MARIANA DE FÁTIMA ALBUQUERQUE PEREIRA

INFLAMMATORY RESPONSE, ANTIOXIDANT, AND GUT MICROBIOTA ALTERATION IN A MURINE MODEL OF INFLAMMATION CHALLENGED WITH *Salmonella enterica* SEROVAR TYPHIMURIUM CONSUMING MILK KEFIR

Thesis submitted to the Science of Nutrition Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

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VIÇOSA - MINAS GERAIS
2023

Ficha catalográfica elaborada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa

T
P436i
2023

Pereira, Mariana de Fátima Albuquerque, 1992-
Inflammatory response, antioxidant, and gut microbiota alteration in a murine model of inflammation challenged with *Salmonella enterica* serovar Typhimurium consuming milk kefir / Mariana de Fátima Albuquerque Pereira. – Viçosa, MG, 2023.
1 tese eletrônica (165 f.): il. (algumas color.).

Texto em inglês.
Inclui apêndice.
Orientador: Maria do Carmo Gouveia Pelúzio.
Tese (doutorado) - Universidade Federal de Viçosa, Departamento de Nutrição e Saúde, 2023.
Inclui bibliografia.
DOI: https://doi.org/10.47328/ufvbbt.2023.390
Modo de acesso: World Wide Web.

1. Kefir - Aspectos nutricionais. 2. Probióticos. 3. Leite fermentado. 4. Camundongos como animais de laboratório. 5. *Salmonella enterica*. 6. Inflamação. 7. Sistema imunitário. 8. Ácidos graxos voláteis. 9. Microbioma gastrointestinal. 10. Eixo encéfalo-intestino. I. Pelúzio, Maria do Carmo Gouveia, 1958-. II. Universidade Federal de Viçosa. Departamento de Nutrição e Saúde. Programa de Pós-Graduação em Ciência da Nutrição. III. Título.

CDD 22. ed. 637.146

Bibliotecário(a) responsável: Bruna Silva CRB-6/2552

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APPROVED: April 19, 2023.

Assent:

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Data: 27/06/2023 11:05:02-0300
Verifique em https://validar.iti.gov.br

Mariana de Fátima Albuquerque Pereira
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Documento assinado digitalmente
gov.br
MARIA DO CARMO GOUVEIA PELUZIO
Data: 27/06/2023 10:26:18-0300
Verifique em https://validar.iti.gov.br

Maria do Carmo Gouveia Peluzio
Adviser

ACKNOWLEDGEMENTS

To God for helping me not to give up, enlightening me and guiding me during my doctorate. Your strength was essential to me and inspires me daily.

To my parents, Marília e Luiz, for being my first masters and who make so much effort to allow my dreams to come true. Thank you for understanding when I was away, for your trust, love, and daily dedication.

To my sister, Juliana, for her companionship, patience, friendly words, affection, and daily help.

To Vinicius for his understanding, love, patience, and daily encouragement.

To my family, especially my grandparents for protection from heaven, uncles and aunts for prayers and cousins for their encouragement. Especially my cousins Marcela and Palloma, both for their friendly shoulder and companionship, and Marcela also for their knowledge and shared laboratory support.

To my advisor, Profª. Maria do Carmo Gouveia Peluzio for being an excellent professional and friend. Thank you for trusting me over these years and for always saying that everything would be fine allowing this work to be completed.

To my co-supervisors, Tiago Mendes, Leandro Licursi, and Reggiani for their guidance and availability making the development of this work viable.

To my research partners at the Nutritional Biochemistry Laboratory who welcomed me and made my daily life lighter and special: Toninho, Larissa, Andressa, Bruna, Iasmin, and Gabriela. May our friendship last for many years.

To all my friends of Viçosa, especially Flávia, Matheus, Rafael, Maria Luísa, and Rafaela, for the good moments and friendship.

To my work partners at Prefeitura Municipal de Viçosa, especially Marcella, Emanuele, and Fernanda for helping me manage to reconcile the work of being a nutritionist with the doctorate and the support.

To the employees, students, professors, members, and partners of the Experimental Nutrition Laboratory (DNS-UFV), Experimental Pathology Laboratory (DBA-UFV), Molecular Immunovirology Laboratory (LIVM-UFV), and Laboratory of Biochemical Analyzes (DBB-UFV), Biomolecules Nucleus, and Microscopy Nucleus (UFV).

To the Federal University of Viçosa, for the opportunity to make friends, complete the postgraduate course, and the opportunities as a researcher.

To the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), for granted the scholarship.

To all people whom directly or indirectly contributed to this work, thank you very much!

“The only place success comes before work is in the dictionary”. (Albert Einstein)

ABSTRACT

PEREIRA, Mariana de Fátima Albuquerque, D.Sc., Universidade Federal de Viçosa, April, 2023. **Inflammatory response, antioxidant and gut microbiota alteration in a murine model of inflammation challenged with *Salmonella enterica* serovar Typhimurium consuming milk kefir**. Adviser: Maria do Carmo Gouveia Peluzio. Co-advisers: Tiago Antônio de Oliveira Mendes, Reggiani Vilella Gonçalves and Leandro Oliveira Licursi.

