sabor e aroma específicos a bebida. Os grãos podem ser adicionados novamente ao leite, e o processo repetido (RATTRAY; O'CONNEL, 2011).

Várias propriedades probióticas do kefir já foram relatadas na literatura, bem como seus efeitos como agente antimutagênico, anticarcinogênico, hipocolesterolêmico e anti-inflamatório. Também são descritos efeitos sobre o perfil lipídico, controle glicêmico e da pressão arterial (DE LIMA et al., 2017; KLIPPEL et al., 2016; OSTADRAHIMI et al., 2015; PRADO et al., 2016; RATTRAY & O'CONNEL, 2011; SHARIFI et al., 2017; TUNG et al., 2018; YAMANE et al., 2018). Os metabólitos presentes na fração não microbiana do kefir, produzidos durante a fermentação, também apresentam relevância na proteção da mucosa intestinal contra patógenos (HAMET et al., 2016; IRAPORDA et al., 2017; VINDEROLA et al., 2006).

Segundo a Agência Nacional de Vigilância Sanitária (ANVISA) probióticos são definidos como microorganismos vivos capazes de melhorar o equilíbrio microbiano intestinal, produzindo efeitos benéficos à saúde do indivíduo. A quantidade mínima viável de probióticos deve estar situada na faixa de 10^8 a 10^9 Unidades Formadoras de Colônias (UFC), na recomendação diária do produto pronto para o consumo. Valores menores podem ser aceitos, desde que sua eficácia seja comprovada pelo fabricante (ANVISA, 2002).

Entre os possíveis mecanismos de ação atribuidos aos probióticos, tem-se (Figura 5) (CIORBA, 2012; MALEKI et al., 2016; VARANKOVICH; NICKERSON; KORBER, 2015):

- Competição por nutrientes e por sítios de adesão, denominada exclusão competitiva.
- Alteração do metabolismo microbiano, por meio do aumento ou da diminuição da atividade enzimática.
- Estímulo da imunidade do hospedeiro, por intermédio do aumento dos níveis de anticorpos e da atividade dos macrófagos.

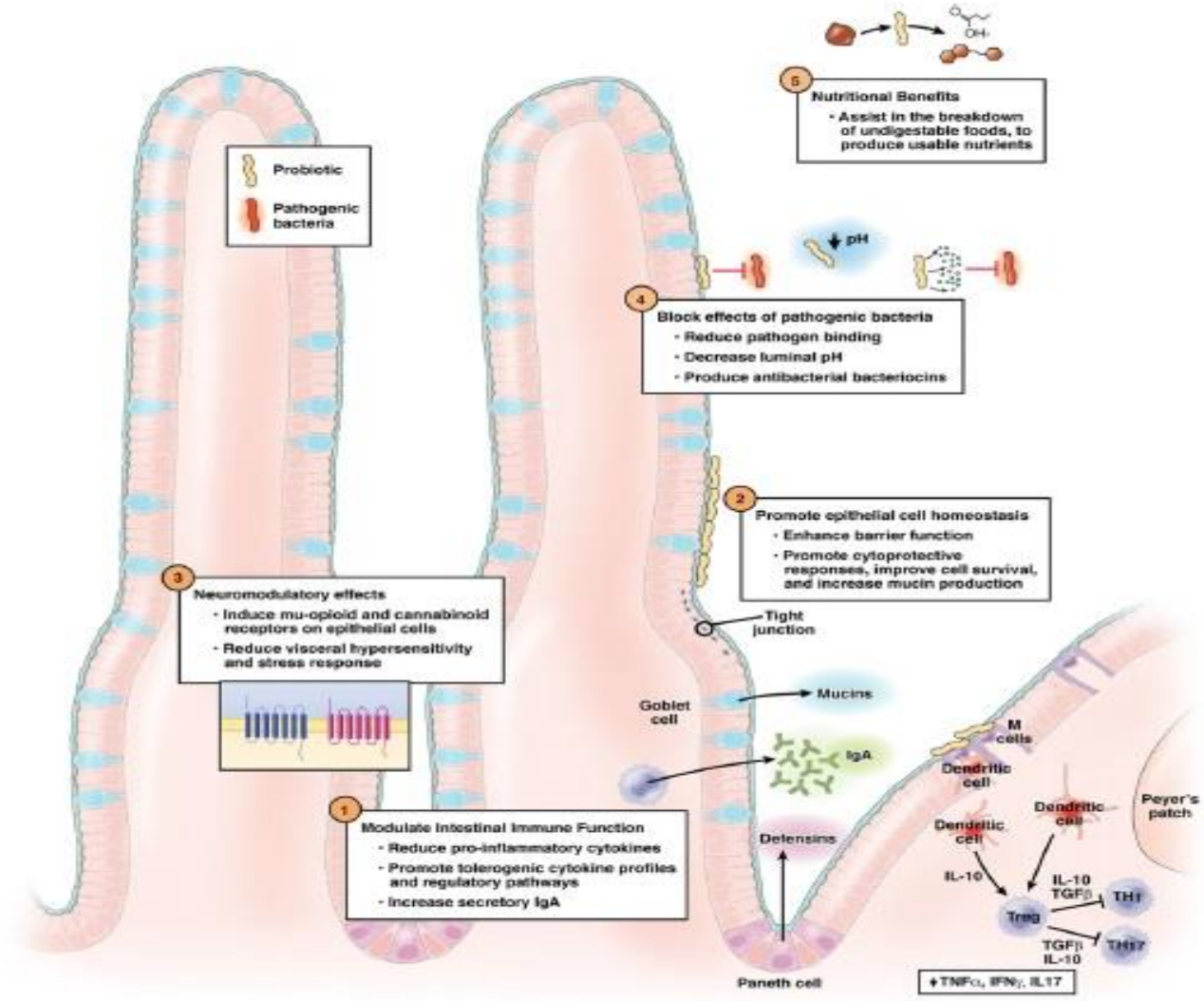


Figura 5. Mecanismos de ação dos probióticos no trato gastrointestinal. ‘’A Gastroenterologist’s Guide to Probiotics’’ (Ciorba, 2012).

Segundo a legislação brasileira vigente (BRASIL, 2007), tem-se como definição para o kefir como o “produto resultante da fermentação do leite pasteurizado ou esterilizado, por cultivos ácido lácticos elaborados com grãos de kefir, *Lactobacillus kefir*, espécies dos gêneros *Leuconostoc*, *Lactococcus* e *Acetobacter* com produção de ácido láctico, etanol e dióxido de carbono. Os grãos de kefir são ainda constituídos por leveduras fermentadoras de lactose (*K. marxianus*) e leveduras não fermentadoras de lactose (*S. onisporus*, *S. cerevisiae* e *S. exiguus*), *Lactobacillus casei*, *Bifidobaterium spp*. e *Streptococcus salivarius ssp. thermophilus*”.

Os microrganismos mais comumente isolados de grãos de kefir compreendem os gêneros *Lactobacillus* (*L. brevis, L. casei, L. kefiri, L. acidophilus, L. plantarum, L. kefiranofaciens subsp. kefiranofaciens, L. kefiranofaciens subsp. kefirgranum, L.*

parakefir), *Lactococcus* (*L. lactis subsp. lactis*), *Leuconostoc* (*L. mesenteroides*), *Acetobacter, Kluyveromyces* (*K. marxianus*) e *Saccharomyces* (CHEN et al., 2008; DERTLI; ÇON, 2017; NALBANTOGLU et al., 2014).

Os probióticos também têm sido associados a prevenção de câncer através de mecanismos como o estímulo do sistema imunológico, diminuindo a incidência de infecções, regulando a inflamação intestinal e ligando-se a compostos tóxicos (MALEKI et al., 2016).

No caso das leveduras, a capacidade de aglutinar patógenos, resistir ao pH ácido e aos sais biliares do trato gastrointestinal estão entre os mais importantes critérios para sua pré- seleção como probióticos (GARCÍA-HERNÁNDEZ et al., 2012).

O consumo do kefir é estimulado por sua longa história de efeitos benéficos à saúde, o alimento ocupa um importante lugar na dieta humana, principalmente no Sudoeste da Ásia, Europa, America do Norte, Japão, Oriente Médio, Norte da África e Rússia (SARKAR, 2008). No Brasil, ainda é pouco conhecido, sendo elaborado à nível doméstico (FARNWORTH, 2005; FARNWORTH; MAINVILLE, 2008; MIGUEL et al., 2011).

3.5 Microbiota Intestinal

Existem pelo menos 100 trilhões de microrganismos vivendo no trato gastrointestinal humano, incluindo bactérias, vírus, fungos e protozoários, que constituem a microbiota. A microbiota intestinal humana é um ecossistema complexo, com uma biomassa de aproximadamente 1,5 kg. Ademais, as composições de microrganismos são variadas em diferentes partes do intestino, incluindo cólon ascendente, cólon distal, íleo proximal e jejuno, e eles são críticos para o funcionamento adequado, homeostase e saúde, incluindo a digestão dos alimentos, biossíntese de vitaminas, respostas comportamentais e proteção contra patógenos (SEARS; GARRETT, 2014). A maioria das bactérias endógenas em adultos saudáveis são representadas pelos dois filos, *Firmicutes* e *Bacteroidetes*, que representam aproximadamente 90% da microbiota. A microbiota pode trabalhar com o hospedeiro para promover saúde, mas pode também iniciar ou promover a doença (ZOU; FANG; LEE, 2018).

As novas tecnologias que permitem analisar em grande escala o perfil genético e metabólico da comunidade microbiana do intestino, tem permitido uma melhor

compreensão da composição e funções da microbiota intestinal humana (MARCHESI et al., 2016).

Evidências emergentes mostram que a disbiose intestinal pode levar à alteração da fisiologia do hospedeiro, resultando nos processos patogênicos de diferentes doenças. A microbiota intestinal pode promover o desenvolvimento e a progressão do câncer colorretal por diferentes processos, incluindo a indução de um estado inflamatório crônico, alterando a resposta imune e a dinâmica celular, a biossíntese de metabolitos tóxicos e genotóxicos, afetando o metabolismo do hospedeiro (Figura 6) (TSILIMIGRAS; FODOR; JOBIN, 2017; YU; FANG, 2015; ZOU; FANG; LEE, 2018).

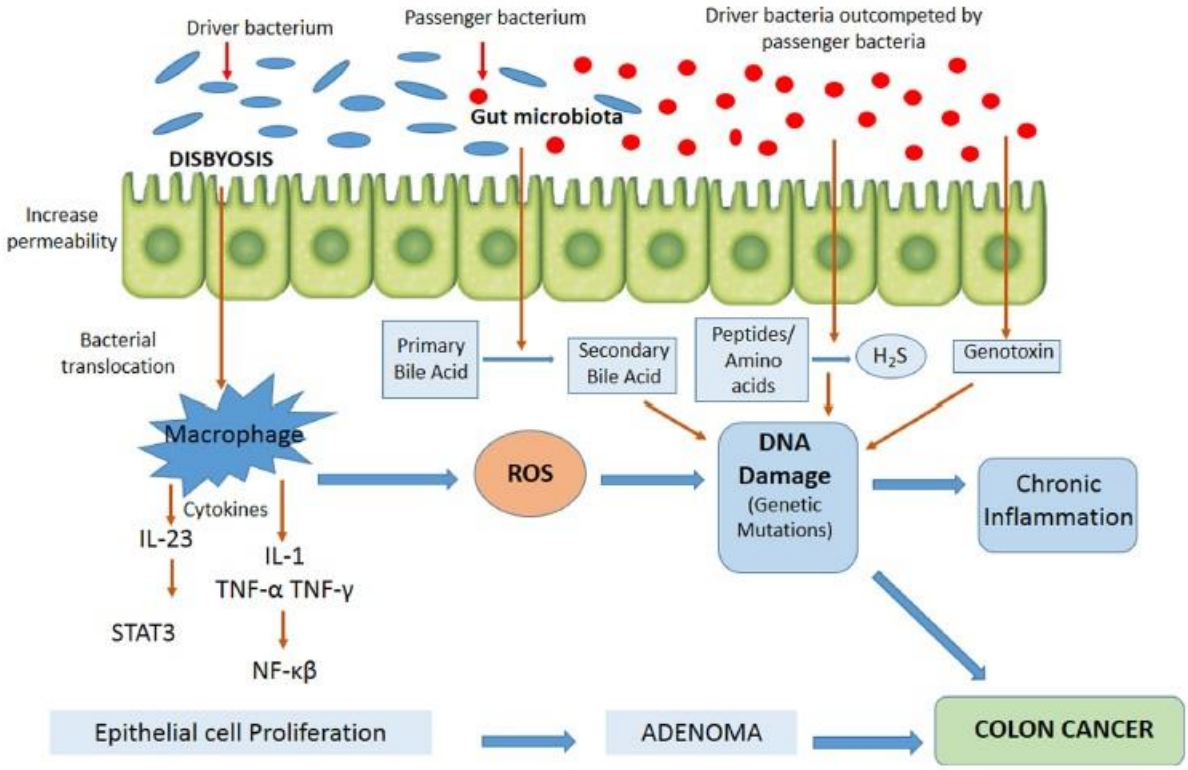


Figura 6. Possíveis mecanismos microbianos envolvidos na promoção do câncer colorretal (Nistal et al., 2015).

Sugere-se que a dinâmica e função da microbiota pode ser influenciada por muitos fatores, incluindo genética, dieta, idade e agentes toxicológicos como fumaça de cigarro, contaminantes ambientais e drogas. A ruptura deste equilíbrio, chamada disbiose, está associada com uma infinidade de doenças, incluindo doenças metabólicas, doença inflamatória intestinal, doença pulmonar obstrutiva crônica,

periodontite, doenças de pele e distúrbios neurológicos. A importância da microbiota para a saúde humana também levou ao surgimento de novas abordagens terapêuticas a manipulação intencional da microbiota, seja restaurando funções ausentes ou eliminando agentes nocivos (SCOTTI et al., 2017).

Em trabalho que analisou a composição da microbiota intestinal em modelo animal de câncer de colón induzido por DMH, ao realizar o sequenciamento da região V3 do gene 16S rRNA, foi evidenciada diferenças significativas na composição microbiana do lúmen intestinal entre os grupos controle e tumoral. Com maior abundância de *Firmicutes*, e redução de *Bacteroidetes* e *Spirochetes* em ratos induzidos ao tumor (ZHU et al., 2014).

É conhecido que em condições estáveis a microbiota modula o desenvolvimento e a função de diversas células imunes, assim como a síntese de interleucinas (IL). Desta forma, alterações na microbiota e no sistema imune do hospedeiro, gerado por exemplo, por fatores nutricionais, podem levar a inflamação intestinal e ao câncer. Estas alterações também contribuem para a geração de inflamação subclínica evidenciada na obesidade, uma vez que o LPS age em receptores do tipo Toll-Like 4 (TLR4), ativando a via do NF-kappa B e a transcrição subsequente de citocinas inflamatórias, tais como o fator de necrose tumoral (TNF-α), interleucina 6 (IL-6) e interleucina 1 (IL-1) (CANI et al., 2007; SANZ & MOYA-PEREZ, 2014). Essas alterações também parecem estar implicadas na carcinogênese colorretal (JOSHI et al., 2015).

Estudo conduzido em humanos por Ortiz-Andrellucchi et al. (2008), o consumo materno de *Lactobacillus casei* durante o período pós-parto foi capaz de modular a resposta imune materna, com redução das concentrações de TNF-α no leite, e diminuição da incidência de episódios gastrointestinais no bebê.

O desenvolvimento da microbiota intestinal perinatal é influenciado por múltiplos fatores, incluindo idade gestacional, tipo de parto, microbiota materna, método de alimentação infantil, genética e fatores ambientais, como a dieta de seguimento. A diversidade microbiana aumenta rapidamente durante os primeiros meses da infância. Ao nascer, a microbiota é aeróbica, com baixo número e baixa diversidade. Dentro de alguns dias, o ambiente intestinal torna-se anaeróbico resultando em crescimento de bactérias como *Bifidobacterium*, que é o gênero dominante no intestino do lactente nos primeiros meses de vida (BEZIRTZOGLOU, 1997; EDWARDS, 2017; MOHAJERI et al., 2018).

Fatores que promovem a microbiota saudável em neonatos incluem parto vaginal, parto a termo, aleitamento materno, e exposição a uma variedade de microrganismos. Em contraste, cesariana, parto prematuro, fórmula infantil e a exposição a antibióticos tem um impacto negativo na diversidade e composição da microbiota em lactentes. Prematuros demonstram colonização tardia da microbiota intestinal com *Bifidobacterium*, e têm alta prevalência de *Enterobacteriaceae*, *Staphylococcus* e *Enterococcaceae* (COLLADO et al., 2014; RODRIGUEZ et al., 2015).

Desta forma, a colonização intestinal do recém-nascido é essencial para a maturação, estabelecimento e manutenção da barreira da mucosa intestinal. Existem evidencias que a colonização microbiana inicial exerce forte impacto sobre a saúde do lactente e do indivíduo adulto. Em condições normais, a microbiota materna é a principal fonte para colonização do trato gastrointestinal do recém-nascido, e posteriormente o consumo alimentar contribuirá na sua instalação. Desta forma, o leite materno apresenta-se com grande relevância neste processo (KALLIOMAKI et al., 2001; GOHIR et al., 2015; PENDERS et al., 2006; SJÖGREN et al., 2009).

4. OBJETIVOS

4.1. Objetivo geral

Avaliar os efeitos do consumo materno de kefir durante a lactação e sua continuidade até a puberdade sobre a microbiota intestinal, parâmetros inflamatórios e a suscetibilidade à carcinogênese colorretal induzida na progênie adulta de ratos Wistar, programados pela superalimentação no período neonatal.

4.2. Objetivos específicos

- Realizar uma revisão sistemática da literatura sobre o papel dos probióticos em modelos murinos de carcinogênese colorretal.

