UNIVERSIDADE FEDERAL DE MINAS GERAIS
PROGRAMA INTERUNIDADES DE PÓS-GRADUAÇÃO EM BIOINFORMÁTICA

Larissa Gabriela Morais de Ávila

CARACTERIZAÇÃO DE KEFIR DE LEITE E IMPACTO DO SEU CONSUMO EM MODELO MURINO DE INFECÇÃO POR SALMONELLA TYPHIMURIUM

Belo Horizonte

2023

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Tese apresentada ao Programa Interunidades de Pós-graduação em Bioinformática da Universidade Federal de Minas Gerais como requisito parcial para obtenção do título de Doutor em Bioinformática.

Orientador: Dr. Tiago Antônio de Oliveira Mendes

Coorientadores:
Dra. Maria do Carmo Gouveia Pelúzio
Dra. Maria Cristina Dantas Vanetti
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ATA DE DEFESA DE TESE

LARISSA GABRIELA MORAIS DE ÁVILA

Às quatorze horas do dia **03 de abril de 2023**, reuniu-se, através de videoconferência, a Comissão Examinadora de Tese, indicada pelo Colegiado do Programa, para julgar, em exame final, o trabalho intitulado: **"Caracterização de kefir de leite e impacto do seu consumo em modelo murino de infecção por *Salmonella typhimurium*"**, requisito para obtenção do grau de Doutora em **Bioinformática.** Abrindo a sessão, o Presidente da Comissão, **Dr. Tiago Antonio de Oliveira Mendes**, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra à candidata, para apresentação de seu trabalho. Seguiu-se a arguição pelos Examinadores, com a respectiva defesa da candidata. Logo após a Comissão se reuniu, sem a presença da candidata e do público, para julgamento e expedição de resultado final. Foram atribuídas as seguintes indicações:

Professor(a)/Pesquisador(a)	**Instituição**	**Indicação**
Dr. Tiago Antonio de Oliveira Mendes - Orientador	Universidade Federal de Viçosa	**Aprovada**
Dra. Alinne Pereira de Castro	Universidade Católica Dom Bosco	**Aprovada**
Dra. Bruna Cristina dos Santos Cruz	Universidade Federal de Viçosa	**Aprovada**
Dr. José Miguel Ortega	Universidade Federal de Minas Gerais	**Aprovada**
Dra. Mariana de Moura e Dias	Centro Universitário Governador Ozanam Coelho	**Aprovada**

Pelas indicações, a candidata foi considerada: **Aprovada**

O resultado final foi comunicado publicamente à candidata pelo Presidente da Comissão. Nada mais havendo a tratar, o Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora.

Belo Horizonte, 03 de abril de 2023.

Documento assinado eletronicamente por **Tiago Antônio de Oliveira Mendes**, **Usuário Externo**, em 03/04/2023, às 18:37, conforme horário oficial de Brasília, com fundamento no art. 5º do Decreto nº 10.543, de 13 de novembro de 2020.

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RESUMO

Devido à problemática global da resistência antimicrobiana em bactérias, e a falta de sucesso e investimento em pesquisas que procuram novos antibióticos, são necessárias novas alternativas eficazes para reduzir infecções bacterianas. A Organização Mundial da Saúde, alerta que doenças transmitidas por alimentos contaminados (DTA 's) matam 420 mil pessoas no mundo por ano e uma em cada dez pessoas no mundo adoecem após sua ingestão. Entre as DTA´s, as bactérias do gênero Salmonella são a segunda maior causadora de infecções no Brasil. Uma possível solução é o uso de probióticos, que são alimentos com microrganismos viáveis capazes de conferir benefícios à saúde, quando administrados em dose pré-estabelecida. O Kefir, um probiótico originário do Cáucaso e Península Balcânica, é um alimento fermentado, em leite ou água, cuja fermentação, alcoólica e láctica, é conduzida pelos grãos de Kefir ou por fermento de Kefir. Este produto possui atividades antimicrobiana, anti-inflamatória e antioxidante; auxilia no tratamento de síndrome do intestino inflamado e doenças diarréicas. Neste trabalho foi realizada a caracterização microbiologica e físico-quimica de uma bebida láctea fermentada por kefir produzida em condições domésticas (25°C ± 2°C por 24 horas, sem agitação em aerobiose), bem como seu consumo por camundongos C57BL/6 e qual a relação com o eixo intestino-cérebro. Esse eixo descreve a participação de ambos órgãos em uma comunicação contínua e bidirecional. Novas tecnologias permitiram demonstrar que o estímulo digestivo pode ativar regiões do cérebro, assim como sintomas psicológicos podem causar transtornos na função gastrointestinal. Além disso, foi avaliado o efeito do consumo de Kefir neste modelo murino anterior ao desafio com *Salmonella enterica* subs. *enterica* Typhimurium. Todos os experimentos foram acompanhados de análise metagenômica para caracterização da comunidade bacteriana sob as condições avaliadas. A abundância relativa foi calculada e inicialmente, foi observado que animais tratados com Kefir tiveram maior abundância relativa de bactérias da família Lachnospiraceae e do gênero Lachnoclostridium, e menor abundância relativa de enterobactérias, como *Helicobacter* e *Shigella/E.coli*. Alguns benefícios previamente associados com o uso de kefir foram também encontrados, como aumento na produção de butirato e aumento de atividade de enzimas que participam do processo anti-oxidativo. Porém, diante da infecção, os animais alimentados com Kefir apresentaram maior mortalidade do que os animais tratados com água (grupo controle), associados a efeito inflamatórios (houve

aumento de IFN-ɤ e diminuição de IL-10) que comprometeram o controle da infecção, levando a falta de resposta imune, permitindo que *Salmonella typhimurium* causasse uma infecção grave. Desta forma, o uso indiscriminado de probióticos, especialmente em casos de infecções, devem ser utilizados com cautela, pois também podem estar associados a efeitos tóxicos ou indesejados.

Palavras-chave: Kefir; Metataxonômica; Metagenômica; *Salmonella* Typhimurium;Probiótico; Microbiota fecal; Diversidade bacteriana.

ABSTRACT

Due to the global problem of antimicrobial resistance in bacteria, and the lack of success and investment in research looking for new antibiotics, new effective alternatives are needed to reduce bacterial infections. The World Health Organization warns that diseases transmitted by contaminated food (Foodborne diseases) kill 420,000 people worldwide per year and one in ten people in the world become ill after ingestion. Among Foodborne diseases, bacteria of the genus Salmonella are the second major cause of infections in Brazil. A possible solution is the use of probiotics, which are foods with viable microorganisms capable of conferring health benefits when administered in predetermined doses. Kefir, a probiotic originating in the Caucasus and the Balkan Peninsula, is a fermented food, in milk or water, whose alcoholic and lactic fermentation is carried out by kefir grains or kefir yeast. This product has antimicrobial, anti-inflammatory and antioxidant activities; assists in the treatment of inflamed bowel syndrome and diarrheal diseases. In this work, the microbiological and physical-chemical characterization of a kefir-fermented milk drink produced in domestic conditions was carried out (25°C ± 2°C for 24 h, without agitation in an aerobic environment), as well as its consumption by C57BL/6 mice and the relationship with the intestine-brain axis. This axis describes the participation of both organs in continuous and bidirectional communication. New technologies made possible to demonstrate that digestive stimulation can activate regions of the brain, just as psychological symptoms can cause disturbances in gastrointestinal function. Furthermore, the effect of Kefir consumption in this murine model before the challenge with *Salmonella enterica* subs. *enterica* Typhimurium. All experiments were accompanied by metagenomic analysis to characterize the bacterial community under the evaluated conditions. The relative abundance was calculated and, initially, it was observed that animals treated with Kefir had a higher relative abundance of bacteria from the Lachnospiraceae family and the genus *Lachnoclostridium*, and a lower relative abundance of enterobacteria such as Helicobacter and Shigella/E.coli. Some benefits previously associated with the use of kefir were also found, such as an increase in the production of butyrate and an increase in the activity of enzymes that participate in the anti-oxidative process. However, given the infection, animals fed with Kefir showed higher mortality than animals treated with water (control group), associated with inflammatory effects (there was an increase in IFN-ɤ and a decrease in IL-10) that compromised the infection control, leading to a lack of immune response,

allowing Salmonella typhimurium to cause a severe infection. Thus, the indiscriminate use of probiotics, especially in cases of infections, should be used with caution, as they may also be associated with toxic or unwanted effects.

Keywords: Kefir. Metataxonomic; Metagenomic; *Salmonella* Typhimurium; Probiotic; Fecal microbiota; Bacterial diversity.

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SUMÁRIO

1. INTRODUÇÃO

Foodborne diseases, que é traduzido literalmente como Doenças transmitidas por alimentos, são doenças transmitidas por alimentos que foram contaminados em qualquer passo da cadeia produtiva, seja nas etapas de produção, processamento, distribuição, comércio, preparação e consumo. Existem mais de 200 doenças transmitidas por alimento que podem ser causadas por bactérias, vírus, parasitas ou substâncias químicas, como metais pesados. A maioria delas está associada a problemas intestinais, principalmente diarreia, podendo causar também sintomas neurológicos, ginecológicos e imunológicos. É um problema de saúde pública que afeta o mundo inteiro, embora ocorra principalmente em países subdesenvolvidos e em desenvolvimento (OMS, 2018).

Nos Estados Unidos, Austrália e União Europeia, *Salmonella* não-tifóide é a segunda maior causa de infecções, sendo a terceira causa na Coreia (Lee & Yoon, 2021). A maioria dos países subdesenvolvidos ou em desenvolvimento possuem poucos dados sobre essas doença, o controle da qualidade dos alimentos e sua produção, gera uma perda econômica ao país, alimentando um ciclo de pobreza (OMS, 2015). Além disso, o clima tropical presente favorece a proliferação de parasitas e produção de suas toxinas. Obviamente, muitos dos habitantes desses países vivem abaixo da linha da pobreza, o que leva a baixo acesso a água tratada, tratamento do esgoto, alimento de qualidade, remédios, desnutrição. Isso tudo favorece a um ciclo vicioso, onde esses indivíduos estão mais propensos a doenças, sintomas mais graves e consequentemente, a morte (OMS, 2015).

Embora o tratamento dos sintomas da salmonelose na maioria das vezes seja apenas repouso e hidratação, os sintomas podem evoluir e ser necessário o uso de antibióticos intravenosos (Ministério da Saúde, 2022). Infelizmente, no Brasil já foram identificadas cepas com resistências a beta-lactâmicos, sulfonamida, estreptomicina, tetraciclina, gentamicina e cefalosporina (Voss-Rech et. al., 2020). Considerando a tendência de mais cepas resistentes surgirem e a baixa probabilidade de novos antibióticos serem descobertos (Sutherland & Barber, 2017), tratamentos alternativos se tornam uma possibilidade atrativa.

Probióticos tem efeitos benéficos em seus consumidores, tais como: redução de inflamação, aumento na produção de IgA e na atividade de células ‘natural killers’, inibição de alergias, neutraliza toxinas e proteção contra patógenos (ASHAOLU, T. J., 2020). Estudos mostram o efeito antimicrobiano de microrganismos isolados de kefir

em determinadas quantidades contra *S. enteritidis* e *S*. Typhimurium (MIAO et al., 2014, 2016; JEONG et al., 2018; ABATEMARCO JÚNIOR et al., 2018), nos levando a conjecturar que a bebida kefir poderia ter efeito bactericida em caso de infecção por *Salmonella*.

***Salmonella* ssp.**

1.1 Classificação

De acordo com estudos moleculares, *Salmonella* ssp. podem ser classificadas em *Salmonella bongori* e *Salmonela enterica*, conforme recomendado pelo Centro de colaboradores da Organização Mundial da Saúde e adotada pelo *Centers for Disease Control and Prevention* (CDC) (BRENNER et al., 2000).

A gama de subespécies pertencentes à espécie *S. enterica* podem ser agrupadas em subespécies I, II, IIIa, IIIb, IV e VI. Cada subespécie pode ser subdividida em sorovares, também conhecidos como sorotipo, de acordo com testes sorológicos de seus antígenos somáticos (O), capsulares (Vi) e flagelares (H) (TRABULSI; ALTERTHUM, 2008; RYAN et al., 2017). Além disso, as subespécies (subs.) são classificadas como tifoides e não tifoides, para fins clínicos, sendo a maioria dos sorovares correspondente ao último grupo. *Salmonella* tifoides englobam *S.* Typhi e *S.* Paratyphi, enquanto as não-tifoides englobam principalmente *S*. Typhimurium e *S.* Enteritidis (JAY et al., 2008). A Figura 1 demonstra um resumo das classificações do gênero *Salmonella* em espécies, subespécies e sorovares. Por convenção, o nome do sorovar é escrito com a primeira letra maiúscula, sendo as demais minúsculas, e não se aplica itálico (RYAN et al., 2017).

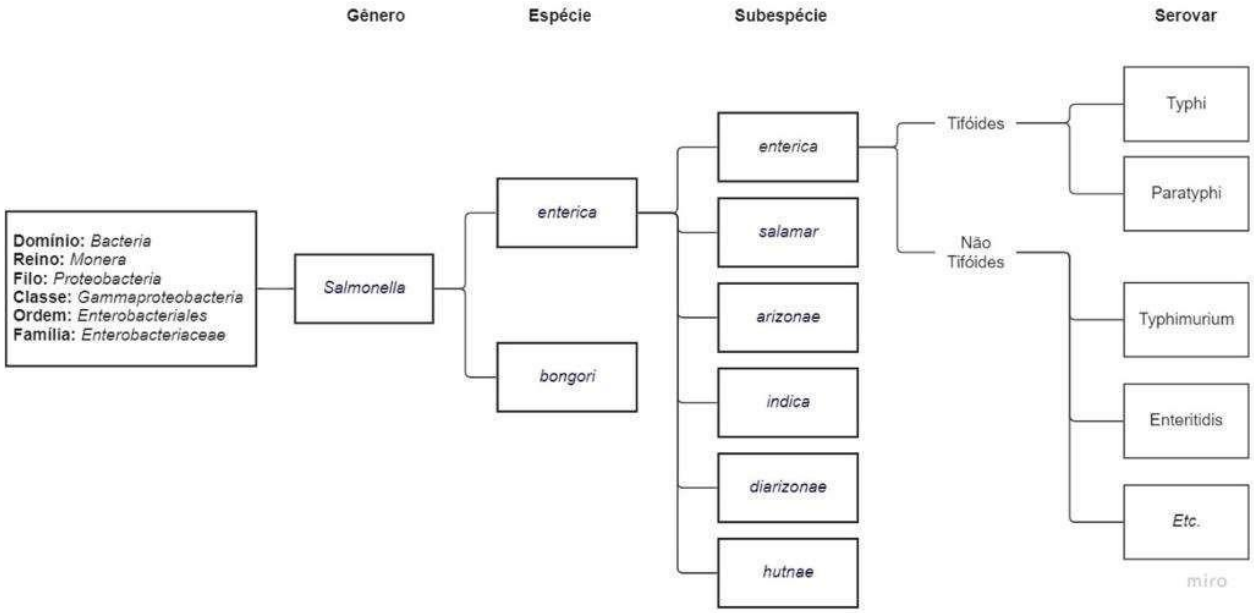

Figura 1 – Taxonomia de *Salmonella* ssp. Simões, M. J.

Salmonella enterica subs. *enterica* é de maior interesse, pois é capaz de infectar animais de sangue quente, sendo seu habitat natural o trato intestinal de animais. Mais de2600 subtipos dessa subespécie já foram reportados (Tabela 1) (CARNEIRO et al., 2020).

Tabela 1 - Quantidade de sorovares em *Salmonella* spp. (ISSENHUTH-JEANJEAN et al., 2014,modificado).

Espécie/subespécie	**2659**
S. *enterica*	**2637**
subsp. *enterica*	1586
subsp. *salamae*	522
subsp. *arizonae*	102
subsp. *diarizonae*	338
subsp. *houtenae*	76
subsp. *indica*	13
S. bongori	**22**
Total	2659

Considerando a taxonomia de *Salmonella* spp., a nomenclatura completa seria composta por seis palavras: gênero (1), espécie (2), palavra “subespécie” ou abreviado por “subsp” (3), nome da subespécie (4), palavra “sorovar” (5), nome do sorovar (6) (RYAN et al., 2017). O alvo de estudo deste trabalho é a *Salmonella enterica* subsp *enterica* sorotipo Typhimurium e, nas menções subsequentes, designaremos como *S.*

Typhimurium, omitindoos nomes das espécies e subespécies, conforme Guibourdenche et al., (2010).

A *S*. Typhimurium é um grupo de bactérias da família *Enterobacteriaceae*, gram- negativas, anaeróbias facultativas, produtoras de H_2S e não fermentadoras de lactose (HEGAZY e HENSEL, 2012; LEVINSON, 2016). No entanto, esse gênero de bactérias pode transformar-se em fermentador de lactose por aquisição de plasmídeos do tipo lac+ (TRABULSI; ALTERTHUM, 2008).

S. Typhimurium são patógenos invasivos-inflamatórios de grande importância clínica, sendo capaz de infectar uma ampla diversidade de hospedeiros, incluindo humanos e animais (LEVINSON, 2016; GARMORY, BROWN e TITBALL, 2002).

1.2 Salmonelose

Aproximadamente 10% da população mundial é adquiri com Doenças de Transmissão Hídrica e Alimentar (DTHA), por ano. Segundo a Secretaria da Vigilância em Saúde *Salmonella* spp. é o principal patógeno causador da DTHA no Brasil, podendo levar o indivíduo infectado a óbito, esegundo maior no mundo. Nesse caso, podemos reportar como surto de *Salmonella* a infecção de dois ou mais indivíduos pela mesma fonte de contaminação (SECRETARIA DA VIGILÂNCIA EM SAÚDE, MINISTÉRIO DA SAÚDE, 2021).

Existem 2 grupos de salmonela: a *Salmonella* tifóide engloba o único grupo que apresenta apenas humanos como reservatório e, como a própria classificação indica, culmina em febre tifoide (ACHESON; HOHMANN, 2001; TRABULSI; ALTERTHUM, 2008). O contágio ocorre por contato com doentes por trato respiratório e pele lesionada ou ingestão de água contaminada, raramente ocorre por ingestão de alimentos contaminados. Mesmo pessoas assintomáticas podem eliminar fezes contaminadas por semanas, aumentando a chance de transmissão em caso de esgotos não tratados. Já os sorovares não tifoides podem ser transmitidos por água contaminada, alimentos irrigados por água contaminada e sem higienização adequada, e por via oro-fecal e contato com animais, incluindo bovinos, aves, porcos e tartarugas. Os alimentos fontes de *Salmonella* são ovos, aves e seus derivados e frutos do mar (de FREITAS NETO et al., 2010). Alguns surtos da doença foram relacionados a contaminação de espinafre, frutas, tomate, amendoim e brotos (GAL-MOR et al.,

2014).

Dentre os principais sintomas relacionados a infecções por *S.* Typhimurium, podemos destacar diarreia, gastroenterite, sepse, desenvolvimento de doenças sistêmicas e morte em imunossuprimidos, diabéticos e tumorais (ACHESON; HOHMANN, 2001; TRABULSI; ALTERTHUM, 2008). Ademais, os sintomas variam com a subespécie, sorovar e quantidade ingerida de *S. entérica* (OCHOA & RODRÍGUEZ, 2005).

Salmonelose está diretamente relacionada à falta de saneamento básico, dificuldade de acesso a água e má qualidade da água para consumo, que podem ser impulsionados por distribuição de chuvas, gestão e exploração inadequada de recursos. Além disso, higienização incorreta de alimentos, preparo incorreto (cozimento insuficiente) utensílios de cozinha e do próprio consumidor também são importantes fatores propulsores de infecções por *Salmonella* (SECRETARIA DA VIGILÂNCIA EM SAÚDE, MINISTÉRIO DA SAÚDE, 2021).

Em 2017, na América Latina e Caribe, os índices de casos são de 2 (1·5 to 2·6) habitantes a cada 100.000 habitantes, e o nível de mortalidadesão de 1,46 habitantes por milhão (0·81 to 2·40) (STANAWAY et al., 2019). De fato, até 2021, 16% da brasileira foi identificada como sem acesso a água tratada, o que facilita ainfeccção pela doença (SUDRÉ, 2020). Em 2022, mais de 100 casos suspeitos de infecções por *S.* Typhimurium foram reportados na Europa, principalmente em crianças menores de dez anos, ao consumirem chocolate da marca kinder ovo. Apesar de nenhum óbito ter sido registrado, os infectados foram hospitalizados, levando a suspensão da comercialização dos produtos no Brasil pela Agência Brasileira de Vigilância Sanitária (ANVISA e Agência O Globo, Exame, 2022). Os casos também foram reportados em outros países e o risco de propagação foi considerado moderado.

Em 2016, foram atribuídas 700.000 mortes por ano, em todo mundo devido resistência a antibióticos (Figura 2). Estima-se que até 2050, serão 10 milhões de mortes (O'NEILL, J. 2016). Em bactérias que possuem Elementos Genéticos Móveis (EGM), como plasmídeos conjugativos, transposons, sequência de inserção e Ilhas de Patogenicidade (IP), aumenta-se a propagação de genes de resistência (ROWE-MAGNUS, 2001). A primeira cepa de *Salmonella* resistente foi encontrada na década de 60, com apenas resistência a cloranfenicol. Desde então, o número de cepas resistentes (a um ou vários antibióticos) tem aumentado cada vez mais, devido ao uso errôneo e/ou excessivo do medicamento e pelo fácil acesso ao medicamento

(ANGELO et.al., 2016). Quando a *Salmonella* é resistente à primeira linha de antibióticos como ampicilina, cloranfenicol e trimetoprim-sulfametoxazol, ela é considerada multirresistente; (ENG et. al., 2015).

Os dados sobre resistência bacteriana mudam muito entre países. No Reino Unido, *Salmonella* foram identificadas pela *United Kingdom Health Security Agency* (UKHSA) e relatadas como resistentes a seis classes de antibióticos: aminoglicosídeos (canamicina, espectinomicina, estreptomicina e gentamicina), fenicol, penicilinas, tetraciclinas, trimetoprima e sulfonamidas (ROCHA, 2022).

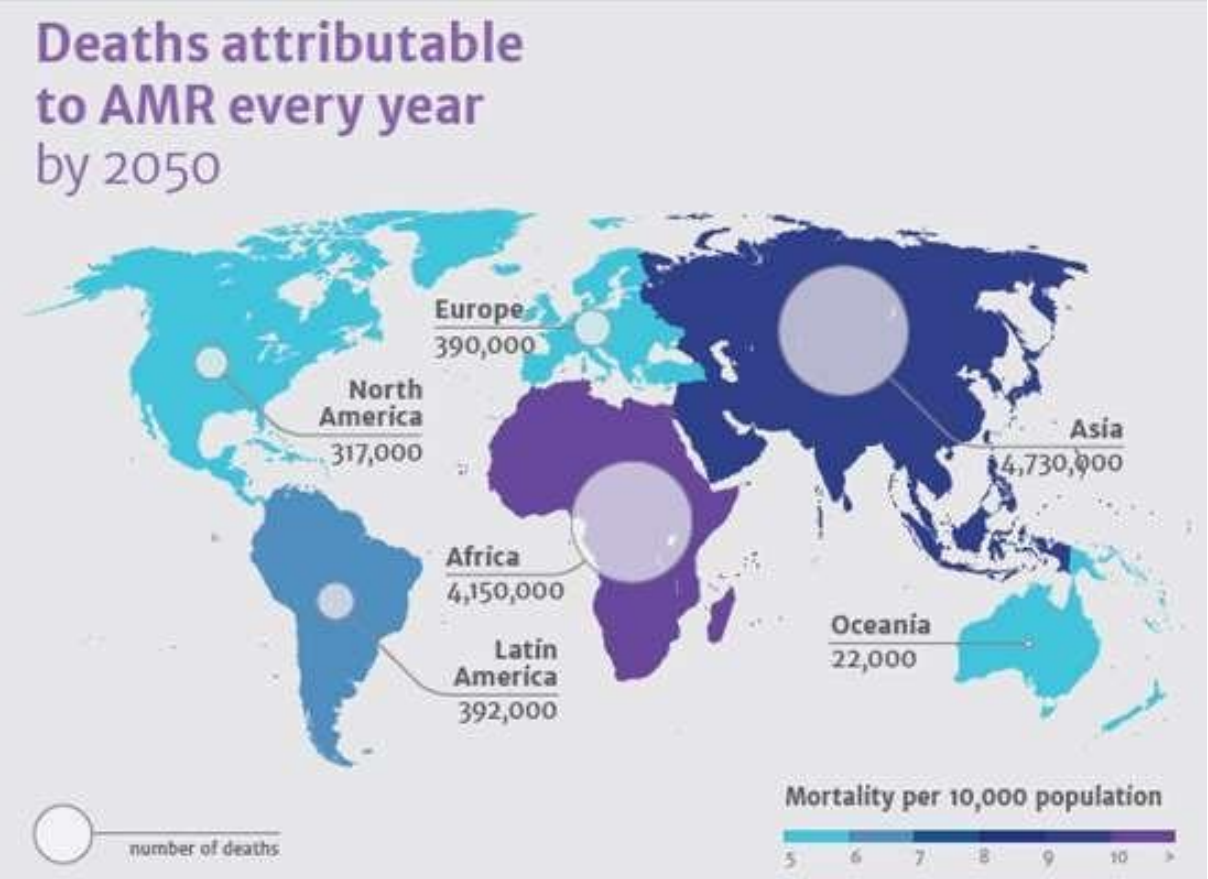

Figura 2: Mortalidade associada à resistência antimicrobiana para cada 10.000 habitantes prevista para 2050, demonstrada por continente. Fonte: O'Neill, J. (2017).