The use of probiotics has been suggested as a possible bacterial prophylaxis against *Salmonella* infections because some probiotic strains have protective effects. The combination of several mechanisms is described in the literature, such as the production of antibacterial substances against *Salmonella* and the increase of the host immune response against infection in the intestinal mucosa. Among the microorganisms used in the production of probiotic fermented beverages, the kefir culture stands out. Kefir is a fermented beverage composed of a microbial community that lives in a symbiotic association. Studies report that the use of milk kefir has anti-inflammatory, antimicrobial, antioxidant activities, in addition to increasing the concentration of short-chain fatty acids (SCFAs), altering the microbiota, among other benefits to intestinal health. An unbalanced composition of the intestinal microbiota, known as dysbiosis, is involved not only in intestinal diseases, but also in pathologies of other organs, such as the brain. Then, through the production of hormones, immunological factors and metabolites, the intestinal microbiota can modulate the intestinal environment and interfere with the behavior and function of the host's central nervous system. However, there are still gaps regarding the role of kefir in the mechanisms of action in acute infections and safety. Thus, the general objective of our work is to investigate the effect of milk kefir consumption on the immune system, antioxidant and intestinal microbiota composition of wild-type C57BL-6 and IL-10 knockout mice on health and oral infection by *Salmonella enterica* serovar Typhimurium. In manuscript 1, the objective was to review data from studies, from the last 10 years in different databases, with murine models on the role of kefir against inflammation and the main response mechanisms involved in this process. In total, 23 studies were included in this review. The experimental design of manuscript 2 was carried out with C57BL6 mice (n=20) subdivided into groups that received 0.1mL of water or 0.1mL (10% w/v) of kefir. The

kefir underwent maturation for 48 hours, and was then administered via orogastric route, to the animals for 4 weeks. In the manuscript 3 experiment, C57BL-6 wild type (WT) (n=40) and C57BL-6 IL-$10^{-/-}$ (KO) (n = 40) mice were subdivided into eight experimental groups treated or not with kefir. In the first 15 days, the water groups received filtered water (0.1 mL) while the kefir groups received milk kefir (0.1 mL, 10% w/v) via orogastric route. Then, two groups of each strain received a single dose (0.1 mL) of *S.* Typhimurium inoculum (ATCC 14028, dose: 10^6 CFU/mL). According to the revised data (Manuscript 1), kefir was shown to act reducing inflammation, initially by alternating between innate, Th1 and Th2 responses, reducing pro-inflammatory cytokines while increasing anti-inflammatory ones. In addition, it also acts in the mediation of immunomodulatory and protective effects through the numerous metabolites and organic acids produced and secreted by kefir in the intestinal microbiota, and has also been shown to act as an antihypertensive, antioxidant, antitumor and hypocholesterolemic and hypoglycemic action, factors that contribute to reducing inflammation. In manuscript 2, the aim was to analyze the microbiota profile of milk kefir and its effect on metabolism, oxidative stress, and the microbiota-gut-brain axis in a healthy murine model. Milk kefir drink exhibited high antioxidant potency compared to milk and *in vivo* increased antioxidant enzymes. The composition of the microbiota of the drink and the fecal microbiota were different, but composed mainly of SCFA-producing bacteria (*Comamonas* in the drink; *Lachnospiraceae* and *Lachnoclostridium* in mice). In metabolism, kefir improves liver function, reduces triglycerides and uric acid. The increase in cerebral and fecal SCFAs and the antioxidant effect found are associated with the change in the intestinal microbiota caused by kefir, which indicates that kefir positively influences the gut-microbiota-brain axis and contributes to the preservation of intestinal and brain health. In manuscript 3, our objective was to evaluate the role of IL-10 in inflammation and gut microbiome in mice consuming milk kefir and orally challenged with *Salmonella enterica* serovar Typhimurium. Kefir was observed to prevent systemic infections only in IL-$10^{-/-}$ mice, which were able to survive, regulate cytokines, and control colonic inflammation. The abundance in *Lachnospiraceae* and *Roseburia* and the higher production of SCFA in the pre-infection showed that kefir has a role in intestinal health and protection, colonizing and offering competition for nutrients with the pathogen, in addition to acting in the regulation of *Salmonella*

infectivity only in the lack of IL-10. These results demonstrate the role of IL-10 in the prognosis of salmonellosis and how milk kefir can be used in acute infections.

Keywords: Milk kefir. Probiotic. Fermented milk. C57BL-6 mice. Salmonella enterica serovar Typhimurium. Inflammation. Anti-inflammatory pathways. Immune system. Bacterial diversity. Short-chain fatty acids. Microbiota-gut-brain axis. Fecal microbiota. Kefir microbiota.

RESUMO

PEREIRA, Mariana de Fátima Albuquerque, D.Sc., Universidade Federal de Viçosa, abril de 2023. **Resposta inflamatória, antioxidante e alteração da microbiota intestinal em modelo murino de inflamação desafiado com *Salmonella enterica* sorovar Typhimurium consumindo kefir de leite**. Orientador: Maria do Carmo Gouveia Peluzio. Coorientadores: Tiago Antônio de Oliveira Mendes, Reggiani Vilella Gonçalves e Leandro Oliveira Licursi.