- Avaliar os efeitos do consumo materno de kefir na lactação e sua continuidade até a puberdade sobre o estado nutricional, marcadores inflamatórios, microbiota intestinal, tumores e características histopatológicas do cólon da prole adulta.

5. METODOLOGIA

5.1 – Modelo experimental

Todos os procedimentos seguiram os preceitos éticos para o uso e cuidado de animais experimentais. O projeto foi aprovado pela Comissão de Ética para o Cuidado e Uso de Animais Experimentais (CEUA) da Pró-reitoria de Pesquisa da Universidade Federal de Juiz de Fora (UFJF) (nº21/2016).

Ratas *Wistar* (*Rattus norvergicus, albinus*) (3 meses), nulíparas, mantidas em biotério com temperatura (22±2°C), umidade (55±10%) e ciclo claro-escuro (07-19h) controlados foram acasaladas na proporção de 3 fêmeas para 1 macho e tiveram livre acesso a ração comercial e água filtrada (VIEIRA et al., 2018). Ao nascimento, as ratas lactantes com suas respectivas proles, foram divididas randomicamente em quatro grupos experimentais (RODRIGUES et al., 2009):

1) Grupo Controle (C): cuja ninhada foi ajustada para 10 filhotes, e a rata lactante recebeu ração comercial + administração de água (1mL/dia) por gavagem durante a lactação (n= 6 ninhadas; 60 filhotes machos).

2) Grupo Controle Kefir (CK): cuja ninhada foi ajustada para 10 filhotes, e a rata lactante recebeu ração comercial + administração de kefir (1mL/dia – 10^8 UFC/ dia) por gavagem durante a lactação (n= 6 ninhadas; 60 filhotes machos).

3) Grupo Superalimentado (S): cuja ninhada foi ajustada para 3 filhotes, e a rata lactante recebeu ração comercial + administração de água (1mL/dia) por gavagem durante a lactação (n= 21 ninhadas; 63 filhotes machos).

4) Grupo Superalimentado Kefir (SK): cuja ninhada foi ajustada para 3 filhotes, e a rata lactante recebeu ração comercial + administração de kefir (1mL/dia – 10^8 UFC/ dia) por gavagem durante a lactação (n= 21 ninhadas; 63 filhotes machos).

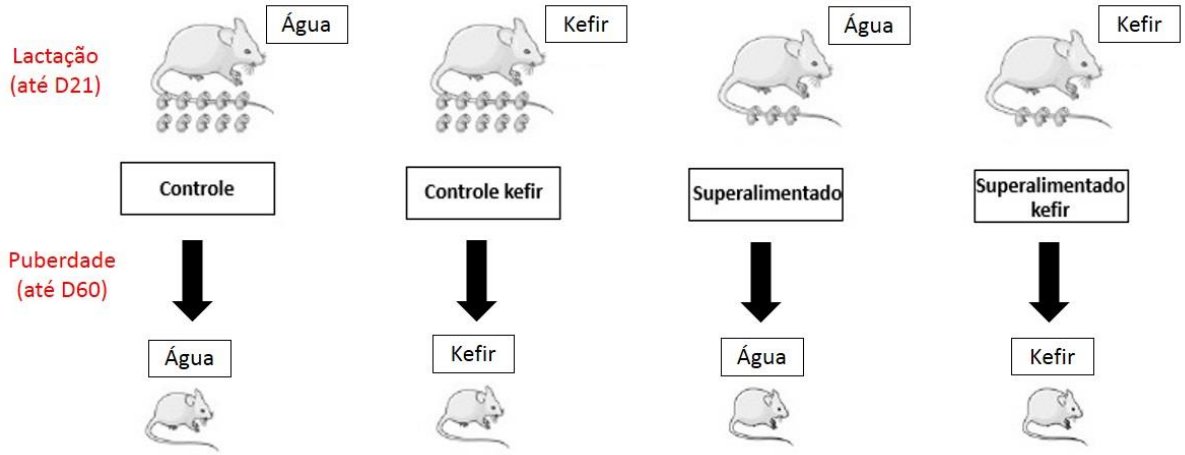


Figura 7. Modelo experimental.

A via de administração e a quantidade a ser utilizada do kefir de leite, foram estabelecidas considerando estudos que avaliaram que esta dosagem é considerada segura e apresenta efeitos desejáveis (ROSA et al., 2017). Ressalta-se que foi utilizado o número de UFC considerada pela legislação vigente (ANVISA, 2002) como tendo ação probiótica.

Durante a lactação, as ratas receberam ração comercial (Nuvilab®, Paraná, Brasil) e água *ad libitum*. A eutanásia das ratas lactantes ocorreu ao final da lactação (21 dias). Nesse período, as proles dos grupos C, CK, S e SK seguiram recebendo por gavagem o mesmo protocolo de tratamento de suas respectivas mães, ao desmame até 60 dias de idade. Após este período, foram submetidos à indução da carcinogênese colorretal, conforme descrito abaixo (item 5.2). Para a avaliação da evolução da tumorigênese, os animais foram eutanasiados após 24 semanas da última aplicação da DMH (240 dias de idade).

Para eutanásia, os animais foram mantidos em jejum por 8 horas e anestesiados com uma combinação de Xilazina (10 mg/Kg de peso corporal) e Cetamina (90 mg/Kg de peso corporal). Foram excisados os tecidos, intestino delgado e cólon, ceco, tecido adiposo (epididimal e retroperitoneal), e fígado.

5.2- Indução da carcinogênese colorretal

Foi utilizada a 1,2 dimethilhidrazina (DMH, Sigma Chemical CO, Mo, EUA) para indução da carcinogênese do cólon preparada imediatamente antes do uso, dissolvendo em solução de NaCl 0,9% com 1,5% de EDTA e 10 mM de citrato de sódio trifosfato, e pH final ajustado para 8 (LARANJEIRA et al., 1998). Os animais induzidos receberam quatro injeções de DMH, na dosagem de 40mg/kg de peso corporal, via intraperitoneal (i.p.) num intervalo de tempo de duas semanas com dias alternados (RODRIGUES et al., 2002). Desta forma, a DMH foi aplicada nos dias 46, 48, 52 e 54 após o desmame (67, 69, 73 e 75 dias de idade) (MOHANIA et al., 2014).

O manuseio e aplicação da DMH foram realizados com equipamentos de proteção individual (EPI) e os resíduos do carcinógeno descartados conforme recomendação para resíduos tóxicos.

5.3 – Obtenção e preparo do kefir de leite

O método de produção da bebida kefir ocorre pela adição direta dos grãos ao substrato de preferência. No presente estudo, foi empregado o leite pasteurizado integral como substrato e utilizados grãos de kefir, oriundos de manipulação familiar existentes no Laboratório de Bioquímica Nutricional do Departamento de Nutrição e Saúde (DNS) da Universidade Federal de Viçosa (UFV), Minas Gerais, Brasil. Para o cultivo foi seguido rigorosamente o protocolo experimental garantindo a qualidade do kefir a ser ofertado aos animais.

Os grãos de kefir congelados a -20ºC foram ativados e cultivados diariamente durante o período de tratamento dos animais. Os grãos foram inoculados na proporção de 1:10 em leite pasteurizado integral (Benfica®, Juiz de Fora, MG, Brasil), em recipiente de vidro esterilizado, e mantidos em estufa a 25°C± 2°C, durante 24 horas, em meio aeróbio. Posteriormente, os grãos foram separados do leite fermentado utilizando-se uma peneira e lavados com água destilada. A tamisagem foi realizada com peneira, sob assepsia. Os grãos retidos na tamisagem foram novamente inoculados ao leite, repetindo as etapas descritas. O leite fermentado fresco foi ofertado aos animais (OTLES & CADINGI, 2003; CZAMANSKI, 2003; ROSA et al., 2017).

Durante todo o tratamento dos animais, foi realizada periodicamente, duas vezes por semana, a contagem de bactérias ácido-láticas (BAL) totais e de leveduras do kefir, garantindo a oferta da contagem microbiológica programada.

A contagem de BAL foi realizada pelo método de plaqueamento em superfície por meio da técnica de microgotas a partir de diluições decimais seriadas (IBBA; ELASKY, 2016). Na superfície de cada placa, contendo o meio ágar de Man, Rogosa e Sharpe (MRS), foram inoculados 20 µL das diluições decimais (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}) do kefir. As placas foram incubadas a 37 °C por 24-48h horas em estufa para a contagem de unidades formadoras de colônia (UFC). A contagem de leveduras também foi realizada pelo método de plaqueamento em superfície a partir de diluições decimais seriadas. Na superfície de cada placa contendo ágar de batata e dextrose (BDA) acidificado com solução de 10 % de ácido tartárico, foram inoculados 100 µL das diluições decimais (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}) do kefir. As placas foram incubadas a 25 °C em estufa incubadora durante 5 dias.

A partir da fórmula: (média final da contagem de UFC x fator de diluição)/alíquota utilizada para o plaqueamento, determinou-se a quantidade de UFC/mL de kefir (BRASIL, 2003).

Além da análise microbiológica, foi determinada a composição centesimal do kefir de leite. Para tanto, foram realizadas análises em triplicatas por métodos já descritos pela Association of Official Analytical Chemist – AOAC (1989, 2005), e já padronizados no Laboratório de Composição e Valor Nutricional de Alimentos do Departamento de Nutrição da UFJF. Foram analisados: umidade por secagem direta da amostra em estufa a 105 ºC; determinação do teor de cinzas, realizado por incineração em mufla a 550 ºC e posterior resfriamento em dessecador até a temperatura ambiente; teor de lipídeos totais foi obtido por secagem da amostra em estufa a 105 ºC, seguido por extração com éter, em extrator do tipo Soxhlet, e posterior remoção do solvente, por destilação; teor de proteínas foi determinado pelo método de Kjeldahl (AOAC, 1990). A quantificação de carboidratos foi determinada pelo cálculo da diferença percentual, subtraindo-se do total da soma de umidade, cinzas, lipídeos e proteínas.

5.4 – Avaliação do estado nutricional e adiposidade

O consumo de ração pelas ratas lactantes foi monitorado diariamente por toda a lactação (21 dias) e de seus filhotes, após o desmame até o dia da eutanásia, de 4 em 4 dias.

A massa corporal (MC) das ratas lactantes e das proles foi acompanhada diariamente durante a lactação. Após o desmame, a MC dos filhotes foi aferida de 4 em 4 dias até a eutanásia.

O cálculo do coeficiente de eficácia alimentar (CEA) foi estabelecido pela relação entre o ganho de peso/consumo alimentar (NERY et al., 2011).

A adiposidade foi avaliada pela pesagem da gordura das regiões epididimal, visceral e retroperitoneal.

5.5 – Concentração de citocinas e óxido nítrico no homogenato de cólon

Para análise de citocinas no cólon, 100 mg de tecido de cada animal foi homogeneizado em 1 ml de tampão PBS contendo 0,05% de Tween 20, 0,5% de albumina de soro bovino e inibidores de proteases (0,01 mM de EDTA e 20 UI de aprotinina A) utilizando um homogeneizador. O homogenato resultante foi centrifugado (10.000 rpm por 10 min. a 4°C) e o sobrenadante empregado em teste de Imunoensaio Enzimático (ELISA). As concentrações de interleucina-1 (IL-1β) (faixa de sensibilidade do teste: 63-4000 pg/mL), IL-6 (faixa de sensibilidade do teste: 31-2000 pg/mL), Interferon (IFN-γ) (faixa de sensibilidade do teste: 63-4000 pg/mL), e Tumor Necrosis Factor Alpha (TNF-α) (faixa de sensibilidade do teste: 63-4000 pg/mL), foram mensuradas com uso do kit de ELISA (PeproTech Inc., Rocky Hill, NJ, USA) sanduíche para citocinas seguindo as instruções do fabricante. Os resultados finais foram expressos em pg/mL.

A dosagem da acumulação total de óxido nítrico (NO) no tecido do cólon foi realizada com base no método desenvolvido por Miranda et al. (2001), que se baseia na redução de nitrato pelo cloreto de vanádio III em nitrito combinado com a detecção do nitrito total pela reação de Griess.

5.6 – Contagem e categorização dos focos de criptas aberrantes (FCA) e tumores intestinais

O cólon foi removido para quantificação e categorização dos FCA. Após a retirada, o intestino foi lavado em solução salina fisiológica, aberto longitudinalmente, medido e dividido em três seguimentos iguais. O tecido foi colocado em placas de isopor, e fixado em formol a 10% por 48 horas. Assim, foi realizada a contagem dos tumores e registrada sua localização. Para a contagem dos FCA, os seguimentos foram corados em solução de azul de metileno a 0,1% por 30 segundos e lavados em tampão fosfato. A contagem das lesões foi realizada por microscopia óptica por dois avaliadores treinados, de forma independente. A categorização dos FCA foi realizada considerando o número de criptas aberrantes por foco, assim, focos com 1, 2, 3, 4 criptas e focos com 5 ou mais criptas (BIRD, 1987).

5.7 – Análise histopatológica

Os tecidos intestinais (cólon) foram fixados em formol e previamente preparados para análise histopatológica. As lâminas foram coradas com hematoxilina e eosina (H&E) e examinadas por um patologista experiente e cegado em microscópio óptico quanto à presença de infiltrado de células inflamatórias, hiperplasia, perda de células caliciformes e criptas irregulares.

Os tecidos do cólon fixados em formol foram desidratados, embebidos em parafina e fatiados em seções de 5 µm. As seções foram hidratadas e coradas com H&E. A pontuação microscópica foi realizada conforme o método descrito por Faramarzpour et al (2019). As lâminas histológicas foram examinadas em microscópio óptico quanto a gravidade de edema, inflamação e danos à cripta. Os escores de gravidade do edema foram: 0 = edema ausente no cólon; 1 = edema leve na mucosa; 2 = edema na mucosa e submucosa; 3 = edema em toda a parede do cólon; e 4 = edema grave em toda a parede do cólon. Os escores de gravidade da inflamação foram: 0 = nenhum; 1 = leve; 2 = moderado; e 3 = grave. Os escores de dano à cripta foram: 0 = nenhum; 1 = 1/3 basal danificado; 2 = 2/3 basal danificado; 3 = criptas perdidas e epitélio superficial presente; e 4 = criptas e epitélio perdidos.

5.8 – Caracterização da microbiota intestinal

Amostras cecais foram escolhidas aleatoriamente entre os grupos controle (n = 5), controle kefir (n = 5), superalimentado (n = 5) e superalimentado kefir (n = 5) para a análise da microbiota intestinal. O DNA genômico total foi extraído de amostras de fezes coletadas diretamente do ceco em tubos estéreis, e utilizou-se o kit de extração de DNA genômico (MagaZorb® DNA Mini-Prep Kit, Promega), conforme instruções do fabricante. O DNA foi inicialmente avaliado pela razão 260/280-nm com o NanoDrop 1000 espectrofotómetro (Thermo Fisher Scientific, Wilmington, DE, EUA). Após uma pré-seleção, as amostra passaram pelo sistema Bioanalyzer para controle de qualidade de alta sensibilidade e precisão. Para cada amostra um sequenciamento de alto rendimento na plataforma Illumina MiSeq (Illumina, San Diego, CA, EUA) foi realizado na empresa GenOne Biotechnologies (Rio de Janeiro, Brasil). As regiões V3-V4 do gene 16S rRNA bacteriano foram sequenciadas e os dados obtidos, atribuídos e analisados.

A filtragem de qualidade dos dados brutos de sequenciamento foi realizada para obter tags limpas de alta qualidade, que foram posteriormente analisadas usando o software QIIME (Quantitative Insights Into Microbial Ecology) com configurações padrão. Um conjunto de sequências em um nível semelhante de 97% foi agrupado em uma Unidade Taxonômica Operacional (OTU) pelo *pipeline* UPARSE, e usando Mothur como algoritmo de atribuição e Silva como banco de dados de referência. Uma sequência foi escolhida como representante de cada OTU para anotar informações taxonômicas.

Usando o Pipeline do Ribosomal Database Project (RDP) (http://pyro.cme.msu.edu/index.jsp; Cole et al., 2009), as sequências foram processadas. O RDP-Classifier foi utilizado para a classificação taxonômica das sequências representativas de cada OTU. A diversidade alfa (Chao1, Shannon, Simpson e Dominance) foi quantificada usando o software Past.

5.9 – Análise estatística

Os dados foram analisados pelo programa estatístico *GraphPad Prism* 5 e expressos como média ± erro padrão da média. O teste de normalidade Kolmogorov-Smirnov foi aplicado. Para valores com distribuição normal a análise de variância uni-

ou bivariada foram utilizados para análise. Testes não-paramétricos foram realizados para os valores que não apresentaram distribuição normal. As diferenças foram consideradas significativas quando $p<0,05$.

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7. RESULTADOS

ARTIGO 1 – Preclinical evidence of probiotics in colorectal carcinogenesis: a systematic review

Publicado no periódico Digestive Diseases and Sciences (Fator de impacto: 2.937).