Nos Estados Unidos, é estimado que cepas resistentes de Salmonella spp. causam 100.000 infecções anualmente (ANGELO et.al., 2016). Em um meta-estudo no Brasil que acessou banco de dados com informações de isolados nos anos de 1995 a 2004, mostrou um alto nível de resistência a sulfonamidas, ácido nalidíxico e tetraciclina em isolados de aves, enquanto o de humanos tem resistência a sulfonamidas, tetraciclina e ampicilina (Voss-Rech et. Al., 2020). Além disso, o

surgimento de cepas resistentes a fluoroquinolonas e cefalosporinas de terceira geração vem ocorrendo no mundo todo.

A gravidade da doença depende da expressão de genes codificadores de fatores de virulência (ADELBERG et al., 2005). As infecções por *S.* Typhimurium são moduladas por tais genes, localizados em ilhas de patogenicidade, denominadas ilhas de patogenicidade de *Salmonella* (IPS). As ilhas de patogenicidade (IP) são definidas como *cluster* de genes inseridos no DNA bacteriano, envolvidos na virulência de *Salmonella* spp. e bactérias patogênicas. Os genes destas IP serão expressos em determinada etapa da infecção, como por exemplo *operons* de fímbrias e flagelos, e de resistência a antibióticos. O número, local e genes codificados pelas IP's variam em cada sorovar e podem gerar diferentes especificidades no hospedeiro, bem como diferente patogênese (MARCUS, et al., 2000). Nas IP 's, esses genes muito provavelmente foram adquiridos por transferência horizontal, visto que ocorrem dentro de elementos genéticos móveis, como plasmídeos e fagos lisogênicos (FONTES et al., 2012).

Durante o processo infeccioso, genes para indução de fímbrias e adesinas, bem como fatores de virulência induzidos pela IPS-6 são expressos na etapa denominada de adesão (Figura 3.A). Posteriormente, o citoesqueleto é rearranjado com auxílio dos genes da IPS-1, induzindo expansão das microvilosidades na membrana de células intestinais e propiciando a penetração da bactéria formando um vacúolo celular na etapa de invasão (Figura 3.b). O vacúolo inicial sofre maturação, transformando-se em vacúolo tardio (Figura 3.c). *Salmonella* inicia o processo de replicação após algumas horas (Figura 3.d), seguido por indução de apoptose celular (OLIVEIRA et al., 2013; LÓPEZ et al., 2012). Até o momento já foram catalogadas 21 IPS, sendo as IPS-1 e IPS-2 com maior papel na invasão, sobrevivência e multiplicação intracelular. Interessantemente, apenas a *S.* Typhi (tifoide) possui a IPS-7, e que a IPS-8, IPS-15, IPS-18, IPS-20 e IPS-21 estão ausentes em S. Typhimurium e S. Enteritidis (WISNER et al., 2012).

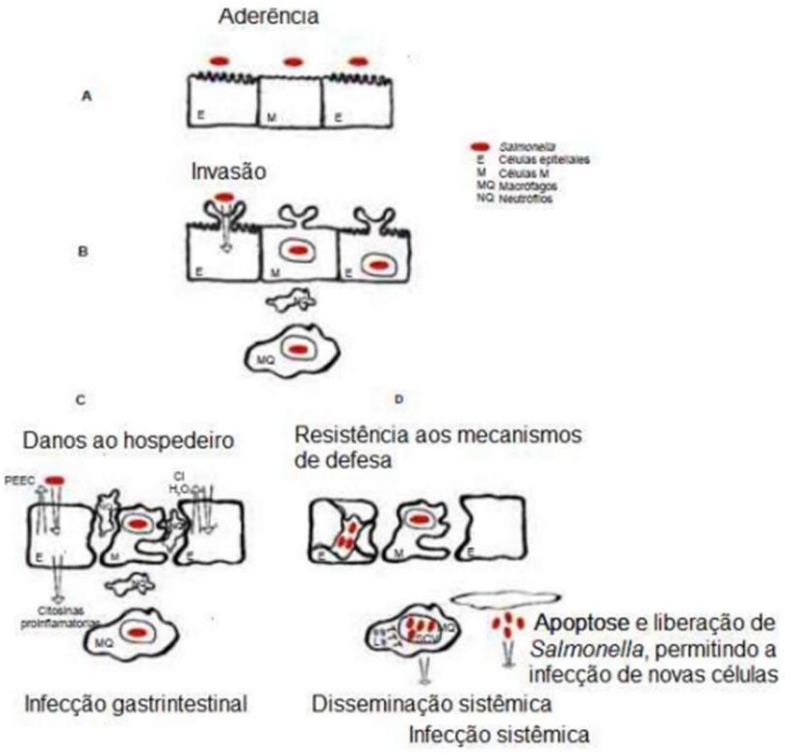

Figura 3 – Etapas de infecção por *Salmonella* spp. (OLIVEIRA et al., 2013). E=Células epiteliais, M=Células M, MQ=Macrófagos, NQ=Neutrófilos, ● = Salmonela

Alguns sintomas da Salmonelose podem ser tratados de maneira simples. No entanto, o quadro pode piorar, necessitando de prescrição de antibióticos (MINISTÉRIO DA SAÚDE, GOV, 2022). Apesar dos antibióticos possuírem grande espectro de ação e serem eficazes, o uso descontrolado pode representar problemas graves, como toxicidade não- seletiva, efeitos colaterais, e o desenvolvimento de resistência (WALSH, 2003). Considerando tais fatores, estratégias alternativas para profilaxia contra microrganismos patogênicos, principalmente *Salmonella* spp., têm sido pesquisadas, tais como o uso de probióticos (GILL & CROSS, 2002).

1.2.1 Alimentos Fermentados

Pasteur definiu fermentação como ‘a vida que ocorre sem oxigênio’, embora soubesse que havia muito mais complexidade ao termo (PASTEUR, 1895). Posteriormente o termo foi ampliado para processos bioquímicos em que carboidratos são decompostos por microrganismos em anaerobiose (MOAT, 2002). Fermentação também pode ser definida com uma reação em que o composto orgânico serve como aceptor e doador de elétrons, enquanto na indústria significa usar bactérias ou células eucarióticas com objetivo de

obtenção de um produto, como produtos alimentícios ou drogas (ISAPP, 2023).

Ainda podemos diferenciar o processo de fermentação, caso o aceptor final de elétrons são moléculas orgânicas, como piruvato, ou acetil-coA, enquanto no processo da respiração, o aceptor final é o oxigênio (CAMPBELL, 2004). A Associação Científica Internacional para probióticos e prebióticos definiu alimentos fermentados como “alimentos produzidos através do crescimento microbiano desejado e conversões enzimáticas de componentes alimentares’’ (MARCO et al., 2021).

A fermentação é um método conhecido há muito tempo, havendo evidências de sua existência em diferentes lugares, como um pão encontrado na região da Jordânia há 14 mil anos (ARRANZ-OTAEGUI, 2018), recipientes para fermentação de grãos para obtenção de cerveja entre 10 a 13 mil anos AC (HAYDEN et al., 2012), como também, egípcios e sumérios fermentavam grãos amiláceos (SAMUEL, 1996), para fabricar bebidas com cevada e um tipo arcaico de trigo, além de claro, pão, que tiveram seus resíduos encontrados em tigelas próprias para misturar (SAXENA, 2015). Em torno de 6.000 A.C., arqueólogos encontraram evidências na Geórgia, região dos Cáucasos, bebidas alcóolicas feitas de frutas, arroz e mel (ANAGNOSTOPOULOS & TSALTAS, 2019). Jarros com resquícios de vinho encontrados no Irã foram datados em 7000 A.C, como também forte evidência de bebidas fermentadas na Babilônia, em 3000 A.C. (MCGOVERN et al., 2003)

Com o passar dos anos, o hábito da fermentação trouxe diversos produtos fermentados pros dias de hoje, como iogurte, cerveja, vinho, saquê, shoyu, kefir, kombucha, queijo, salsichas, picles, peixe, pães e creme azedo (conhecidos em inglês, respectivamente como sourdough bread e sour cream) (COPETTI, 2019), (STEINKRAUS, 1997). O principal benefício da fermentação é que cria um ambiente tão hostil, que impossibilita o crescimento de outras cepas, inclusive as patogênicas, devido formação de álcoois, ácidos orgânicos e bacteriocinas, característica bastante útil na época em que não existia refrigeração, facilitando também o transporte desses produtos e aumentando aumento o tempo de validade do alimento (BRIGGS et al., 2004). Além disso, a transformação de alimentos gera novos sabores e texturas, o que tem atraído novos consumidores (COPETTI, 2019).

Fermentações podem fornecem produtos com maiores níveis de nutrientes, e melhorar sua disponibilidade ideal para pessoas com deficiência nutricional, especialmente em países subdesenvolvidos (STEINKRAUS, 1997). Esses microrganismos fermentadores podem produzir diversos nutrientes, como: aminoácidos, vitaminas (como tiamina, riboflavina, ácido fólico, B12). O Ácido lático produzido no produto tem função de reduzir citocinas pró-inflamatórias, enquanto o lactato diminui espécies reativas de oxigênio nos enterócitos

(IRAPORDA, 2015).

O consumo do alimento fermentado inclui os microrganismos presentes nele, e caso o consumo seja diário, ocorre a formação de uma população transiente, em conjunto a população que já habita o trato intestinal do consumidor. O aumento desses microrganismos pode ser de até 10000 vezes, levando ao acúmulo de benefícios que esses microrganismos podem levar, como: produção de ácidos graxos de cadeia curta (Short-chain fatty acids – SCFAs), que mantem a integridade da barreira intestinal e a produção de muco, diminuem o risco de cancer colorretal diminuindo a inflamação (PENG et al, 2009; O'KEEFE, 2016). Os receptores de SCFAs, estão presentes na proteína G, renomeados em receptor de ácidos graxos livres, como FFAR2 e FFAR3, e presentes em células do sistema imune, nervoso e da mucosa intestinal (Bolognini et al, 2016). Um dos mecanismos de ação descoberto é a ação das SCFA's na formação mais rápida das junções oclusivas das células epiteliais, formando junções estreitas, através da ativação de AMPK (proteína quinase ativada por AMP), que usualmente regulam vias metabólicas no metabolismo de ácidos graxos, glicose e síntese de proteínas. Esse resultado sugere uma possível ligação entre os SCFA's e uma sinalização para o desenvolvimento da barreira intestinal (PENG et al, 2009). Existem evidências que os SCFA's atravessem para o sistema nervoso central e que podem ter propriedades neuroativas, embora os mecanismos de como isso ocorrem ainda precisem ser elucidados (SILVA et al, 2020).

Outro benefício do consumo de leite fermentado, por exemplo, é a elevação da quantidade de proteína de choque térmico 70, que tem função antioxidante e é um agente anti-inflamatório, que também diminui danos e dores musculares ao melhorar o metabolismo de glicose quando há exercício intenso por suprimir o estresse oxidativo (AOI et al, 2007; IWASA et al, 2013). Além disso, estudos mostram que leite fermentado pode prevenir osteoporose, diabetes e doenças cardiovasculares, promovendo a saúde do intestino e modulação do sistema imune. Dentro dos fermentados, existe um grupo específico chamado de probióticos que serão detalhados no próximo tópico e que também se mostram promissores.

1.2.2 Probióticos

O termo probiótico surgiu na década de 60 e é a combinação de *pro* (de origem latina: a favor de) e *bios* (de origem grega: vida). Esta denominação aparece em

oposição ao termo antibiótico. A OMS definiu em 2014 que probióticos são microrganismos viáveis que conferem benefícios à saúde do hospedeiro, quando administrados em dose adequada. No geral, probióticos são considerados seguros, embora seu uso em crianças, idosos, grávidas, imunocomprometidos deva ser realizada com precaução. É amplamente indicado que a comunidade médica considere mais seu uso para prevenir e/ou tratar doenças ou disbioses e alergias, considerando que os efeitos colaterais são comumente passageiros e leves, como inchaço e flatulência. Em casos raros, o seu uso pode provoca diarreia e erupções cutâneas geralmente em casos de alergia ao produto (OMS, HUNGIN et al., 2018).

Apesar das vantagens inerentes aos probióticos, muitos aspectos em relação ao uso desses microrganismos ainda são desconhecidos. Por exemplo, em termos de profilaxia, a dose a ser aplicada, o tempo de ação e as possíveis interações probiótico-alimento no intestino ainda dependem de mais estudos (MARKOWIAK, ŚLIŻEWSKA et al., 2017, DE MELO PEREIRA, et al., 2018). Embora sejam benéficos para o uso em geral, adultos e crianças imuno-comprometidos ou doentes podem ser mais propensos a infecções fúngicas ou bacterianas. (RONDANELLI et. al., 2017) Em alguns raros casos, também é possível que genes de resistência a antibióticos presentes nas bactérias dos probióticos, acabem sendo transferidos para outras bactérias (AGAMENNONE, ET. AL., 2018).

Zendeboodi e colaboradores (2020) propuseram uma nova definição, visto que também já foi comprovado o benefício do uso de microrganismos não-viáveis (e metabólitos gerados por eles, quando viáveis) para a saúde do hospedeiro. Segundo o autor, a distribuição dos probióticos seria em três classes: i) probióticos verdadeiros: célula probiótica viável e ativa; ii) pseudo-probiótico: célula probiótica viável, porém inativa, na forma vegetativa ou de esporo; iii) probiótico fantasma: célula probiótica mortas/inviáveis. Todas conferem benefícios ao hospedeiro. Neste estudo, além dos autores terem englobado novas definições do termo probiótico, foi proposta uma nova classificação dos probióticos de acordo com o local de ação: (i) interno: *in vivo*; quando está dentro do corpo, (ii) externo: *in vitro*, quando está fora do corpo, sendo produzido (ZENDEBOODI et al., 2020). Dentre os microrganismos designados como probióticos destacam-se as bactérias ácido-láticas (BAL), incluindo *Lactobacillus* spp., *Enterococcus* spp. e *Streptococcus* spp., dentre outros (DIDARI et al., 2014). Além disso, *Bifidobacterium* e algumas linhagens de leveduras, como *Saccharomyces* spp., também são capazes de atuar como probióticos (DIDARI et al., 2014; ZENDEBOODI

et al., 2020, BRASIL et al., 2018).

Uma gama de efeitos positivos já foi reportada em decorrência do uso de probióticos, tais como auxiliar a terapia contra doença celíaca, prevenção de doenças inflamatórias do intestino, exacerbações pulmonares em crianças com fibrose cística e eczema, infecções agudas do trato respiratório superior, estímulo da motilidade intestinal, proteção intestinal contra bactérias patogênicas, neutralização e inibição de toxinas microbianas, modulação do sistema imune, regulação das vias metabólicas e ação anti-tumorigênica (de SOUSA MORAES et al., 2014; CREMON et al., 2018; VALDES et al., 2018; ASHAOLU, T. J.,2020; KHANEGHAH et al., 2020).

Na alimentação animal, os probióticos são fonte de nutrientes e enzimas e auxiliam na síntese de vitaminas do complexo B, manutenção da microbiota intestinal normal e produção de ácidos graxos voláteis, H_2O_2 e outros inibidores de bactérias patogênicas (MUSA et al., 2009; ROSA et al., 2017; KHANEGHAH et al., 2020). A nível macro, alguns probióticos aumentam o ganho de peso diário, eficiência alimentar e produção de leite, além de estimularem o desempenho, a saúde e o crescimento animal (KALAVATHY, et al., 2003).

Embora ainda não estejam totalmente elucidados, grupos de pesquisa sugerem que os mecanismos de ação dos probióticos estão relacionados à microbiota digestiva, envolvendo alteração do pH intestinal, a síntese de antimicrobianos, enzimas e defensinas, e a competição por nutrientes e sítios de ligação (dos REIS et al., 2017; BAJAJ et al., 2021). De fato, a profilaxia contra *Salmonella* ssp. é atribuída a um conjunto de ações, como atividade antibacteriana e produção de imunoglobulina A (IgA) e outras células do sistema imune (RIJKERS et al., 2011; FARAG, et al., 2020).

Diante dos inúmeros benefícios advindos do uso de probióticos, a demanda por alimentos com propriedades probióticas vem aumentando. Nesse contexto, a cultura de Kefir, utilizada na produção de bebidas lácteas e leites fermentados, têm ganhado destaque (FARAG, et al., 2020).

1.2.3 Kefir

O Kefir, originário do Cáucaso e Península Balcânica, é um alimento fermentado e probiótico dependendo da quantidade de microorganismos viáveis, cuja fermentação, alcoólica e láctica, é conduzida pelos grãos de Kefir ou por fermento de

Kefir (PRADO et al., 2015). O Kefir pode ser classificado em Kefir lácteo e não lácteo de acordo com o fermentado e sua produção pode ser feita tanto pela adição de grãos de Kefir (método tradicional ou doméstico) quanto por culturas, comercializadas em pó (método industrial) (KAVAS, 2015). Em ambos processos, o Kefir é obtido por diminuição do pH e coagulação do alimento, puro ou adicionado de outros produtos alimentícios, como derivados lácteos.

Os grãos de Kefir, envoltos por uma matriz polissacarídica e protéica, apresentam composição variável de acordo com a origem, sendo constituídos por associação simbiótica entre: i) BAL, tais como *Lactobacillus kefir*, *Lactobacillus casei*, *Lactococcus* ssp., *Leuconostoc* spp., *Acetobacter* spp., *Bifidobacterium* ssp. e *Streptococcus salivarius* subsp *thermophilus*; ii) bactérias ácido-acéticas; iii) leveduras fermentadoras de lactose, como *Kluyveromyces marxianus*; iii) leveduras não fermentadoras de lactose: *Saccharomyces omnisporus*, *Saccharomyces cerevisiae; Saccharomyces exiguus* (BRASIL, 2007; FARAG et al., 2020; GAO, ZHANG, 2019).

Os grãos do Kefir, massas gelatinosas irregulares de coloração branca a amarelada, podem ser fermentar uma gama de produtos lácteos fermentados, como soro de leite, queijo e líquidos açucarados e obtidos por peneiração do leite para serem utilizados novamente (GAO, LI, 2016). Como resultado do processo fermentativo, há a produção de ácidos orgânicos de cadeia curta, principalmente ácidos láctico e acético, álcool etílico, peptídeos, antibióticos, diacetil, exopolissacarídeos (e exemplo do quefirano), bacteriocinas, H_2O_2 e CO_2, gerando aroma e textura características, sabor levemente ácido (pH entre 3,3 e 5,5) ao leite (POGAČIĆ et al., 2013; ROSA et al., 2017; FARAG et al., 2020).

Embora o número mínimo de microrganismos no produto final possa variar de acordo com o país, é de senso comum que os microrganismos devem estar viáveis, ativos e abundantes durante o prazo de validade do item. Segundo o Ministério da Agricultura, Pecuária e Abastecimento (MAPA) a contagem mínima de unidades formadoras de colônias (UFC) estabelecida para o Brasil é de 10^7 e 10^4 UFC/g de bactérias lácticas totais e leveduras, respectivamente (BRASIL, 2007).

O grande interesse para população na utilização do Kefir está relacionado a várias características, dentre elas o elevado valor nutricional (usualemente proteínas presentes no alimento base onde é fermentado), o baixo custo, poucas contra indicações e de riscos à saúde humana, bem como seu efeito probiótico. Polissacarídeos e bacteriocinas produzidos pelo Kefir auxiliam no controle de

patógenos deterioradores, sendo importantes na indústria alimentícia (FARAG et al., 2020). De fato, BAL isoladas têm sido utilizadas como bioconservantes em alimentos (CLEUSIX et al., 2007; CORR et al., 2007).

O valor nutricional do Kefir é resultado das porcentagens de carboidratos, proteínas, aminoácidos essenciais e lipídeos, além dos micronutrientes, incluindo minerais e vitaminas. Cálcio, potássio, magnésio, zinco, cobre e ferro são minerais presentes no Kefir que compõem o sangue e ajudam a modulação do metabolismo celular (FARAG et al., 2020). A composição química pode variar, sendo influenciada pelo tipo de alimento base para fermentação e pela microbiota, dentre outros fatores (SIMOVA, et al., 2006). De fato, o fermentado, tipo de microbiota, quantidade de cultura inicial e condições de fermentação, como temperatura, tempo e armazenamento, podem afetar as características químicas, microbiológicas e sensoriais do Kefir (BARUKČIĆ et al., 2017; ROSA et al., 2017).

Inúmeras bioatividades do Kefir já foram relatadas (Figura 4), incluindo atividades antimicrobianas, controle do metabolismo da glicose, produção de citocinas, melhora da digestibilidade alimentar e da intolerância à lactose, sinalização celular, antiproliferativa, hipocolesterolêmica, antioxidante, cicatrizante, anti-hipertensivo e modulação do sistema imune (CHEN et al., 2015; ROSA et al., 2017; FARAG et al., 2020; AZIZI et al., 2021; WULANSARI et al., 2021). Além disso, sabe-se que os efeitos do Kefir são dependentes da origem e microbiota presente nos grãos bem como do tempo de fermentação (LEITE et al., 2013). Devido aos benefícios associados ao Kefir, seu consumo tem sido estimulado em muitos países (LEITE et al., 2013; KIVANÇ, YAPICI, 2016).

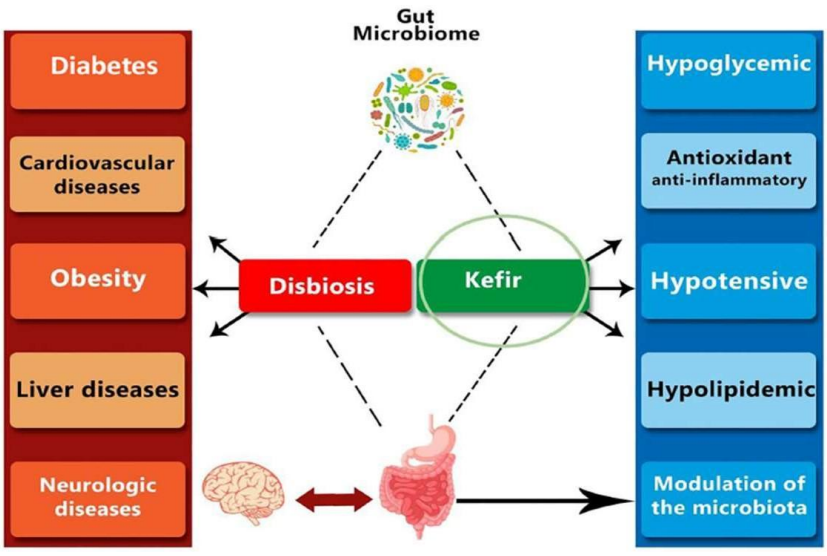

Figura 4 – Efeitos do uso de Kefir na microbiota intestinal e nos distúrbios metabólicos (PELUZIO etal., 2021).

Dentre as aplicações medicinais do Kefir, destaca-se a atuação sobre a microbiota intestinal. Tal efeito é consequência de um conjunto de fatores, incluindo inibição direta de patógenos por metabólitos (ácidos, peptídeos e bacteriocinas). As atividades antimicrobiana são capazes de diminuir a competição entre microrganismos patogênicos e benéficos, favorecendo o estabelecimento da microbiota normal (FARAG et al., 2020; PELUZIO et al., 2021). Somado a isso, considerando que alterações na microbiota intestinal, como disbioses, predispõem o organismo a desenvolver distúrbios metabólicos (Figura 4), a modulação da microbiota tem se mostrado uma excelente estratégia para prevenção e controle de doenças (PELUZIO et al., 2021). De fato, a alteração nas microbiotas fecal e intestinal está associada a inúmeros efeitos positivos, como diminuição de diarreia e enterocolite (HAMET et al., 2016; HSU et al. 2018).

O exopolissacarídeo kefiran, produzido por *Lactobacillus* ssp. isolados de grãos de Kefir, aumentou a população de *Bifidobacterium* em camundongos, auxiliando o desempenho físico, dentre outros fatores (BOLLA et al, 2013). Tal molécula também tem sido associada a atividades antimicrobiana, antioxidante, anti-inflamatórias (por supressão de síntese de citocinas e degranulação de mastócitos) e antitumorais (FURUNO E NAKANISHI, 2012; JENAB et al., 2020; TAN et al., 2020).

O Kefir já foi reportado como inibidor de vários microrganismos, incluindo *Escherichia coli*, *Salmonella enteritidis* e *Aspergillus* (ISMAIEL et al., 2011; ABDEL-MOGHEITH et al., 2017). Macuamule e colaboradores (2016) demonstraram inibição de *Mycobacterium bovis* no leite, agente causador de tuberculose bovina que pode ser transmitido aos humanos pelo consumo de leite e derivados. Apesar dos estudos relacionados à atividade antimicrobiana do Kefir, muitos autores têm focado na utilização de microrganismos isolados do Kefir contra patógenos, incluindo *S. enteritidis* e *S.* Typhimurium (MIAO et al., 2014, 2016; JEONG et al., 2018). Neste contexto, *Lactobacillus diolivorans* 1Z inibiram o crescimento de *S.* Typhimurium *in vivo*, com taxa de sobrevivência de 70% de camundongos infectados (ABATEMARCO JÚNIOR et al., 2018).

Em relação à atividade anti-inflamatória, Rosa e colaboradores (2017) demonstraram que ratos hipertensos suplementados com Kefir aumentaram a expressão de interleucina 10 (IL-10) no tecido adiposo, após dez semanas de suplementação.