O uso de probióticos tem sido sugerido como uma possível profilaxia bacteriana contra infecções por *Salmonella* porque algumas cepas probióticas têm efeitos protetores. A combinação de vários mecanismos é descrita na literatura, como a produção de substâncias antibacterianas contra *Salmonella* e o aumento da resposta imune do hospedeiro contra infecções na mucosa intestinal. Dentre os microrganismos utilizados na produção de bebidas fermentadas probióticas, destaca-se a cultura de kefir. O kefir é uma bebida fermentada composta por uma comunidade microbiana que vive em uma associação simbiótica. Estudos relatam que o uso do kefir de leite possui atividades anti-inflamatória, antimicrobiana, antioxidante, além de aumentar a concentração de ácidos graxos de cadeia curta (SCFAs), alteração da microbiota, entre outros benefícios à saúde intestinal. Uma composição desequilibrada da microbiota intestinal, conhecida como disbiose, está envolvida não apenas em doenças intestinais, mas também em patologias de outros órgãos, como o cérebro. Então, através da produção de hormônios, fatores imunológicos e metabólitos, a microbiota intestinal pode modular o ambiente intestinal e interferir no comportamento e função do sistema nervoso central do hospedeiro. No entanto, ainda existem lacunas sobre o papel do kefir nos mecanismos de ação na segurança do seu uso em infecções agudas. Assim, o objetivo geral do nosso trabalho foi investigar o efeito do consumo de kefir de leite no sistema imunológico, antioxidante e composição da microbiota intestinal de camundongos C57BL-6 do tipo selvagem e IL-10$^{-/-}$ na saúde e na infecção oral por *Salmonella enterica* sorovar Typhimurium. No manuscrito 1, o objetivo foi revisar dados de estudos, dos últimos 10 anos em diferentes bases de dados, com modelos murinos sobre o papel do kefir contra a inflamação e os principais mecanismos de resposta envolvidos neste processo. No total, 23 estudos foram incluídos nesta revisão. O delineamento experimental do manuscrito 2 foi realizado com

camundongos C57BL-6 (n=20) subdivididos em grupos que receberam 0,1mL de água ou 0,1mL (10% p/v) de kefir. O kefir passou por maturação por 48 horas, sendo então administrado por via orogástrica, aos animais por 4 semanas. No experimento do manuscrito 3, camundongos C57BL-6 selvagem (WT) (n=40) e C57BL-6 IL-$10^{-/-}$ (KO) (n=40) foram subdivididos em oito grupos experimentais tratados ou não com kefir. Nos primeiros 15 dias, os grupos água receberam água filtrada (0,1 mL) enquanto os grupos kefir receberam kefir de leite (0,1 mL, 10% p/v) por via orogástrica. Em seguida, dois grupos de cada linhagem receberam uma única dose (0,1 mL) do inóculo de *S.* Typhimurium (ATCC 14028, dose: 10^6 UFC/mL). De acordo com os dados revisados (Manuscrito 1), o kefir demonstrou atuar na redução da inflamação inicialmente através da alternância entre as respostas inata, Th1 e Th2, reduzindo citocinas pró inflamatórios ao mesmo tempo que aumenta as anti-inflamatórias. Além disso, também atua na mediação de efeitos imunomoduladores e protetores por intermédio dos inúmeros metabólitos e ácidos orgânicos produzidos e secretados pelo kefir na microbiota intestinal, e ainda demonstrou atuar como, anti-hipertensivo, antioxidante, ação antitumoral e hipocolesterolêmico e hipoglicemiante, fatores que contribuem para redução da inflamação. No manuscrito 2, o objetivo foi analisar o perfil da microbiota do kefir de leite e seu efeito no metabolismo, estresse oxidativo e no eixo microbiota-intestino-cérebro em modelo murino saudável. A bebida kefir de leite exibiu alta capacidade antioxidante em comparação com o leite e *in vivo* aumentou as enzimas antioxidantes. A composição da microbiota da bebida e da microbiota fecal foram distintas, mas composta principalmente por bactérias produtoras de SCFAs (*Comamonas* na bebida; *Lachnospiraceae* e *Lachnoclostridium* nos camundongos). No metabolismo, o kefir melhorou a função hepática, reduziu os triglicerídeos e o ácido úrico. O aumento de SCFAs cerebrais e fecais e o efeito antioxidante encontrado estão associados à mudança na microbiota intestinal causada pelo kefir, o que indica que o kefir influencia positivamente o eixo intestino-microbiota-cérebro e contribui para a preservação da saúde intestinal e cerebral. No manuscrito 3, nosso objetivo foi avaliar o papel da IL-10 na inflamação e microbioma intestinal em camundongos consumindo kefir de leite e desafiados oralmente com *Salmonella enterica* sorovar Typhimurium. Observou-se que o kefir preveniu infecções sistêmicas apenas em camundongos IL-$10^{-/-}$, os quais foram capazes de sobreviver, regular citocinas e controlar a inflamação do cólon. A

abundância em *Lachnospiraceae* e *Roseburia* e a maior produção de SCFA na pré-infecção mostraram que o kefir tem um papel na saúde e proteção intestinal, colonizando e oferecendo competição por nutrientes com o patógeno, além de atuar na regulação da infectividade da *Salmonella* apenas na falta de IL-10. Esses resultados demonstram o papel da IL-10 no prognóstico da salmonelose e como o kefir de leite pode ser utilizado em infecções agudas.

Palavras-chave: Kefir de leite. Probiótico. Leite fermentado. Camundongos C57BL-6. *Salmonella enterica* sorovar Typhimurium. Inflamação. Vias anti-inflamatórias. Sistema imune. Diversidade bacteriana. Ácido graxo de cadeia curta. Eixo microbiota-intestino-cérebro. Microbiota fecal. Microbiota do Kefir.

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LIST OF ACRONYMS AND ABBREVIATIONS

4T1 Cell	Transplantable Mammary Tumor Cell Line.
ABTS	2,2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid.
ACF	Aberrant Crypt Focus.
ALT	Alanine Aminotransferase.
AOAC	Association of Official Analytical Chemists.
AOX	Alternative Oxidase.
APOE	Apolipoprotein E.
ARRIVE	Animal Research: Reporting of In Vivo Experiments.
AST	Aspartate Aminotransferase.
ASV	Amplicon Sequence Variants.
BALB/C	Albino Mice Strain.
BW	Body Weight.
C	Control Group.
C57BL/6	C57 Black 6 Strain.
CAPES	Coordination for The Improvement of Higher Education Personnel.
CAT	Catalase.
CBA	Cytometric Bead Array.
CCL20	Chemokine 20 Linker.
CD1	Cluster of Differentiation 1 Balb/C Strain.
CD36	Platelet Glycoprotein 36.
CD4+	Cluster of Differentiation 4 – T Help Lymphocytes.
CD68	Platelet Glycoprotein 68.
CD8+	Cluster of Differentiation 8 - Cytotoxic T Lymphocytes.
CEUA	Ethics Committee on Animal Experimentation.
CFU	Colony Forming Units.
CNPQ	National Council for Scientific and Technological Development.
CNS	Central Nervous Systems.
CONCEA	National Council for The Control of Animal Experimentation.
CRP	C-Reactive Protein.
CTL	Control.
CTLK	Kefir Control.