ARTIGO 2 – Kefir regulates inflammatory cytokines and reduces DMH-associated colorectal cancer in adult Wistar rat offspring

Preclinical evidence of probiotics in colorectal carcinogenesis: a systematic review

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Abstract

Background: Colorectal cancer, the second major cause of cancer deaths, imposes a major health burden worldwide. There is growing evidence that supports that the use of probiotics is effective against various diseases, especially in gastrointestinal diseases, including the colorectal cancer, but the differences between the strains, dose and frequency used are not yet clear. Aims: Perform a systematic review to compile the results of studies carried out in animal models and investigated the effect of probiotics on colorectal carcinogenesis. Methods: Studies were selected in PubMed/MEDLINE and Scopus according to the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines. Search filters were developed using three parameters: probiotics, colorectal cancer, and animal model. Results: From a structured search, we discovered 34 original articles and submitted them to a risk of bias analysis using SYRCLE's tool. The studies show a great diversity of models, most were conducted in rats (55.8%) and used 1,2 dimethylhydrazine (DMH) as the drug to induce colorectal carcinogenesis (61.7%). The vast majority of trials investigated Lactobacillus (64%) and Bifidobacterium (29.4%) strains. Twenty-six (86.6%) studies found significant reduction of lesions or tumors in the animals that received probiotics. The main methodological limitation was the insufficient amount of information for the

adequate reproducibility of the trials, which indicated a high risk of bias due to incomplete characterization of the experimental design. Conclusions: The different probiotics strains showed anticarcinogenic effect, reduced the development of lesions and intestinal tumors, antioxidant and immunomodulatory activity, and reduced fecal bacterial enzymes.

Keywords: Colorectal neoplasms; carcinogenesis; probiotics; animal model; systematic review

1. Introduction

Chronic non-communicable diseases are responsible for the majority of global deaths, and cancer represents an important cause of morbidity and mortality worldwide. Cancer incidence is related to the westernization of lifestyle, and social and economic transition in countries'.[1] The GLOBOCAN 2018, published by the International Agency for Research on Cancer (IARC), estimated the occurrence of 18.1 million new cases and 9.6 million cancer deaths worldwide in 2018. For colorectal cancer, 1.8 million new cases and 881,000 deaths are estimated to occur in 2018, representing the third neoplasia in incidence and second in cause of mortality, with average case fatality higher in countries with lower HDI (Human Development Index). [2, 3]

With multifactorial etiology, cancer is associated to genetic factors, nutrition and inflammatory processes. The increase in incidence of colorectal cancer is associated with changes of dietary patterns, obesity, and factors related to lifestyle.[4] There is convincing evidence that shows that processed meat, alcoholic drinks and accumulation of body fat increase the risk of development of the disease. On the other hand, physical activity is a protective factor.[4-6]

There is evidence that indicates the role of diet in the development of colorectal cancer. Dietary compounds may influence pathways by which carcinogens are metabolized and epigenetic changes that lead to cancer development.[4, 7] There is indication that some probiotics strains can affect the host's immunologic response,

stimulating anti-inflammatory cytokines, antioxidants and anti-carcinogenic compounds.[8] In this respect, probiotics are associated with anticancerous and antimutagenic activity.[9,10] According to the Food and Agriculture Organization/World Health Organization (FAO/WHO), probiotics are defined as ‘’live microorganisms which when administered in adequate amounts confer a health benefit on the host’’.[11] However, the differences between the strains, doses and frequency used, as well as the mechanisms by which they exert their effects are not yet clear.

Given the difficulty of studying the effects of several treatments, including nutritional aspects, in colorectal carcinogenesis, preclinical models for colorectal carcinogenesis are used to induce lesions similar to colorectal cancer in humans, being widely used in experimental studies.[12] However, as the findings of preclinical studies often originate from relatively small experiments and are quite heterogeneous, they may not always be applicable in a translational context to enhance human health and well-being. [13,14] Based on this, the objective of the present study was to systematically review the preclinical evidence in a qualitative manner (unlike the widely used narrative reviews). We believed that a study like this might provide us with reliable and solid new evidence on whether or not probiotic supplementation could be beneficial in the context of colorectal carcinogenesis. We performed a critical analysis of preclinical studies in order to improve the quality of the reports and to prevent the spreading of methodological failures, which could compromise the development of future clinical studies.

2. Methods

The systematic review was elaborated according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyzes – PRISMA [15], whose methods include data source and search, study selection, eligibility criteria, data extraction, analysis of results and risk of bias. The protocol was registered at the International Prospective Register of Systematic Reviews - PROSPERO (registration number: CRD42018111201).

2.1 Search strategy

The bibliographic search was performed using the electronic databases MEDLINE (PubMed platform - https://www.ncbi.nlm.nih.gov/pubmed) and Scopus

(https://www.scopus.com/home.uri), admitting only studies in animal models. The keywords for the construction of the filters followed three criteria: probiotics AND colorectal cancer AND animal models (Supplementary file S1). The hierarchical distribution of the MESH terms was the strategy used to develop the filter on the PubMed platform. We applied a standardized filter in the Scopus platform for animal studies and the same PubMed search strategy was adapted and used. Views, comments, notes and unpublished studies were not included.

No restrictions were imposed for language or date of publication. The bibliographies of the eligible studies were checked manually to find possible publications of interest.

2.2 Selection of studies

We included all the original experimental studies that evaluated the administration of probiotics in an animal model (in vivo) of colorectal carcinogenesis. Prespecified eligibility and exclusion criteria were set using the PICOS (Population, Intervention, Comparison, Outcome and Study design) strategy. The following exclusion criteria were used:

1) Studies that analyzed the associated effect of probiotics with prebiotics and/or nutritional supplements;

2) In vitro studies;

3) Descriptive studies, such as annals of congresses, editorials, letters, case reports and review works;

4) In vivo studies with humans were also excluded. Abstracts or unpublished reports have been disregarded.

The evaluation of the eligibility of the studies was performed independently by two reviewers (P.G.A.B. and S.C.P.D.L). In the case of disagreements, another group of reviewers (R.V.G., M.C.G.P and R.D.N.) decided whether the study met the inclusion and exclusion criteria. Inclusion or exclusion was verified by evaluating the full text of potentially relevant studies.

2.3 Extraction and synthesis of data

A detailed examination of the studies was carried out in order to evaluate the strength of the evidence and the validity of its inclusion in this review.

Data extraction and compilation tables were developed according to the following information: (i) Publication characteristics: authors, publication year and country; (ii) Characteristics of the experimental model (animal model, sex, age, number of animals, control group and carcinogenic model) and main characteristics of the intervention (strain, dose and duration); (iii) Effects of probiotics and its main outcomes. When essential information was absent, the authors were contacted in order for us to obtain it. The outcomes on the development of aberrant crypt foci (ACF), intestinal tumors, fecal enzymes activity, antioxidant activity, and immune markers were analyzed and presented. The data was subsequently compared and conflicting information was identified and corrected after discussion among the reviewers.

2.4 Risk of bias

The risk of bias was analyzed using the SYRCLE tool (Systematic Review Centre for Laboratory animal Experimentation), based on Cochrane Collaboration (RoB 2.0), which aims at evaluating the methodological quality of the studies. This instrument was adjusted for bias aspects that play a specific role in animal intervention studies. The objective was to establish consistency and to avoid discrepancies in the evaluation of methodological quality in the field of animal experimentation. In order to increase transparency and applicability, signaling issues have been formulated to facilitate judgment, based on the following areas: 1. Random sequence generation. 2. Baseline characteristics. 3. Allocation concealment. 4. Random housing. 5. Investigator blinding. 6. Blinding of outcome assessment. 7. Blinding of outcome. 8. Incomplete outcome data. 9. Selective outcome reporting. 10. Ethical considerations. [16]

3. Results

3.1.1 Study Selection

Initially 778 references were found in the databases. By reviewing titles and abstracts, 738 citations were excluded for different reasons (human model, in vitro

studies, intervention or outcome not pertinent, review articles, other diseases). Forty articles were selected for full text examination, then nine studies were excluded due to insufficient data (n= 3), full text not available (n= 1), other tumors (n= 1) and other treatments (n= 4). Three additional citations were included by manual search after research in the references of the articles that were initially included. Figure 1 shows the flow diagram of the study selection process.

3.1.2 Study Characteristics

Thirty-four studies with different probiotic preparations were selected and reviewed. The selected studies were performed in 12 different countries, most of them in the USA (23.5%) followed by India (17.6%), Argentina (11.7%), Canada (8.8%) and Japan (8.8%). All studies were published in English. The articles selected for this qualitative review show a great diversity of models, age of animals (21 days to 20 weeks old) and duration of treatment (7 days to 36 weeks). Most of the studies were conducted in rats (55.8%), and the remainder in mice (44.2 %). The proportion of the animals' sex was 52.9% male (n = 18), 26.4% female (n = 9), 11.8% both (n = 4), and three studies omitted the animals' sex (8.9%).

The majority of studies investigated the *Lactobacillus* (64%) and the *Bifidobacterium* (29.4%) genera (Table 1). For the dietary treatment, either as isolated strain, combined formulation, or probiotic food (e.g., kefir), the works mostly used commercial bacterial cultures, and reported no microbiological counting. Out of the 34 studies included, 25 used only isolated strains (73.5%), 3 investigated the effect of two or more combined strains (8.8%), and 6 evaluated probiotic foods (17.7%).

3.2.1 The effect of probiotics in the development of lesions and intestinal tumors

Probiotics have been tested individually or in combination with different concentrations. Our results showed that the majority of studies offered the probiotic source daily (91%). Thirty studies (88.2%) presented results of development of ACF, early pre-cancerous lesions, and/or intestinal tumors after the subjects were exposed to the carcinogen. In 26 studies (86.6%), there was a significant reduction of lesions or tumors in the animals that received probiotics.

Of the articles included, twenty-one (61.7%) used 1,2 dimethylhydrazine (DMH) as the inducing drug of colorectal carcinogenesis, and the doses varied from 15 to

40mg/kg. The second most used drug was azoxymethane (AOM) (17.6%). Out of the 34 studies, 27 (79.4%) utilized chemically induced models by DMH or AOM, and the usage duration of these chemicals carcinogens, were of 14.3% for the administered for up to 2 weeks; 47.6% between 3 and 19 weeks and 38.1% for 20 or more weeks. For AOM, 50% of the studies used the carcinogen for up to 2 weeks, the remainder between 3 and 19 weeks. Application frequency occurred once a week (77.7%) or twice a week (22.3%).

The studies that have evaluated the effects of the *Lactobacillus* genus (*acidophilus*, *casei*, *fermentum*, *delbrueckii*, *gasseri*, *rhamnosus*, *plantarum* species), observed reductions in lesions [17, 18, 27–30, 19–26] in the intestinal tumors. [31, 32, 41, 42, 33–40] Table 2 presents the main effects of different probiotics' strains on the development of histopathological parameters in the studied models.

In the groups supplemented with milk fermented by L. bulgaricus and S. thermophiles or isolated L. bulgaricus [43, 44], the strains had no significant effect on the incidence of tumors. It was also shown that the effects depended of the dose, meaning that the protective effect of Lactobacillus bulgaricus was only detected in those animals treated with the higher dose. [44] When the administration of Bifidobacterium was evaluated, almost every study showed inhibition in the development of ACF compared to the control groups. [17–19, 23, 45] Using genetically modified strains (Streptococcus thermophilus and Lactococcus lactis subsp. cremoris), the analysis of histologic damages showed the highest scores in the samples obtained from the DMH and L. lactis group. Mice that received genetically modified lactic acid bacteria showed decreased damage scores compared to the DMH group. [46] Seven studies worked with fermented foods [26, 34, 38, 39, 43, 47, 48], including kefir (2), yogurt (3), probiotic curd (1) and Dahi (1), showing that the oral administration of milk and soy milk kefirs inhibited tumor growth significantly. [34] When the use of yogurt was evaluated, only one study observed results in the development of intestinal tumors, signaling that the yogurt diet significantly reduced the number of colorectal tumors induced by DMH in male rasH2 mice. [39]

3.3 The secondary effects of probiotics

3.3.1 Antioxidant activity

Five studies evaluated the antioxidant activity exerted by probiotics. [25, 35, 46, 48, 49] Two studies of the same group assessed the effects of different genetically modified lactic acid bacteria. Mice that received a catalase-producing L. lactis strain (L. lactis KAT) presented an increase in catalase activity in samples taken from small and large intestines. H2O2 concentrations were slightly lower in samples from animals that were supplemented with L. lactis KAT as opposed to L. lactis NZ or no bacterial supplementation (DMH group) [35]. The same pattern was observed for Streptococcus thermophilus and Lactococcus lactis subsp. cremoris - which produced antioxidant enzymes (catalase or superoxide dismutase).[46] The evaluation of the antioxidant capacity of the probiotic strains revealed that the malondialdehyde level was significantly lower in animals that received probiotics when compared to those that received DMH alone. The DMH treatment of animals significantly decreased the amount of glutathione and the activities of the enzymes glutathione-S-transferase, superoxide dismutase, catalase and glutathione peroxidase. These changes appear to be reversed by probiotic supplementation. [25, 49]

3.3.2 Fecal bacterial enzymes

The activity of fecal bacterial enzymes was evaluated in 6 studies, demonstrating decrease within all included studies. Fecal bacterial activity of β-glucuronidase declined significantly in animals that received probiotics. [17–19, 24, 25] Whole yoghurt maintained the enzyme levels lower or similar to control. [47] Bacterial β-glucosidase activity was reduced by the administration of different probiotics, including Lactobacillus acidophilus, Lactobacillus rhamnosus and Bifidobacterium bifidum. [24, 25] Were also observed significant decrease of nitroreductase activity in groups that received Lactobacillus casei and plantarum. [25] In large intestine fluid, whole yoghurt feeding decreased or maintained enzyme levels, similar to the non-treatment control. These mice were fed with yoghurt cyclically again after eight weeks. The animals from yoghurt-DMH-yoghurt group showed lower nitroreductase activity compared to the group that received only DMH. [47]

3.3.3 Immune system

Cytokines and immune cells were evaluated in 6 studies, presenting different results, but in general the probiotics seem to modulate immune response. In model Apc (Min/+) mice, results show that the average levels of inflammatory cytokine IL-6 reduced after the mice received L. acidophilus. [37] In the same group, mice receiving a probiotic yogurt formulation containing microencapsulated live Lactobacillus acidophilus showed higher concentrations of CD8 cells than the tissue of animals in the control group. Relatively similar Mac-1 expressions were found in animal tissues from both control and treatment groups, 6.02 and 5.43% similarity, respectively. Results suggest that oral administration of the probiotic formulation may lower the expression of markers directly related to intestinal inflammation. [40] Mice receiving L. casei had significantly decreased MCP-1 and TNF-α pro-inflammatory cytokines levels in the intestinal samples and had increased levels of the anti-inflammatory cytokine IL-10, compared to other groups. [27, 30] The mucosal IgA were verified by Liu et al[27], indicating that feeding milk kefir and soy milk kefir significantly increased the total IgA level in the tissue from the small intestine.

3.3.4 Protein expression

Four studies have evaluated the expression of proteins involved in carcinogenesis, demonstrating that B. longum significantly suppressed the expression ras-p21 in colonic mucosa. [33] In another paper, the authors observed that the expression cleaved caspase-3 was reduced, 11.36 in control group vs. 6.09% in treatment group (L. acidophilus).[40] Probiotic supplementation was able to restore the normal expression of both p53 and Bcl-2 after DMH administration. [49] The administration of L. acidophilus + L. fermentum (46.2±3.4%), led to reduced aberrant β-catenin signaling and nuclear staining of β-catenin when compared to saline group (54.7±0.9%), meaning that the aberrant β-catenin signaling in the tumors was suppressed by probiotic strains administration. [29]

3.4.1 Study quality and risk of bias

The results of our risk of bias assessment of the 34 studies included in this systematic review are shown in Figure 2. None of the studies presented a low risk of bias in all the methodological criteria and reached the desired quality. Considering

each criterion analyzed individually, none of the studies reported information as investigator blinding, and declared the sample calculation for the number of animals used. However, most of the studies displayed food availability information during the experiment, use of standardized diets, management conditions, details of animal allocation and experimental groups, food consumption and body weight. Mortality information and comments on study limitations were poorly addressed.

4. Discussion

Our results indicated that a large variety of probiotics strain were effective in reduction of development of lesions and intestinal tumors in animal models. The vast majority of the studies evaluated the administration of Lactobacillus and/or Bifidobacterium genera, which is supported by the safety evidence of these strains. [50, 51] Furthermore, the probiotics presented other benefits, such as modulation of the immune system, antioxidant activity and decrease of fecal bacterial enzymes, all associated with the main result. This data thus provides evidence that probiotics can act as an effective strategy on prevention of colorectal cancer. The results in Table 2 are consistent with this.

Despite the fact that the studies included in this review show wide methodological variability, some common ground was observed. Murine models were the main animal model used for the study of carcinogenesis. The use of animals provides lower costs, allowing more controlled and careful analysis of the outcome measures, which is particularly important because it presents itself as a viable tool for research regarding colorectal cancer, a disease that has great relevance in the world morbidity and mortality. [52] Male animals were often chosen, which may have occurred due to sex-specific mechanisms. However, the relationship between sex and the development of colorectal cancer is not completely clear. [53, 54]

A wide variation was found for the age of the animals used in the experimental models. The vast majority of studies used animals of 5 or more weeks of age. However, some studies did not report the age of the animals. It is interesting to note that the relevance of the preclinical model for age-dependent carcinogenesis, since the cancer is predominantly a disease of elderly people. [56, 57] The studies included in this review evaluated animals between 3 and 20 weeks of age, but only two studies evaluated the consumption of probiotic in the early stages of life, as in post-weaning.