Além disso, Kefir e isolados de Kefir são capazes de induzir a diminuição de: i) produção de interleucina-1 beta (IL-1β), citocina inflamatória; ii) marcadores oxidativos; iii) fator de necrose tumoral (TNF)/IL-10, iv) interleucina 6 (IL-6); v) secreção de interleucina 8 (IL-8) e iv) atividade da enzima hialuronidase (ROSA et al., 2017; SANTANNA et al., 2017; SEO et al., 2018).

Destacando a atividade antioxidante, Kefir adicionado a suco de maçã aumentou os efeitos antioxidantes atribuídos a elevação do conteúdo fenólico total (SABOKBAR et al., 2015). Propriedades antioxidativas também foram evidenciadas por demais grupos de estudo (OZCAN et al., 2009; CHEN et al., 2016; KARAÇALI et al., 2018; YILMAZ-ERSAN et al., 2018).

Embora, o consumo deste alimento é incentivado mediante seus benefícios, principalmente como inibidor de crescimento de outros microrganismos e processos oxidativos algumas consequências do uso de Kefir, bem como seus mecanismos moleculares e microrganismos envolvidos, ainda permanecem obscuros,sendo necessário o aprofundamento do conhecimento acerca o tema (ROSA et al., 2017).

1.3.1 Métodos computacionais para análise de microbiota

A identificação de microorganismos em populações é complexa e difícil. Entre os métodos que podem ser aplicados para esta tarefa estão métodos de coloração, microscopia ótica para se identificar a morfologia, cultura em meios específicos, uso de PCR para identificação de presença ou ausência de genes específicos e testes bioquímicos. Porém, para se aplicar a maioria desses testes, é necessário a cultura desses microorganismos. É estimado que apenas 1% dos organismos são cultiváveis, visto nosso desconhecimento de suas necessidades nutricionais para o crescimento. Ainda há a dificuldade em saber se existem fatores específicos necessários, como íons, ou metabólitos, que muitas vezes podem ser produzidos por outros organismos, sejam eles micro ou macronutrientes (MOREIRA, 2015).

A evolução do sequenciamento foi rápida, desde a possibilidade de sequenciar um fragmento pequeno como na metodologia de Sanger (SANGER et al., 1977), passando pela não- obrigatoriedade de clonar o gene de interesse em um vetor, até o uso de sequenciadores de alto desempenho (LAVER et al., 2015). O problema da impossibilidade de se cultivar certos organismos foi resolvida pela metagenômica, em

que se sequencia o conteúdo genético de micro-organismos extraídos diretamente de um determinado ambiente, seja solo, rio, mar, ou dentro de um organismo, como um ser humano, ou animal (KENNEDY et al., 2007). Os objetivos podem ser os mais diversos: descobrir o efeito do aumento da temperaturano oceano, a falta de um cátion na biosfera de um ambiente, a mudança da microbiota em um acidente ambiental, o efeito colateral de um uso de remédio na microbiota animal humana, ou mesmo a diferença causada pela dieta animal(KENNEDY et al., 2007).

Para se fazer a caracterização de um meio, primeiramente é extraído o DNA presente nesse ambiente, e seguida pela escolha entre duas metodologias (Tabela 2): a metagenômica em si, conhecida como shotgun, onde fragmentos de DNA são produzidos aleatoriamente, seguindo pelo sequenciamento (BREITWIESER et al., 2019). Após várias rodadas de sequenciamento, múltiplas leituras das sequências são obtidas e programas de computador são utilizados para remover possíveis erros das sequências e então, sobrepô-las de maneira a formar sequências contínuas maiores. O ideal é que tenham várias sequências iguais, para que possíveis erros de sequenciamento sejam excluídos, assim como mutações aleatórias induzidas pelos métodos de amplificação de sequência utilizados, que não representem o material genético inicial (LAPIDUS & KOROBEYNIKOV, 2021).

A outra possibilidade é a metataxônomica, em que se escolhe um marcador genético, que será amplificado e sequenciado para identificação da microbiota por comparação a um banco de dados que possui diversas sequências de referência destes marcadores para vários micro-organismos. Usualmente para procariotos é usado o marcador 16s, que é um gene de RNA ribossomal, para fungos são usados nas regiões ITS, e não há um marcador ideal para vírus (WHON, 2021).

O ideal para um marcador é que ele seja extremamente necessário para a vida do organismo, de modo que ele terá trechos altamente conservados, que serão essenciais para exercer sua função. Também é necessário que certas partes sejam variáveis, para que seja possível definir a espécie ou gênero através desses nucleotídeos diferentes (SEGAL et al., 2019)

Quadro 1: vantagens e desvantagens de metagenômica por shotgun ou metataxonômica. Fontes: Nam et al, 2023

	Vantagens	Desvantagens
Metagenômica por Shotgun	-Provê mais informações funcionais -Vírus podem ser detectados -Provê menor viés devido uso de*primers* aleatórios	-Mais caro -Possui custo computacional maior -Provê menos informação taxonômica -Pode haver contaminação por DNA do hospedeiro
Metataxonômica	-Possui Preço mais acessível -Mais rápido -Possui custo computacional menor - Provê maior número de observações -Provê informações taxonômicas abundantes	-Provê pouca informação funcional -Não detecta vírus

Pelo método de metagenômica por shotgun, é possível obter o sequenciamento do genoma completo de vários microorganismos, a identificação de vários genes, e a função e importância desse gene, consequentemente é possível obter a informação das funções que esse microorganismo pode te fornecer. Porém, há contaminação da amostra com DNA do hospedeiro, sendo necessário retirar essas sequências (BREITWIESER et al., 2019). Na metataxonômica, não é possível obter a os genes presentes no organismo , logo diminui-se a informação de funcionalidade. Erroneamente alguns pesquisadores pensam que se há mais determinada bactéria, provavelmente nesse ambiente está ocorrendo mais reações que essa bactéria é capaz de fazer. Porém essa correlação é muito fraca, pois pode se ter várias bactérias que possuem genes parecidos ou que são capazes das mesmas funções, mas que não estão expressando esses genes nesse momento.

A metataxônomica fornece muita informação sobre a taxonomia, porém há um viés: depende de qual região você escolhe como gene marcador, e de qual banco de dados você irá usar. Há bancos de dados que possuem diferentes informações sobre determinadas regiões de genes marcadores, e existem melhores regiões que são mais bem caracterizadas para determinada análise (MCLAREN et al., 2019). Por exemplo, sabe-se que em determinada região existem muitas bactérias sulfurosas, porém não há banco de dados com muitas informações sobre as sequências de 16S rDNA de bactérias sulfurosas. Logo, é melhor escolhermos outro marcador, que irá nos informar

bactérias sulfurosas que estão nessa região. Caso não escolhamos outra região, podemos subestimar as bactérias sulfurosas deste local (BREITWIESER et al., 2019).

A metataxonômica também nos usufrui mais números de amostras/dados. Se em uma corrida de sequenciamento obtemos por exemplo, um mega de nucleotídeos sequenciados, significa que em um sequenciamento conseguimos obter milhares de regiões em torno de 250 nucleotídeos (quantidade obtida pelo miSeq), consequentemente milhares de sequências de 16s (ILUMINA). Caso fossemos fazer sequenciamento de genomas completos, que tem em torno de 100 mil a 5 milhões de nucleotídeos, conseguiríamos sequenciar 10 genomas ou menos. Pelo mesmo motivo, é possível conseguir um maior número de reads que confirmem sua observação na taxonômica. pois em um sequenciamento é obtido muito mais reads de uma sequência de 250 pb's do que de um genoma completo.

O DNA Ribossomal 16s é muito bem descrito na literatura com um bom marcador de taxomia para procariotos (Figura 5). Tem função primordial, visto que o ribossomo é necessário para vida do organismo e é muito conservado. Suas sequências estão em vários bancos de dados extensos, fazendo com que muitos pesquisadores o usem para seus estudos. O DNA ribossomal 16S possui regiões muito conservadas, e regiões variáveis. As regiões conservadas servem para se utilizar primers, de modo que seja possível amplificar essa região, e as regiões variáveis, como o próprio nome diz, variam, e por essas diferenças que é possível usá-las para diferenciar táxons diferentes. Porém, algumas vezes não é possível diferenciar espécies parecidas como *Shigella flexneri* de *E. coli* (HILTON et al., 2016) ou *E. coli* O157:H7 de *E. coli* K-12 (WEINSTOCK, 2012).

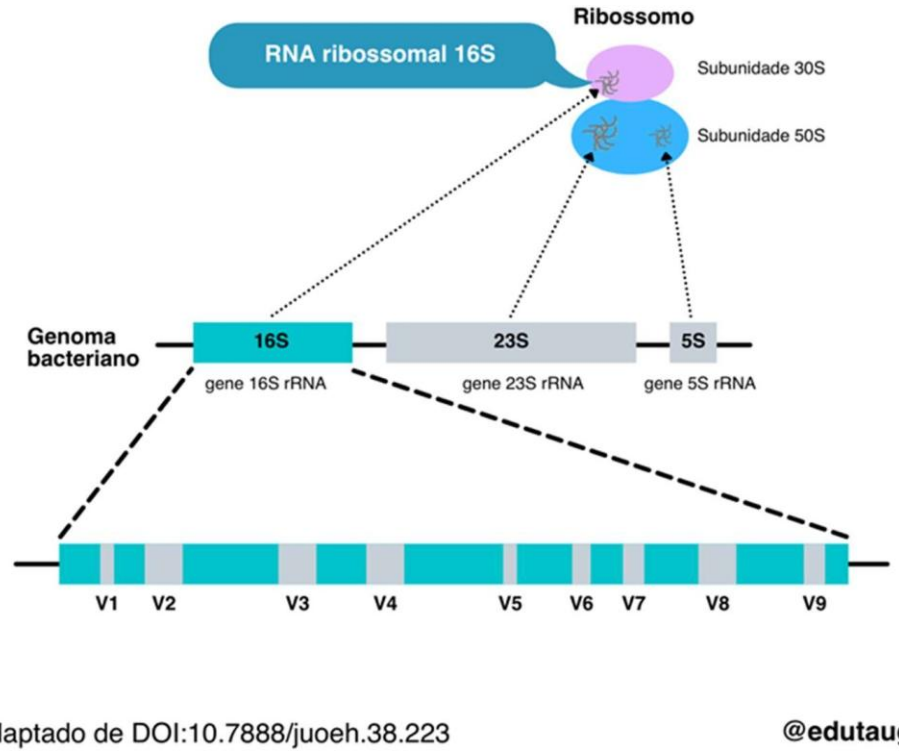

Figura 5: Genes ribossomais em bactéria. Foco na subunidade 16s e regiões hiper variáveis. As regiões Variáveis estão destacadas em cinza, denominadas de V1 a V9.

1.3.2 Desenho experimental

No primeiro experimento, analisamos a microbiota da bebida kefir e para confirmar nossa hipótese, bem como sua retenção e efeito modulador da microbiota de camundongos que consumiram o produto por meio de coleta de fezes após 23 e 24 dias de tratamentos com kefir ou com água (grupo controle).

No segundo experimento, os animais foram infectados com *Salmonella* Typhimurium no 15º dia para avaliar os impactos do consumo deste probiótico durante o curso da infecção (Figura 6).

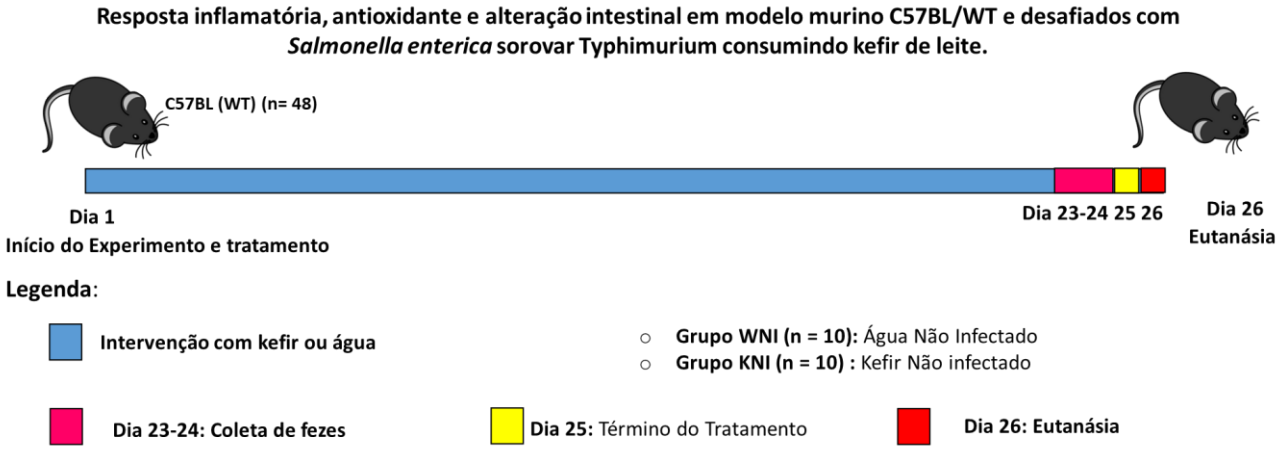

Figura 6: Esquema do experimento 1: Animais tratados com kefir ou água foram divididos em 2 grupos. Intervenção com água e kefir durante todo o experimetno exceto no dia da eutanásia. Autoria: Mariana Albuquerque e Larissa Ávila.

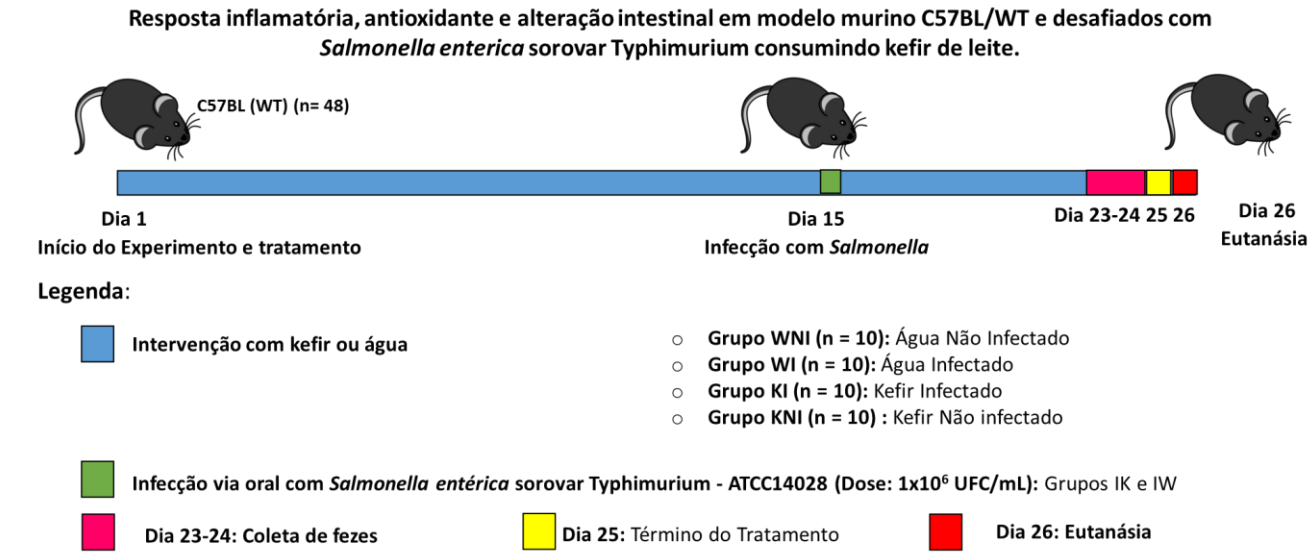

Figura 7: Esquema do experimento 2 : Animais tratados com kefir ou água foram divididos em 4 grupos, 2 deles infectados com Salmonella Typhimurium no 15º dia. Intervenção com água e kefir durante todo o experimetno exceto no dia da eutanásia. Autoria: Mariana Albuquerque e

Larissa Ávila.

1.4 OBJETIVOS

1.4.1 Objetivos Geral

O presente trabalho objetivou caracterizar a bebida Kefir fermentada pelo Kefiran (grãos de kefir) em leite produzida domesticamente e o efeito do uso da bebida em camundongos C57BL/6J saudáveis e em camundongos infectados com *Salmonella Typhimurium.*

1.4.2 Objetivos Específicos

-Padronizar a produção da bebida de Kefir em leite

-Caracterizar fisico-quimicamente a bebida

-Caracterizar microbiologicamente a bebida por meio de análise metagenômica

-Caracterizar a comunidade bacteriana e marcadores bioquímicos do camundongo tratado com Kefir

-Avaliar o efeito do consumo de Kefir para infecção por Salmonela Typhimurium em camundongos:

- O efeito do consumo na produção de SCFA's

- Investigar a carga fecal de Salmonella e a possível translocação sistêmica do patógeno.

-Caracterizar a microbiota do camundongo tratado com Kefir e água, com e sem infectação com *Salmonella* Typhimurium

-Observar efeitos na inflamação intestinal

-Examinar a mortalidade dos animais infectados após o consumo de kefir.

2. Artigo 1: Milk kefir changes bacterial communities and influences the microbiota-gut-brain axis by increasing short-chain fatty acids in healthy mice

Applied Microbiology and Biotechnology

Milk kefir changes bacterial communities and influences the microbiota-gut-brain axisby increasing short-chain fatty acids in healthy mice.

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Abstract:	Kefir is a fermented beverage made of a symbiotic microbial community that stands outfor health benefits. Although its microbial profile still little explore, its effects on modulation of gut microbiota and production of short-chain fatty acids (SCFAs) seems to act improving brain health. This work aimed to analyze the microbiota profile of milk kefir and its effect on metabolism, oxidative stress, and in the microbiota-gut-brain axisin a murine model. The experimental design was carried out using C57BL6 mice (n=20) subdivided into groups that received 0.1mL water or 0.1 mL (10% w/v) kefir. The kefir proceeded to maturation for 48 hours, and then it was orally administered, via gavage, to the animals for 4 weeks. Physicochemical, microbiological, antioxidant analyzes, and microbial profiling of milk kefir beverage were performed as well as growth parameters, food intake, serum markers, oxidative stress, antioxidant enzymes, SCFAs, and metagenomics were analyzed in the mice. Milk kefir had 76.64 ± 0.42% offree radical scavenging and the microbiota composed primarily by the genus Comamonas. Moreover, kefir increase catalase and superoxide dismutase (colon), SCFAs in feces (butyrate), and in the brain (butyrate and propionate). Kefir reduced

	triglycerides, uric acid, and affected the microbiome of animals increasing fecal butyrate-producing bacteria (Lachnospiraceae and Lachnoclostridium). Our results onthe brain and fecal SCFAs and the antioxidant effect found are associated with the change in the gut microbiota caused by kefir, which indicates that kefir positively influences the gut-microbiota-brain axis and contributes to the preservation of gut andbrain health.
Suggested Reviewers:	Fermin Milagro Universidad de Navarra fmilagro@u nav.es Fermin Milagro leads the research group on Nutriomics and Biomarkers in the Centerfor Nutrition Research, University of Navarra (Pamplona, Spain). More than 250 scientific articles and H-factor=48
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	Lukasz Grzeskowia k Freie Universitat Berlin lukgrz@me .com He is a Research Scientist at Freie Universität Berlin. He has experience in the areas of intestinal microbiology, microorganisms vs hosts in humans and animals, microbialecology, solid foods (probiotics and prebiotics) and nutrition. It has skills to develop multidisciplinary research, combining the areas of biology, molecular analysis and evaluation of microbiota and microbiological products.
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Opposed Reviewers:	

Cover Letter

January, 1st, 2023

To Alexander Steinbüchel
Editor- in-Chief of Applied Microbiology and
BiotechnologyDear Editor,

The manuscript submitted entitled “Milk kefir changes bacterial communities and influences the microbiota-gut-brain axis by increasing short-chain fatty acids in healthy mice in healthy mice.” on behalfof my co-workers Mariana Pereira, Larissa Ávila, Gabriela Alpino, Bruna Cruz, Lucas Almeida, Jordana Simões, Andressa Bernardes, Iasmim Campos, Andréa Ribon, Tiago Mendes, and Maria do Carmo Peluziodemonstrates how the microbiota profile of milk kefir and its daily consumption affects the modulation of the metabolism, oxidative stress, and the microbiota-gut-brain axis in a healthy murine model.

Kefir as a fermented beverage made of a symbiotic microbial community stands out for its effects on oxidative stress, modulation of gut microbiota, and production of beneficial metabolites, such as short-chain fatty acids (SCFAs). Because kefir has a probiotic potential and can be easily produced at home, this beverage has been nowadays associated as a psychobiotic food that can improve brain disease symptoms via gut-microbiota-brain axis alteration, especially through SCFAs production. These SCFAs are produced by bacterial fermentation in the colon, inducing different local beneficial responses and leading to brain signaling via the microbiota-gut-brain axis. In this sense, kefir can modulate aspects of the gut-microbiota- brain axis, acting not only as a probiotic but also as a psychobiotic when bioactive compounds can systematically reach the brain through the modulation of the fecal microbiota and its metabolites. However, the kefir microbiota composition and its effect on the axis are still little explored demonstrating the importance of our work. For that reason, the milk kefir beverage in this work showed great antioxidant capacity and great microbiota composition by increasing SCFAs-producing bacteria, such as the genus *Comamonas* in the beverage and *Lachnospiracea* and *Lachnosclostridium* in the mice. Our results on the brain and fecal SCFAs as well as the antioxidant effect found in the colon and brain are associated with the change in the gut microbiota caused by kefir, which indicates that kefir positively influences the gut- microbiota-brain axis and it directly contributes to the preservation of gut and brain health.

The authors followed standard practices, which include adhering to ethical guidelines for research involving animals (ARRIVE guidelines) and ethics approval from the institutional ethics committee as described in the manuscript and supplementary material. We declare that this manuscript is original, has not been published before, and is not currently being considered for publication elsewhere. Furthermore, as the Corresponding author, I confirm

that the manuscript was read and approved for submission by all the named authors.
Thank you for your time and we look forward to hearing from you soon.

Regards,

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1.4.2 **Title:** Milk kefir changes bacterial communities and influences the microbiota-gut-brain axis by increasing

1.4.3 short-chain fatty acids in healthy mice.

3

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36

2.1 Abstract

Kefir is a fermented beverage made of a symbiotic microbial community that stands out for health benefits. Although its microbial profile still little explored, its effects on modulation of gut microbiota and production of short-chain fatty acids (SCFAs) seems to act improving brain health. This work aimed to analyze the microbiota profile of milk kefir and its effect on metabolism, oxidative stress, and in the microbiota-gut-brain axis in a murine model. The experimental design was carried out using C57BL6 mice (n=20) subdivided into groups that received 0.1mL water or 0.1 mL (10% w/v) kefir. The kefir proceeded to maturation for 48 hours, and then it was orally administered, via gavage, to the animals for 4 weeks. Physicochemical, microbiological, antioxidant analyzes, and microbial profiling of milk kefir beverage were performed as well as growth parameters, food intake, serum markers, oxidative stress, antioxidant enzymes, SCFAs, and metagenomics were analyzed in the mice. Milk kefir had 76.64 ± 0.42% of free radical scavenging and the microbiota composed primarily by the genus Comamonas. Moreover, kefir increase catalase and superoxide dismutase (colon), SCFAs in feces (butyrate), and in the brain (butyrate and propionate). Kefir reduced triglycerides, uric acid, and affected the microbiome of animals increasing fecal butyrate-producing bacteria (Lachnospiraceae and Lachnoclostridium). Our results on the brain and fecal SCFAs and the antioxidant effect found are associated with the change in the gut microbiota caused by kefir, which indicates that kefir positively influences the gut-microbiota-brain axis and contributes to the preservation of gut and brain health.

Keywords: Kefir; probiotic; bacterial diversity; SCFA; microbiota-gut-brain axis; fermented milk; fecal microbiota

Statements and Declarations: The authors declare no conflict of interest.

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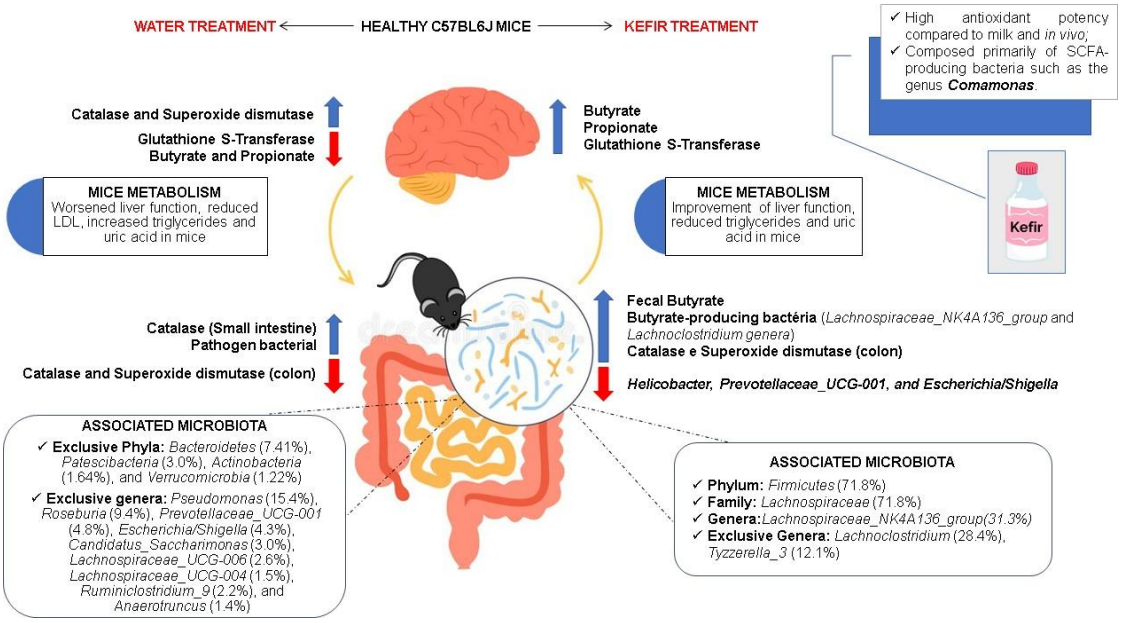
Graphical abstract



Key Points

- Milk kefir modulates fecal microbiota and SCFA production in brain and colon
- Kefir treatment increase the abundance of SCFA-producing bacteria
- Milk Kefir increases antioxidant enzymes and influences the metabolism of mice

2.2 Introduction

Traditional kefir is a by-product of milk resulting from double fermentation, alcoholic and lactic, provided by kefir grains, which are conglomerates of living organisms constituting microecosystems, that present complex symbiotic processes (Kivanç and Yapici 2016; Rosa et al. 2017).