CTNBIO	National Technical Commission on Biosafety.
CXCL10	Chemokine Linker 10 With C-X-C Motif.
CYP	Cytochrome P450 3A1.
D	Day.
DAI	Disease Activity Index.
DBCA	Brazilian Practice Guideline for The Care and Use of Animals for Scientific and Didactic Purposes.
DECS	Health Science Descriptors.
DGAT-1	Diacylglycerol O-Acyltransferase 1.
DLM	Deltamethrin.
DM	Diabetes.
DMH	1, 2-Dimethylhydrazine.
DMK	Diabetes Kefir.
DNA	Deoxyribonucleic Acid.
DPPH	2,2-Diphenyl-1-Picryl-Hydrazyl-Hydrate.
DSS	Dextran Sodium Sulfate.
DTI	Diretoria de Tecnologia de Informação.
ENOS	Endothelial Nitric Oxide Synthase.
EP	Experimental Periodontitis.
EU	European Union.
EWAT	Epididymal White Adipose Tissue.
F	Female.
FAPEMIG	Fundação de Amparo a Pesquisa Do Estado De Minas Gerais.
FASN	Fatty Acid Synthase.
FUNED	Fundação Ezequiel Dias.
G	Giardia.
G-CSF	Granulocyte Colony-Stimulating-Factor.
GIT	Gastrointestinal Tract.
GK	Giardia Kefir.
GLUT2	Glucose Transporter 2.
GLUT5	Glucose Transporter 5.
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor.
GPX	Glutathione Peroxidase.

GSH	Reduced Glutathione Level.
GST	Glutathione S-Transferase.
H&E	Hematoxylin & Eosin.
H_2S	Hydrogen Sulphide.
Hb	Hemoglobin.
HD	Hypercholesterolemic Diet.
HDK	Hypercholesterolemic Diet with Kefir.
HDL	High-Density Lipoprotein.
HFCS	High-Fructose Corn Syrup Group.
HFD	High-Fat Diet.
HFDABK	High-Fat Diet With A-B Kefir.
HOMA-B	Homeostatic Model Assessment B.
HPLC	High-Performance Liquid Chromatography.
HR	Heart Rate.
ICAM	Intracellular Adhesion Molecules.
IFN-γ	Interferon Gamma.
IGF-1	Insulin-Like Growth Factor-1.
IL	Interleukin.
INOS	Inducible Nitric Oxide Synthase.
IR	Ischemia Reperfusion.
IRR-K	Irradiated Group Treated with Kefir.
IRS-1	Insulin Receptor Substrate 1.
K + IR	Kefir + Ischemia/Reperfusion.
K	Kefir Group.
K4 +EP	Milk Kefir with 4 Days of Fermentation plus Experimental Periodontitis.
KI	Wild Type Infected Kefir.
KIKO	IL-$10^{-/-}$ Infected Kefir.
KNI	Wild Type Uninfected Kefir.
KNIKO	IL-$10^{-/-}$ Uninfected Kefir.
KO	Knockout
LAB	Lactic Acid Bacteria.
LDL-C	Low Density Lipoprotein Cholesterol.
Ldlr	Low-Density Lipoprotein Receptor.

LPO	Lipid Peroxidation.
LPS	Lipopolysaccharide.
M	Male.
MAP	Mean Arterial Pressure.
MC	Macrophages.
MCP-1	Macrophage Chemoattractant Protein-1.
MCT	Monocarboxylate Transporters.
MDA	Malondialdehyde.
Mesh	Medical Subject Headings.
Mets	Metabolic Syndrome.
MHC II	Major Histocompatibility Complex II.
MK Group	Milk Kefir Group.
MLN	Mesenteric Lymph Nodes.
MMP-9	Matrix Metalloproteinase 9.
Mogat-1	Monoacylglycerol Acyltransferase.
MPN/G	Most Probable Number/ Per Gram.
MPO	Myeloperoxidases.
Mrna	Messenger Ribonucleic Acid.
MS + Kefir	Metabolic Syndrome Treated with Kefir.
NC Group	Negative Control Group.
ND	Normal Diet.
NF-κβ	Transcript Factor Kappa Betta.
NMDS	Non-Metric Multidimensional Scale Analysis.
NO	Nitric Oxide.
NRF-2	Nuclear Factor Erythroid 2–Related Factor 2.
NS	Not Specified.
OGTT	Oral Glucose Tolerance Test.
OXLDL	Anti-Oxidized LDL.
PAMP	Pathogen-Associated Molecular Patterns.
PBS	Phosphate-Buffered Saline.
PC Group	Positive Control Group.
PC	Carbonyl Protein.
PCR	Polymerase Chain Reaction.