[18, 26] This is particularly relevant, as it indicates the lack of information on the use of probiotics in critical periods of development and their long-term effect, since it is known that early exposure to different substances can program the individual's in adulthood, beneficially or not. [58]

The studies analyzed presented a great variability in relation to carcinogens, mainly the type, dose and frequency in which these compounds are administered to animals. There is evidence that high doses can affect the number and growth features of ACF and of tumor outcome. The ACF are the earliest visible lesions in the colon and rectum and are considered potential precursors of colorectal cancer, being also identified in patients at a high risk of colorectal cancer. [12] The two chemical substances with carcinogenic potential that were the most used were DMH and AOM, an active metabolite of DMH, which enables the chemopreventive and chemotherapeutic study of other compounds, such as probiotics.[59–61] The intestinal mucosal injury DMH-induced involves a sequential process with gradual increase in the number of ACF, which may lead to the development of colorectal cancer. The majority of these tumors develops mutations in the β-catenin gene, which is similar to hereditary nonpolyposis colorectal cancer, with inactivation of the β-catenin destruction complex, generally by APC (adenomatous polyposis coli) mutations. [59, 62] The use of genetically modified animals in the study of colorectal carcinogenesis was also observed, especially of Apc (Min/+) mice, in which the Apc gene is the homolog of human APC gene. Due the fact that its standard molecular and pathologic structure is similar to human familial adenomatous polyposis, it is widely use to study the development, treatment, and prevention of colorectal cancers that contain somatic APC mutations. [52, 63, 64]

In this review, the most common investigated genera were Lactobacillus followed by Bifidobacterium, once lactic acid bacteria represent the main microorganisms added to probiotic products. [65] The two lactic acid bacteria employed in the production of yogurt from milk are the Streptococcus thermophilus and Lactobacillus delbrueckii subsp. bulgaricus. These bacteria appear to be involved in the prevention of carcinogenesis and in immune stimulation, decreasing colorectal cancer risk. [65–67] Included in the classification of fermented foods, the kefir, a probiotic fermented milk with a complex composition of bacteria and yeasts in a polysaccharides matrix, has demonstrate anti-proliferative, anti-inflammatory, and anti-

mutagenic activity. [68, 69] These effects are consistent with the results found in the studies included in this systematic review. [34, 48]

Our findings indicate that several of the studies did not submit or could not establish the dose administered. [17, 18, 40, 43, 46–48, 70, 19, 20, 22, 27, 32–34, 39] This lack of information was caused by not counting the colony forming units (cfu) or by ad libitum offer of probiotic supplementation in water or in experimental diets, that led to estimations of consumption, but not the exactly values. The use of probiotic microorganisms must grant health benefits to the host in the studied dosage and duration of use. It is not possible to establish a general minimum dose because each strain is effective at a specific dose. [71] The different probiotics differ in depending on the way the complex interactions between food, microbiota, microorganism, and intestinal mucosa takes place. [72] Surprisingly, this important information was often underreported in this review, hindering the studies reproducibility and representing an important indicator of heterogeneity among the preclinical models.

Some of the studies included analyzed the expression of markers involved in carcinogenesis, as ras-p21, cleaved caspase-3, Mac-1, Ki-67, β-catenin, E-cadherin, p53, Bcl-2, Bax, caspase-9 and caspase-3. [29, 33, 40, 49] The identification of these markers plays an important role in the early diagnosis and identification of colorectal cancer and in the development of prevention strategies. With the exception of a few specific cases, colon and rectum cancers have remarkably similar patterns of genomic alteration. In almost all tumors there are diverse alterations involving the TGF-β and p53 pathways [62], reinforcing the results found in studies included in this review.

In this sense, some protective effects are attributed to probiotics, including the maintenance or enhancement of intestinal barrier function, explained in part by the increase of the expression of the genes involved in tight junction signaling in intestinal epithelial cells.[73] Other mechanisms that relate to probiotic action involves the fecal bacterial enzymes, including β-glucuronidase, β-glucosidase and nitroreductase, which catalyze the release of procarcinogenic substances in the intestine. The alteration of the intestinal metabolism by modulating the activity of these bacterial enzymes may be one of the possible mechanisms by which probiotics may reduce the risk for the onset of colorectal cancer.[74] According to the results presented,

consumption of probiotics reduced fecal bacterial enzymes in all studies that evaluated this effect. [17, 18, 20, 24, 25, 47]

Another important mechanism is in the relation of the intestinal mucosa and the host-microbiota. Although the intestinal microbiota was not the focus of the included studies [75, 76], three studies evaluated fecal bacteriology and observed that the probiotic strains were recovered in the feces of all the rats that were given probiotic supplementation.[32] In another study, the L. acidophilus group had fecal pH, aerobic bacteria and E. coli count reduced[24], and when comparing the concentration of probiotics strain in feces before and after treatment, significant increase was found (L. acidophilus 4 to 74% and B. bifidum 1 to 36%).[30] These effects may be mediated by adherence to enterocytes, intestinal pH reduction and mechanisms of competition with bacterial pathogens. [24, 32, 74]

Probiotic bacteria also show immunomodulatory activity, stimulating production of IL-10 and IgA in intestinal epithelial cells and decreasing pro-inflammatory pathways (via reduction of IL-1β, IL-6 and TNF-α).[75, 77, 78] Several mechanisms have already been related to the modulation of intestinal barrier function, include the innate and adaptive defense responses, such as of IgA, Toll–like receptors, cytokines, gut associated lymphoid tissues and signaling pathways. [75, 77, 79] Some of the mechanisms were verified in the studies we reviewed, such as the reduction in levels of IL-6 and TNF-α and the increase of IL-10 and IgA. [27, 30, 34, 37]

Complementarily, a few probiotics show antioxidant activity, inhibiting the generation of reactive oxygen species (ROS), such as superoxide ions, free radicals and peroxides. These reactive species in excess result in oxidative stress and they can lead to damage in the cellular structure and in its constituents (DNA, RNA, proteins and lipids). Therefore, oxidative stress has an important role in diseases of the gastrointestinal tract, including inflammatory bowel diseases and colon cancers. Thus, reduction of its levels may represent an effective strategy against the development of tumors.

As a result of the high variability of experimental designs and of the finding of methodological bias, the preclinical evidence for the probiotics is still delicate and inconclusive. The data heterogeneity, with different strains, doses and duration of treatment, as well as the extremely diverse induction model of colorectal

carcinogenesis represent a limitation for evidence availability. The risk of bias analysis (SYRCLE) was performed individually as a way to ensure the validity of the findings and assessing the methodological quality of the studies, demonstrating that the application of standard protocols is essential to the reproducibility and synthesis of results.

5. Conclusions

The probiotics were effective in preventing colorectal cancer and the development of pre neoplastic lesions, demonstrating that their effects and the metabolic pathways involved are diverse and depend on the probiotic strain administered, on the dose and on the duration. The limited methodological description, incomplete characterization of protocols and outcomes was a limitation we found, as well as the methodological heterogeneity in studies. We believe that our critical analysis can promote new preclinical research with lower methodological bias, enable researchers to determine the exact mechanisms by which probiotics act, along with their long-term effects and more importantly guide public health policies that may have an impact on the reduction of colorectal cancer worldwide.

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Table 1. Characteristics of the experimental model and intervention of the studies regarding the use of probiotics in studies of colorectal carcinogenesis in animal models.

Author, year	***Country***	***Animal model***	***Sex***	***Age (weight)***	***Nº animals/ Group***	***Control group***	***Strain***	***Dose***	***Duration (probiotic)***	***Carcinogenesis model***
Goldin and Gorbach, 1980[17]	USA	F344 rats	♂	6-8 wk	11-22	Meat diet	*L. acidophilus*	10^{10} - 10^{11} cells	20/36 wk	DMH (20mg/kg) s.c.
Shackelford et al, 1983[18]	USA	F344 rats	♀	4 wk	28	Commercial diet	*L. bulgaricus* *Streptococcus thermophilus*	?	20 wk	DMH (20mg/kg) s.c.
Kulkarni and Reddy, 1994[19]	USA	F344 rats	♂	5 wk	11	Semi-purified diet (AIN-76A)	*B. longum*	1.5 and 3% in diet ($2x10^{10}$ cells/g)	13 wk	AOM (20mg/kg) s.c.
Abdelali et al, 1995[20]	France	Sprague-Dawley rats	♂	26 d	6	Commercial diet	*B*. ?	~ 6 x 10^9 cells	4 wk	DMH (25mg/kg) i.p.
Goldin et al, 1996[21]	USA	F344 rats	♂	?	8-21	Experimental diet (5%/20% corn oil)	*L. GG*	1% in diets ~ 2-4 x 10^{10} cells/d	24/27 wk	DMH (20mg/kg) s.c.
Challa et al, 1997[22]	USA	F344 rats	♂	7 wk ?	15	Semi-purified diet (AIN-76A)	*B. longum*	0.5% in diet (1 x 10^8 cells/g)	13 wk	AOM (16mg/kg) s.c.

Singh et al, 1997[23]	USA	F344 rats	♂	5 wk	12	Semi-purified diet (AIN-76A)	*B. longum*	2% in diet ~ 2 x 10^{10} cells/g	16 wk	AOM (15mg/kg) s.c.
Balansky et al, 1999[24]	Bulgaria	BD6 rats	♂/♀	16-20 wk (180-220g)	30-32 ?	Commercial diet	*L. bulgaricus* (FFM.B144 or FFM.B5)	1.3g/2.5g/animal FFM.B144: 4 x 10^{7} cfu/g FFM.B5: 3 x 10^{6} cfu/g	8 months	DMH (21mg/kg) s.c.
Rao et al, 1999[25]	USA	F344 rats	♂	6 wk	12	Semi-purified diet (modified AIN-76A)	*L. acidophilus*	2%/ 4% in diet 4.2 x 10^{9} cells/g	10 wk	AOM (15mg/kg) s.c.
Gallaher and Khil,1999[26]	USA	Wistar rats	♂	?	15-20	Semi-purified diet (modified AIN-76A) + skim milk	*B.* ?	10^{8}-10^{9} cfu/animal	3.5 to 5 wk	DMH (15mg/kg) gavaged
Liu et al, 2002[27]	Taiwan	ICR mice	♀	6-7 wk (24±0.8g)	10	Commercial diet + water	Kefir ?	?	30 days	S180 tumor cells in saline (1x10^{8} cells/ml) 0.2 ml s.c.
Tavan et al, 2002[28]	France	F344 rats	♂	?	15	Commercial diet + 20% water	*B. animalis* *Streptococcus thermophilus*	5.4±1 x 10^{8} cfu/day	15 wk	HAA (115 µl) in diet

De Moreno and Perdigón, 2005[29]	Argentina	BALB/c mice	?	? (25-30g)	45-50	Commercial diet + skim milk	Yogurt (*L. delbrueckii* + *Streptococcus thermophilus*)	? 2×10^8 cells/ml	10 days (cyclically)	DMH (20mg/kg) s.c.
Lee et al, 2007[30]	Korea	F344 rats	♂	5 wk (185±10g)	9	Commercial diet	*Bacillus polyfermenticus*	3×10^6 cfu/d in diet	10 wk	DMH (30mg/kg) s.c.
De Moreno et al, 2008[31]	Argentina	BALB/c mice	♂/♀	6 wk (25-30g)	30-35	Commercial diet	*Lactococcus lactis* NZ/KAT	1×10^9 cfu/d	6 months	DMH (20mg/kg) s.c.
Takagi et al, 2008[32]	Japan	BALB/cByJ mice	♀	6 wk	12	Commercial diet	*L. casei* Shirota *L. fermentum* *L. acidophilus* *L. plantarum* *L. reuteri* *L. rhamnosus*	~ 10^9 cells/mg cells 0.005% (w/w)	12 wk	3-Methylcholanthrene (1mg/0.1 ml) s.c.
Cenesiz et al, 2008[33]	Turkey	BALB/c mice	♂/♀	12 wk (average 31.5g)	5	Commercial diet	Kefir ?	? 50% (w/v) ad libitum instead of water	13 wk	AOM (5mg/kg) s.c.
Urbanska et al, 2009[34]	Canada	C57BL/6J-*Apc* Min/+ mice	♂	7-8 wk (20-25g)	11	Commercial diet + saline	*L. acidophillus*	10^{10} cfu/ml	8, 10 and 12 wk	*Apc* (Min/+)

Kumar et al, 2010[35]	India	Rats ?	?	10 wk	25	Experimental basal diet	Probiotic curd Probiotic cultures: *L. acidophilus* + *L. casei*	?	15 wk	DMH (20mg/kg) s.c.
Narushima et al, 2010[36]	Japan	rasH2 mice	♂/♀	8 wk	?	Commercial diet + non-fermented milk	Yogurt (*L. delbrueckii* + *Streptococcus salivarius*)	?	3 wk	DMH (20mg/kg) s.c.
Foo et al, 2011[37]	Taiwan	ICR mice	♂	6 wk	5-18	Semi-purified diet (AIN-76A) + skim milk	*B. longum* *L. gasseri*	*B. longum* ~ 5 x 10^9 cfu/g *L. gasseri* ~ 1 x 10^{11} cfu/g	15/24 wk	DMH (20mg/kg) i.m.
Chang et al, 2012[38]	Korea	F344 rats	♂	5 wk (average 130g)	15	High-fat diet (HF)	*L. acidophilus*	2 x 10^9 cfu/ml	10 wk	DMH (20mg/kg) i.m.
Verma and Shukla, 2013[39]	India	Sprague Dawley rats	?	? (100-150g)	6	Commercial diet + saline	*L. rhamnosus* *L. casei* *L. acidophilus* *L. plantarum* *B. bifidum*	1 x 10^9 cfu	7 wk	DMH (20mg/kg) i.p.

Urbanska et al, 2014[40]	Canada	C57BL/6J-Apc Min/+	♂	5-6 wk	24	Commercial diet + saline	*L. acidophilus* + 2% yogurt	?	17 wk	*Apc* (Min/+)
Mohania et al, 2014[41]	India	Wistar rats	♂	3 wk	24	Experimental basal diet + buffalo milk (BM)	Dahi culture *L. acidophilus* + *B. Bifidum*	Dahi culture 1% 2 x 10^9 cfu/g, *B. bifidum* and *L. acidophilus* each	8, 16 and 32 wk	DMH (40mg/kg) s.c.
Verma and Shukla, 2014[42]	India	Sprague Dawley rats	♂	? (100-200g)	8	Commercial diet	*L. acidophilus* *L. rhamnosus*	1 x 10^9 lactobacilli	19 wk	DMH (20mg/kg) i.p.
Walia et al, 2015[43]	India	Sprague Dawley rats	♀	? (125-175g)	6	Commercial diet	*L. plantarum* *L. rhamnosus*	10^{10} cells	8 and 16 wk	DMH (30mg/kg) s.c.
Shin et al, 2016[44]	Japan	BALB/c mice	♀	5 wk	6	Commercial diet	*L. plantarum*	10 mg	3 wk	Meth-A tumor cells (1 x 10^6 cells) s.c.
Lenoir et al, 2016[45]	Argentina	C57BL/6 mice	♀	6 wk (22-25g)	30-35	Commercial diet	*L. lactis* *L. casei*	1% in the drinking water (average 1±0.4 x 10^9 cfu/mouse)	3, 4, 5 and 6 months	DMH (20mg/kg) s.c.
del Carmen et al, 2017[46]	Argentina	BALB/c mice	♀	6 wk (22-25g)	10	Commercial diet	*Streptococcus thermophilus** *Lactococcus lactis subsp. cremoris**	1 x 10^{10} cfu/ml in the drinking water (average intake ~ 3ml/animal/d	3 to 6 months	DMH (20mg/kg) s.c.

Irecta-Nájera et al, 2017[47]	Mexico	BALB/c mice ♀	14-16 wk (25±2g)	10	Commercial diet	*L. casei*	10^6 cfu	31 wk	DMH (20mg/kg) s.c.
Kahouli et al, 2017[48]	Canada	C57BL/6J Apc Min/+ ♂	4 wk	5	Commercial diet + saline	*L. acidophilus* + *L. fermentum*	1 x 10^{10} cfu	12 wk	*Apc* (Min/+)
Walia et al, 2018[49]	India	Sprague Dawley rats ♀	? (125-200g)	6	Commercial diet	*L. plantarum* *L. rhamnosus*	2 x 10^{10} cells	16 wk	DMH (30mg/kg) s.c.
Agah et al, 2018[50]	Iran	BALB/c mice ♂	6-8 wk	9-10	Commercial diet	*L. acidophilus* *B. bifidum*	1 x 10^9 cfu/g (1.5 g probiotics in water)	5 months + 10 days	AOM (15mg/kg) s.c.

Abbreviations: wk week; d day; ? absent or unclear information; USA United States of America; ♂ Male; ♀ Female; L. *lactobacillus*; B. *bifidobacterium*; DMH 1,2 dimethylhydrazine; AOM azoxymethane; s.c. subcutaneous; i.m. intramuscular; i.p. intraperitoneal; HAA heterocyclic aromatic amines; Apc (Min/+) germ line mutations in the APC gene that lead to spontaneously development of neoplasms; ICR Institute of Cancer Research; HF: high-fat diet resembling the one of some Western human population; Curd culture: *Lactococcus lactis* biovar. diacetylactis; Dahi culture: *Lactococcus lactis ssp. cremoris* and *Lactococcus lactis ssp. lactis* biovar diacetylactis; Yogurt culture: L. delbrueckii subsp. bulgaricus and *S. thermophilus*; *Lactococcus lactis* NZ isogenic non-catalase-producing strain; *Lactococcus lactis* KAT catalase-producing *L. lactis* strain; (*) genetically modified strains.

Table 2. Effects of different probiotics on the development of histopathological parameters in animal models of colorectal carcinogenesis.