The microbiota composition of kefir grains is susceptible to numerous variations, which may derive from several factors, such as the origin and storage of kefir grains, milk type (substrate), the microbiological composition of grains, processing conditions, grain/milk ratio, and environmental conditions such as fermentation time and temperature (Garofalo et al. 2015; Hatmal et al. 2018; Farag et al. 2020; Brasiel et al. 2021). Although there is no consensus on the composition of the kefir microbiota, some microorganisms most commonly isolated from kefir grains include the genera Lactobacillus, Lactococcus, Leuconostoc, Acetobacter, and yeasts such as Kluyveromyces and Saccharomyces (Farag et al. 2020; Brasiel et al. 2021).

Regarding its nutritional value, its benefits are mainly attributed to its rich nutritional composition, which includes macro and micronutrients, and essential amino acids (Rosa et al. 2017). Since natural kefir grains are unique and multispecific, kefir beverage is a distinct fermented dairy product, which has demonstrated promising probiotic effects due to the production of lactic acid and other metabolites from bacteria and yeasts, such as short-chain fatty acids (SCFAs) (Bengoa et al. 2019; Gao and Zhang 2019).

SCFAs are mainly produced from bacterial fermentation in the colon and activate different local beneficial responses. They can also reach brain signaling via the microbiota-gut-brain axis (Silva et al. 2020). This axis is a bi-direccional complex communication system between the gastrointestinal tract (GIT), the microorganisms that inhabit it, and the peripheral and central nervous systems (CNS) (Mörkl et 109 al. 2020).

An unbalanced composition of the gut microbiota, known as dysbiosis, has been involved not only in gut diseases but in pathologies of other organs, such as the brain (Wang and Wang 2016; Chong et al. 2019; Silva et al. 2020; Mörkl et al. 2020). Then, through the production of hormones (Silva et al. 2020), immunological factors (Mörkl et al. 2020), and metabolites (Wang and Wang 2016; Wouw et al. 2020), the gut microbiota may modulate the gut environment and interfere with the behavior and function of the host's CNS (Wang and Wang 2016; Chong et al. 2019; Wouw et al. 2020).

Although some of the mechanisms of action of kefir are still unclear, it acts as a modulator of the gut and fecal microbiota, mainly via SCFAs productions as a strategy with the potential to modulate the microbiota-gut-brain axis (Silva et al. 2020; Mörkl et al. 2020). Furthermore, studies shed light on the antimicrobial action (Rosa et al. 2017; Tenorio-Salgado et al. 2021; Liu et al. 2022), hypocholesterolemic effects (Wang et al. 2009; Arslan 2015; Rocha-Gomes et al. 2018, Bourrie et al. 2018), antioxidant (Chen et al. 2015; Chen et al. 2020; Güven et al. 2021) and anti-inflammatory effects of kefir (Leite et al. 2013; Seo et al. 2018; Chen et al. 2020), and all these features together uphold the increasing kefir consumption worldwide.

Culture-independent methods, such as the high-throughput sequencing of the 16S rRNA gene, have recently been used to analyze kefir diversity and microbial structure, safety, and colonization of microbial communities in the hosts (Dertli and Ahmet 2017). Due to the probiotic potential of kefir and easy production at home, the present study aimed to analyze the microbiota profile of milk kefir and its daily consumption on modulation of the metabolism, oxidative stress, and the influence in the microbiota-gut- brain axis in a healthy murine model.

2.3 **Materials and Methods**

2.3.1. Milk Kefir Beverage Preparation

The kefir grains used in the study were obtained from a household in Viçosa, located in the Zona da Mata Mineira, Minas Gerais, Brazil. The production of fermented milk was performed at the Nutritional Biochemistry Laboratory (LABIN), Department of Nutrition and Health, Federal University of Viçosa (UFV). Initially, the grains were daily activated, cultivated for a few weeks, and inoculated at a concentration of 10% w/v in ultra-high temperature (UHT) whole cow's milk (Cotochés, batch MG1 AM 5). The jars were kept at room temperature, 25°C ± 2°C for 24 h, without agitation in an aerobic environment. Subsequently, the grains were recovered by sieving, washed with distilled water, and inoculated into milk, repeating the steps described above for a new production of kefir. Then, the fermented kefir milk produced proceeded to maturation under refrigeration at 7°C ± 2°C for 24 h, and was administered orally to the experimental animals via gavage. Samples were collected for physicochemical, microbiological, and antioxidant analyses (Supplemental Fig. S1).

2.3.2 Characterization of Milk Kefir Beverage

2.3.2.1. Nutritional Composition

The determination of the nutritional composition of kefir was performed in triplicate by the methods described by the Association of Official Analytical Chemists (AOAC) (AOAC 1989).

The carbohydrates were calculated by the difference between moisture, ash, lipids, and proteins (IAL 1985). Both analyses were made in triplicates. The nutritional composition of kefir is described in Supplemental Table S1.

2.3.2.2 Microbiological Analysis

The lactic acid bacteria (LAB) count was performed in triplicate by serial dilution of kefir milk followed by plating on Man, Rogosa & Sharpe (MRS) agar plates (Acumedia, Michigan, United States -USA) according to Reis (2015). Fungi and yeasts were counted in triplicate by the surface plating method using the pour plate technique and incubated at 28°C for 7 days (Reis 2015). The count of thermotolerant coliforms and Salmonella was performed in triplicate according to the protocol described in Normative Instruction number 62 of August 2003 of the Ministry of Agriculture, Cattle and Supplying (Ministério de Agricultura, Pecuária e Abastecimento, MAPA) and ISO 6579 /IDF 93: 2001 (Brasil 2003). The counting of LAB, fungi and yeasts, thermotolerant coliforms, and Salmonella spp. for fermented beverages is described in Supplemental Table S2.

2.3.3.3 Antioxidant Analysis of Kefir

Kefir samples (6 g) were mixed with 60 mL of aqueous methanolic solution (70:30%, v/v) at room temperature in the dark for 4 h with a magnetic stirrer. The extracts were centrifuged at 1420g for 10 minutes and filtered, and the supernatants were used for ABTS (2,2'-Azino-Bis-3-Ethylbenzothiazoline-6- Sulfonic Acid) and DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) spectrophotometer analysis according to Sahin et al. (2012).

The total antioxidant capacity (ABTS•+ radical cation) was measured by mixing 0.5 mL of kefir extract and 3.5 mL of ABTS solution. This mixture stood for three minutes at room temperature, and the absorbance at 734 nm was recorded using a microplate reader (Multiskan GO). The DPPH discoloration assay was carried out in methanolic DPPH solution (0.2 mM), with absorbance adjustment to standardize the control solution. Then, 0.8 mL of kefir extract and 0.4 mL of a methanolic solution containing DPPH radicals were mixed to give a final concentration of DPPH radicals of 0.2 mM in a test tube. Absorbance readings were recorded using a spectrophotometer (Multiskan GO) at 517 nm after the samples being kept in the dark for 30 minutes. The calibration curve of Trolox was made for both analyses using different concentrations (10, 20, 30, 50, 70, 90, 110, 130, and 150 μM). The results were expressed in milligrams of Trolox equivalents (TE) per 100 mL of sample and by a percentage of free radical scavenging (%) = [1-(sample absorbance at 517 nm) / (methanol absorbance at 517 nm)] × 100

(Supplemental Table S3).

2.3.3.4. Total DNA extraction of milk kefir beverage

DNA from milk kefir beverage was extracted based on the CTAB (cetyltrimethylammonium bromide) method (Chen et al. 2011). The quality and quantification of the DNA were analyzed by Nanodrop with calculation of the ration 260/230 and 260/230, and the Polymerase Chain Reaction (PCR) with 16s rRNA primers was performed to check for impurities.

2.3.4. *Experimental design*

2.3.4.1. Animals and ethical aspects

Twenty healthy male C57BL-6 mice at eight weeks old, weighing approximately 22g, were obtained from the Central Animal House of the Health and Biological Sciences Center of the Federal University of Viçosa (UFV). The sample size was calculated according to Mera et al. (1998), and no animals were excluded. A confidence level of 95% was used ($\alpha < 0.05$), obtaining a value from the distribution table t (two-tailed) = $t\alpha/2 = 1.7171$ with a statistical power of 95% and degrees of freedom (df)= 24-2 = 22. Mice were randomized by weight using the online Research Randomizer® software available at <https://www.randomizer.org>. The animals were divided into two experimental groups (n=10/group) namely: the water group that received a commercial diet and orogastric gavage with filtered water (0.1mL) and the kefir group that received a commercial diet and orogastric gavage with milk kefir (0.1mL, 10% w/v). The volume administered was adjusted based on daily consumption of previous probiotic and kefir studies, gastric capacity of mice and to consider attainable a human daily intake of milk kefir of approximately 4.5 mL/ Kg for humans of 70 kg (Wouw et al. 2020; Dos Santos Cruz, et al. 2020).

All animals were housed collectively in polyethylene cages in the Experimental Nutrition Laboratory during the entire experiment. The animals were kept in a room with controlled temperature (22 ± 2º C), humidity (60-70%), and a 12-h light/dark cycle. Filtered water and a commercial diet (Presence®) were provided ad libitum during the experimental design period. The animals were weighed weekly in the morning, and feces were collected.

The euthanasia procedure was performed with an experienced support team. The animals were individually placed in a transparent sealed box for anesthetic saturation, i.e., 3 to 5 minutes of isoflurane Isoflorine®, Cristalia, Itapira, Brazil), using a simple circuit with a flowmeter coupled to an oxygen cylinder (3% to 5% mixture of isoflurane and oxygen). This procedure increased the expected effects of sedation and deep anesthesia; then, physiological parameters

and reflexes were evaluated to determine the degree of anesthesia sensitivity minimizing the stress of manipulation.

Fasting blood was obtained from the abdominal artery by total exsanguination, centrifuged, and the serum was stored in an ultra-freezer at -80oC. During the euthanasia, the organs (brain and intestines) were removed, separated, washed in phosphate buffer solution (PBS), weighed, and stored in an ultra-freezer at -80oC for further analysis.

The experimental protocol was approved by the National Technical Commission on Biosafety (CTNBio) and by the Ethics Committee on Animal Experimentation (CEUA) of the Federal University of Viçosa (Universidade Federal de Viçosa, Minas Gerais, Brazil), under process number 37/2020, in compliance with current legislation (Law No. 11,794, of October 8, 2008). The experiment was conducted following the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines and the normative resolutions issued by the National Research Council's Guide for the Care and Use of Laboratory Animals, as well as the National Council for the Control of Animal Experimentation (CONCEA), the Brazilian Practice Guideline for the Care and Use of Animals for Scientific and Didactic Purposes (DBCA) and the Guidelines for the Practice of Euthanasia recommended by CONCEA.

2.3.4.2. Analysis of growth parameters, somatic indices, and food intake

The animals were weighed on a digital scale (Marte Slim, model M 2K), individually, weekly, on a fixed day (Monday). After euthanasia, the brain and intestines were collected, separated, washed in PBS, weighed on a digital scale (Marte Slim, model M 2K), and stored at -80º C. Somatic indices were calculated according to the following equations: IS = W / AW x (100), where: IS = somatic index; W = organ weight; and AW = animal weight (Supplemental Table S4). Food intake was determined based on the amount (g) of diet offered by subtracting leftovers (g) not ingested. The quantification of leftovers was performed weekly on an electronic scale (Marte Slim, model M 2K).

2.3.4.3. Serum biochemical analysis

After euthanasia, fasting blood was used in the analysis of serum biomarkers: aspartate aminotransferase (AST) (U/L), alanine aminotransferase (ALT) (U/L), total protein (g/dL), globulin (g/dL), low-density cholesterol (LDL) (mg/dL), triglycerides (mg/dL), uric acid (mg/dL), total cholesterol (mg/dL), high-density cholesterol (HDL) (mg/dL), alkaline phosphatase (U/L), urea (mg/dL), creatinine (mg/dL), glucose (mg/dL), calcium (mg/dL), and albumin (g/dL). The analysis was performed in an automated clinical chemical analyzer (BS-

200 old, Mindray®) using commercial kits (Bioclin®).

2.3.4.4. Analysis of oxidative stress and antioxidant enzymes

Colon, small intestine, and brain samples were homogenized in phosphate buffer (0.1M, pH 7) and centrifuged at 10000 g at 4°C for 10 minutes. The supernatant was used to evaluate the oxidation products, malondialdehyde (MDA) (intestines and brain) (Buege and Aust 1978; Wallin et al. 1991), and the pellet was used in carbonylated protein (colon) (Levine et al. 1990). The activity of the antioxidant enzymes catalase (CAT) (Hadwan and Abed 2016), superoxide dismutase (SOD) (Dieterich et al. 2000), and glutathione S-transferase (Habig et al. 1974) was also evaluated in the brain and intestines. Results were normalized by the total protein concentration of the supernatant (Lowry et al. 1951).

2.3.4.5. Quantification of SCFAs in feces, brain, and small intestine

The fecal SCFAs extraction was based on the method of Smiricky-Tjardes et al. (2003) with modifications. Freshly excreted feces were stored at -80ºC until processing. Stool samples of approximately 50mg were weighted and homogenized in 950 µl of Mili-Q water and kept for 30 minutes on ice, being homogenized every 5 minutes. Afterward, the samples were centrifuged at 10000 g, for 30 minutes, at 4ºC, three times, collecting the supernatants each time between centrifugations. The final supernatants were filtered through a syringe filter (0.45µm) and placed in a vial. The brain and small intestine were used according to a methodology adapted from Siegfried et al. (1984). Tissues were homogenized with Mili-Q water followed by the addition of calcium hydroxide, cupric sulfate, vortexing, and freezing. Then, the samples were thawed and centrifuged, the supernatant was reserved, and concentrated sulfuric acid was added. Finally, the final supernatant was filtered through a syringe filter (0.22 µm) and placed in a vial.

The analysis was performed using high-performance liquid chromatography (HPLC), in a Shimadzu chromatograph (Shimadzu of Brazil, São Paulo-SP), coupled to ultraviolet (UV) detector model SPD-20A VP. It used a wavelength of 210 nm, HPX 87H column (Aminex), 300x7.8mm, and pre-column of the same phase (Bio-Rad Brazil Laboratories, Rio de Janeiro-RJ), run flow of 0.6 mL/minute, run duration of 50 minutes, column pressure of 75 kgF, oven temperature of 32ºC and injected volume of 10 µL. The mobile phase was acidified water (0.005 M sulfuric acid).

2.3.4.6. Metataxonomic analysis of the fecal microbiota

Fecal DNA extraction was performed with 30 ± 2 mg of stool samples according to a protocol from Zhang et al. (2006). The quality and quantity of the extracted nucleic acids were verified by NanoDrop with calculation of the ration 260/230 and 260/230, absence of degradation smear in the 0.8% agarose electrophoresis, and positive amplification by PCR using 337F and 518R 16s rRNA primers for V3 hypervariable region to detect 16S rRNA (Park et al. 2021). Subsequently, the samples were sent to the company responsible for the sequencing, on an Illumina HiSeq 2500 platform. The V3-V4 hypervariable region of the 16S rRNA gene was amplified and the qualities of the sequences obtained were evaluated using the FastQC package (version 1.44.3) (Wang and Qian 2009; Caporaso et al. 2012). The low-quality sequences were trimmed using the Trimmomatic program (Version 0.36) and the high-quality sequences were analyzed using the DADA2 package (Version 1.14.1) implemented on the R platform (Version 1.2.1335). Then, they were aligned to the Silva 16S rRNA database (Version 138.1), and the evaluated taxonomic interest levels were the phylum, family, and genus. The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

2.3.5 *Statistical Analysis*

The data were submitted to the Kolmogorov-Smirnov or Shapiro-Wilk normality test. Body weight was submitted to Anova One-Way repeated measures. Total food intake, weekly food intake, body weight gain, serum biomarkers, oxidative stress biomarkers, antioxidant enzymes, and SCFAs were analyzed using the unpaired T-test with Welch's correction, whereas statistical differences were represented by (*) with a 5% of significant level ($p < 0.05$). A non-metric multidimensional scale analysis (NMDS) was performed on the R platform (Version 1.2.1335) to analyze variability between experimental groups. Alpha diversities were compared by Shannon and Simpson indices, while the Chao 1 index was used to assess bacterial richness between groups. The relative abundance and comparison of specific taxa were carried out by unpaired T- test analysis with Welch's correction. The software used to statistical analyses and graphs was GraphPad® Prism, version 8.3.0. (San Diego, California, USA).

2.4. **Results**

Microbial profiling and antioxidant activity of milk kefir beverage

2.4.1 Bioinformatics analysis

Bioinformatics analysis and microbiota profiling were performed by sequencing the V3-V4 hypervariable region of the 16S rRNA gene. A total of 2,649,259 sequences were obtained for

kefir beverage with a 465-length amplicon. Low-quality and chimeric sequences were removed. For kefir beverage samples, 2,647,956 high-quality readings were obtained with an average of 882,652 sequences for each sample. The sequences of kefir beverage were assigned to 781 amplicon sequence variants (ASVs). These results suggest the good quality of the extracted DNA and sequencing, which probably covered most of the microbial diversity.

2.4.2. Milk kefir microbiota

The relative abundances of bacteria at the phylum, family, and genus levels identified are reported in Fig. 1a-c. The prevalent bacterial phyla in the kefir samples were Proteobacteria (81.3%), Firmicutes (17.1%), and Bacteroidetes (1.6%) (Fig. 1a). Burkholderiaceae was the most abundant family found (54.0%). Other seven families were also predominant, such as Enterobacteriaceae (16.2%), Carnobacteriaceae (11.7%), Pseudomonadaceae (8.3%), Enterococcaceae (3.4%), Moraxellaceae (2.9%), Staphylococcaceae (1.9%) and Bacteroidaceae (1.6%) (Fig. 1b). Regarding the genera found in kefir, Comamonas represented most of its composition (54.0%), followed by Hafnia-Obesumbacterium (13.2%), Carnobacterium (11.7%), Pseudomonas (8.3%), Enterococcus (3.4%), Acinetobacter (2.9%), Staphylococcus (1.9%), Serratia (1.6%), Bacteroides (1.6%) and Buttiauxella (1.4%) (Fig. 1c).

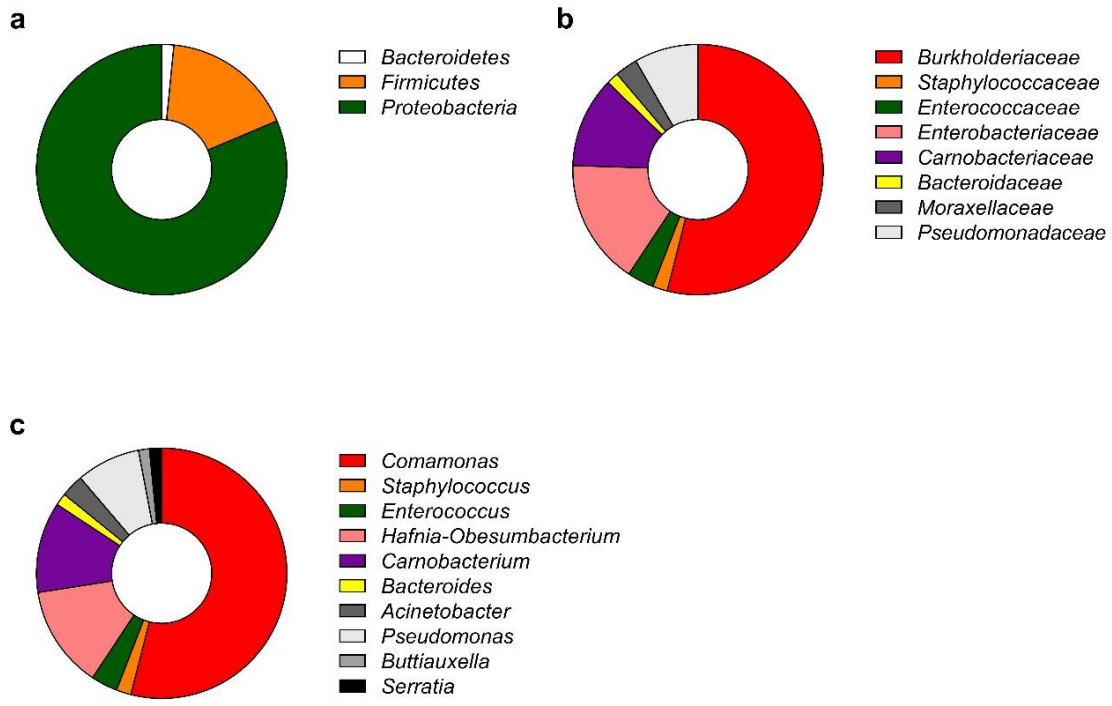


Fig. 1 Relative abundance distribution of major phyla (a), families (b), and genera (c) found in the milk kefir beverage (10%w/v) (top 50 bacteria). The analysis was made in triplicate (n=3 kefir sample).

2.4.3. Kefir increases free radical scavenging ability

The ability of the sample to quench free radicals was measured using the ABTS method, and the results showed that the scavenging capacity of milk kefir was greater than that of UHT whole milk ($p=0.0033$) (Supplemental Table S2). The DPPH method also showed the higher ability of fermented milk to scavenge free radicals compared to UHT milk ($p < 0.0001$). Furthermore, kefir had a percentage of $76.64 \pm 0.42\%$ of free DPPH radical scavenging.

2.4.4 *Effect of kefir in the C57BL-6 mice*

2.4.4.1 Effect of kefir on growth parameters, food intake, and organs

During the experiment, the average weekly body weight and total weight gain between the experimental groups were homogeneous (Fig. 2a-b). The administration of kefir did not exert a differential effect on the weight of the animals over the weeks, and no differences were observed in food consumption between the experimental groups (Fig. 2c-d). No significant differences were observed in the somatic indices (Supplemental Table S4).

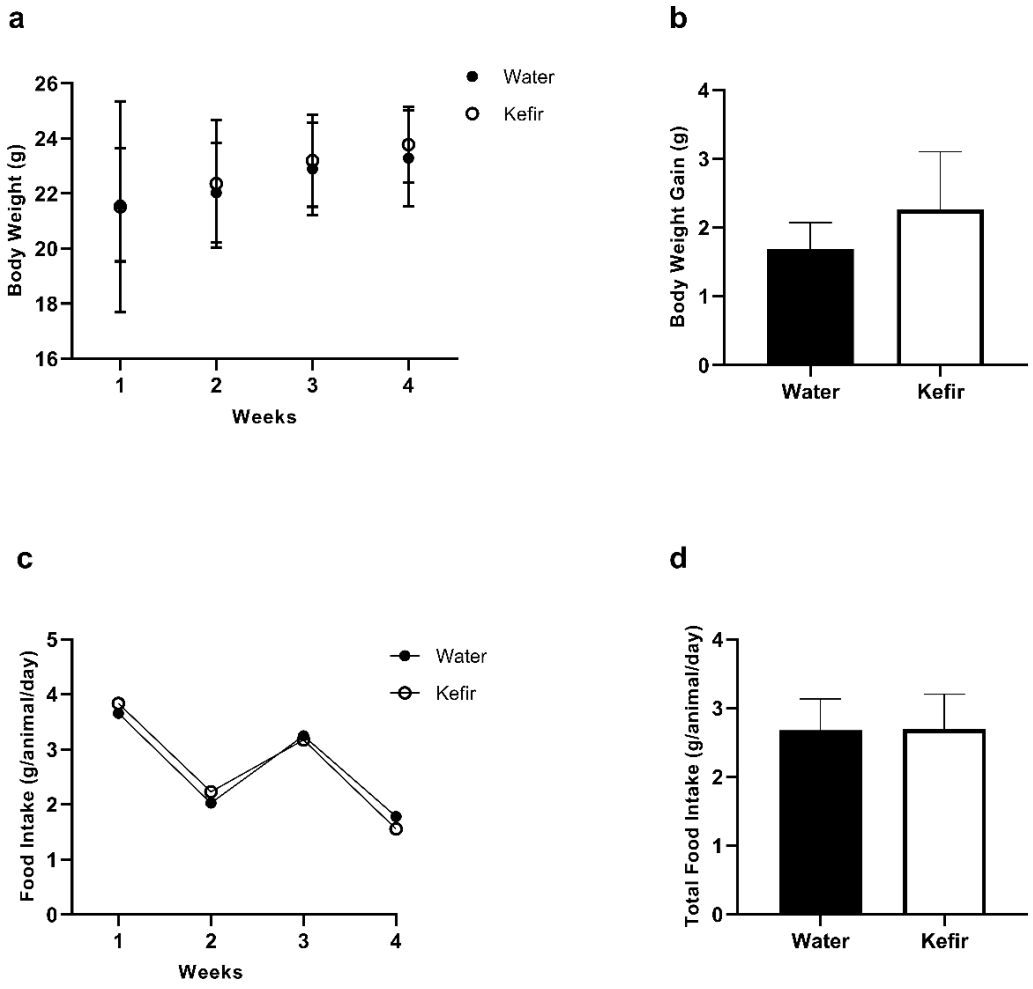


Fig. 2 The effect of treatments on growth parameters and food intake of C57BL6 mice. (a) Body weight (g); (b) Body weight gain(g); (c) Food Intake (g/mice/day); (d) Total Food Intake. Weight data are expressed as mean ± Standard error media (n = 10 mice/group). The results of weekly food consumption are represented as the mean ± mean standard error of each experimental group (the means referring to the food consumption pool of the groups/animal number (1 pool/week)). Statistical differences between groups were analyzed using the unpaired T-test with Welch's correction (Body weight gain, food intake, and total food intake) and ANOVA One-way repeated measures (Body weight), in which (*) represents significant
differences ($p < 0.05$)

2.4.4.2 Effect of kefir on serum biomarkers, oxidative stress, and antioxidant enzymes

Oral administration of kefir reduced the liver enzymes ALT (Fig. 3a) and AST (Fig. 3b), total proteins (Fig. 3c), and globulins (Fig. 3d.) compared to the water group. Although the oral administration of milk kefir reduced triglyceride (Fig. 4f) and uric acid concentration (Fig. 3g), LDL level was increased (Fig. 3e). Parameters such as alkaline phosphatase (Supplemental Fig.