PFC	Prefrontal Cortex.
PFT	Novel Kefir Product.
PGC-1	Peroxisome Proliferator-Activated Receptor-Gamma Coactivator-1.
PICOS	Parameter, Population, Intervention, Comparison, Outcomes, Study Design.
PP	Aggregated Lymphoid Nodules.
PP	Peyer Patch.
PPAR-A	Peroxisome Proliferator-Activated Receptor Alpha.
PRISMA-P	Preferred Reporting Items for Systematic Reviews.
Pt	Platelet.
PVN	Hypothalamic Paraventricular Nucleus.
RBC	Red Blood Cells.
RPWAT	Retroperitoneal White Adipose Tissue.
RVLM	Rostral Ventrolateral Medulla.
SCFA	Short-Chain Fatty Acid.
SD	Standard Deviation
SHR	Spontaneously Hypertensive Rats.
SIGA	Iga-Secretion.
SOD	Superoxide Dismutase.
SRA	Sequence Read Archive.
SREBP-1	Sterol Regulatory Element-Binding Protein 1c.
STZ	Streptozotocin.
TAC	Total Anti-Oxidant Capacity.
TBARS	Thiobarbituric Acid Reactive Substances.
TC	Total Cholesterol.
TG	Triacylglycerols.
TE	Trolox Equivalents.
TLR- 4	Toll-Like Receptor-4.
TLRS	Toll-Like Receptors.
TNF-A	Tumor Necrosis Factor Alpha.
UHT	Ultra-High Temperature.
US	United States
VLDL	Very Low-Density Lipoprotein.

W	Week.
WBC	White Blood Cells.
WDABK	Western Diet with A-B Kefir.
WI	Wild Type Infected Water.
WIKO	IL-10$^{-/-}$ Infected Water.
WNI	Wild Type Uninfected Water.
WNIKO	IL-10$^{-/-}$ Uninfected Water.
WT	Wild Type.

SUMMARY

1. INTRODUCTION

The use of probiotics has been suggested as a possible bacteria-prophylaxis against *Salmonella* infection, due to the protective effect conferred by some probiotic strains against the challenge in mice with *Salmonella* (Hill et al, 2014). The protection conferred by probiotics, in the experimental model of murine salmonellosis, is probably attributed to the combination of several switches, such as, for example, the production of antibacterial substances against *Salmonella* and the increase in the host's immune response against the infection, observed mainly by the increase in production of specific IgA antibodies against the pathogen in the intestinal mucosa, as well as increased activity of immune system cells (Dias, 2011; Rattray, O'Connel, 2011; Dias et al. 2012; Farag, et al., 2020).

Salmonellosis is considered the most widespread zoonosis in the world. *Salmonella* has its transmission cycle involving virtually all vertebrates and its transmission is associated with food and water intake. Its control represents a challenge for public health, because of the emergence of new serovars, both in developing and industrialized countries (Brasil, 2011).

Considering that the main route of transmission of *Salmonella* is in the food chain, its presence in animals raised for commercial purposes, products of animal origin such as meat, milk, and eggs, in addition to agricultural products, for example, vegetables and fruits, points to this microorganism as the most incident and relevant etiological agent of intestinal infections (Shinohara et al., 2008). Contamination of fecal origin for agricultural products is due to exposure to contaminated water; for milk and eggs, contamination occurs through direct exposure, and for meat, it usually occurs during slaughter operations (Brasil, 2011). This causes losses of millions of dollars for the industry, particularly cattle, pork, and poultry, both for the domestic market and for export, and in some countries, rigidity in sanitary inspection represents a constant need for quality (Mastroeni & Grant, 2011).

Except for the few serovars adapted to the human species, in this context, the treatment of salmonellosis, classically, involves the use of antimicrobials belonging to the fluoroquinolone class. However, the frequent therapeutic use, as well as the use as a growth promoter of antimicrobials in production animals, has led to an increase

in the number of infections by resistant and multidrug-resistant *Salmonella* (Knodler et al., 2010; WHO, 2012).

Given this scenario, the demand for foods that promote health and well-being has been increasing. Among the foods that meet this demand, those with functional properties have attracted the attention of consumers and the food industry. Among functional foods, probiotics stand out (Rosa et al., 2017), as they have positive effects on the composition of the intestinal microbiota and the individual's general health (Desland et al., 2012; Mackowiak, 2013).

Fermented dairy products, however, are generally good food matrices for probiotics (Martins, E.et al., 2013; KIVANÇ, YAPICI, 2016). Among the microorganisms used in the production of probiotic fermented beverages, the kefir culture stands out. Kefir is a food originating in the Caucasus obtained from the fermentation of milk by kefir grains or fermentation (BRASIL, 2007). Kefir grains are generally described as a symbiotic association between yeast, lactic acid bacteria and acetic, acid bacteria, surrounded by a matrix of polysaccharides (Pogačić et al., 2013; Rosa et al., 2017).

Currently, kefir consumption is increasing in many countries, including Brazil, due to its sensory properties, popular wisdom, and scientific evidence associated with beneficial effects on human health (Leite et al., 2013; Kivanç & Yapici, 2016). Previous studies on kefir reported its antimicrobial action, mainly related to the production of organic acids, peptides (bacteriocins), carbon dioxide, hydrogen peroxide, ethanol, and diacetyl; SCFAs produced during kefir fermentation 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-directional complex communication system between the gastrointestinal tract (GIT), the microorganisms that inhabit it, and the peripheral and central nervous systems (CNS) (Mörkl et 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 & 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 & Wang 2016; Wouw et al. 2020), the gut micro-

biota 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).

In addition, other benefits as the immunomodulation is due through the formation of bioactive peptides during fermentation or digestion processes, cytokine production, and stimulation of different signaling pathways; antitumor effects with which cancer prevention and early-stage tumor suppression are associated; hypocholesterolemic effects, among which are associated with the inhibition of exogenous cholesterol absorption in the small intestine, by the incorporation of lipid molecules into bacterial cells and by the suppression of the reabsorption of bile acids; in addition to antioxidant effects, attenuating lipid peroxidation and recruitment of free radicals (Leite et al., 2013; Arslan, 2015; Chen et al., 2015; Farag et al., 2020). However, studies also point out that the fermentation time of kefir influences its effectiveness and its activities, as well as its origin, due to the different microorganisms present in the grains (Leite et al., 2013).