Probiotic strain	Effect	Sample size	Outcomes	Percentage of inhibition (p value)*
Lactobacillus acidophilus	Reduction	n = 9	Incidences of colonic lesions [50]	57% ($p < 0.05$)
		n = 20	Colon cancer [17]	37% ($p < 0.02$)
		n = 12	Total number of ACF in colon [25]	29-39% ($p < 0.01 – 0.001$)
		n = 15	Total number of ACF in colon [38]	41.1% ($p < 0.05$)
		n = 6	Percentage of ACF [39]	96% ($p < 0.05$)
		n = 11	Adenomas in large intestine [34]	12.5-50% ($p > 0.05$)
	Without alteration	n = 8	Incidence of tumor [42]	0% (p ?)
Lactobacillus rhamnosus	Reduction	n = 21	Small intestinal tumors [21]	28.5% ($p < 0.02$)
		n = 6	Percentage of ACF [39]	98% ($p < 0.05$)
		n = 8	Incidence of tumor [42]	11.12% (p ?)
		n = 6	Tumor incidence [43]	33.4% (p ?)
Lactobacillus bulgaricus	Without alteration	n = 28	Colon tumor [18]	0% ($p > 0.05$)
Lactobacillus casei	Reduction	n = 6	Percentage of ACF [39]	45% ($p < 0.05$)
		n = 5	Number of damage score [45]	45% ($p < 0.01$)
		n = 10	Number of ACF [47]	68.1% ($p < 0.01$)
Lactobacillus plantarum	Reduction	n = 6	Percentage ACF [39]	89% ($p < 0.05$)
		n = 6	Tumor incidence [43]	50% (p ?)
Bifidobacterium longum	Reduction	n = 11	Number of ACF [19]	43-53% ($p < 0.01-0.001$)
		n = 10	Total number of ACF in colon [22]	23.3% ($p < 0.05$)
		n = 12	Intestinal tumor incidence [23]	31.2% ($p < 0.05$)
		n = 9	Number of microadenomas and adenomas [37]	35-43% ($p < 0.05$)
Bifidobacterium bifidum	Reduction	n = 6	Percentage ACF [39]	74% ($p < 0.05$)
		n = 9	Incidences of colonic lesions [50]	27% ($p > 0.05$)
Bifidobacterium longum + Lactobacillus gasseri	Reduction	n = 9	Number of ACF [37]	25-30% ($p < 0.05$)
Lactobacillus acidophilus + fermentum	Reduction	n = 5	Intestinal polyp [48]	40% ($p < 0.05$)
Mix[1]	Reduction	n = 10	Number of tumors [46]	100% (p ?)

ACF aberrant crypt foci; n number of animals in the treatment groups; ? absent or unclear information.
[1]Mix: Genetically modified *S. thermophilus* strain that produces the antioxidant enzymes catalase and superoxide dismutase, combined with *L. lactis* IL-10. *Results extracted from the original studies.

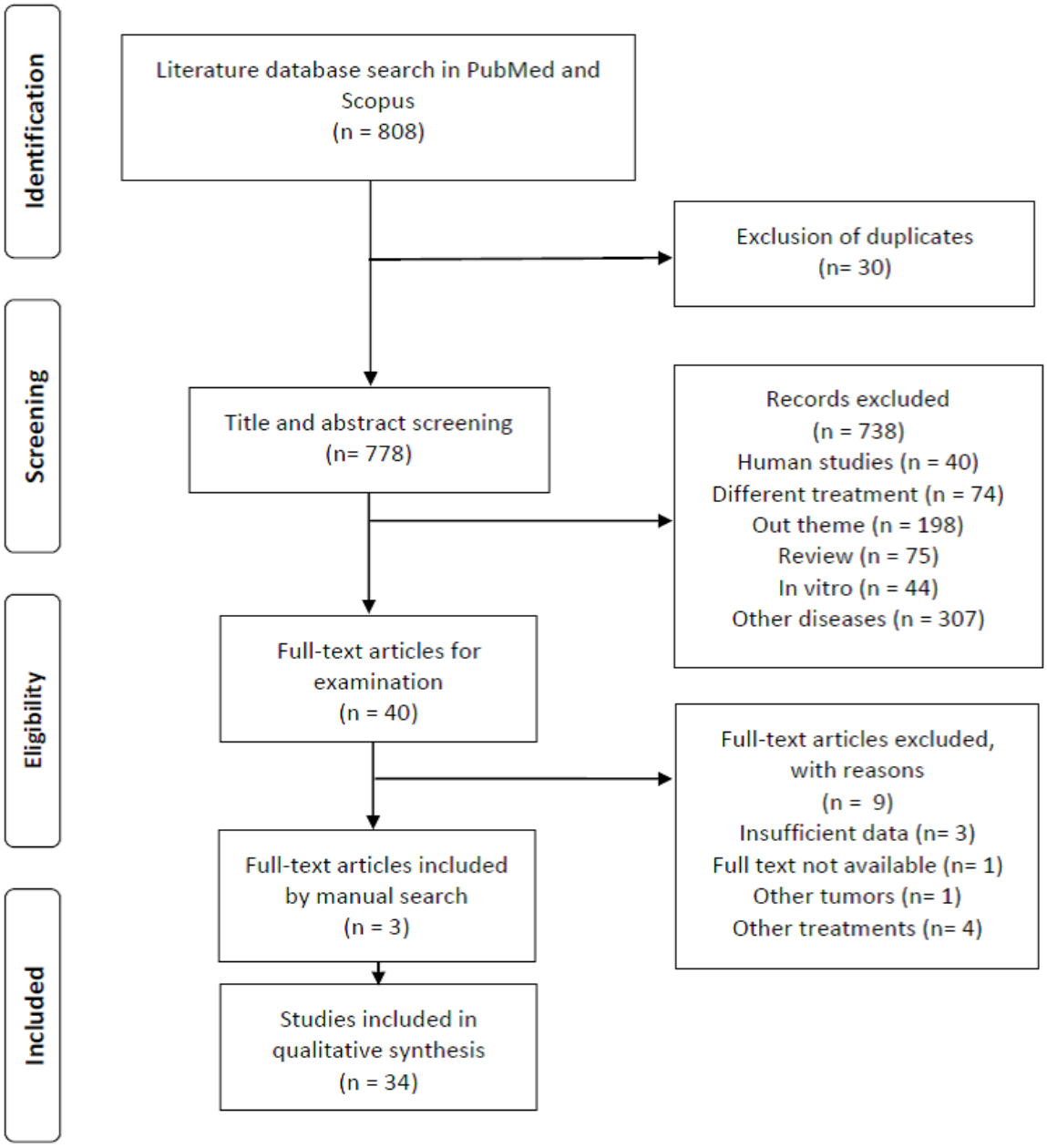

Figure 1. Flow diagram of the search results of our systematic literature review. Based on "Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement". www.prisma-statement.org From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009).

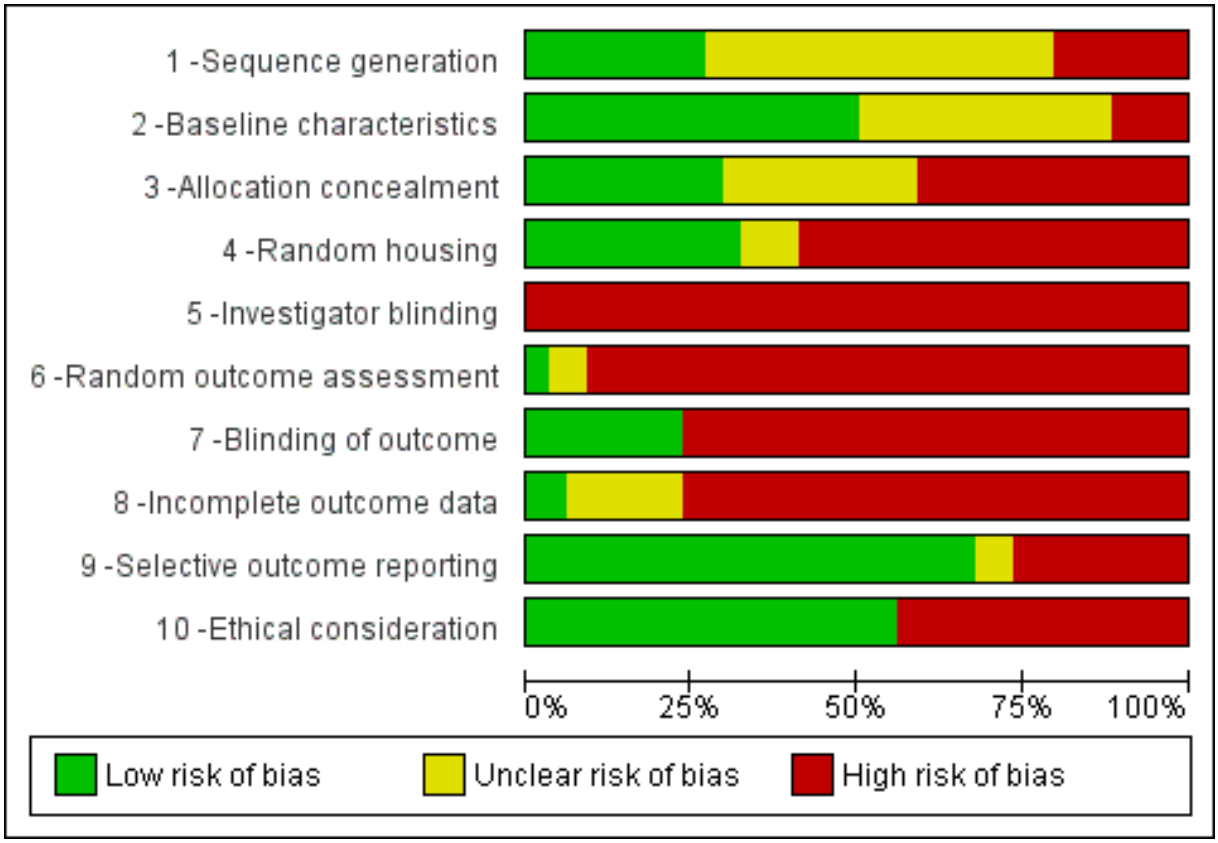

Figure 2. Evaluation of the animal studies using SYCLE's risk of bias tool for animal studies.

Kefir regulates inflammatory cytokines and reduces DMH-associated colorectal cancer in adult Wistar rat offspring

Abstract

Nutritional changes during critical periods of development, as lactation and puberty, have an impact on the risk of developing disease in adult life. In this sense, the neonatal overfeeding may result in altered programming leading to increased susceptibility to obesity, inflammation, and related complications. This study investigated the programming effects by kefir/overfeeding during the lactation and puberty period on adulthood offspring in 1,2 dimethylhydrazine (DMH)-induced experimental colon carcinogenesis, about adiposity, inflammation, gut microbiota and colorectal cancer development. Lactation Wistar rats were assigned to four groups: Control (NL, n=7 pups); Kefir control (KNL, n=8 pups); Overfeeding (SL, n=7 pups); Kefir overfeeding (KSL, n=7 pups). Dams in the NL and SL groups were given 1 ml distilled water by gavage once per day. For the other test groups, animals were given 1 ml of milk kefir (10^8 cfu/mL) by gavage once per day during the 21 days of lactation. After weaning, all pups continued receiving the same maternal treatment (water or kefir) until 60 days of age. In adulthood (24 weeks after the last application of DMH), the SL group showed biggest sum of adipose tissues compared to NL (+53.83%; $p < 0.001$), KNL (+48.85%; $p < 0.001$) and KSL (+20.04%; $p < 0.01$) groups. The kefir suppressed significantly the tumor number, even in the overfeeding group (KSL: -71.43%; $p < 0.01$). There was increased of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) in colon tissue of SL group. For nitric oxide production was observed an increase in SL rats, but was reduced by kefir administration (KSL group) (-69.9%, $p < 0.001$). We investigated for the first time the effects of kefir consumption during critical periods of development and identified its ability to reduce colon tumors, tissue damage, and proinflammatory cytokines, decrease adiposity and modulate the intestinal microbiota of adult offspring.

Keywords: Colorectal neoplasms; kefir; overnutrition; microbiota; inflammation.

Introduction

The “developmental origins of health and disease” (DOHaD) hypothesis proposes that environmental conditions during fetal and early post-natal development influence lifelong health through permanent effects on growth, structure and metabolism, known as ‘programming’. Environmental conditions that are experienced in early life can profoundly influence human biology and long-term health. The window of developmental plasticity extends from preconception to early childhood and involves epigenetic responses to environmental changes, which exert their effects during life-history phase transitions. The epigenetic responses influence development, cell- and tissue-specific gene expression, and could be transmitted transgenerationally (1,2).

A model widely used for induction of overfeeding in newborn rat pups is to reduce the litter size for newborn rat pups to 3 pups/dam (small litter; SL). Due to the increased consumption of milk, SL rats were overweight as well as hyperinsulinemic, hyperleptinemic and hyperglycemic during the suckling period. In the post-weaning period, SL rats demonstrated hyperphagia and maintained increased body weight gain throughout life, insulin resistance, and increased oxidative stress (3–5).

It is known that excessive adipose tissue accumulation, mainly visceral, triggers a series of metabolic and immune changes that contribute to the generation of low-grade chronic inflammation and also been associated with dysbiosis and increased intestinal permeability, which may contribute to perpetuation of inflammation(6), which represents a favorable microenvironment for tumor development(7).

The gut dysbiosis mediated inflammation and the consecutive regulation of innate and adaptive immune responses might constitute a link with the initiation, development and progression of cancer (8,9). Data show that various metabolites derived from the microbiota might control several factors playing a key role in the regulation of epigenetics (10). The composition of gut microbiota affects the health status of the host, specifically associated with the development of obesity, creating a microenvironment favorable to neoplastic development. The microbiota is involved in the energy balancing, intestinal integrity, and immunity against invading pathogens; thereby microbiota controls the overall health status of the host (11,12). It was already demonstrated that a significant difference in intestinal bacterial exists between healthy rats and colorectal cancer animals (13).

Data published by the International Agency for Research on Cancer (IARC), estimated the occurrence of 18.1 million new cases and 9.6 million cancer deaths worldwide in 2018. For colorectal cancer, 1.8 million new cases and 881,000 deaths are estimated to occur in 2018, representing the third neoplasia in incidence and second in cause of mortality (9,14,15). There is evidence that indicates the role of diet in the development of colorectal cancer. Dietary compounds may influence pathways by which carcinogens are metabolized and epigenetic changes that lead to cancer development(16,17). There is an indication that some probiotics strains can affect the host's immunologic response, stimulating anti-inflammatory cytokines, antioxidants and anti-carcinogenic compounds (18,19).

For this reason, the kefir, probiotic fermented milk, produced from grains and containing a complex mixture of bacteria, yeasts in association with a matrix composed of protein, and polysaccharide, has gained evidence. Health effects attributed to the consumption of kefir include modulation of intestinal microbiota, antioxidant action, immunomodulation, and metabolic effects (20,21). Previous studies suggest that the probiotics, especially lactic acid bacteria have different anticancer properties. The mechanisms involved include the interaction with several cellular pathways and regulate biological processes (antioxidative process, apoptosis and proliferation), activation of macrophages and phagocytosis and nitric oxide (NO) production, secretion of cytokines, and suppressed Th2 immune response and activated Th1 immune response that induce anti-allergic effect (22–25).

Thus, concerning the long history of kefir, its probiotic effects, ease of preparation and low cost, we investigated for the first time the effects of its consumption during critical developmental periods about intestinal microbiota, inflammatory biomarkers and the development of colorectal carcinogenesis in adulthood progeny.

Materials and methods

All procedures involving animals were conducted in accordance with local regulations (Brazilian Council for Control of Animal Experimentation, CONCEA, Brazil). The study was approved by the Ethical Committee for Animal Handling of the Federal University of Juiz de Fora at Minas Gerais, Brazil (protocol 21/2016).

Experimental design and procedures

Three-month-old Wistar rats (200–250 g) were obtained from the Center of Reproduction Biology of the Federal University of Juiz de Fora, Minas Gerais, Brazil. On the first day after the birth of the puppies, the litters were adjusted to three males pups for each dam (small litter, SL), and ten pups for each dam were considered normal litter (NL)(3). At the beginning of the study (birth), there were no differences between the offspring of groups in body weight (NL: 6.15±0.05; KNL: 5.97±0.06; SL: 6.08±0.05; KSL: 6.20±0.04). All animals were housed in plastic cages under controlled conditions of humidity (44-65%), light (12h light/dark cycle) and temperature (22±2°C). All rats had free access to normal rat chow and water.

To evaluate the effects of kefir intake of kefir/overfeeding during lactation, the rats with their offspring were randomly divided into four groups: Control (NL, n=7 pups); Kefir control (KNL, n=8 pups); Overfeeding (SL, n=7 pups); Kefir overfeeding (KSL, n=7 pups). Offspring from different litters per group were used to avoid litter effects (NL: 6 litters; SL: 7 litters). Dams in the NL and SL groups were given 1 ml distilled water by gavage once per day. For the other test groups, animals were given 1 ml of milk kefir (10^8 cfu/mL) by gavage once per day during the 21 days of lactation. After weaning, all pups continued receiving the same maternal treatment (water or kefir) until 60 days of age (Figure 1). In addition to their respective treatments, all animals were allowed free access to standard rodent chow (Nuvilab®, Paraná, Brazil) and drinking water *ad libitum*.

At 67 days of age, started to the induction of colorectal carcinogenesis was performed. All animals received an intraperitoneal injection of 1,2-dimethylhydrazine (DMH, Sigma-Aldrich, St. Louis, MO, USA), at a dose rate of 40 mg/kg body weight, twice weekly for 2 consecutive weeks. DMH was prepared fresh before use dissolved in 0.9% saline solution containing 1 mM EDTA and 10 mM sodium citrate, pH 8 (26).

At 240 days old, 24 weeks after the last DMH injection, the animals were euthanized with a lethal dose of Ketamine (90 mg/kg) and Xylazine (10 mg/kg), and the tissues and feces were collected and stored in the freezer at - 80°C until analysis.

Preparation and analysis of milk kefir

Kefir grains used in the study were obtained from the Department of Nutrition and Health, Universidade Federal de Viçosa, Viçosa, Brazil. Prepared with pasteurized whole milk (Benfica®, MG, Brazil) was added to kefir grains on a 1:10 (m/v), and was incubated at 25±2 °C for 24h. At the end of the incubation, the grains were separated from the kefir drink by filtration through a plastic sieve and washed. This procedure was repeated daily for the fresh kefir during the treatment period (20). A sample of milk kefir was collected and analyzed for the chemical composition according to reference methods of the Association of Official Analytical Chemist - AOAC (27).