S2c), urea (Supplemental Fig. S2d), creatinine (Supplemental Fig. S2e), albumin (Supplemental Fig. S2h), total cholesterol (Supplemental Fig. S2a), HDL (Supplemental Fig. S2b), glucose (Supplemental Fig. S2f), and calcium (Supplemental Fig. S2g) were not altered in mice fed with kefir.

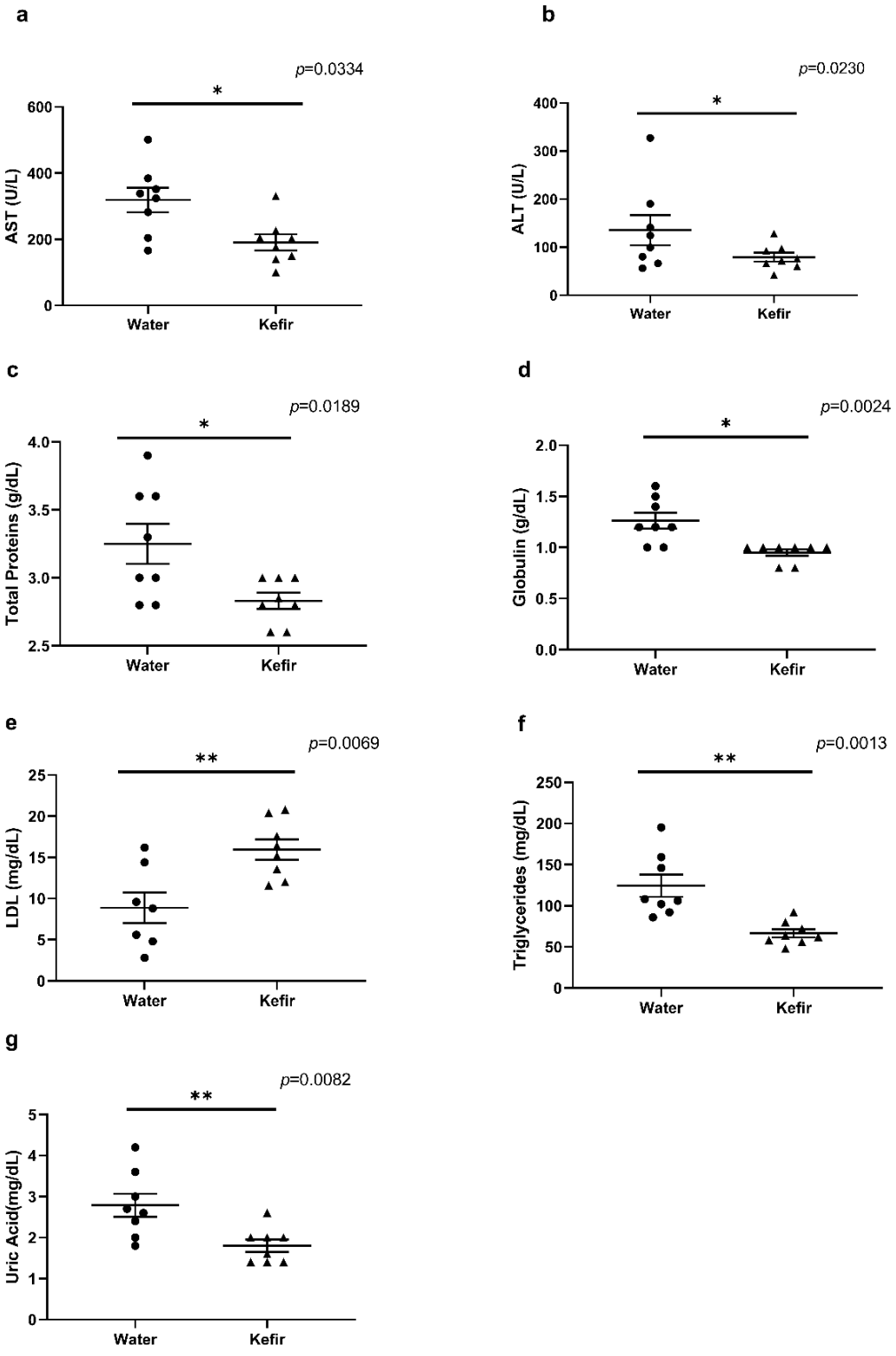


Fig. 3 The effect of treatments on the concentration of serum biomarkers in C57BL6 mice (a) AST (U/L); (b) ALT (U/L); (c) Total Proteins (g/dL); (d) Globulin (g/dL); (e) LDL (mg/dL); (f) Triglycerides (mg/dL); (g) Uric Acid (mg/dL). Data are expressed as mean ± mean standard error (n = 8 mice/group). Statistical differences between groups were analyzed using the unpaired T-test with Welch's correction, in which (*) represents significant differences with $p < 0.05$, (**) $p < 0.01$ Regarding oxidative stress biomarkers and antioxidant enzymes, no significant differences were observed concerning MDA in all organs ($p > 0.05$) (Fig. 4a, f, j), protein carbonylation in the colon ($p=0.6207$) (Fig. 4b), and GST (Fig. 4e, i) in the colon ($p=0.7215$) and small intestine ($p=0.8069$). However, kefir increased SOD ($p=0.0406$) (Fig. 4c) and CAT($p=0.0329$) (Fig. 4d) concentrations in the colon compared to water group, whereas in the small intestine, kefir reduced CAT ($p=0.0246$)(Fig. 4h) and no differences in the SOD ($p=0.5927$)(Fig. 4g) were observed. In the brain, the effect of kefir was opposed to the colon, reducing brain SOD ($p=0.0171$) (Fig. 4k) and brain CAT ($p=0.0249$) (Fig. 4l) compared to the water group and increased brain GST ($p=0.0022$) (Fig. 4m).

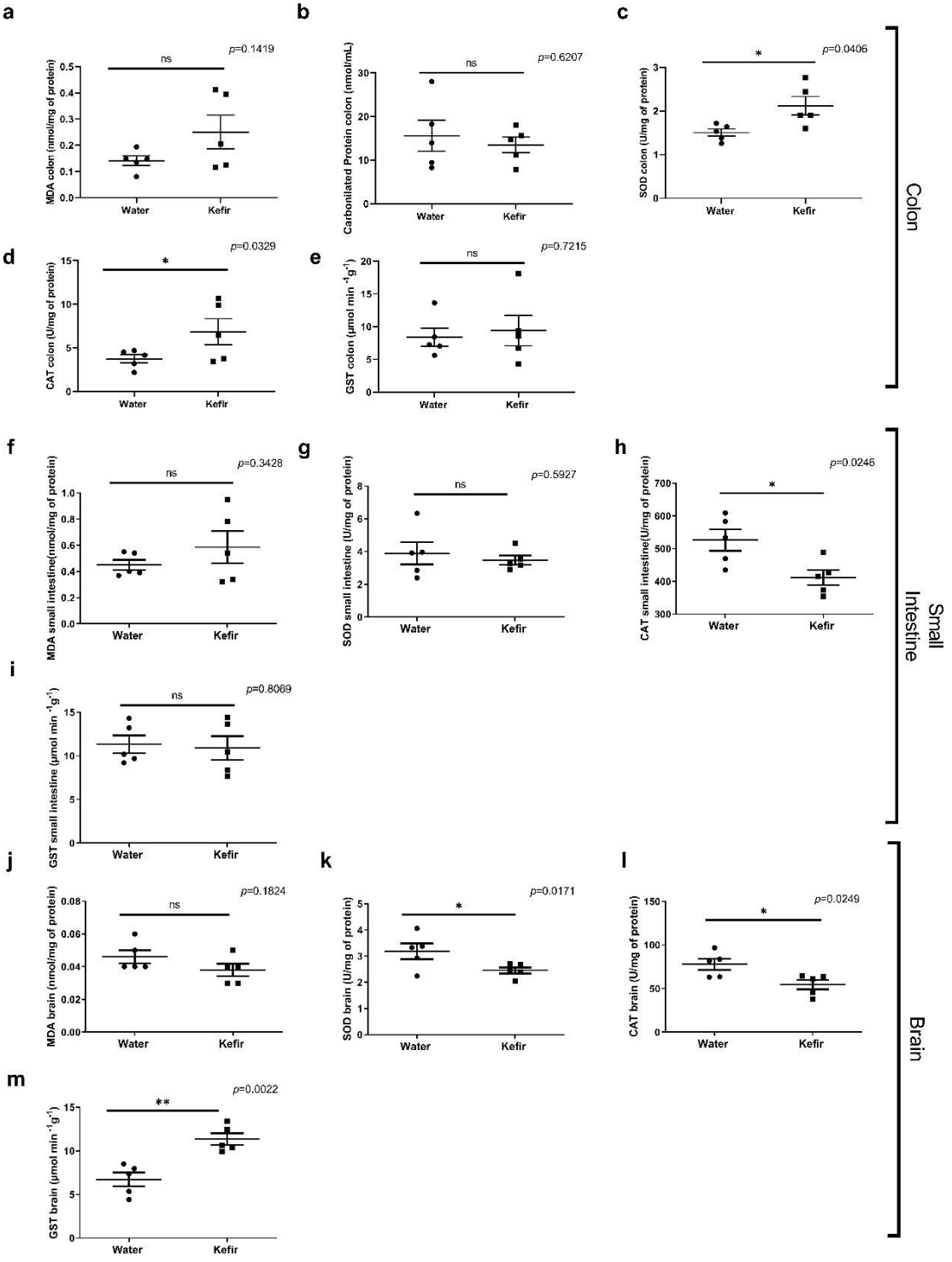


Fig. 4 The effect of treatments on biomarkers of oxidative stress and antioxidant enzymes on the colon, small intestine, and brain in C57BL6 mice. Data were expressed as mean ± mean standard error (n = 5 mice/group). (a) MDA (Malondialdehyde) colon; (b) Carbonylated Protein colon; (c) Superoxide dismutase (SOD) colon; (d) Catalase (CAT) colon; (e) Glutathione s-transferase colon (GST); (f) MDA small intestine; (g) SOD small intestine; (h) CAT small intestine; (i) GST small intestine; (j) MDA brain; (k) SOD brain; (l) CAT brain; (m) GST brain. Statistical differences between groups were analyzed using the unpaired T-test with Welch's correction, in which (*) represents significant differences (p <

0.05), (**) p<0.01

2.4.4.3 Effect of kefir on the SCFAs production in the brain, feces, and small intestine

Oral administration of kefir increased butyrate (p=0.0289) and propionate (p=0.0160) in the brain of the kefir group compared to the water group (Fig. 5a-b), but brain acetate was not altered by kefir consumption (p=0.1352)(Fig. 5c). On fecal SCFAs production, the treatment with kefir increased butyrate (p=0.0400) (Fig. 5d) but not altered concentractions of acetic acid (p=0.0504) (Fig. 5e), and propionic acid concentrations were not found in the samples. Regarding the fatty acids in the small intestine, no significant differences were observed in propionic acid (p=0.0647) and acetic acid (p=0.0289) between the water and kefir groups (Fig. 5f-g), and butyric acid was not found in the samples.

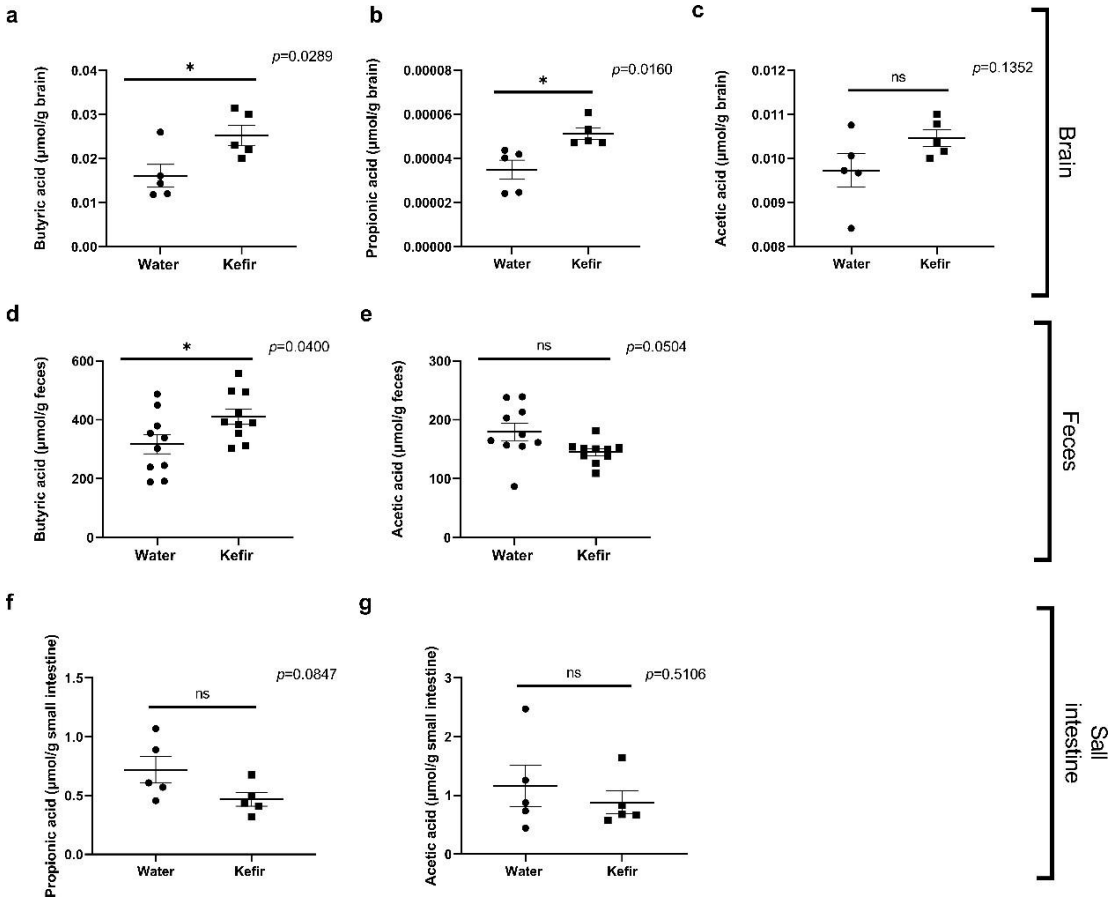


Fig. 5 The effect of treatments on SCFAs concentration on the brain, feces, and small intestine in C57BL/6 mice. (a) Butyric acid (µmol/g of the brain); (b) Propionic acid (µmol/g of the brain); (c) Acetic acid (µmol/g of the brain). (d) Butyric acid (µmol/g of feces); (e) Acetic acid concentrations (µmol/g of feces); (f) Propionic acid concentrations (µmol/g of small intestine); (g) Acetic acid concentrations (µmol/g of small intestine). Data are expressed as mean ± mean standard error (n = 5 mice/group to

brain and small intestine; n=10 mice/group to feces). Statistical differences between groups were made using the unpaired T-test, where (*) represents significant differences ($p < 0.05$)

2.4.4.4. Diversity and metatoxonomic analysis of fecal microbiota

A total of 1,377,816 sequences from fecal DNA were obtained with a 465-length amplicon. Low-quality and chimeric sequences were removed. For the fecal DNA, 1,377,129 high-quality readings were obtained, with an average of 229,521.5 sequences for each sample. The sequences of fecal DNA were assigned to 766 amplicon sequence variants (ASVs). These results suggest the good quality of the extracted DNA and sequencing, which probably covered most of the microbial diversity.

A non-metric multidimensional scale analysis (NMDS) was performed to analyze the observed variability. The NMDS plot (Fig. 6a) showed that the distances between samples from the water group are smaller than the kefir group samples, meaning that the water group presents greater microbial homogeneity.

Therefore, mice fed with water were more similar to each other than the kefir treated mice.

Alpha diversities were compared between experimental groups by Shannon and Simpson indices (Fig. 6b-c), while the Chao 1 index was used to assess bacterial richness (Fig. 6d). Thus, there was no statistically significant difference in terms of bacterial diversity or bacterial richness between animalstreated with water or kefir ($p > 0.05$).

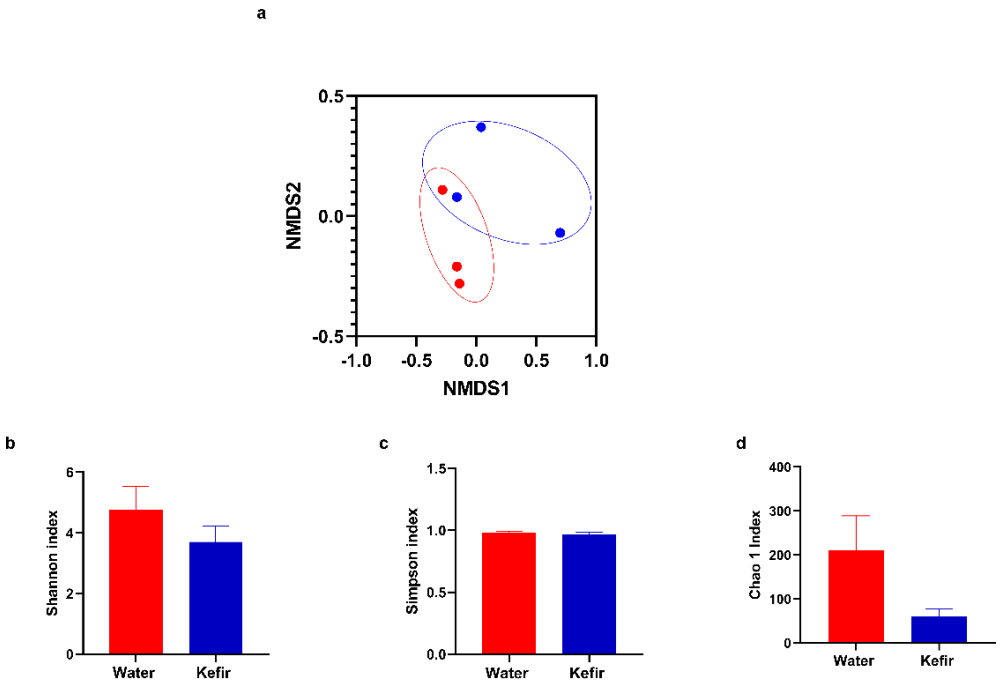


Fig. 6 Non-metric multidimensional scaling (NMDS) plot (a) of Metagenomic sequencing data in mice

treated or not with kefir. Red dots represent water treatment; Blue dots represent kefir treatment. (b, c) Alpha bacterial diversity; (d) Bacterial Richness (n= 9, distributed in 3 pools with 3 mice per group) Statistical differences between groups were analyzed using the unpaired T-test, in which (*) represents significant differences ($p < 0.05$)

At the phylum level, Firmicutes, Proteobacteria, and Epsilonbacteraeota were the most predominant among the fecal samples retrieved from both groups (Fig. 7a). After treatment of kefir, the relative abundance of Firmicutes was higher in the kefir group compared to the water group (71.8% vs. 423 48.2%,1.49-fold, p=0.0130) and lower for the phyla Proteobacteria (24.2% vs. 29.8%, 0.8-fold, p=0.0268), and Epsilonbacteraeota (4.1% vs. 8.7%, 0.47-fold, p=0.1410) (Fig 7a). The phyla Bacteroidetes (7.41%), Patescibacteria (3.0%), Actinobacteria (1.64%), and Verrucomicrobia (1.22%) were found exclusively in the stool samples of animals that received water compared to animals that received kefir.

At the family level, the Lachnospiraceae family was more abundant among the top fifity bacteria in the group that received kefir compared to water (71.8% vs. 42.5%, 1.7-fold, p=0.0160), followed by Helicobacteraceae, which had a reduction in the relative abundance in the group that received kefir compared to the group treated with water (4.1% vs. 8.7%, 0.47-fold, p=0.0180). The families Pseudomonadaceae (15.4%), Ruminococcaceae (5.7%), Prevotellaceae (4.8%), Enterobacteriaceae (4.3%), Saccharimonadaceae (3.0%), Muribaculaceae (2.6%), Eggerthellaceae (1.6%), Puniceicoccaceae (1.2%) were found exclusively in the group that received water (Fig. 7b).

Among the fifty most abundant genera, thirteen genera have selected the groups (Fig. 7c). The genus most found in both groups was Lachnospiraceae_NK4A136_group, but it was more abundant in the group that received kefir (31.3% vs. 17.4%, 1.8-fold, p=0.0205); however, for Helicobacter, kefir reduced the relative abundance of this strain compared to animals that received water (4.1% vs. 8.7 %, 0.47-fold, p=0.0955). Of all the genera identified, nine of them, such as Pseudomonas (15.4%), Roseburia (9.4%), Prevotellaceae_UCG-001 (4.8%), Escherichia/Shigella (4.3%), Candidatus_Saccharimonas (3.0%), Lachnospiraceae_UCG-006 (2.6%), Lachnospiraceae_UCG-004 (1.5%), Ruminiclostridium_9 (2.2%), and Anaerotruncus (1.4%) were identified only in the group that received water. In the treatment with kefir, two other exclusive genera were found, e.g., Lachnoclostridium (28.4%) and Tyzzerella_3 (12.1%).

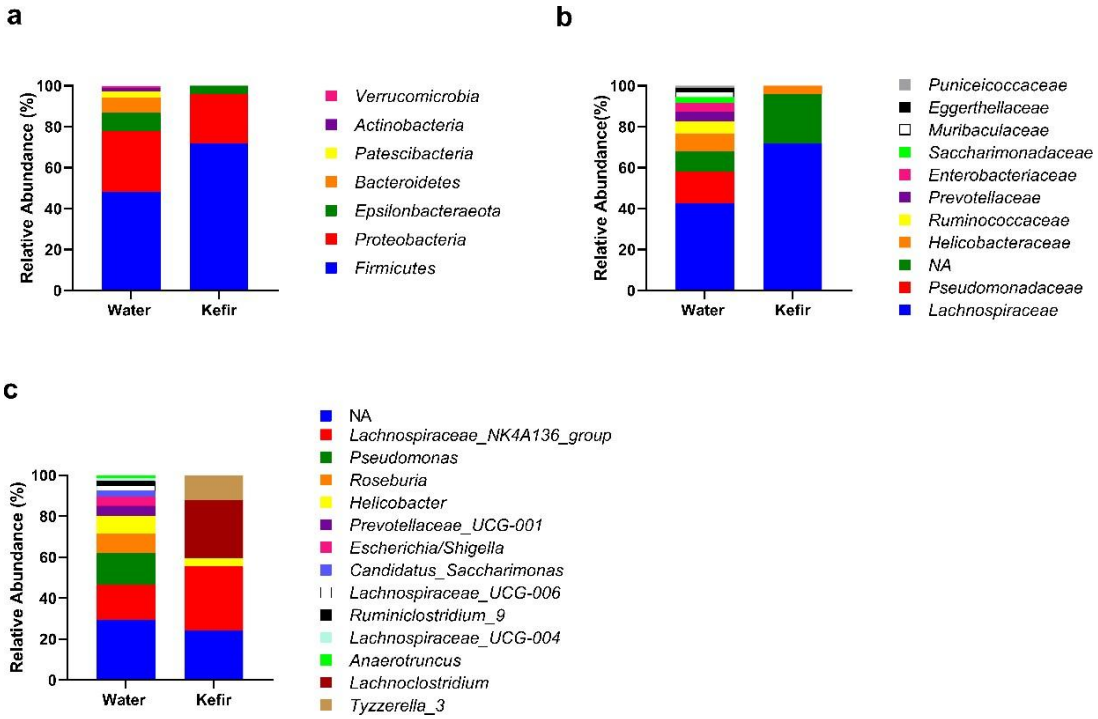


Fig. 7 Relative abundance of phyla (a), families (b), and genera (c) across the water and kefir experimental groups (10%w/v) (top 50 bacteria). The taxa were sorted by the decreasing order of average relative abundance (n= 9, distributed in 3 pools with 3 mice per group). Statistical differences between groups were analyzed using the unpaired T-test with Welch's correction, in which (*) represents significant differences ($p < 0.05$). NA means not available.