In this way, the number of studies that evaluate its health benefits as previously mentioned, as well as the effect on the regular consumption of kefir, is increasing, however, questions remain about its performance in mechanisms of action in the hosts (Rosa et al., 2017). Therefore, as kefir can have probiotic effects also cheap and easily produced at home, the present study becomes necessary to verify whether the regular consumption of whole milk kefir would be able to act on the immune system, antioxidant, as well as on the intestinal composition and metabolism of C57BL wild-type and IL-10 knockout mice orally challenged with *Salmonella enterica* serovar Typhimurium.

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2. OBJECTIVES

2.1. General objective

To investigate the effect of milk kefir consumption on the immune system, antioxidant, and gut microbiota composition of wild-type C57BL-6 and IL-10 knockout mice on health and oral infection by *Salmonella enterica* serovar Typhimurium.

2.2. Specific objectives

2.2.1. Manuscript 1

- Review the data from studies with murine models on the role of kefir against inflammation and the main response mechanisms involved in this process.
- Reports the variability between studies and different ways of inducing inflammation.

2.2.2. Manuscript 2

- To characterize physicochemical and microbiological aspects, antioxidant capacity, and microbiota composition of milk kefir beverage;
- To assess the effect of kefir on growth parameters, food intake, and organs (colon, small intestine and brain);
- To evaluate the effect of kefir on serum biomarkers;
- To quantify oxidative stress biomarkers and verify the activity of antioxidant enzymes (catalase, superoxide dismutase and glutathione S-transferase) in the small intestine, colon, and brain;
- To quantify the SCFAs production in the brain, feces, and small intestine;
- To evaluate the diversity and composition of fecal microbiota.

2.2.3. Manuscript 3

- To assess animal parameters (weight), survival, and food intake;
- To evaluate the fecal microbiota;
- To quantify fecal short-chain fatty acids (SCFA);
- To assess the profile of the immune response in the colon (cytokines);
- To assess histopathological inflammation in the colon;
- To correlate gut microbiota and inflammation.

3. LITERATURE REVIEW

3.1. Manuscript 1

Manuscript published in Nutrition Reviews (Impact Factor: 6.846). https://doi.org/10.1093/nutrit/nuad052.

Article Type: Systematic Review

Title: Anti-inflammatory pathways of kefir in murine model: a systematic review

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Title: Anti-inflammatory pathways of kefir in murine model: a Systematic Review

Abstract

Context: Kefir consumption has been associated with immune response modulation, antioxidant, and anti-inflammatory effects. **Objective:** The objective of this systematic review was to investigate the role of kefir against inflammation and the main response mechanisms involved in this process in a murine model. **Data Sources:** The searches were searched in the PubMed, Science Direct, and LILACS databases. Only murine model studies, according to PRISMA guidelines, published in the past 10 years were included. **Study Selection:** Only articles about original and placebo-controlled experiments in murine models used to investigate the anti-inflammatory mechanisms of kefir were considered. Of the articles found, 349 were excluded according to the following criteria: duplicate articles (n = 99), off-topic title and abstract (n = 157), reviews (n = 47), studies in vitro (n = 29), and studies with humans (n = 17). In total, 23 studies were included in this review. **Data Extraction:** Two independently working authors assessed the risk of bias and extracted data from the included studies. **Results:** Kefir consumption had positive effects on inflammation modulation. The main mechanisms involved were the reduction of pro-inflammatory and molecular markers; reduction in inflammatory infiltrate in tissues, serum biomarkers, risk factors for chronic diseases, and parasitic infection; composition and metabolic activity change of intestinal microbiota and mycobiota; activation of humoral and cellular immunity; and modulation of oxidative stress. **Conclusions:** Kefir modulates the immune system in different experimental models, among other secondary outcomes, to improve overall health. The beverage reduces inflammation through the alternation between innate, Th1, and Th2 responses, reducing levels of pro-inflammatory cytokines while increasing those of anti-inflammatory ones. In addition, it also mediates immunomodulatory and protective effects through the numerous molecular biomarkers and organic acids produced and secreted by kefir in the intestinal microbiota. The health-promoting effects attributed to kefir may help in the different treatments of inflammatory, chronic, and infectious diseases in the population.

Key words: anti-inflammatory pathways, immune system, inflammation, kefir, mouse, probiotic.

INTRODUCTION

Inflammation is characterized by the body's response to an injury that may result from the development of various changes or even diseases. Anti-inflammatory agents are drugs or substances that reduce the intensity of the inflammatory process and have a broad spectrum of action, especially in some diseases. Commercial anti-inflammatory agents such as steroidal and nonsteroidal compounds are highly efficient in the treatment of inflammatory processes, but they are also associated with many side effects. Among natural substances, there is increasing research on the use of probiotics as anti-inflammatory agents.[1] These are defined as live microorganisms that, when administered in adequate amounts, exert a beneficial effect on the health of the host.[2] Studies involving probiotics and inflammatory diseases have already been developed, such as in cases of allergies,[3] dermatitis,[4] respiratory,[5] gastrointestinal conditions,[6] and cancer.[7]