Periodically, twice a week, during the whole period of the treatment of animals was determined the concentration of viable microorganisms in kefir appropriate dilutions in 0.85% physiological saline. The homogenized samples were serially diluted, and were then plated using specific media by the pour plate method. The MRS (Man-Rogosa-Sharpe) agar for lactic acid bacteria (LAB) was used for the enumeration of bacteria in respective plates. The plates were incubated at 37°C for 24–48 h aerobically. For yeast count the sample was grown on potato dextrose agar (PDA) slants at 25 °C for five days. The number of colonies was counted as colony forming unit cfu/mL. The experiment was done in triplicate(28).

The kefir used for the animal treatment has been analyzed in the laboratory and contained 2.9g/100g fat, 3.1g/100g protein, 4,1g/100g lactose, 0.7g/100g ash, and 89.2g/100g moisture. The pH ranged between 4.14 and 4.3. Throughout the period the count of 10^8 cfu/mL of LAB and 10^6 cfu/mL for yeasts was maintained, thus meeting the values proposed by international body Codex Alimentarius (29).

Nutritional evaluation, adiposity and anatomical characteristics of organs

Body mass (BM) of the offspring were monitored daily during lactation. The food intake (FI) and BM were evaluated once every 4 days after weaning until they were 240 days old. Food conversion efficiency (FCE) was calculated, dividing the body weight gain by food consumption.

Total body fat was measured as the sum of the following individual fat pad weights: epididymal fat + retroperitoneal fat + visceral fat. The adiposity index was

calculated as (total body fat/final BM) × 100. The adiposity index was used as a measure of adiposity (30).

The hepatosomatic index was obtained by dividing the liver weight by the BM of the animal x 100. After intact removal of the colon from the abdominal cavity, colon length was measured from the ileocecal valve to the anus in a relaxed position without stretching, using a millimeter ruler.

Tumors counting and analysis of aberrant crypt foci

A thorough necropsy was then made, and the vital organs including the liver, the spleen, the small bowel intestinal, the brain, and the colon were scrutinized for lesions and metastatic deposits. The number, tumor incidence (percentage number of animals having tumors) and location of tumors were assessed.

After removal, the colon was washed in saline solution, opened along the mesenteric margin, placed in paraffin plates with the mucous facing the top of the plate, and fixed. Following fixation, the number of aberrant crypts foci (ACF) were determined in the proximal, medial and distal segments, and were also expressed for the entire colon; stained with 0.1% methylene blue for 30 seconds to quantify aberrant crypt foci under a BX-60 light microscope (Olympus, Tokyo, Japan) with a magnification of 10x. The number of ACF was counted, as described by Bird(31). The ACF categorization was based on the number of aberrant crypts per focus: 1, 2, 3, 4 and foci with five or more aberrant crypts (AC/focus≥5). All tissue was assessed for two independent assessors in a blind fashion.

Histological evaluation of the colon

Colon was fixed in 10% buffered formalin for 48h. For histological examination, the fixed tissues were embedded and sectioned at 5 mm intervals. Tissue was stained with standard hematoxylin and eosin (H&E) for light microscopic examination, and examined under a light microscope (with 10x and 40x magnification). Two independent assessors in a blind fashion reviewed tissue sections. Any discrepancy between these two investigators was resolved through the reevaluation until a consensus of opinion was reached. Histopathologic evaluation was performed according to the three parameters edema, inflammation and crypt damage severities (32).

Cytokine measurement

For measurement of the cytokine levels, 100 mg tissue (medial colon) from each animal was homogenized in 1 ml PBS buffer containing 0.05% Tween 20, 0.5% bovine serum albumin and protease inhibitors (0.01 mM EDTA) and 20 IU aprotinin A, using a tissue homogenizer (PHD Equipamentos MTDHH4; Piracicaba, SP, Brazil). The resulting homogenate was centrifuged (13.500 rpm for 20 min. 4°C) and the supernatant was stored at -80°C for further cytokine quantitation. Interleukin-1 (IL-1β) (assay sensitivity: 63-4000 pg/mL), IL-6 (assay sensitivity: 31-2000 pg/mL), interferon gamma (INF-γ) concentrations, and tumor necrosis factor alpha (TNF-α) were measured by the cytokine sandwich ELISA kit (PeproTech Inc., Rocky Hill, New Jersey, USA) following the manufacturer's instructions. The results of colonic tissue cytokine levels were expressed as pg/mL.

Determination of tissue nitric oxide

Measurement of total nitric oxide (NO) accumulation in colonic tissue was performed based on the method developed by Miranda et al., which is based on the reduction of nitrate by vanadium III chloride to nitrite combined with detection of total nitrite by Griess reaction(33).

Microbiota analysis using 16s rRNA high-throughput sequencing and bioinformatics

Caecal samples were randomly chosen from the groups of NL (n = 5), KNL (n = 5), SL (n = 5), and KSL (n = 5) for gut-microbiota analysis. DNA was isolated from samples using the MagaZorb® DNA Mini-Prep Kit (Promega, Madison, WI, EUA). A high-throughput sequencing on the Illumina MiSeq platform for each sample was performed at the GenOne Biotechnologies enterprise (Rio de Janeiro, Brazil). Hypervariable regions of V3–V4 of bacterial 16S rRNA genes were sequenced and data obtained, assigned and analysed.

On the DNA samples extracted were performed highly accurate and precise DNA electrophoresis with the Bioanalyzer DNA analysis and used to amplify a small (~300 bp) fragment of the 16S rRNA gene using the primers F515 (50 GTGCCAGCMGCCGCGGTAA30) and R806 (50GGACTACHVGGGTWTCTAAT30) for further high-throughput sequencing. PCR reactions and 16S sequencing were

performed at the Molecular Research LP (MRDNA, Shallowater, Texas USA). The MiSeq instrument (Illumina, San Diego, USA) was used for sequencing the 16S amplicons following the manufacturer's instructions. This technology has been used in several studies and is recommended by the Earth Microbiome Project (33). Raw 16S data were obtained and analyzed using the freely available bioinformatics pipeline QIIME v.1.8 with default parameters. MRDNA conveniently provides users with files containing joined reads (full.fasta and full.qual files). These files were combined in one single fastq file using QIIME.

A set of sequences at a similar level of 97% was grouped into one operational taxonomic unit (OTU). The OTU table generated by this approach was used for all diversity and taxonomic analyses. In this study we used the v. 13_5 of the GreenGenes OUT representative 16S rRNA sequences as the reference sequence collection. The phylogenetic method UniFrac (Unique Fraction metric) was used to investigate differences in microbial communities. We conducted an analysis of the Good's coverage, diversity estimator (Shannon), and rarefaction curve. Statistical analysis of Bray–Curtis dissimilarities were calculated using the relative abundances of bacterial genera using Adonis function in R (version 3.2).

Statistical analysis

Data were analyzed by statistical program GraphPad Prism version 5 (GraphPad Software, Inc., La Jolla, CA, USA) and expressed as means ± standard error of the mean (SEM). The normality of the data was evaluated by the Kolmogorov-Smirnov test. For parametric analysis, one-way ANOVA followed by Newman-Keuls post-test determined the differences between the groups. Non-parametric Kruskal-Wallis test, followed by Dunn's multiple comparisons test was applied for values that do not present normal distribution. The differences were considered statistically significant when $p < 0.05$.

Results

Nutritional evaluation, adiposity and anatomical characteristics of organs

During the lactation, both SL and KSL offspring showed higher BM gain compared to groups NL and KNL ($p < 0.05$) from the beginning of lactation (D) until 21 days (Figure 2A). At weaning, the offspring from the SL and KSL groups presented

greater BM compared to the NL group (+34.84% and +30.24%, respectively, $p < 0.001$). These rats from small litters continued to weigh more than those from NL pups throughout the study (Figure 2B). At the end of the study, SL rats remained weighed more than NL rats (+21.78%; $p < 0.01$), KNL (+15.43%; $p < 0.05$) and KSL (+10.98%; $p > 0.05$) (Figure 2C). The animals have shown a great variation of FI, with SL and KSL group had a higher food intake during the part of the study. Groups NL and KNL presented a sporadic increase of FI along with the experiment (Figure 2D). No significant differences between groups have been observed in the food conversion efficiency (not presented).

In adulthood, the SL group showed biggest sum of adipose tissues compared to NL (+53.83%; $p < 0.001$), KNL (+ 48.85%; $p < 0.001$) and KSL (+20.04%; $p < 0.01$) groups. The animals of SL group maintained larger liver weight compared to NL (+26.02%; $p < 0.01$) and KNL (+22.59%; $p < 0.01$) groups (Figure 3A and C). No differences were found between the groups in the adiposity index and for the hepatosomatic index (Figure 3B and D).

Table 1 presents the results of weight of the cecum, colon weight and length, colon weight/length ratio. The caecal weight was significantly higher in KSL group compared to NL (+34.7%; $p < 0.05$), KNL (+61.26; $p < 0.001$) and SL (+30.85%; $p < 0.05$).

Tumors number and aberrant crypt focus

We investigated the effect of kefir on tumor growth in the DMH-induced colon cancer model. No tumors developed in the KNL group within the entire length of the experimental time. The kefir suppressed significantly the tumor growth, even in the overfeeding group (KSL: -71.43%; $p < 0.01$) (Figure 4A). The reduction of tumors was observed predominantly in the medial part of the colon where the majority of tumors were formed (Figure 4B). A representative image of tumors in the colon is shown in Figure 4C. The tumor incidence was 100% in animals of SL group, with a percentage of reduction of both, NL and KSL of 71.43% (table 2).

The colon mucosa of rats in the four DMH—groups, show any microscopical alterations compatible with the presence of ACF (Figure 5). The number of preneoplastic lesions in the colon did not present significant differences between

groups (total ACF; $p > 0.05$). ACF followed a regional distribution along the colon that was similar in all groups. Noteworthy, there were also no differences in the number of ACF considering the segments of the colon, proximal, medial and distal ($p > 0.05$) (Table 3).

The number of AC in each focus or crypt multiplicity was also determined (Table 3). Most of the lesions were formed by five or more crypt (49.5%), followed by the number of foci containing 3 crypts (15.7%), while thereafter, the foci comprising 2, 1 and 4 crypts were progressively lower, appearing at a 12%, 11.8% and 11%, respectively ($p > 0.05$).

Histological findings

Histopathological examination of colon tissues revealed severe loss of mucosal architecture associated with severe inflammatory cell infiltration and submucosal edema in SL rats. The colon of rat from group KSL showing moderate mucosal inflammatory cells infiltration associated with moderate edema; The colon of rat from group NL showing slight submucosal edema with inflammatory infiltration, and glandular dilation; Colon of rat from group KNL showing slight mucosal inflammatory cells infiltration, with no hyperplasia. The administration of kefir prior to DMH appear reduce hyperplasia and inflammatory cell infiltration (Figure 6). To evaluate the overall impact of the treatment procedure, after 24 weeks of induction, histology of the colon lesions was analyzed and compared with that of control animals.

Cytokine and nitric oxide measurement

Inflammatory cytokines showed a strong decrease in the concentration of IL-1β in the KNL group compared with NL (-66.65%; $p < 0.001$). The KSL group also presented diminution in the cytokine concentration versus SL group (-52.48%; $p < 0.001$) (Figure 7A). The KSL rats showed a decline in IL-6 compared to SL group (-14.35%; $p < 0.01$), that has also been observed for the KNL versus NL groups (-76.23%; $p < 0.001$) (Figure 7B). KNL animals had a decreased TNF-α production versus NL (-82.08%; $p < 0.001$) (Figure 7C). In addition, the release of IFN-γ, a cytokine critical to innate and adaptive immunity, and that functions as the primary activator of macrophages, was reduced in the KNL group compared to NL and SL groups (-15.5% and -17.87%, respectively, $p < 0.01$) (Figure 7D). For nitric oxide production was

observed an increase in overfeeding rats, but was reduced by kefir (KSL group) (-69.9%; $p < 0.001$). The KNL group also presented a reduction of the NO versus NL (-71.75%; $p < 0.001$) (Figure 7E).

Characterization of gut microbiota

To investigate the role of kefir and overfeeding in modulating the gut microbiota composition we evaluated whether kefir treatment under a murine model of colorectal cancer may induce changes in specific bacterial populations. The 16S rRNA amplicons obtained from DNA samples extracted from caecal contents were sequenced, resulting in 1,436,821 high quality sequences, ranging between 63,155 and 79,576 with an average value of 71,841 sequences per sample. Reads were clustered into 2,168 OTU based on 97% nucleotide sequence identity between reads. To assess whether sampling provided sufficient OTU coverage to describe the bacterial composition of each sample accurately, individually based rarefaction curves were generated for each sample (Figure S1). We used the Venn diagram to show the interrelationship of OTU in the caecal samples among different groups (Figure 8A).

According to the taxonomic results, for all groups, the three most commonly found phyla were Bacteroidetes, Firmicutes and Proteobacteria. Bacteroidetes accounting 61.62, 46.26, 47.16 and 23.34% of the gut microbiota in NL, KNL, SL and KSL groups, respectively, was the most predominant phylum in NL, KNL, and SL. While Firmicutes followed by Proteobacteria were the most predominant phylum in the KSL group. The overall microbial composition for each group at the phylum and family level is shown in Figure 8. At the genus level, our studies found the microbial composition differed significantly between the groups (Figure 9A).

The microbial alpha diversity was estimated based on the originally observed count values prior to any pre-processing. Figure 9B presents the Shannon index of the groups. The value of Good's coverage for each group was over 93%, indicating that the 16S rRNA sequences identified in the groups represent the majority of bacteria present in the study samples.

Discussion

Epidemiological and animal studies have indicated that changes during early life can have lasting effects on adulthood, which represent a critical window for

development (35–37). In the present study, we used a well-established animal model of reduction of litter size to study for the first time the role of neonatal overfeeding/kefir consumption on the development of colorectal cancer, and their relation to gut microbiota and inflammation. Neonatal overfeeding led to the greatest weight gain during the lactation (SL and KSL groups), which remained throughout life, programming to overweight in adult life (240 days), without consistent changes in food intake, but only in SL offspring, indicating a long-term protective effect of kefir.

The overfeeding animals also developed a greater sum of adipose tissue at adulthood, but in the KSL group occurred reduction in fat deposition compared to the SL group, demonstrating that early postnatal nutrition influences the inflammatory phenotype of adipose tissue induced by kefir. Gao et al. (38) investigated the role of milk kefir and observed that its administration for 8 weeks reduced the gain in body weight and abdominal fat mass of the rats. Other work has also identified that kefir peptides may act as an anti-obesity agent to prevent body fat accumulation and obesity-related metabolic diseases on high fat diet (HFD)-induced obesity in rats(39). Indeed, obesity is considered as the leading risk factor for metabolic diseases. Studies show that body fat is as a risk factor for the development of several cancers, including colorectal cancer. Among the mechanisms that associate obesity and cancer is chronic low-grade inflammation(12,40).

In addition, we observe that animals whose mothers received kefir during lactation and the offspring maintained their consumption at puberty, presented a reduction in the number and incidence of colon tumors induced by DMH. This animal model is one of the most frequently used for the study of chemopreventive agents since it develops morphological and histological features similar to those observed in colorectal cancer, which is sporadic and the most common in humans (41,42). Interestingly, the administration of the kefir totally inhibited neoplastic lesions in KNL group, these animals also shown lower adipose tissue than the KSL group, an important component that has a strong connection with inflammation(43). The protective effects of kefir have already been confirmed in previous studies with a murine model of colorectal carcinogenesis (44,45). However, this is the first study to assess the impact of their consumption during critical periods of development, and no adverse effects were observed. These results are consistent with the lack of toxicity reported for the kefir in animal models (25,46) and humans (47).

We have not found significant differences in ACF counts between groups; however, animals of the KNL group show a greater number of preneoplastic lesions. We believe that this can have been reflected in the delay in the development of tumors in this group, since animals have not presented tumors.

To the best of our knowledge, this is the first report to demonstrate the impact of kefir in critical period of development (lactation and puberty) to reduce DMH-induced colon tumors. We observe that neonatal overfeeding increase the tumor number (SL group), which was reversed by kefir administration (KSL group). The greater fat deposition in animals SL can justify the tumor development increased. It is known that excessive adipose tissue accumulation is associated with an increased risk of cancer, especially colon cancer(12).

The adipose tissue, especially the visceral, secretes various growth factors and cytokines that play a role in the low-grade, chronic inflammatory state that is linked to their obesity and subsequent cancer risk(48). Besides the function of energy homeostasis, the adipose tissue also acts as an endocrine organ, releasing hormones, growth factors and adipokines, the cell signalling proteins produced by adipose tissue(49). Epithelial cells, when stimulated, can produce immunomodulatory mediators that can interfere with neoplastic phenotypes such as angiogenesis and cell growth and survival(50). With the consequent release of inflammatory cytokines and adipokines from adipocytes and infiltrating immune cells, there are clear links between inflammation and dysregulated metabolic pathways, such as TNF production correlating with insulin resistance(49).

In this respect, we find a higher concentration of inflammatory cytokines, IL-1β, IL-6 and TNF-α in SL group, animals that also had greater tumor development and accumulation of adipose tissue. These results reinforce the role of adipose tissue in the state of the chronic inflammatory state characterized by progressive infiltration of macrophages and other immune cells, and that entails the increased adipose secretion of proinflammatory cytokines such as TNF, IL-1β and IL-6(51). The inflammation is an important hallmark of cancer, is related to cancer development through the production of free radicals, suppression of the immune system or aberrant cell signaling and upregulation of proliferative and anti-apoptotic pathways as well as angiogenesis and cell migration(52).