2.5 Discussion

Changes in overall microbiota of milk kefir beverage and gut microbiota has been reported previously (Carasi et al. 2015; Kin et al. 2017; Hsu et al. 2018; Gao et al. 2019; Bellikci-Koyu et al. 2019; Yilmaz et al. 2019; Du et al. 2021). The cited studies reported that kefir grains can modulate the gut microbiota by altering hosts bacterial communities, contributing to gut health. These changes are expected and can vary according to the type of milk used, concentration of grains/milk, and either the traditional or industrial fermentation procedure. In our milk kefir beverage, the phyla Proteobacteria, Firmicutes, and Bacteroidetes were reported, which are commonly found in studies with isolated probiotics and milk kefir from different parts of the world (Dobson et al. 2011; Gao and Zhang 2019; Brasiel et al. 2021; Du et al. 2021; Tenorio-Salgado et al. 2021).

The microbiota can be diverse among hosts as observed in our study since the mice's fecal

microbiota was different from kefir beverage. Fecal microbiota was dominated by three main phyla: Firmicutes, Proteobacteria, and Epsilonbacteraeota as was corroborated by the works of Hugon et al. (2015) and Dos Santos Cruz et al. (2020). The mice fed with kefir presented a specific intestinal microbiota composition, which could act positively on the gut-brain axis through increases in SCFAs. Notably, none of the bacterial strains present in the kefir beverage microbiota were detected in the gut microbiota of mice receiving kefir, indicating that the milk kefir microbiota did not colonise to high levels. This fact has parallels with the fact that probiotics most frequently do not colonise the gut (Derrien et al. 2015, Wouw et al. 2020). Gut microbiota coexists in true symbiosis with the host playing the main role as a key element for well-being and brain health (Cryan et al. 2019; Peluzio et al. 2020; Wuow et al. 2020). The homeostasis of the intestinal microbiota and good function of the gut-brain axis depends on the characteristics of the host and environmental conditions (Cani et al. 2008; Conlon and Bird 2015; Bourrie et al. 2016; Cryan et al. 2019) and a dysfunction of this axis may be a relevant contributor to many diseases of the nervous system, such as depression, anxiety, and demencia (Chong et al. 2019; Barbosa and Barbosa 2020; Mörkl et al. 2020).

The most predominant genus in our kefir beverage, Comamonas, has been known for its versatile catabolic abilities (Wu et al. 2018). This genus can catabolize a wide range of organic substrates, including amino acids, carboxylic acids, steroids, and aromatic compounds. It has been reported that species of the genus Comamonas are part of the human gastrointestinal tract (Ricaboni et al. 2017). However, Comamonas has not yet been widely studied in humans, and their role in the intestinal microbiota of animals is still unclear. Furthermore, colonic fermentation of exopolysaccharides (EPS) produced by LAB in kefir beverage increases the proportion of microorganisms belonging to the genus Comamonas, as well as a substantial decrease in the proportion of enterobacteria (Kim et al. 2015; Bengoa et al. 2020), which corroborate for the results in the mices of our study.

The reduction of Enterobacteria, such as Shigella/Escherichia coli and Helicobacter, which are pathogenic, and the increment of the Lachnospiraceae family and Lachnoclostridium genus were found in our kefir-treated mice. While the presence of H. pilori in the gastric microbiome has been linked with peptic ulcer disease and gastritis, the low pH promoted by milk kefir intake may limit the types of microbes that live in the gastric lumen and select for acid-resistant bacterial populations, reducing pathogens (Hollister et al. 2014; Rosa et al. 2017; Tenorio-Salgado et al. 2021; Liu et al. 2022). Lachnospiraceae colonizes the intestinal lumen being some of the main producers of SCFAs and their impact on the host physiology is often inconsistent across different studies (Biddle et al. 2013; Sheridan et al. 2016; Peluzio et al. 2020). However,

a recent study found that the administration of probiotics and prebiotics increased levels of the genus Lachnospiraceae_NK4A136_ group, which also produces SCFAs and plays an important role in relieving colitis (Wang et al. 2019).

Among Firmicutes, the Lachnospiraceae family is also known to be able to hydrolyze diet-derived polysaccharides and other sugars producing SCFAs (Biddle et al. 2013; Wong et al. 2014; Sheridan et al. 2016). Nonetheless, Lachnoclostridium is a genus found in our treated kefir mice that can alter liver metabolism and improve the absorption of SCFAs, which could affect the brain and behavior (Silva et al. 2020). In addition, the genus Lachnoclostridium includes organisms from the Lachnospiraceae family and several clostridial clusters, such as Clostridium XIVa (Yutin and Galperin 2013; Guo et al. 2020). Clostridial cluster XIVa is known as a significant part of the human gut microbiota (Lopetuso et al. 2013; Guo et al. 2020), and it can exert anti-inflammatory effects and plays a role in homeostasis. In addition, via its components and metabolites, especially butyrate, the clostridial cluster XIVa can maintain intestinal health (Guo et al. 2020).

Butyric acid, or butyrate, is related to the decrease of DNA damage in colonocytes maintaining the intestinal barrier (Silva et al. 2020; Mörkl et al. 2020), which was found in higher concentrations in fecal SCFA in our work on mice treated with kefir. The class clostridia, to which Lachnoclostridium and Lachnospiraceae belong can induce Treg cells, which produce interleukin-10, suppressing inflammation (Atarashi et al. 2011; Dandashi et al. 2021). Moreover, among SCFAs, butyrate stands out because of its wide array of biological functions, such as ability to influence brain functions. It seems that butyrate produced via bacteria inhibits the translocation of the transcription factor of NF-kβ from the cytoplasm to the nucleus, decreasing the NF-kβ DNA binding activity, and reducing the transcription of pro-inflammatory factors (Inan et al. 2000; Alpino et al. 2022).

In our study, mice that received kefir showed increased butyrate (feces and brain) and propionate (brain) compared to untreated animals. This fact can be explained because EPS, a bioactive compound of kefir, is metabolized by gut microbiota, increasing propionic and butyric acid, two SCFAs that have been associated with improvement in gut health and neuroprotective effects (Bengoa et al. 2020). In addition, SCFAs are mainly produced in the colon, where are taken up by colonocytes via monocarboxylate transporters (MCTs), expressed in abundance in endothelial cells, generating ATP and energy for these cells in healthy individuals (Schönfeld and Wojtczak 2016; Dalile et al. 2019). Furthermore, SCFAs are known to influence in numerous physiological functions such as mucus production, gastrointestinal mobility, and immunity, maintaining the integrity of the intestinal epithelial barrier (Lewis et al. 2010;

Gonzalez et al. 2018; Dalile et al. 2019). Gut microbial-derived SCFAs are also increasingly implicated in emotional processing and behaviour, as butyrate and the increase abundance in Lachnospiracea has been shown to ameliorate cognitive impairments, tryptophan metabolism, and reduce anxiety/stress in mice (Liu et al. 2015; Wouw et al. 2018; Sun et al. 2019; Alpino et al. 2022).

SCFAs can also be produced in the small intestine but can be rapidly metabolized by passive diffusion. That may explain the lack of differences between experimental groups in our work. In a reduced fraction, they reach the peripheral circulation, where they translocate to other tissues, such as the brain (Gonzalez et al. 2018). Since SCFAs can cross the blood-brain barrier, probably via MCTs, they seem to play an important role in the development of the brain as well as the preservation and plasticity of CNS (Sarkar et al. 2016; Silva et al. 2020). Although the mechanisms involved remain unknown, some animal studies show that SCFAs influence behavior and neurological activities, exerting neuroactive properties, which is interesting for the prevention and adjuvant therapy of neurological and psychological disorders due to their neuroprotective functions. (Silva et al. 2020; Alpino et al. 2022).

Consequently, the effect caused by the consumption of milk kefir on the composition of the intestinal microbiota of healthy mice is due to a combination of factors, such as the direct inhibition of pathogens by SCFAs (main butyrate) and the competitive exclusion of pathogens in the intestinal mucosa by SCFA-producing bacteria favoring benefic bacterias in microbiota composition (Rosa et al. 2017; Farag et al. 2020; Peluzio et al. 2020; Al-Mohammadi et al. 2021). In addition, kefir starter cultures themselves and the metabolites of microorganisms formed during its fermentation lead to the formation of not only SCFAs but also the release of new antioxidant micronutrients associated with milk and antioxidant enzymes (Ylmaz-Ersan et al. 2018). These components have antioxidant effects by scavenging free oxygen or nitrogen radicals, chelating pro-oxidative metal ions, inhibiting lipid peroxidation, and ascorbate autoxidation as demonstrated in our work in the beverage (ABTS and DPHH) analyses and in previously studies (Leite et al. 2013; Sabokbar and Khodaiyan 2016; Karaçali et al. 2018; Seo et al. 2018; Chen et al. 2020).

Furthermore, we found that kefir maintained normal lipid peroxidation (gut and brain) and protein oxidation concentrations (colon) in our mice. It increased CAT and SOD antioxidant enzymes in mice's colon, whereas in the small intestine, kefir reduced CAT. Corroborating our results, Ozcan et al. (2009) evaluated the effect of kefir supplementation in rodents induced oxidative stress, and after 6 weeks of treatment, kefir consumption increased glutathione and reduced MDA to levels comparable to the non-induced group. Ghoneum et al. (2020) studied

the protective activities of a novel kefir product (PFT) on 10-month-old oxidative stress-induced mice. This study showed that administration of PFT significantly increased antioxidant enzyme activities of SOD, CAT, and glutathione peroxidase; decreased oxidative stress biomarkers nitric oxide, and malondialdehyde; reversed reductions in total antioxidant capacity, glutathione levels, and anti-hydroxyl radical content. Interestingly, the study found that administration of PFT reversed oxidative changes associated with ageing, hence normalizing the levels to young control mice in the brain. It is still argued that Bacteroides sp. produces antioxidants enzymes, such as SOD and CAT, which destroy toxic oxygen products (Karaçali et al. 2018). These microorganisms were found in the fecal microbiota of our mice and in the kefir beverage. Thus, the results support the hypothesis that kefir is a potential tool to control oxidative stress in the brain and colon because the consumption of kefir was not able to induce oxidative stress by itself and stimulate the activity of enzymes (Rosa et al. 2017; Ghoneum et al. 2020).

Regarding metabolism, several disorders, such as liver lipid accumulation, bacterial and viral diseases, inflammation, and endo and exotoxins, can induce hepatocellular injury and the release of transaminases into the bloodstream (Thrall et al. 2015). Kefir-treated mice reduced AST and ALT compared to the water-treated mice. Therefore, that explains the maintenance of levels or reduction in the absence of diseases. In this way, the globulins reduction and preservation of normal creatinine concentrations in the kefir group can be inferred as a way of balancing the state of homeostasis, which can avoid the stress caused by the experimental conduct itself.

The kefir treatment was shown to reduce uric acid compared to the water group.This effect were related in other studies, which exhibited a reduction in the inflammatory process and gout in vivo with kefir treatment (Dalberth and Palmano 2011; El-Bashiti et al 2017). The consumption of foods rich in purines and alcohol has been consistently linked to cases of hyperuricemia associated with chronic inflammation; however, dairy, fruits, vegetables, and other foods are inversely associated (Ekpenyong and Daniel 2014). For that reason, the reduction of uric acid found in our kefir group demonstrates a lower probability of inflammation in the mice and the risk of development of gout later, adding to the health benefits provided by the use of kefir on metabolism.

Another noteworthy action of kefir is to change the lipid profile. In our study, kefir reduced triglycerides compared to the water-treated group. In agreement with our data, Liu et al. (2006) observed that triglyceride concentrations were reduced by a diet based on milk kefir or soy kefir in hamsters for 8 weeks. When comparing the kefir treatment with the water group, an increase

in LDL is observed. Thus, effects on non-HDL fractions may be associated with the inhibition or not of exogenous absorption of cholesterol in the small intestine by incorporation of lipid molecules into bacterial cells and by suppression of bile acid reabsorption (Azizi et al. 2021).
Experimental evaluations sometimes can be expensive to implement because of the wide variety of outcome variables. Given a budget constraint and the limitation of mice tissues, such as the brain and colon to do all analyses, sometimes the best approach, in total sample size, may be a limitation. Another issue found in our study is the high variability of kefir samples which causes its identification a difficult task. Indeed, kefir produces a series of bioactive metabolites, such as butyrate and acetate, increases antioxidant enzymes, and changes the metabolism.
Althogether, our results show that kefir has overall health benefits and modulate aspects of the gut-microbiota-brain axis in health mice. This beverage can act not only as a probiotic but may act as a future psychobiotic reaching the host brain through the modulation of the fecal microbiota and its metabolites. The next step for further studies using kefir should be focused in explain the mechanisms related to neurological diseases and behavior alteration.

2.6 Conclusion

Milk kefir beverage exhibited highly antioxidant potency compared to milk and in vivo by not inducing oxidative stress as well as by increasing antioxidant enzymes. The beverage and gut microbiota composition were distinct but composed primarily of SCFA-producing bacteria, such as the genus Comamonas in the beverage and Lachnospiracea and Lachnoclostridium genus in mice. Our results on the brain and fecal SCFAs as well as the antioxidant effect in the colon are associated with the change in the bacterial communities caused by the kefir intake, which indicates that kefir positively influences the gut-microbiota-brain axis. In addition, kefir promoted growth and adequate food intake. It improved liver function, reduced triglycerides and uric acid reinforcing the general health benefits. More studies need to investigate the effects of kefir and kefir-associated bacterial strains as a psychobiotic food focused on neurological diseases. However, the evidence we provide in this study indicates that kefir directly contributes to the preservation of gut and brain health.

Author Contributions:

A.R., T.M. and M.C.P., Conceptualization; M.P, L.A., G.A., T.M. and M.C.P, Data curation; M.P, L.A., G.A., T.M. and M.C.P., Formal analysis; A.R., T.M. and M.C.P Funding acquisition; M.P, L.A., G.A., B.C., L.A., J.S., A.B., I.C., A.R., T.M. and M.C.P Investigation;

M.P, L.A., G.A., B.C., L.A., J.S., A.B., I.C., A.R., T.M. and M.C.P, Methodology; T.M. and M.C.P., Project administration; A.R., T.M. and M.C.P, Resources; M.P, L.A. and T.M, Software; A.R., T.M. and M.C.P, Supervision; M.P, L.A., G.A., B.C., L.A., J.S., A.B., I.C., A.R., T.M. and M.C.P., Validation; M.P, L.A., G.A., T.M. and M.C.P, Visualization; M.P., L.A., G.A. Roles/Writing - original draft; M.P, L.A., G.A., B.C., L.A., J.S., A.B., I.C., A.R., T.M. and M.C.P., Writing - review & editing. All authors have read and agreed to the published version of the manuscript.

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3. Artigo 2: Oral ingestion of kefir produced in household conditions increases the mortality of C57BL/6 mice infected with *Salmonella enterica* serovar Typhimurium

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Key words: Kefir, probiotics, *Salmonella* Typhimurium, antioxidant

3.1 Abstract:

Salmonellosis is one of the main causes of diarrheal diseases in the world and affect directly the intestinal microbiome. The use of probiotics such as Kefir is showed as an alternative to keep health associated microbiota with different benefits and it consume is expanding. In this work, the effect of infection with *Salmonella* enterica serovar Typhimurium in C57BL/6 mouse model after consumption of kefir-fermented milk drink produced in domestic conditions was evaluated. Previous consumption of kefir resulted in increased mortality of animals after infection, with increased detection of Salmonella in fecal samples. Kefir showed increase of inflammatory cytokines, reduction of inflammatory cells in the colon associated to increase of butyric acid. Analysis of microbiome indicated that animals treated with Kefir had a higher frequency of bacteria from the Lachnospiraceae family and the genus *Lachnoclostridium* and a decrease in enterobacteria, such as *Helicobacter* and *Shigella*/*E.coli*. The increase of inflammatory cytokines after using Kefir may have favored the systemic translocation of pathogen, resulting in a more severe infection. These results suggest that the indiscriminate use of probiotics, especially in cases of infections, should be evaluated with caution, as they may also be associated with toxic or unwanted effects.

3.2 Introduction

Salmonellosis is 1 out of 4 main causes of diarrheal diseases in the world and it is transmitted through contaminated food and water (1). Almost 550 million of cases are reported worldwide annually (1). In the European Union, in 2020, it causes 61 deaths among 53169 laboratory confirmed cases (2). In developing countries, the infection is a common cause of childhood sepsis (3) associated to waterborne cases, due to lack of basic sanitation and house hold storage.

A lso, in these areas, such as tropical Africa, it has a higher rate of children deaths due to bacteremia (4,5,6). The common symptoms are nausea, abdominal cramps, vomiting, headache, myalgias and non-bloody diarrhea, which are usually self-limiting and lasts less than 10 days (7). When the infection turns invasive, is necessary the use of antibiotics. Another problem which affects under developed countries is the expensive price of second-line antibiotics, like ciprofloxacin and ceftriaxone, which could be use if the strain is resistant for the first line antibiotics, such ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole (8, 9).

Alterations in the gut microbiome are related to some gastrointestinal tract (GIT) problems including salmonellosis, and probiotics may prevent them (10). Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host (11). A stable and adequate GIT microbiome is associated to health. For example, the lack of adequate gut colonization in newborns, may lead a later autoimmune disorders, allergies and higher risk of infectious.

Probiotics can also delay the progression of some diseases as: obesity, type 2 diabetes, osteoporosis, autism, inflammatory bowel diseases, diarrheal diseases, lactose intolerance and decrease inflammation processes (10, 12, 13). To be considered a probiotic, the food needs to contain a minimum number of viable microorganisms, at the time of the consumption. The right quantity depends on the regulation of each country (14). Some examples of probiotic foods are kefir, kombucha, natural yogurt, sauerkraut and miso (15).

Kefir milk is a fermented milk produced by inoculating kefir grains originally in cows, goats, sheep, camels, or buffalo milk. (16) It originates from the *Caucasus*, and it is a mixture of bacteria and yeasts living in a symbiotic association (17). The composition

of the kefir grains relies on many factors: where the grains come from, which medium is used to cultivate it, the type and composition of the milk used, the room temperature, the storage conditions, and the techniques used during the process. (8-16)

Kefir has been known by its benefits that includeanticarcinogenic and antimutagenic properties, antimicrobial activity, gastrointestinal better functioning, immunomodulation, anti-inflammatory, hypocholesterolaemic effect (18,19,20). Probiotics are showed to have benefic effects to control GTI pathogen infections.

The mechanisms associated to the benefits including the competition for nutrients decreasing the chances of pathogen's survival, production and secretion of antimicrobial substances, immune system modulation, secretion of acids which decrease the intestine's pH with potential of inhibition some pathogens, improvement of barrier integrity by decreasing membrane's permeability and repairing them after damage, stimulation of neurochemicals and hormones, production of enzymes which can avoid pathogenic adhesion or destroying pathogen's cell membrane, production of short-fatty acids (SCFAs) stimulating production of membrane receptor which boosts immune system; Suppression of Th2 chemokines inhibiting allergies (21,22).

Although many probiotic benefits are known, some mechanisms of actions of the kefir are still unclear. The use of probiotics in healthy adult is considered safe, but it can still be not effective (or even worsening) in specific situations, including for immunocompromised patients due to infection with fungi or bacteria (23, 24), young infants or low birth weight neonates (25, 26) and post antibiotics use can delay gut recovery (27). Additionally, many factors may contribute to the effect of probiotics such as the dosage, the diet and preexisting diseases on the patient, the microorganisms which constitute the probiotic, the number of viable microorganisms, metabolites produced by the probiotic (23). For salmonellosis, there are studies showing that kefir isolated microorganisms are able to improve the response against an infection with *Salmonella* Typhimurium *in vitro* or *in vivo* (28,29,30). However, the impact of infection after kefir oral ingestion, as well as, the mechanism associated to interaction between pathogen and host with continuous consummation of kefir were not previously studied. In the C57/BL6 infected model after Kefir consummation performed in this work demonstrated that the inflammatory cytokines induced by continuous ingestion of Kefir may have contributed to the worsening of the infection. When we are talking about the effect specifically about kefir, in case of infection caused by *Salmonella*; the number of

studies is rare. This is an innovative study, which tests the effect of Kefir ingestion before the infection. We evaluate the animals' immune response, production of SCFA's and the profile of bacterias on the feces. Because Kefir may change the microbiome and immunological environment, the results may not be the same observed when Kefir is only used after de infection.

3.2 Material and Methods

3.2.1 Kefir Production

Kefir grains were obtained from a household in the city of Viçosa, located in the Zona da Mata Mineira, Minas Gerais, Brazil. A total of 20g of kefir grains were inoculated in a sterile glass container in 200 ml of UHT whole cow's milk (10% w/v - Cotochés, batch MG1 AM 5). Kept at 25°C ± 2°C for 24 hours, without agitation, in an aerobic environment. The grains were sieved and washed with filtered water, and reserved for a new production. The prior produced beverage proceeded to maturation, at 7°C ± 2°C, for 24 hours.

3.2.2 *Salmonella enterica* serovar Typhimurium infection

The Salmonella enterica subspecie enterica serovar Typhimurium-ATCC14028 was kindly provided by Prof. Dr. Jacques Robert Nicoli, from Fundação Ezequiel Dias (FUNED, Belo Horizonte, Brazil). The pathogen was cultivated in Brain Heart Infusion broth (BHI, Difco, Sparks, USA) for 18 hours at 37ºC, in aerobic conditions.

3.2.3 Ethics and Biosafety

This experiment was approved under the process number 37/2022, according with the current Brazilian legislation by the Ethics Committee on Animal Experimentation (CEUA) of the Universidade Federal de Viçosa, Minas Gerais, Brazil. The experiment also was conducted following the Animal Research Reporting of In Vivo Experiments (ARRIVE) guidelines, the normative resolutions issued by the National Research Council's Guide for the

Care and Use of Laboratory Animals, as well as the resolutions of the National Council for the Control of Animal Experimentation (CONCEA), the Brazilian Practice Guideline for the Care and Use of Animals for Scientific and Didactic Purposes (DBCA), and the Guidelines for the Practice of Euthanasia recommended by CONCEA.

3.2.4 Mice model of Kefir treatment

The Central Animal House of the Health and Biological Sciences Center of the Universidade Federal de Viçosa (UFV) provided 40 healthy, male, eight weeks old C57BL/6J mice, weighing approximately 22g, which were randomized one day before the experiment, using the online Research Randomizer® software available at <https://www.randomizer.org>. The mice were divided in 4 groups (n=10/group): Uninfected water (NIW), uninfected kefir (NIK), infected water (IW) and infected kefir (IK). During the first 15 days, the groups IW and NIW received 0.1ml of filtered water, while NIK and IK group receive 0.1ml of kefir with 10^9 CFU/mL of total bacteria, orally by gavage. A total of 5 animals were kept in each polyethylene cage under temperature controlled at 22ºC (± 2ºC), humidity at 60-70%, and a 12-hour light/dark cycle with filtered water and commercial diet *ad libidum*. The animals were weighed weekly, and feces collected every 2 days. After 15^{th} day of Kefir treatment, the reminiscences animals of IW and IK groups received the single dose of the 10^6 CFU/mL of pathogen by oral gavage. [14]. The growth curve was made prior the infection to standardize and obtain this dose of bacteria. The number of surviving mice were evaluated daily. For the euthanasia, which happened at the 26^{th} day, the animals were placed individually in a translucid sealed box, until the effects of the anesthesia were visible, and its physiological parameters were tested t evaluate the performance of the anesthesia, and then the exsanguination was performed. During the euthanasia, the colon was removed and it was washed with saline solution, weighed, and stored in a -80ºC ultra-freezer.

3.2.5. Evaluation of the immune response profile in the colon

After oral treatment with Kefir, the Cytometric Bead Array (CBA) (BD™) kit

was used to quantify the cytokines IL-2, IL-4, IL-10, IL-17, TNF-α, and IFN-γ, according to the manufacturer protocol. A phosphate buffer was added to 50 mg of the colon, kept on ice and were ground in a tissue homogenizer (IKA®, model T10 basic). The samples were centrifuged at 10000 x g for 10 minutes at 4°C and the supernatant was stored. The beads were prepared in diluent solution and then added to a microtube containing 25 µL of the sample and 17 µL of the detector solution and incubated for 2 hours. 1ml of washingsolution was added and centrifugation was performed at 1800 x g for 5 minutes,at 4°C, and approximately 800 µL of the supernatant was discarded. The remaining volume was passed in the flow cytometer. The FCAP Array Software v3.0 was used to build a ten-point calibration curve and to process the data.

3.2.6. Histopathological analysis of the colon

Microscope slides were obtained from 6 animals/group. Carnoy's solution was used to preserve the mice's colon fragments, and then, cut transversally, washed in increasing concentrations of alcohol, dried, and impregnated in paraffin. A semiautomatic microtome was used to section the tissues, which were fixed on glass, and stained by Hematoxylin & Eosin (H&E). Photographs of the slides were taken with 40x magnification using a microscope coupled with a digital camera (Zeiss®, Inc.), and the number of fields with inflammatory cells were counted through the Image J software. The percentage was obtained by dividing the number of fields with inflammatory cells by the total number of fields (300 fields/microscope slides).

3.2.6. Quantification of fecal short-chain fatty acids (SCFA)

SCFA extraction was based on the method of Smiricky-Tjardes et al (2003)[18] with modifications. 50 mg of stool samples were weighed and homogenized in 950µl of deionized water every 5 minutes, for 30 minutes.The samples were kept in ice during this process, and then centrifuged at 10000 x g, for 30min, at 4°C, three times. The supernatant was collected and filtered using 0.45 µm filter. A calibration curve of acetic and butyric acids (SUPELCO®) was made to evaluate the concentration in the sample. The chromatograph (Shimadzu of Brazil, São Paulo-SP) was used to do a high-

performance liquid chromatography (HPLC) analysis on a chromatograph (Shimadzu of Brazil, São Paulo-SP). An ultraviolet (UV) detector (model SPD-20A VP) was coupled to the chromatograph.