Kefir is a dairy food considered a complex natural probiotic because of its composition and therapeutic activities. It is a beverage produced from the fermentation of milk by a microbial association of lactic acid bacteria, acetic bacteria, and yeasts present in its grains. These grains, also called kefiran, are formed by a gelatinous matrix containing proteins and polysaccharides, in which microorganisms coexist. Thus, kefir is formed by 2 fractions: a solid portion containing microorganisms and a soluble fraction presenting mainly the products of microbial fermentation. Among these products are lactic acid, acetic acid, ethanol, aromatic compounds, proteins with bioactive peptides, exopolysaccharides, antibiotics, and bacteriocins.[8,9] Regular consumption of kefir is associated with several health benefits, such as modulation of the immune system,[10] cholesterol reduction,[11] lactose digestion, and relief of symptoms resulting from gastrointestinal disorders,[12] antimicrobial,[13] antitumor,[14] antioxidant,[15] and anti-inflammatory activity.[16,17]

In this article, data from studies with murine models on the role of kefir against inflammation and the main response mechanisms involved in this process were reviewed. We also report in this review the variability between studies and different ways of inducing inflammation. A greater knowledge about the molecular and immunological mechanisms associated with the use of kefir may increase the interest of industries, both pharmaceutical and food, in the sense of making available foods and supplements based on this probiotic, consolidating its use by the population.

METHODOLOGY

The protocol for writing this systematic review followed the recommendations described by the Preferred Reporting Items for Systematic Reviews and Meta Analyses Protocols (PRISMA-P) 2020 statement,[18,19] in which the PRISMA guide (PRISMA checklist) was used to present the results of this review (see Table S1 in the Supporting Information online).

Data sources and research

Searches for scientific articles were performed using the US National Library of Medicine and the National Institutes of Health (PubMed), Science Direct, and Latin American and Caribbean Health Sciences Literature (LILACS) databases of articles published in the past 10 years. We used descriptors indexed in the Health Science Descriptors and Medical Subject Headings. The terms used in the searches were *kefir, milk kefir, water kefir, tibicos, kefiran, inflammation and anti-inflammatory agent*, in English and Portuguese. The Boolean operators “AND” and “OR” were used to combine the descriptors. The searches were performed independently by 2 researchers. Only articles about original and experimental studies with rats and mice, and that investigated the anti-inflammatory effects of kefir were considered. In addition, the reference lists of selected articles were manually analyzed to identify other relevant studies that could be included in this review.

Screening and eligibility of studies

The PICOS strategy (Table 1)[20] was used to include studies in this systematic review. After the searches, the initial screening was based on title and abstract. Duplicate articles between databases were excluded. In addition, articles that did not present the main theme, reviews, in vitro studies, studies with humans, letters to the editor, and theses and dissertations were also removed from the analysis. The selected studies were read in full and evaluated for eligibility criteria.

The selected articles were restricted to original works, developed in a murine model and published in English or Portuguese in the past 10 years. The eligibility of articles was independently analyzed by 2 researchers, and divergences were decided by consensus.

Data extraction and synthesis

The variables of interest in the analyzed studies were: general characteristics (ie, article title, authors, publication date, country); characteristics of the experimental model (ie, lineage, number of animals, sex, age, and initial weight of the animals); experimental methods (ie, number of experimental groups, number of animals per group, induction of inflammation, presence of the control group, treatment, dose and duration of the intervention); and main results found in the works. The data were grouped and arranged in tables to facilitate the visualization of the results.

Risk of bias assessment

The experimental articles selected for evaluation in this review were analyzed for risk of bias according to the criteria of the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guide[21]. This guide is based on short descriptions of essential features of experimental model studies in order to improve the reporting standard of animal research. Some of these criteria include theoretical and methodological basis, research objectives, and refinement of analytical methods, statistical draw, sample calculation, and outcome measures.

RESULTS

Selected studies

The flow diagram of the article selection process (Figure 1) was developed according to PRISMA guidelines and specifies the number of articles excluded or included in each step of the protocol. In total, 365 articles were retrieved from the 3 electronic databases searched (PubMed, n = 58; Science Direct, n = 305; and Lilacs, n = 2). Of these articles, 349 were excluded according to the following criteria: duplicate articles (n = 99), off-topic title and abstract (n = 157), reviews (n = 47), studies in vitro (n = 29) and studies with humans (n = 17). After manually checking the reference lists of the 16 previously selected articles, 3 more studies relevant to the topic were found and included for analysis. In the end, 23[22-44] articles were selected to compose this review.

Qualitative data

The selected studies were carried out in 11 countries, most of them in Brazil (39.1%; n = 9)[24,30,32,35-38,40,42], followed by Turkey (17.4%; n = 4)[22,27,39,43]. The other 43.5% (n = 10)[23,25-26,28-29,31,33-34,41,44] were performed in Tunisia, Argentina, Indonesia, Republic of Korea, Canada, Malaysia, Taiwan, Iraq, and Egypt (Table 2)[22-44]. In preclinical studies, the experimental models used were rats (65.2%, n=15)[22-24,26-27,29,32,35-37,39-43] and mice (34.8%, n = 8)[25,28,30-31,33-34,38,44], and the majority were male animals (82.6%, n = 19)[22-27,29,31-33,35-43]. Only 1 study[34] used male and female animals (4.3%). The age of the animals ranged from 2 days to 21 weeks. However, 3 studies (13.0%)[26,42-43] did not mention the age of the animals used in the experiment. The animals' initial body weight was not reported in 39.1% of the studies (n = 9)[22,24-25,28,31,34,36-37,40].