Epidemiologic and experimental studies have already shown an increase expression of IL-6 or other inflammatory markers in serum and tissue samples of patients with cancer or induced animals, besides correlates with poor prognosis(44,53–55). TNF exacerbates inflammation by recruiting inflammatory monocytes and neutrophils, and increasing the production of reactive oxygen species (ROS). The KNL animals showed less IFN-γ concentration in the colon; and although of the complexity of the role of IFN-γ in cancer, your signaling is involved in Th1-mediated immune responses, promotes the development of regulatory T cells, and alters the colonic epithelial barrier. The increased intestinal permeability can drive intestinal inflammation and promote colorectal cancer formation(56).

We also identified an increase of NO in the SL group compared to levels of the KNL and KSL groups, reinforcing the important role of kefir in the normalization of these values. NO can damage DNA, by direct or indirect means; interfere in your repair, and cause post-translational modification, leading to tumor initiation, establishment and progression(57). NO may mediate pro-tumorigenic activities, including capillary leakage, angiogenesis, leukocyte adhesion and infiltration, and eventually, metastasis(58). Therefore, justify the largest number of tumors identified in these overfeeding animals, since it the NO involved in the inflammatory process and carcinogenesis.

The gastrointestinal tract microbiota in development has been gaining evidence as essential to a potent and balanced immune system occurring during mammalian early life. The “hygiene hypothesis” concept involves insufficient microbial exposures early in life that predispose the individual to inflammation-associated pathologies later in life(59). There is suggested that gut bacteria-host signaling is continuous and reciprocal throughout life, constituting a vast gut immune-endocrine-brain signaling axis(40).

The role of the gut microbiota in the pathogenesis of colorectal cancer has been extensively studied, and it is known that dietary habits may cause relevant differences in the gut microbiota structure(60,61). In our study, overfeeding animals (SL) revealed a rise in *Lactobacillus* genera, a member of the *Lactobacillaceae* family and of Firmicutes phylum; which may be justified by the higher food intake of this group (SL), and consequently higher consumption of breast milk, favoring colonization by *Lactobacillus*, since breastfeeding is critical for the establishment of gut

microbiota(62,63). Furthermore, the caecal communities in overfeeding rats differed from the other communities in being less rich in terms of OTU content, and exhibit lower diversity index. Interestingly, the KNL group had the highest Shannon diversity index, and the values of the KSL group approached the control (NL).

In addition, the kefir in the KNL group led to the sharp increase of *Lactobacillus*, microorganisms comprising gram-positive bacteria that produce lactic acid as the major metabolic end-product of carbohydrate fermentation that contributes to the health status(64,65). Indeed, the kefir presents several health benefits, and as involved mechanisms are the anti-inflammatory, anticarcinogenic and antimicrobial activity, with an impact on gut microbiota and in the restoration of the intestinal barrier(66,67). A challenge for comparison of our results was the presence of a wide methodology difference with other studies, as the use of the diseased host, age of animals, caecal samples, age and period of treatment, that substantially influence the outcome. It is important to point out that this is the first study to evaluate the microbiota of adult animals programmed by kefir/overfeeding.

An impoverished microbiota might result in an immune deficit, whereas defects in innate immunity lead to an altered gut microbiota, which might transfer inflammatory and metabolic disease phenotypes upon faecal transplantation. Modulation of inflammatory and metabolic processes by the microbiota presents implications for several diseases beyond the gut, including diabetes, obesity and related complications. The interaction of microbiota, immunity, and metabolism begins in the intestinal epithelium. The immune and metabolic functions of the epithelium are functionally interconnected and inversely regulated; thus, disturbed host metabolism with excess fat storage might arise from defects in innate immunity(59). As microbial, inflammatory and metabolic signaling pathways are interlinked and are influenced by diet, it is suggested that identification and manipulation of the microbiota and/or alteration of the inflammatory response offer new therapies to the management of obesity related disease(70,71).

In conclusion, our data expand the knowledge on the role of kefir/overfeeding in colon cancer. The antitumorigenic effects of kefir are multiple in that it declined the adipose tissue and reduces inflammatory cytokines involved in the tumor microenvironment. These effects are evident with the reduced production of TNF-α, IL-1β, IL-6 and NO in the tissue, which are key molecules that link inflammation with colon

cancer; influenced by changes in mucosal function and structure as well as in the colonic bacterial microbiota. Therefore, knowledge of kefir antineoplastic effects may provide an interesting basis for a new prevention strategy early to colon cancer. Further clinical studies involving human subjects are required to clarify the role of kefir on colorectal cancer prevention and to investigate the potential combined effect of their dietary intake in association with a pharmacological strategy to obtain effective protection.

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Table 1. The caecal weight, colon weight, length and weight/length ratio.

Groups	Caecal weight (g)	Colon weight (g)	Colon length (cm)	Colon weight/length ratio
NL	1.70±0.09*	2.24 ± 0.20	20.13 ± 0.38	0.11 ± 0.008
KNL	1.42±0.10***	2.24 ± 0.21	21.41 ± 0.61	0.10 ± 0.007
SL	1.75±0.18*	2.44 ± 0.26	21.42 ± 0.71	0.11 ± 0.009
KSL	2.29±0.07	2.76 ± 0.21	21.67 ± 0.57	0.12 ± 0.008

Results are expressed as mean ± SEM (n = 6–8) and were analyzed by one-way ANOVA, followed by Newman-Keuls test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. KSL).

Table 2. Effect of kefir/overfeeding in development of colon carcinogenesis in the DMH rat model.

Groups	Colon tumor incidence	Total number of tumors	Percentage of inhibition
NL	57%	0.57 ± 0.20**	- 71.43%
KNL	0%	0**	- 100%
SL	100%	2 ± 0.57	0%
KSL	28%	0.57 ± 0.42**	- 71.43%

Results are expressed as mean ± SEM. Significantly different from SL group by one-way analysis of variance followed by Newman-Keuls test (* $p < 0.05$, ** $p < 0.01$).

Table 3. Effect of kefir/overfeeding on number of ACF in colon, number of crypt per focus and colonic segments.

Groups	Total of ACF	No. of crypts per focus					Segments		
		1	2	3	4	≥5	Proximal	Medial	Distal
NL	15.75±3.63	2.8±1.4	1.6±0.2	2.4±0.4	1.2±0.2	7.6±1.8	5.5±2.6	5.0±2.6	8.6±4.9
KNL	44.71±14.06	3.2±1.2	5.5±2.5	7.1±2.3	4.7±1.2	24.2±7.7	9.3±3.3	28.5±9.3	16.2±5.3
SL	14.33±5.48	4.0±4	1.3±0.3	1.3±0.6	2.3±1.2	6.6±1.8	2.6±2.6	4.0±2.6	7.3±2.8
KSL	17.00±11.12	3.0±1.7	3.0±2.0	3.6±3.1	2.3±2.3	10.0±5.1	0	4.5±4.5	3.0±1.5

Results are expressed as mean ± SEM (n = 6–7) and were analyzed by one-way ANOVA, followed by Newman-Keuls test. No significant differences ($p > 0.05$) were found between groups submitted at the different treatments. ACF aberrant crypt foci.

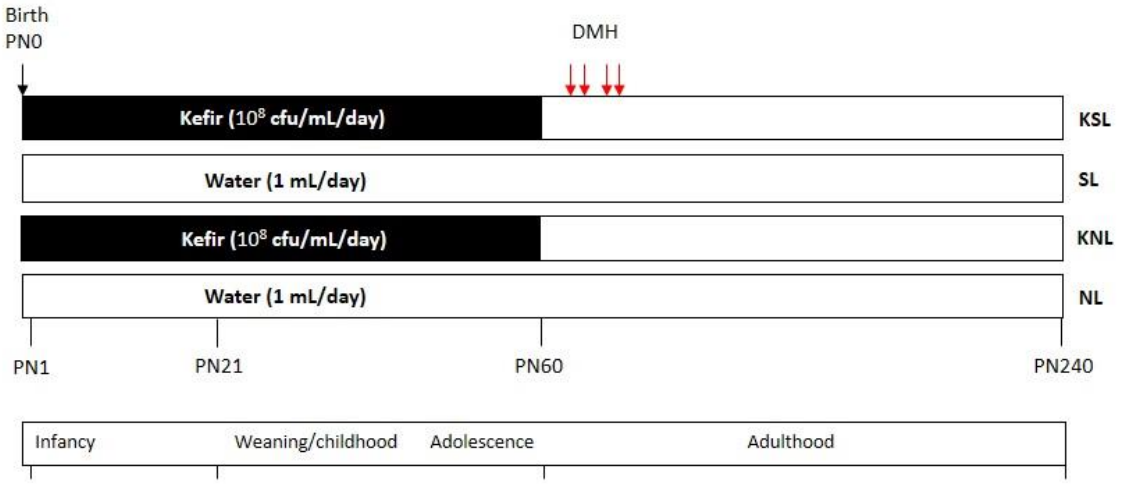

Figure 1. Experimental model. At PN1, litters from both diet dams were adjusted to either ten pups for normal litters (NL) or three pups for small litter (SL) groups. The groups of dams were maintained on treatment kefir or water, control component, for 21 days of the lactation period. Progeny were weaned to the same treatment as their dams. The four groups' litters received the treatment up to PN60 and after maintained on commercial chow until PN240, when the experiments were conducted. Delivery was considered PN0. Offspring (NL, KNL, SL, and KSL) received DMH (red arrows) at postnatal days (PN) 67, 69, 73 and 75. After 24 weeks, gastrointestinal tissues were evaluated for presence of tumors. Colony forming unit (cfu).

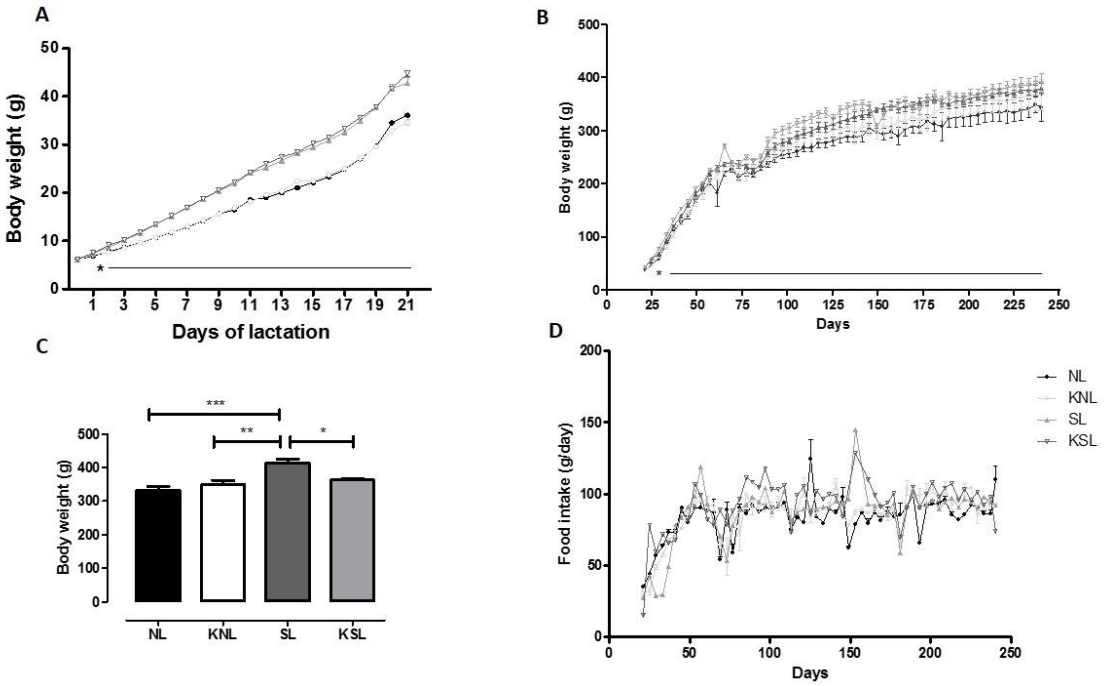

Figure 2. Body weight of Wistar rats during the lactation (**A**); Body weight of the groups that received 1,2-dimethylhydrazine (DMH) twice a week for two weeks until 240 days old (**B**); Body weight at 240 days old (**C**); Food intake at weaning until 240 days old (**D**). Results are expressed as mean ± SEM (n = 6–8) and were analyzed by one-way ANOVA, followed by Newman-Keuls test (* $p < 0.05$, ** $p< 0.01$, *** $p < 0.001$).

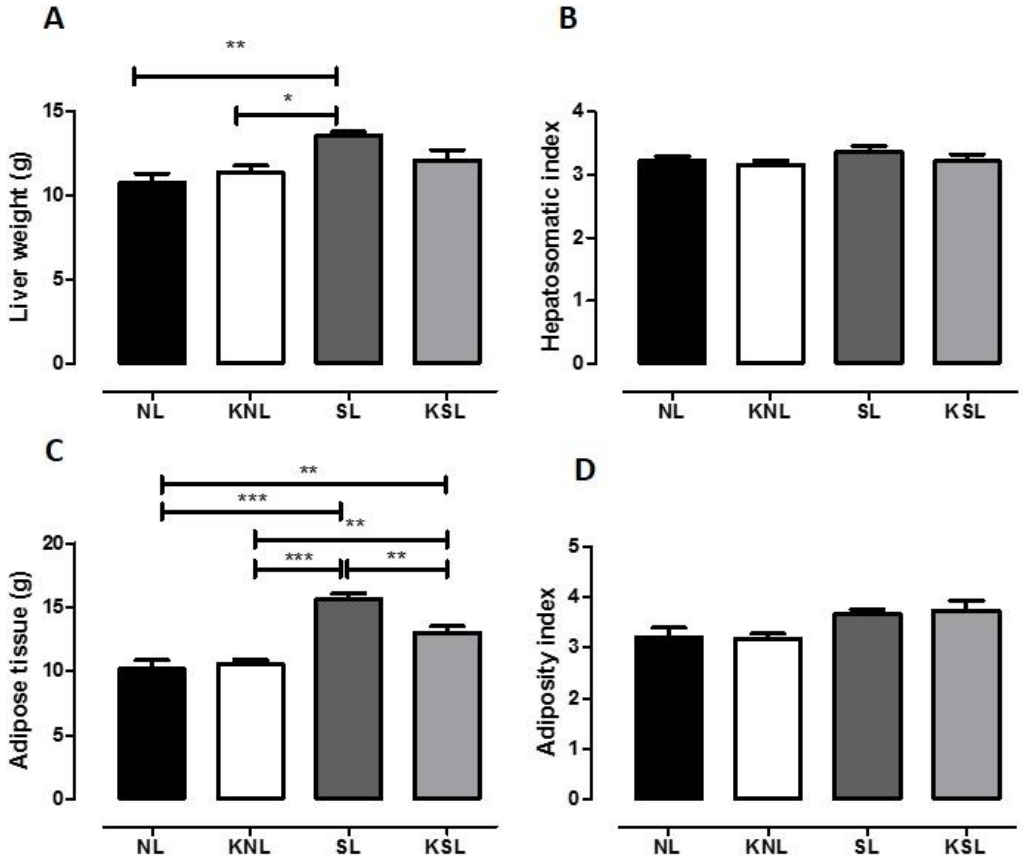

Figure 3. Liver weight (**A**); Hepatosomatic index (**B**); Sum of adipose tissue (**C**); Adiposity index (**D**). Results are expressed as mean ± SEM (n = 6–8) and were analyzed by one-way ANOVA, followed by Newman-Keuls test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

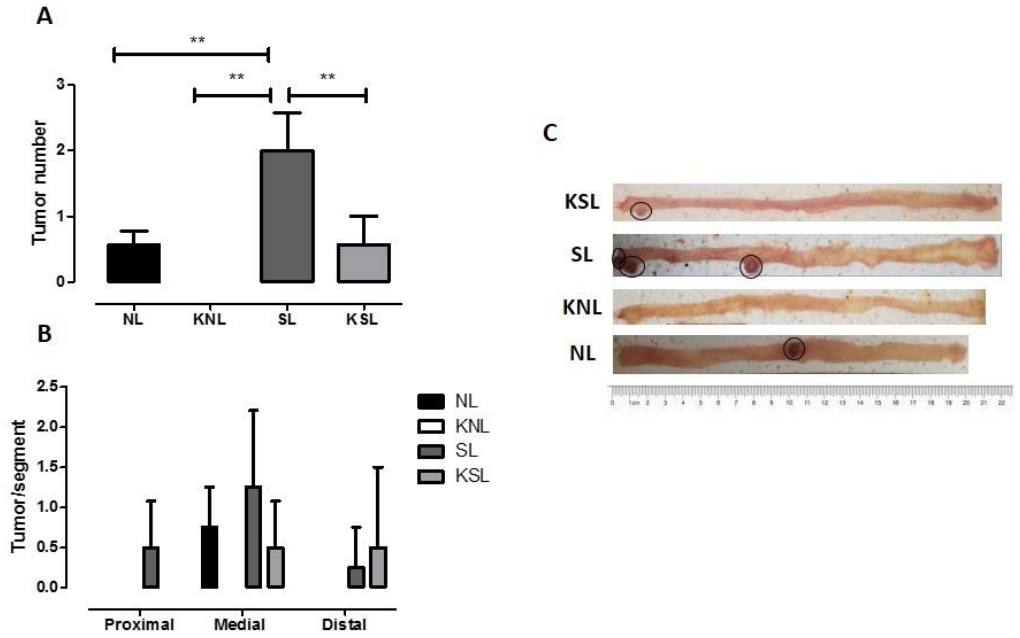

Figure 4. Tumor evaluation. In each colon, tumors were assessed the sum for number all tumor **(A)**, and in each part of colon was calculated **(B)**. Representative histological images of tumors is indicated with a circle **(C)**. Results are expressed as mean ± SEM (n = 7–8) and were analyzed by one-way ANOVA, followed by Newman-Keuls test (** p< 0.01).