3.2.7 Metagenomic analysis

For DNA extraction and analysis, three pools of feces (3 animals/pool) were done. It was collected before the infection, and 15 days after the infection. To extract the DNA, a protocol adapted from Zhang et al., (2006) [15] was used. The samples were quantified using a NanoDrop and the quality of the DNA was tested by performing a PCR for the 16S rRNA gene. Then, the samples were sent to be sequenced in the region V3-V4 of the 16s gene.

The following programs were implemented on R platform (4.1.1): FastQC package (version 1.44.3) was used to evaluate the quality of the sequences, and the low-quality sequences were trimmed by Trimmomatic (version 0.36). DADA2 removed the quimeras and analyzed the amplicon sequence variants and then, aligned to the SILVA 16s rRNA database. The data were normalized calculating the average of the tree samples, divided by the value of the total of the samples, to find the relative percentage. (Table S1). Phylum, family, and gender taxonomic levels were analyzed. From metagenomic data, the quantification of Salmonella in the feces was also performed.

3.2.8 Statistical Analysis

The statistical analysis was made in the in the GraphPad Prism software version 8.00 (San Diego, California, USA and the significance level accepted was 5% ($p<0.05$). To perform the normality of the data, the Kolmogorov- Smirnov test was performed. Then the T-test was used for comparison of two groups and One-Way ANOVA with Tukey post-test was performed to confirm the differences between three or more groups. The result showed the mean and standard deviation. The Long-rank test was conducted to analyze the difference between the survival rates.

3. Results

3.3.1 The oral ingestion of Kefir by C57/BL6 mice induces the production of inflammatory cytokines in the colon

In order to understand the intestinal environment induces by Kefir and which was subsequently challenged with *Salmonella*, the quantification of cytokines was performed in colon samples of mice after oral ingestion of Kefir or water (control). Kefir seems to decreased the anti-inflammatory IL-1 ($p=0.0233$), and increase inflammatory interleukins IFN-ɤ ($p=0.0395$) (Figure 1).

No significant differences were found between experimental groups for IL-2, IL-4, TNF-α and IL-17 concentrations. Therefore, this Kefir treatment induces an inflammatory environment based on cytokine profile.

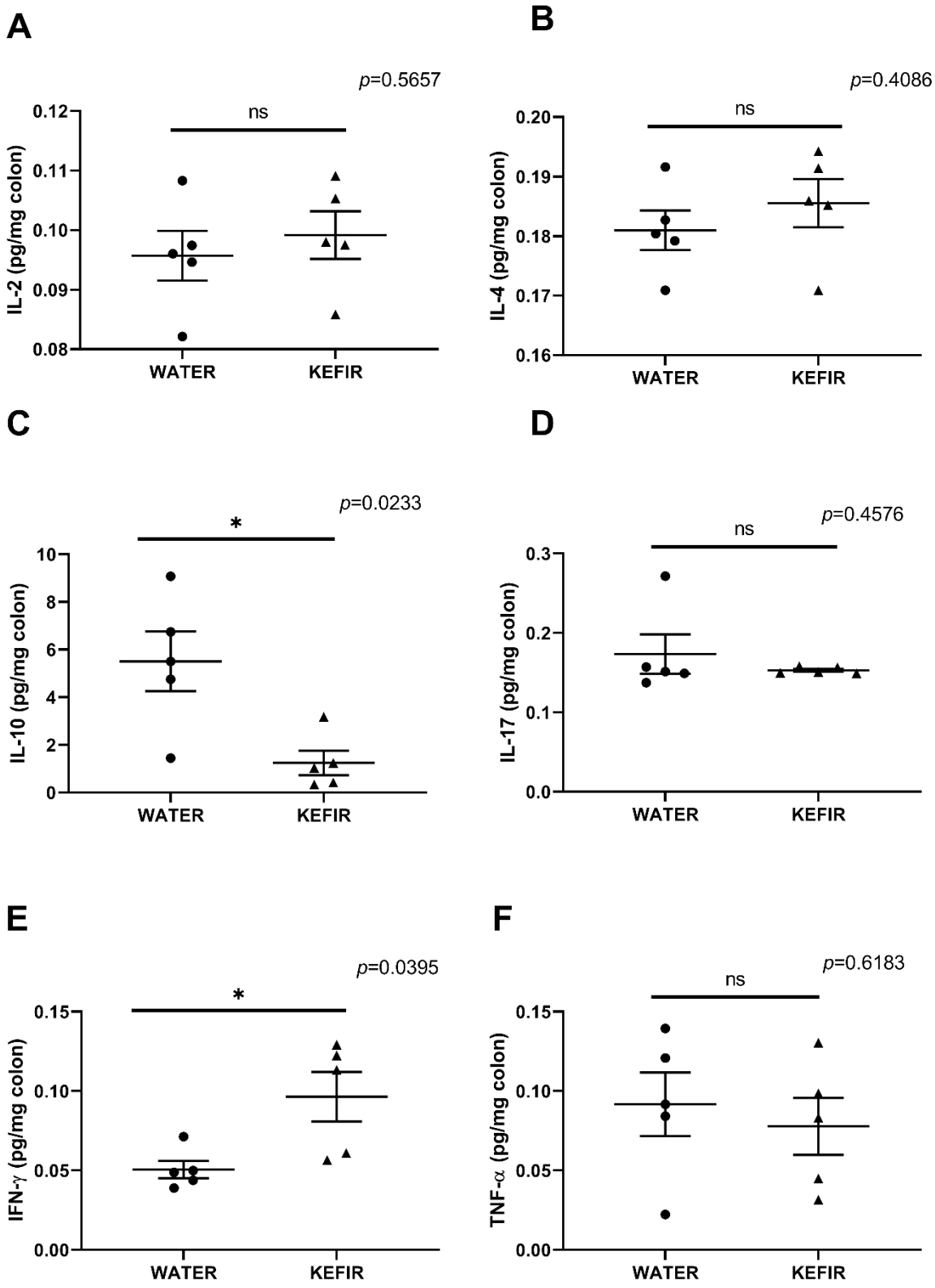

Figure 1. Effect of Kefir treatments on the cytokine concentration in the colon of C57BL/6J mice. (A) IL-2 (pg/mg colon); (B) IL-4 (pg/mg colon); (C) IL-17A (pg/mg colon); (D) IFN-γ (pg/mg colon); (E) TNF-α (pg/mg colon). Data are expressed as mean ± SEM (n = 5 mice/group)). Statistical differences between groups were analyzed using the T-test. Different letters represent

significant differences with $p < 0.05$.

3.3.2 The oral ingestion of Kefir by C57/BL6 mice induces the reduction of Inflammatory cells in the colon

Although the cytokine profile indicated the presence of a more inflammatory environment, the number of quantified immune system cells with inflammatory cells in the colon were reduced (Figure 2). Thus, although there is an inflammatory environment after kefir ingestion, there is also a reduction in the inflammatory cells in the colon. This result suggests that although the number of immune cells is lower in the tissue; these cells are likely to produce more cytokines.

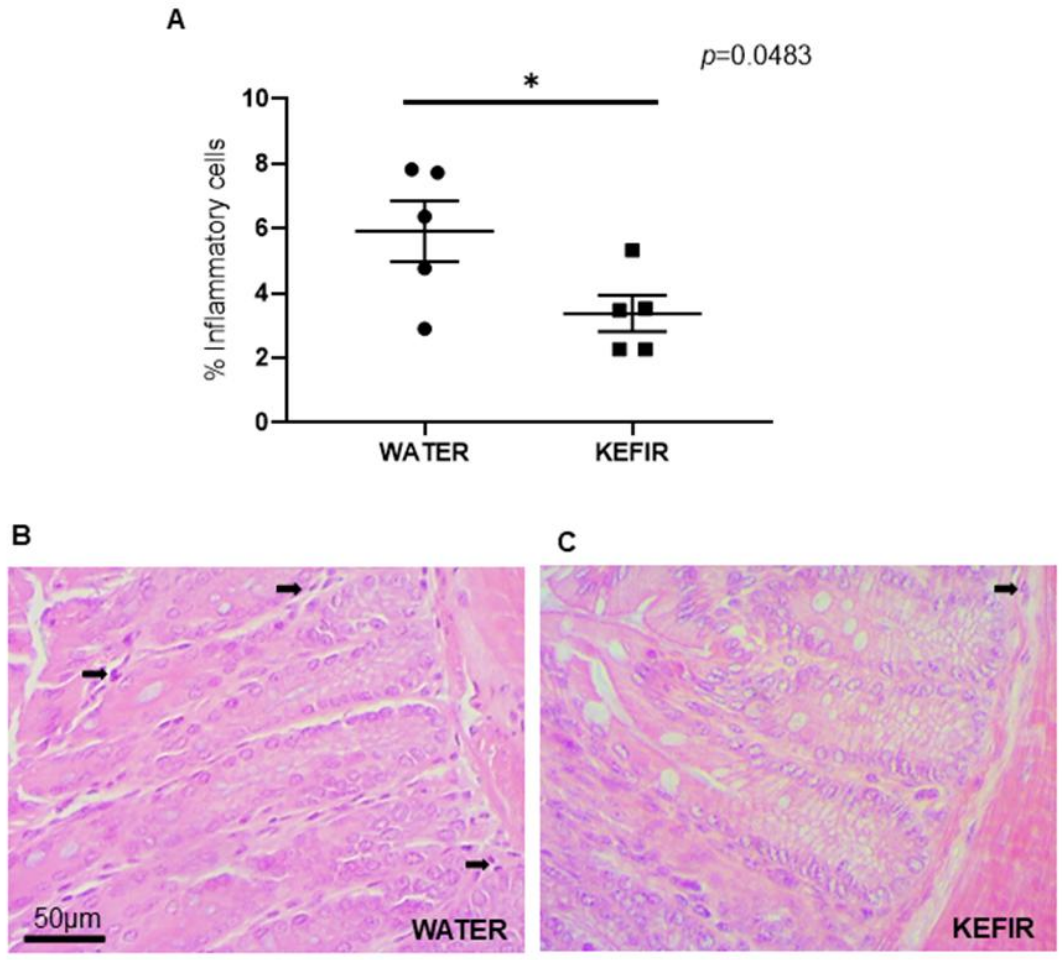

Figure 2. Percentage of inflammatory cells in the colon of C57BL/6J mice after oral ingestion of water or Kefir (A). Representative photomicrographs of colon sections from C57BL-6J mice that received filtered water (B) or kefir (C). Slides stained with H&E at 40× magnification. Black arrows indicate the presence of an inflammatory cell.

3.3.3 Oral ingestion of Kefir by C57/BL5 increases fecal Short Chain Fatty Acid

(SCFA) production

Since Kefir affected cytokines and inflammatory cells and this effect may be associated to intestinal microbiota, the concentration of SCFA, the main metabolites produced in the colon by bacterial fermentation of dietary fibers and resistant starch, was quantified (Figure 3). Animals treated with kefir showed higher concentrations of the butyric acid, both in the first week and in the second (Figure 4C, p=0.0147; Figure 4D, P=0.0400). Furthermore, no differences were observed in the production of acetic acid between treatments (Figure 3A; Figure 3B). Data were obtained in mg/mL (ppm) and transformed into a percentage of fecal mass (mg of FA/mg of feces). The result was expressed in mmol of FA/g of feces.

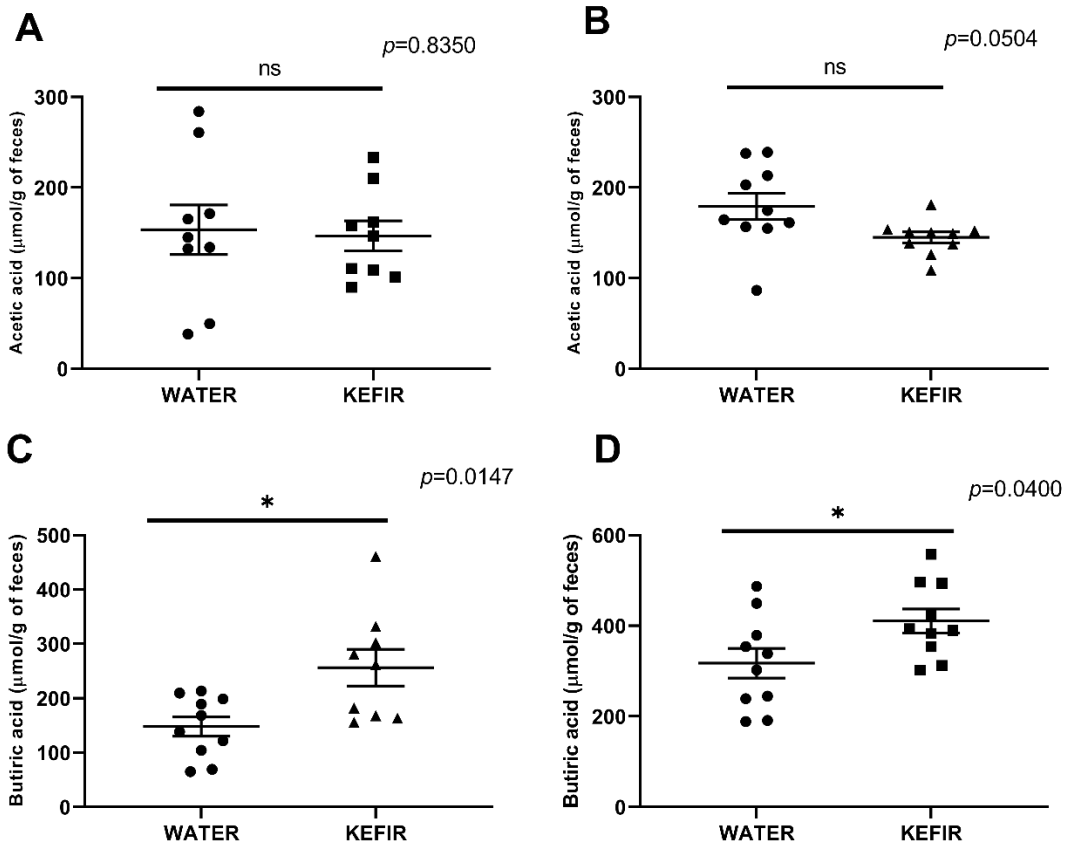

Figure 3. Effect of oral ingestion of Kefir on fecal SCFA concentration in C57BL/6J mice. (A) acetic acid concentrations (µmol/g of feces) in the first experimental week; (B) acetic acid concentrations (µmol/ g of feces) in the second experimental week; (C) butyric acid concentrations (µmol/g of feces) in the first experimental week (D) butyric acid concentrations (µmol/g of feces) in the second experimental week. Data are expressed as mean ± standard deviation (n= 10 mice/group). Statistical differences between groups were analyzed using the T-test.

3.3.4 Previous Kefir ingestion increases the mortality of C57BL/6 mice during infection with *Salmonella*

In order to evaluate the impact modulation of intestine environmental induced by Kefir to infection with *Salmonella enterica* serovar Typhimurium, the survival rate of the C57BL/6J animals after the pathogen challenge was detected (Figure 1). The oral administration of treatments in mice maintained animal survival at 100% for non-infected groups throughout the experimental period, suggesting that the mortality observed was associated only to infection. After 6-day infection, 50% of the animals in the infected group previously treated with water died.

On the other hand, the mortality of infected animal group previously treated with oral administration of kefir was of 90%, value statistically significant higher compared to water treated control group. To evaluate the impact of intestinal environment to Salmonella infection, total 16 amplified from bacteria DNA extracted of animal feces were sequenced by Metagenomics approach and quantification of normalized reads account specific for the genus Salmonella was performed. In the feces of non-infected animals (NIW and NIW) reads of the genus Salmonella was not found, excluding the possibility of infection or contamination of the animals before the experiment.

On the other hand, the normalized quantification of reads for infected animals that previously ingested kefir orally, suggesting that the intestinal environment induced by kefir provided an uncontrolled multiplication of the pathogen, with subsequent translocation and systemic disease, associated with the death of the mice.

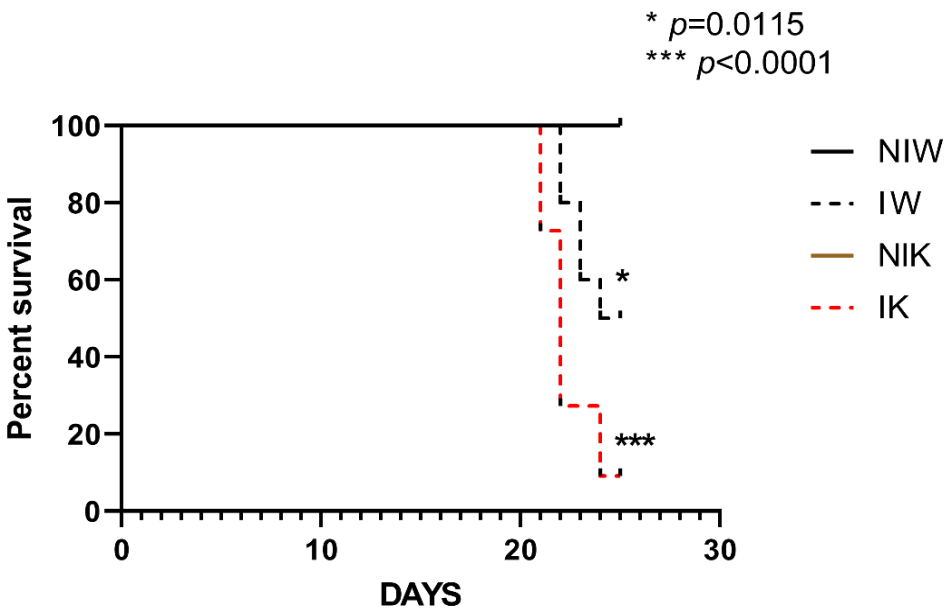

Figure 4. Effect of Kefir oral ingestion on the survival rate of C57BL/6J mice infected with *Salmonella enterica* serovar Typhimurium. Results are shown as percentage of animal survival during the experimental design period (n = 10 mice/group). NIW: Non-infected water group; IW: Infected water; IK: Infected kefir; NIK: Non-infected kefir.

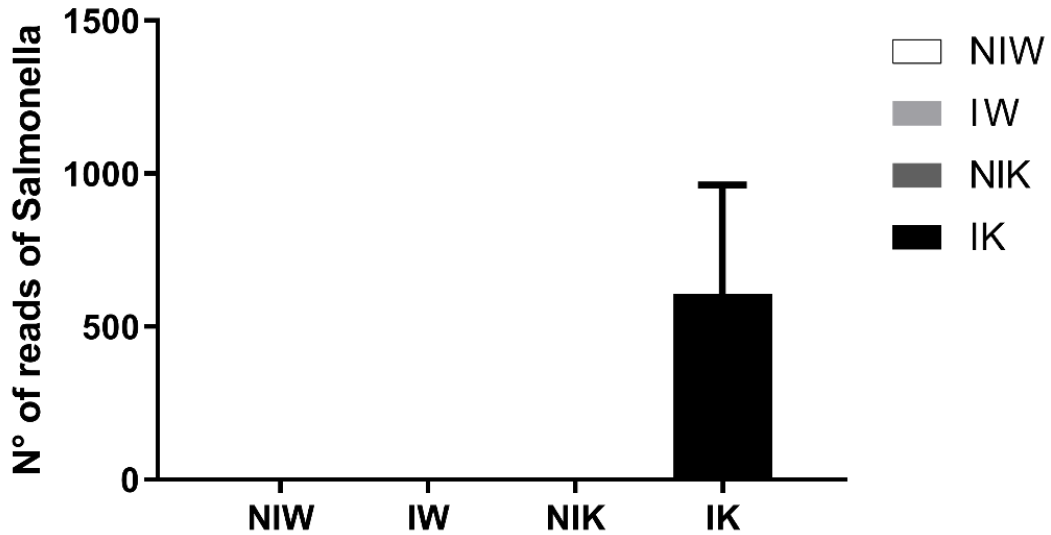

Figure 5. Quantification of Salmonella reads in the DNA extracted from stool samples of C57BL/6J mice infected and uninfected with *Salmonella enterica* serovar Typhimurium. The results are shown in number of normalized reads, with standard deviation and mean of 3 animals per group. NIW: Non infected water group; IW: Infected water; IK: Infected kefir; NIK: Non infected kefir.

3.3.5 Kefir alters the relative abundance in phylum, family and genus bacteria level with difference associated to course of infection

In order to understand the impact of microbiome in the infection course observed for each experimental group, metagenomics analysis was performed (Figure 6). The global differences between samples were measured by non- metric multidimensional scaling (NMDS) plot (Figure 6A. The groups are mostly different from themselves, except for a minimal overlap between non-infected groups, suggesting that there is difference between microbiome compositions that may be associated to infection results obtained. One sample of the group IW was an outstanding outlier, so it was taken it off from the analysis. The difference between microbiome of experimental groups were also analyzed by alpha diversity indexes (Figure 6B-D). All groups show no statistical difference values of these diversity indexes. Despite that, Simpson and Shannon indexes shows more similar values. On the other hand, Chaos 1 index shows a higher variability between to experimental groups, where the water non-infected group had more diversity that infected or/and kefir treated groups.

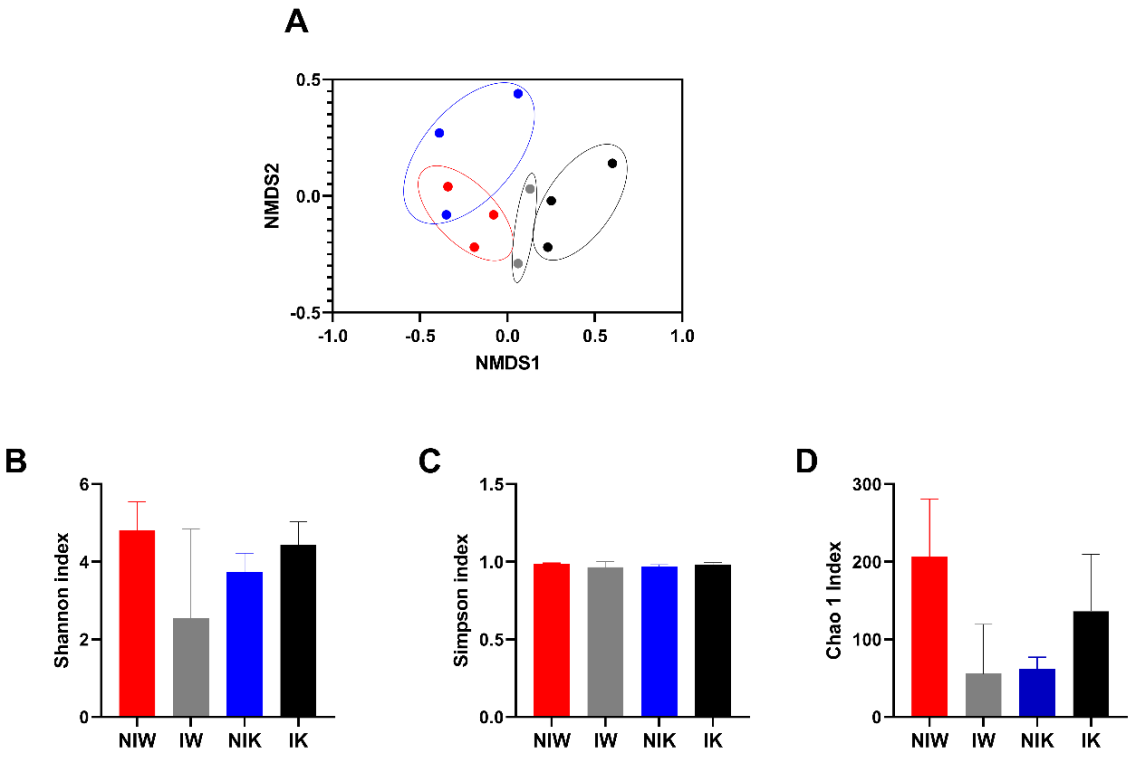

Figure 6. Comparison of microbiome composition and diversity indexes. Non- metric multidimensional scaling (NMDS) plot (a) of Metataxonomic sequencing data of 16s Gene, (b, c) Alpha bacterial diversity; (d) Bacterial Richness. Unpaired T-test was made to analyze statistical differences between the groups. No significant differences ($p<0.05$) were found.