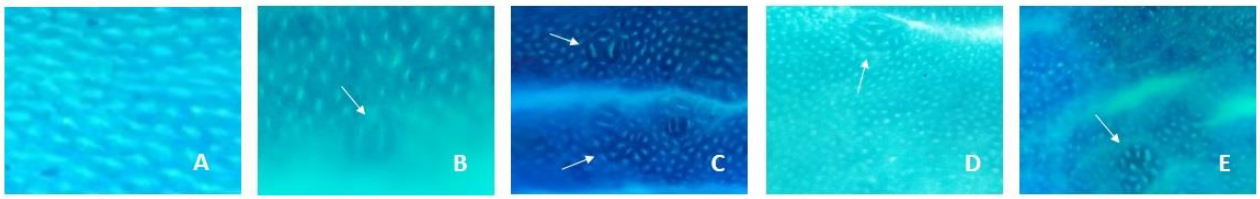

Figure 5. Aberrant crypt foci (ACF) observed under a light microscope after staining of the colon with methylene blue (magnification of x10). The images show the appearance of colon the of animals in (**A**) normal crypts; and a topographic view of ACF indicated by a white arrow (**B, C, D and E)**.

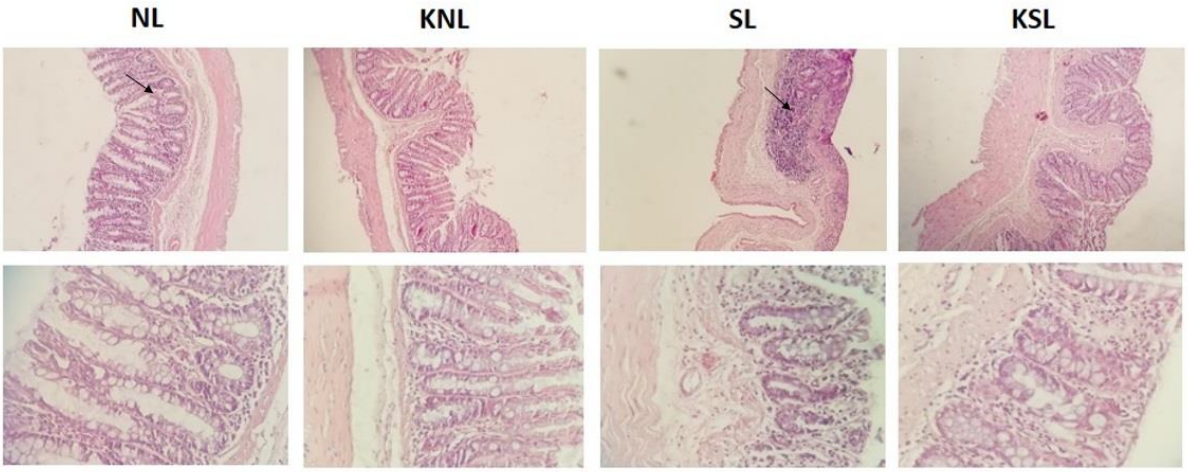

Figure 6. Representative photomicrographs of histological sections of the colon segments of NL, KNL, SL and KSL rat. Upper section, 10x magnification; lower section, 40x magnification. Normal litter NL, Kefir normal litter KNL, Small litter SL, and Kefir small litter KSL.

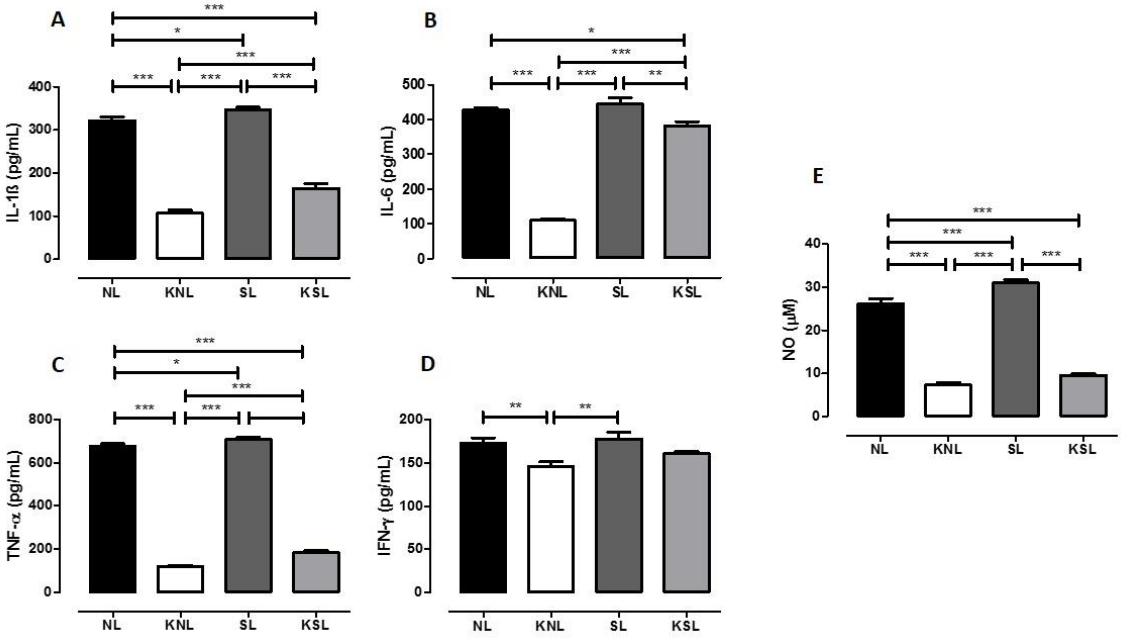

Figure 7. Concentration of inflammatory cytokines, IL-1β **(A)**, IL-6 **(B)**, TNF-α **(C)**, IFN-γ **(D)** and nitric oxide (NO) **(E)**. Results are expressed as mean ± SEM (n = 6) and were analyzed by one-way ANOVA, followed by Newman-Keuls test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

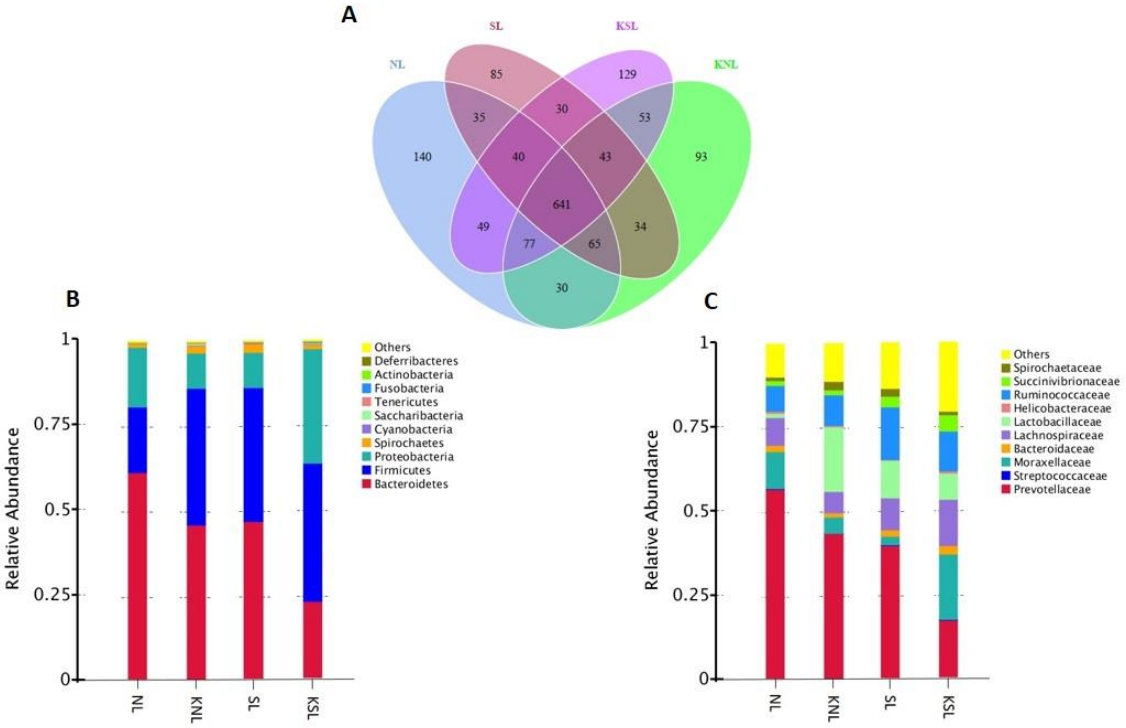

Figure 8. Venn Diagram based on the OTU among the samples of different groups **(A)**. The relative abundance of bacterial phylum **(B)** and family **(C)** in microbiota of each group. ‘‘Others’’ represents the unclassified bacteria.

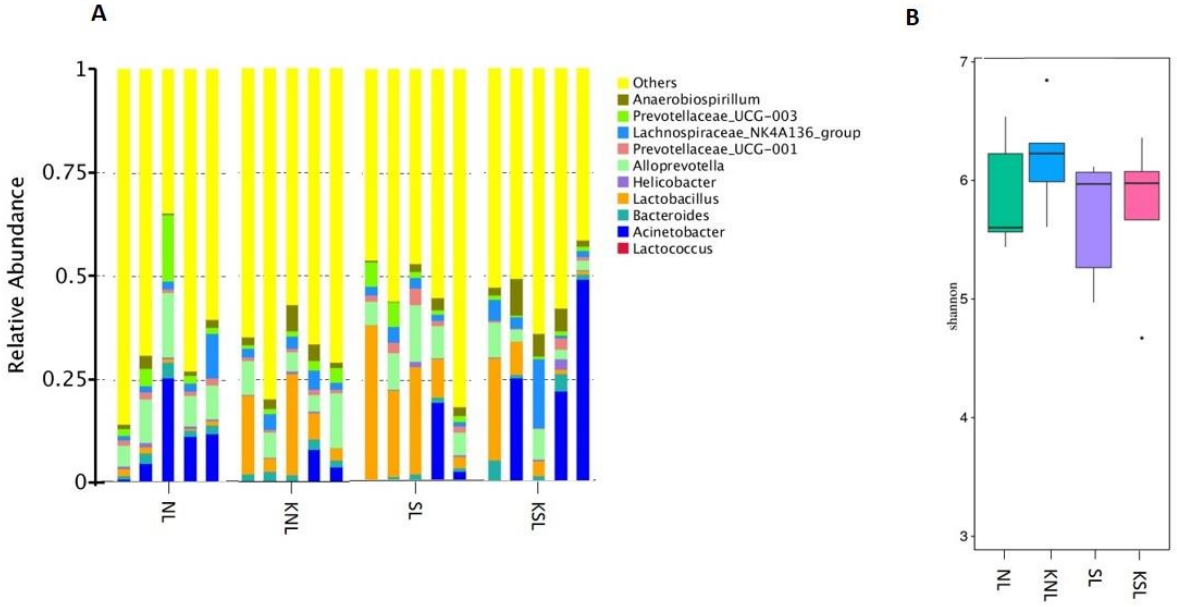

Figure 9. The relative abundance of bacterial genus in microbiota of each sample. ‘‘Others’’ represents the unclassified bacteria **(A)**. Shannon diversity index **(B)**.

Supplementary information

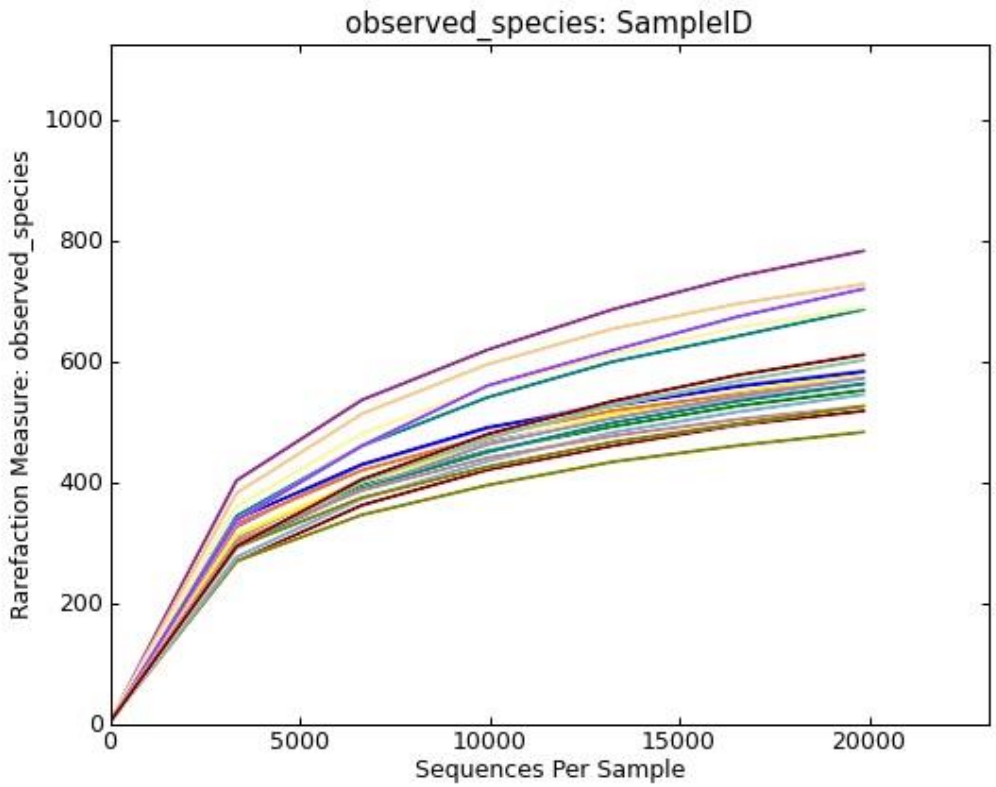

Figure S1. Rarefaction curves generated for each samples.

8. CONCLUSÕES GERAIS

Nossos resultados expandem o conhecimento sobre o papel do kefir e da superalimentação no câncer de cólon. Os efeitos antitumorigênicos do kefir são múltiplos, pois diminuem o tecido adiposo e reduzem as citocinas inflamatórias envolvidas no microambiente tumoral. Esses efeitos são evidentes com a produção reduzida de TNF-α, IL-1β, IL-6 e óxido nítrico no tecido, moléculas-chave que ligam a inflamação ao câncer de cólon; influenciado por alterações na função e estrutura da mucosa, bem como na microbiota bacteriana colônica. Portanto, o conhecimento dos efeitos antineoplásicos do kefir podem fornecer uma base interessante para uma nova estratégia de prevenção precoce ao câncer de cólon. Sugere-se que a ingestão materna de kefir durante a lactação e sua continuidade pela prole no pós-desmame até a pubertade, desempenhe papel essencial na prevenção ao desenvolvimento de tumores de cólon induzidos na prole adulta; contribuindo para integridade da mucosa intestinal e modulando a resposta inflamatória no cólon dos animais adultos. Estudos clínicos adicionais envolvendo seres humanos são necessários para esclarecer o papel do kefir na prevenção do câncer colorretal e a obtenção de proteção eficaz.

ANEXO 1

Aprovação pela Comissão de Ética no Uso de Animais (CEUA) da Universidade Federal de Juiz de Fora (UFJF)

SERVIÇO PÚBLICO FEDERAL
UNIVERSIDADE FEDERAL DE JUIZ DE FORA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

CERTIFICADO

Certificamos que o protocolo nº. 21/2016 – CEUA sobre "Avaliação dos efeitos do consumo materno de kefir na lactação sobre a microbiota intestinal, parâmetros metabólicos, inflamatórios e susceptibilidade à carcinogênese colorretal na progênie adulta de ratos *Wistar*, programados pela superalimentação neonatal" projeto de pesquisa sob a responsabilidade de SHEILA CRISTINA POTENTE DUTRA LUQUETTI com a colaboração de ANA PAULA BORONI MOREIRA, ALINE DE AGUIAR NEMER, NATHÉRCIA PERCEGONI, EDUARDA KINGMA e MAÍRA SCHUCHTER FERREIRA está de acordo com os Princípios Éticos na Experimentação Animal, adotados pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) da PRÓ-REITORIA DE PESQUISA/UFJF, em reunião realizada em 06/12/2016. Para o desenvolvimento da pesquisa serão utilizados 10 mães (90 dias) com ninhadas de 2 dias de 10 machos cada e 26 mães (90 dias) com ninhada de 2 dias de 3 machos cada de ratos Wistar totalizando 214 animais, conforme solicitado e que serão utilizados no período 01/01/2017 a 01/01/2019.

CERTIFICATE

We certify that the protocol nº. 21/2016 – CEUA about "Avaliação dos efeitos do consumo materno de kefir na lactação sobre a microbiota intestinal, parâmetros metabólicos, inflamatórios e susceptibilidade à carcinogênese colorretal na progênie adulta de ratos *Wistar*, programados pela superalimentação neonatal" under responsability of SHEILA CRISTINA POTENTE DUTRA LUQUETTI and collaboration of ANA PAULA BORONI MOREIRA, ALINE DE AGUIAR NEMER, NATHÉRCIA PERCEGONI, EDUARDA KINGMA and MAÍRA SCHUCHTER FERREIRA is in agreement with the Ethical Principles in Animal Research adopted by Brazilian Council for Control of Animal Experimentation (CONCEA) and was approved by the PRÓ-REITORIA DE PESQUISA/UFJF – ETHICAL COMMITTEE FOR ANIMAL HANDLING (CEUA) in 06/12/2016. For the development of the research will be used 10 mothers (90 days) with 2-day litters of 10 males each and 26 mothers (90 days) with 2 days' litter of 3 males each of Wistar rats totalizing 214 animals, will be delivered as requested in the period of 01/01/2017 to 01/01/2019.

Juiz de Fora, 19 de dezembro de 2016

Coordenadora
CEUA

Vice-coordenadora
CEUA