DNA was extracted from the feces of mice treated with kefir or water, and orally challenged and not challenged with S. Typhimurium. Non-infected water (NIW – represented by red dots/bars), Infected water (IW – represented by gray dots/bars), Infected kefir (IK- represented by black dots/bars), Non-infected kefir (NIK – represented by blue dots/bars). To identify taxa associated to each experimental group, initially the sequences were assigned in six phyla: *Actinobacteria*, *Bacteroidetes*, *Epsilonbacteria*, *Firmicutes*, *Patescibacteria* and *Proteobacteria*. *Actinobacteria* and *Patescibacteria* only presented in the non-infected water (NIW) group. The most abundant were *Bacteroidetes* and *Firmicutes*, which showed different patterns comparing infected and non-infected groups. After infection, *Bacteroidetes* groups increased, while firmicutes groups decrease (Supplementary Table S1 and Figure 7). Comparing the genus level of kefir treated groups (NIK vs IK), it was observed that the infected group presented a larger amount of the genus *Bacteroides* and the family *Bacteroidaceae* (p=0.0268 and p=0.0375, respectively; table S1 and figure 6). In the water treated groups (NIW vs IW), the infected group presented a larger amount of the family *Muribaculaceae* (p=0.0398). Regarding the non-infected groups (NIW vs NIK), we can observe that the kefir have caused a decrease of the genus *Roseburia.* (p=0,0476). However, in the infected groups (IK vs NIK), we can observe that the kefir itself have caused a decreased of the genus *Prevotellaceae UCG-001* (p=0,0476). Candidus saccharimonas, Lachnospiraceae_UCG-004, Pseudomonas, *Roseburia*, *Ruminiclostridium* was detected only in the water treated non- infected (NIW) group. *Erysipelatoclostridium* and *Ruminococcaceae*_UCG-014 was detected only in the water treated infected (IW) group. The absence of certain genus was observed in some groups: *Lachnospiraceae*_NK4_A136 was not presence in water treated infected (IW) group. *Alloprevotella*, *Bacteroides*, *Escherichia/Schigella* and *Parasuterella* were not presence in kefir treated non-infected (NIK) group. *Helicobater* and *Prevotellaceae_UCG-001* were not presence in Kefir infected (IK) group. In general, infected groups had less firmicutes and more Bacteroidetes, so the ratio of Bacteroidetes to Firmicutes increased in infected groups (Figure 6).

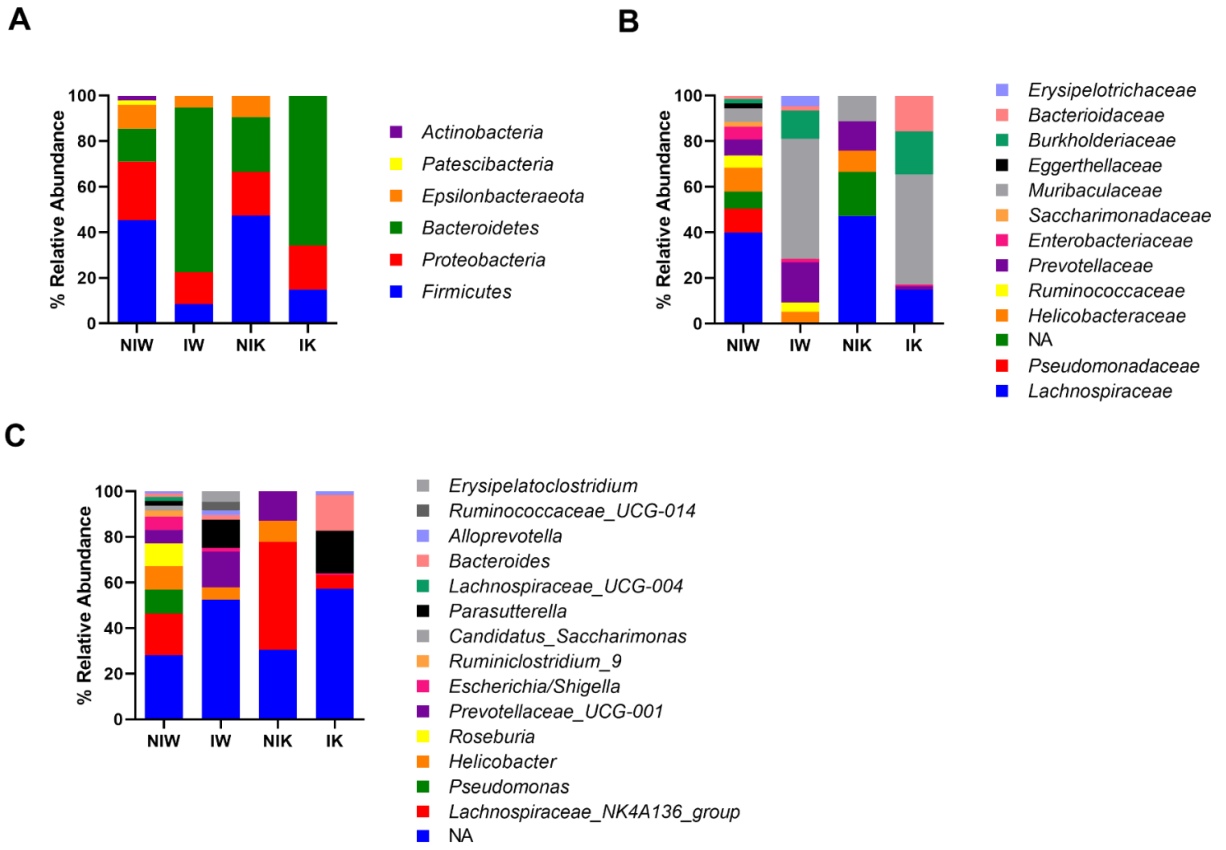

Figure 7. Relative abundance (%) of phylum (A), families (B), and genera (C) (top 50 bacteria) based on the 16s sequence. The DNA was extracted from the feces of mice treated with kefir or water, and orally challenged and not challenged with *S.* Typhimurium. non-infected water (NIW), infected water (IW), infected kefir (IK), non-infected kefir (NIK). NA means not Available.

3.4. Discussion

Nowadays, the scientific works, commercial marketing and community knowledge have mainly highlighted the benefits of the use of probiotics, even though its mechanisms are still unrevealed. Studies of probiotics are limited in different aspects: Some of them are in vitro (30), some test only strains isolated from the probiotic (29, 31, 32, 33) some test in human cells (34), many test them for obese patients (35, 36,37) and of course, the effect of each probiotic can vary if a disease happens. In vitro studies have shown presence of carbonyl compounds, histone, cathelicidin and lactic acid in kefir, which has an antimicrobial effect,

which could be the cause of the reduction of Salmonella Typhimurium and Salmonella Arizonae (30).

Studies can be a little controverse when just on variable changes: Many isolates from kefir have antimicrobial activity against these pathogens E. coli, Salmonella, Escherichia coli O157:H7, Salmonella enterica subsp. enterica serotypes Typhimurium and Enteritidis, Staphylococcus aureus and Listeria monocytogenes (38). However, when testing the possibility of kefir being a medium of growth for pathogen, if accidentally contaminated in the user's home, the outcome is different: many pathogens can grow in the kefir (39). So, kefir and its antimicrobials metabolites may not be enough to kill the pathogens.

Our work shows that kefir can be harmful when the mice get infected with Salmonella Typhimurium (Figure 4). Due to many beneficial of probiotics, we would expect the opposite, especially with kefir treatment. This study shows the unexpected effect of the daily use kefir when mice were infected with Salmonella Typhimurium. Half of the water treated animals died, however, the mortality was more than 90% for the kefir treated group. It is known that some isolates from kefir can increase the production of butyric and acetic acid.

Also, the presence of some exopolysaccharides (EPS), which are present on the bacteria's cell wall, can increase butyric acid concentration (40, 41). Butyrate enhances the junction in the cells of the intestinal barriers, making harder the invasion of pathogens (42) Na Figure 2, kefir treated animals showed smaller percentage of inflammatory cells. Kefir treated groups also produced more butyrate (Figure 3), which not necessarily means more protection.

As shown in Figure 5, the kefir treated infected group had higher level of salmonella detected in the feces, Kefir, as other probiotics are known for its anti-inflammatory properties (9, 43), developing less inflammatory cells as pictured in Figure 3. We would expect more anti-inflammatory interleukins been produced, which happened in IFN-ɤ, however, the opposite happened to IL-10 (Figure 1). This could be explained since each kefir has different microbiota, and studies is done with different kefir and sometimes, different strains of mice. IFN-ɤ increases could be explained by the decrease of IL-10 (since IL-10 has a negative effect on the production of IFN-ɤ), and because also other cells can produce and influence the production of IFN-ɤ).

The increase of early IFN-ɤ produced by NK cells would have a protective effect against salmonella, although this is not seen (44). Interesting, type-1 Interferon (IFN-α and IFN-β), inhibit anti- inflammatory and antimicrobial responses, which was not measured in our study, and could explain these results (45). Salmonella presence could have changed all the earlier scenarios of interleukins.

It is known that Salmonella enterica has a secreted effector (SarA-, Salmonella anti-inflammatory response activator) responsible for producing more IL-10, reducing the production of Reactive Oxygen species, which is essential to kill intracellular invaders (46). So SarA acts by increasing IL-10, raising intracellular replication. Since most of the kefir-treated and infected mice died (IK), we could not analyze their levels of cytokines. We could only expect that if the non-infected Kefir-treated group had kept its levels of cytokines, there would be a better elimination of the intracellular S. Typhimurium and a decrease in the systemic infection.

Still, since more animals treated with kefir died, maybe this decrease was not high enough to be protective, or changed due to the infection (44, 47). The higher complexity of bacteria in the gut microbiota, higher is the protection against Salmonella, since the bacteria forming the gut suppress pathogen growth (48), however, our analysis suggests that complexity among the groups are the same (Figure 7). There was a huge change in the ratio of Bacteroidetes and Firmicutes, which increased after infection, which indicates a dysbiosis (49). In the Kefir treated groups (NIK vs IK), the infected group (IK) presented larger amount of the genus Bacterioides and the family Bacteroidaceae.

Bacteroides can break high molecular weight carbohydrates and produces acetate, succinate, and isovalerate (50, 51). A few species are deeply studied, and a group inside the specie stands: the group ETBF (enterotoxigenic Bacteroides fragilis) is associated with diarrheal disease and may induce chronic inflammation (52). In the water treated groups (NIW vs IW), Infected groups (IW) presented a bigger amount of the family Muribaculaceae, which is little known, since they are uncultivated. This family is known for being utilizers of mucus-derived monosaccharide in the gut (53), production of the Propyanate (54), and its presence is correlated to an enhanced longevity in some cases, however the mechanism is still not uncovered (55), which could have contributed to the better response against salmonella.

Comparing the kefir-treated group to the group treated with water (NIW vs NIK), kefir (NIK) group had a decrease of Roseburia, a genus butyrate producer and associated with weight loss, the butyrate level was larger in the group treated with kefir (56, 57). Prevotellaceae Family is also a butyrate producer, and also had a smaller amount in the Kefir treated group, comparing the infected groups, (IK vs IW) (58). We can assume that the Prevotellaceae or Roseburia were not responsible for the increase of butyrate on the Kefir groups, since butyrate had a larger production.

The majority of Firmicutes are produces butyrate, and some its families as Lachnospiraceae, Ruminococcaceae are the main producers (59, 60). So, the larger ammount of butyrate of the

Kefir treated group could be explained by the presence of this families, or by bacterias we could not identify, even though there was a smaller ammount of Roseburia, comparing the NIW snd NIK groups (61). Although butyrate can maintain the intestinal barrier, it can also inhibit intestinal inflammation (62). As can be seen, (figure 3), kefir treated group (NIK) had less inflammatory cells.

In summary, the increase of inflammatory cytokines after using Kefir resulted in more deaths. Kefir could have favored a bigger systemic translocation of pathogen, resulting in a more deaths, although more studies are needed to assure this assumption. These results suggest that the indiscriminate use of probiotics, especially in cases of infections, should be evaluated with caution, as they may also be associated with toxic or unwanted effects.

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4 DISCUSSÃO INTEGRADORA E CONCLUSÃO

Kefir possui muitos efeitos benéficos incluindo ação antimicrobiana, hipocolesterolêmica, antioxidante e anti inflamatória, como já visto na literatura também comprovado por nosso trabalho. Esses efeitos benéficos tem contribuído para que milhões de pessoas no mundo terem aumentado o consumo dessa bebida. Várias cepas isoladas de probióticos são também descritas e vendidas comercialmente para ter efeitos benéficos generalistas, em sua maioria caracterizadas apenas *in vitro*.

Outro benefício observado em nosso trabalho é que há pouca diferença calórica entre kefir de leite e leite, além do fato da bebida não aumentar o consumo de calorias dos camundongos, o que faz a bebida ser uma boa opção para pessoas com sobrepeso ou obesas. Observamos também que o uso de Kefir pelos camundongos, levou a diminuição de globulinas, proteínas totais, AST e ALT no fígado. Houve diminuição de triglicérides e concentração de ácido úrico, embora o LDL tenha aumentado. Em suma, a bebida kefir apresenta muitos benefícios. É interessante notar, que usualmente, a microbiota presente nos probióticos não coloniza o intestino (DERRIENet al., 2015,). O gênero Comamonas foi predominante no Kefir, porém seu papel exatodentro da microbiota, não é muito claro.

Todos os benefícios citados levaram a expectativa que a presença e modulação de bactérias benéficas na microbiota intestinal poderia levar a redução dos efeitos negativos da infecção por patógenos intestinais, tais com Salmonella. Este efeito de controle de patógeno poderia ser associado a competição por nutrientes moduladas beneficamente por micro-organismos do Kefir, produção de compostos antibacterianoscom atividade sob Salmonella e modulação benéfica do sistema imunológico para controle da infecção. Porém, diferente da hipótese prevista, o consumo de Kefir no desenho experimental utilizado, contribui para aumento da mortalidade dos animais, associada a agravamento da infecção sistêmica. Considerando os efeitos anti- inflamatórios observados pelo tratamento com Kefir, sugere-se que a redução da resposta imunológica inflamatória induzida pelo Kefir pode ter contribuído para redução da eliminação do patógeno pelo sistema imunológico de mucosa. Desta forma, o patógeno teria mais eficiência de multiplicação e translocação no intestino, provocando uma doença mais severa.

Uma evidência desta hipótese é que nos animais que consumiram a bebida, houve o aumento da produção de butirato, conhecido por proteger a parede intestinal contra patógenos. Porém o butirato produzido pela microbiota modulada pode inibir a

translocação do fator de ativação NF-κβ do plasma para o núcleo, diminuindo atranscrição de fatores pró-inflamatórios e gerando uma falta de resposta imune contra a *Salmonella* (INAN et al., 2000).

Embora em condições normais, em sujeitos saudáveis, a maioria dos dados mostram muito mais benefícios que riscos. Quando ocorrem doenças infecciosas, tais como salmonelose, o patógeno pode ser favorecido pelos efeitos fisiológicos do Kefir. Logo é recomendado o acompanhamento de um nutricionista ou médico antes do uso. Probióticos produzidos em casa e de diferentes fontes podem ter efeitos diferentes, então esses devem ser usados com cautela e sob orientação médica e nutricional.

5 PERSPECTIVAS

Para responder as perguntas que esse trabalho deixou, é interessante haver outros estudos, em que haja a caracterização das leveduras presentes no Kefir. Assim como também identificar que fator imunológico é responsável pelo efeito indesejados do Kefir observados neste trabalho e neste desenho expeirmental, em caso de infecção com a Salmonella Typhimurium. O grupo possui interesse a princípio no uso de animais knockout para IL-10, para observarmos os efeitos da falta desta citocina durante o tratamento com Kefir e infecção, uma vez que estas moléculas estão associadas a efeitos anti-inflamatórios induzidos por Kefir. Dessa forma talvez seja possível encontrar a resposta sobre o mecanismo de ação da modulação do sistema imunológico, sobretudo durante o processo de infecção por *Salmonella*.

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APÊNDICE

Material Suplementar do Artigo:

Title: Milk kefir changes bacterial communities and influences the microbiota-gut-brain axis by increasing short-chain fatty acids in healthy mice.

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Supplementary Materials:

- **Table S1** Nutritional composition of UHT* whole milk and kefir (10%w/v) treated or not with kefir;
- **Table S2** Microbiological analysis of milk kefir (10% w/v);
- **Table S3 A**ntioxidant analysis of UHT§ whole milk and kefir samples (10% w/v);
- **Table S4** Somatic index of the organs of C57BL-6 mice treated or not with kefir;
- **Fig. S1** Flowchart of the Production of Milk Kefir (10%w/v);
- **Fig. S2** Effect of treatments on the concentration of serum biomarkers in C57B6 mice treated or not withkefir.

Table S1. Nutritional composition of UHT* whole milk and kefir (10%w/v)

Nutritional composition	UHT Whole milk	Kefir
Moisture (%)	88	89,96
Protein (g/100g)	3,0	3,09
Total Fats (g/100g)	3,0	3,0
Total Carbohydrates (g/100g)	4,4	3,06
Ashes (%)	0,7	0,89
Sodium chloride (%)	0,1	0,17
Fiber (g/100g)	-	-
Energetic Value (Kcal/100g)	56,6	51,60

* UHT: ultra-high temperature

Table S2. Microbiological analysis of milk kefir (10% w/v)

Analysis	Kefir
Lactic Acid Bacteria (CFU*/g)	$1,3 \times 10^8$
Molds and Yeasts (CFU/g)	$0,34 \times 10^4$
Thermotolerant Coliforms (MPN*/g)	< 3,0
Salmonella spp.	Absent /25g

*CFU/g: Colony Forming Unit/ per Gram, MPN/g: Most Probable Number/ per gram

Table S3. Antioxidant analysis of UHT[§] whole milk and kefir samples (10% w/v)

Analysis	UHT Whole Milk	SD[|]	Kefir	SD[|]
ABTS[†] (mg Trolox / 100mL)	609.03	1.80	1362.38[*]	207.72
DPPH ‡ (mg Trolox / 100mL)	670.41	19.26	4224.97[*]	51.63

†ABTS: 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid); ‡DPPH: 2,2-Diphenyl-1-picrylhydrazyl; §UHT: ultra-high temperature; |SD: standard deviation; * Statistical differences between groups were analyzed using the T test, in which (*) represent significant differences $p<0.05$.

Table S4. Somatic index of the organs of C57BL-6 mice treated or not with kefir.

Organs	Water	SD[†]	Kefir	SD[†]
Brain (g)	1.80	0.14	1.78	0.10
Colon (g)	1.19	0.34	1.17	0.21
Small Intestine (g)	5.71	0.53	4.96	0.70

†The somatic index is calculated by dividing the organ weight (g) by the animal weight (g). Data are expressed as mean ± standard deviation (SD) (n = 10 mice/group). * Statistical differences between groups were analyzed using the T test, in which (*) represent significant differences $p<0.05$.

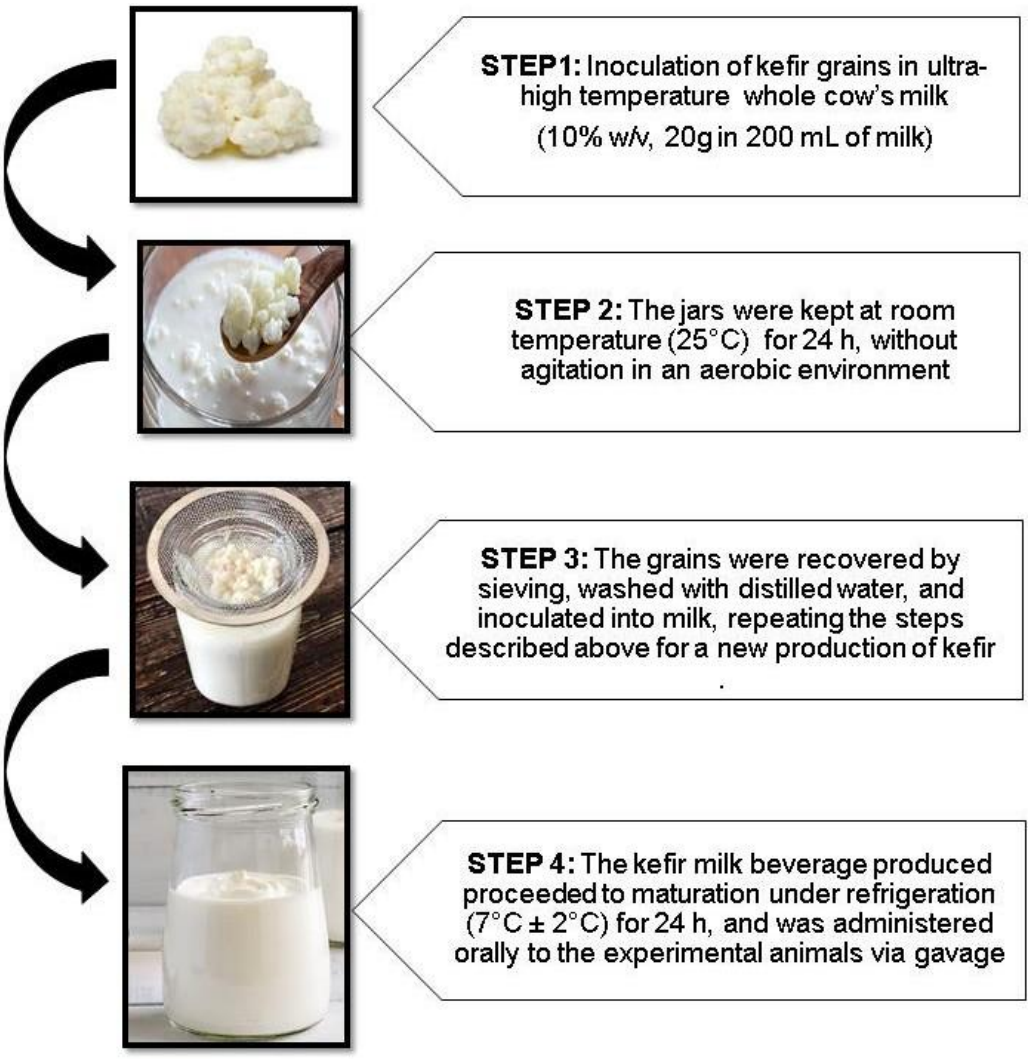

Fig. S1 Flowchart of the Production of Milk Kefir (10%w/v)

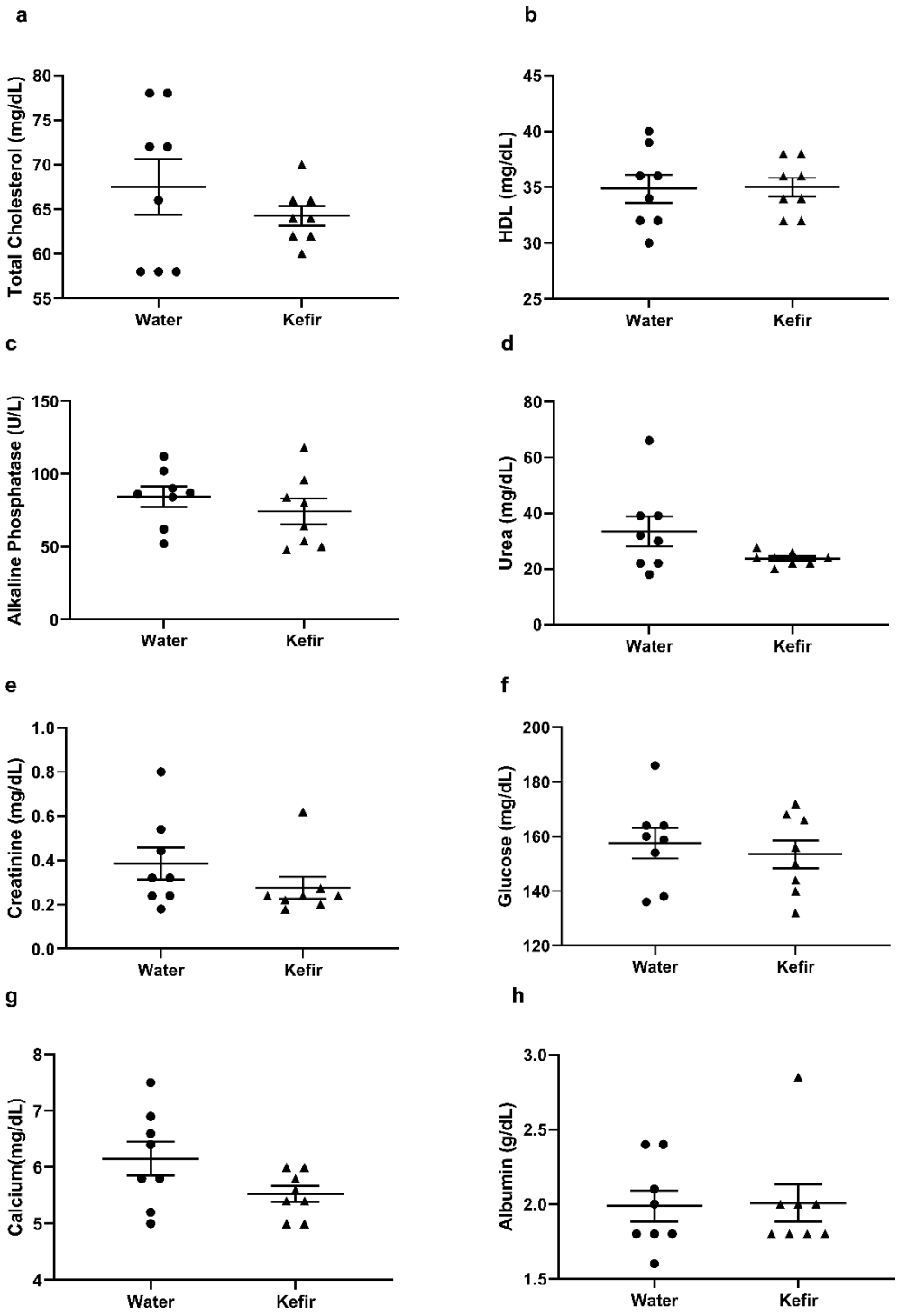

Fig. S2 The effect of treatments on the concentration of serum biomarkers in C57BL-6 mice. (a)Total Cholesterol (mg/dL); (b) High density cholesterol (HDL) (mg/dL); (c) Alkaline Phosphatase (u/L); (d) Urea (mg/dL); (e) Creatinine (mg/dL); (f) Glucose (mg/dL); (g) Calcium (mg/dL); (h) Albumin (g/dL). Data are expressed as mean ± mean standard error (n = 8 mice/group). Statistical differences between groups were analyzed using the unpaired T- test with Welch's correction, in which (*) represent significant differences with $p < 0.05$.