To induce inflammation, 5 studies used diet as an inflammatory factor, 3 of which used high-fat diet (HFD) induction (13.0%),[25,31,38] and 2 used a high-fructose diet (8.7%)[22,27]. Four studies used hyperglycemia through the application of streptozotocin as an induction factor (17.4%)[29,32,35,41]; 3 used injection (Lipopolysaccharides [LPS], 1,2-dimethylhydrazine, and dextran sulfate sodium (13.0%)[34,36,39]; dextran sulfate sodium was also used to induce colitis in animals. In 2 studies (4.3%),[28,33] researchers inoculated animals with *Giardia Intestinalis* and *Entamoeba histolytica* trophozoites. In 1 study,[26] intoxication was induced in animals by deltamethrin fertilizer (4.3%) and by gamma irradiation (4.3%).[23] In 1 study, researchers used breast cancer cells (4T1 cell line) (4.3%)[44]; and 6 studies (26.1%) performed the induction through the presence of diseases such as hypercholesterolemia[30], periodontitis[42], arterial hypertension[24,40], ischemia[43], and metabolic syndrome[37] (Table 3)[22-44].

The most used treatment in the diet of the control groups was water (n = 10 studies; 43.5%)[22-23,27,30,32,35-36,41-42,44] followed by milk (n= 5; 21.7%)[24,31,34,39-40], standard diet (n = 3; 13.0%)[29,38,43], 0.9% saline solution (n=3; 13.0%)[25,33,37], and corn oil (n=1; 4.3%)[26]. Only 1 study used phosphate-buffered saline (PBS) and milk in the control groups (4.3%)[28] (Table 3). All the treatments used were with *in natura* kefir at different dosages, with only 2 studies carried out with water kefir[30,44]. The dose ranged from 0.01mL to 5 mL, according to body weight, with most studies offering higher doses to 1 mL (n = 13; 56.5%)[22,26-27,29,32-33,35-39,41,43]. Three studies, however,

represented doses in other measurement units: 1 administered 10^9 CFU,[25] another used a dose of > 10^5 CFU/mL,[30] and the third[34] used 5 g/L ad libitum.

In 39.1% of the reports (n = 9),[26,28-31,33-34,38,44] treatment was administered orally, but these studies did not specify the form of administration (ie, gavage or addition to water or diet) and the other 60.9% (n = 14)[22-25,27,32,35-37,39-43] reported administration of treatments via orogastric gavage. The duration of the intervention varied considerably from 3 days to 20 weeks (Table 3).

Main results

The preclinical studies we reviewed that were conducted with rodents had a diversity of outcomes related to the modulation of inflammation. Among these, in general, they were mainly related to their anti-inflammatory activity, as well as antioxidant and antimicrobial activity, using histopathological, immunological, inflammatory and molecular markers; and evaluation of microbial activity, microbiota, and mycobiota, among others (Table 4).

Among the 23 articles evaluated, 14 studies (60.9%)[22-23,25-26,30-31,33,35,38-40,42-44] highlighted anti-inflammatory activity in histopathological analysis of the intestine[22,33,39-40], liver[22-23,25-26,31], kidney[35,43], lung[43-44], and adipose tissue[25]. In the intestine, kefir demonstrate normal structure in colon and little increase in goblet cells; no pathological changes were noticed in animals infected with *Entamoeba* histolytica[33] contributing to reduce inflammation. Specifically in the ileum, a study[22] found that the intensity of immunostaining of NF-κB and inducible nitric-oxide synthase (iNOS), as well as necrotic degeneration and macrophage infiltration, in rats fed a high concentration of fructose were reduced after treatment with kefir. Furthermore, the authors determined an increase in the expression of occludin and claudin-1 in the ileum of rats supplemented with kefir. Thus, it was indicated that kefir alleviated inflammation and reconstituted the function of tight junctions in the intestine[22]. In addition, it was observed that in a colitis model[39], kefir treatment resulted in reduced disease scores in animals given dextran sulfate sodium. In animals with arterial hypertension treated with kefir[40], it was observed that kefir partially reversed the pathophysiological changes of the intestinal barrier, increasing the number of Paneth cells per crypt as well as reducing the thickness of the tunica muscularis and improving the morphology of the intestinal barrier in these animals. In this way, kefir could reduce mucosal

permeability and systemic inflammation, protecting the barrier function and suppressing inflammatory agents, thus providing the maintenance of the physiological state of the intestine.

Regarding the hepatic histopathological changes, treatment with kefir resulted in decreased lymphocytic infiltration, severely reducing liver damage in animals treated with kefir associated with deltamethrin and moderately reduced liver failure in hepatocytes with the preventive use of kefir[26]. There also is a reduction of CD68 in Kupffer cells, and degeneration and lipid vacuoles in animals fed a high concentration of fructose[22]. Another study, however, observed that animals treated with kefir and ionizing radiation showed a slight activation of Kupffer cells, but these had normal structure of the hepatic lobules[23]. In HFD models, kefir treatment has been shown to slightly reduce ballooning[25] and mild steatosis in the liver,[31] resulting in lower liver injury scores, and to reduce the area of adipocytes in epididymal and retroperitoneal adipose tissue[25].

The anti-inflammatory activity of kefir is also highlighted in histopathological analyses in the kidney[35,43] and lung[43-44]. Reduction of leukocyte infiltration, presence of edema, and hemorrhage in both tissues were observed in animals with ischemia and reperfusion that received co-treatment with kefir. Other outcomes such as normal architecture of the lungs and alveoli and reduction of necrotic cells were also observed[43]. When using water kefir treatment, 1 study demonstrated reduction in tumor size and lung volume[44].

In addition to these tissues, 3 studies evaluated other sites, such as the aorta[30,38] and the periodontium[42]. They found that treatment with kefir did not reduce the area of aorta affected by atherosclerosis and there no reduction in lesion size in the aortic valve in the hypercholesterolemic model[30]. In another study, treatment with kefir after 4 days of fermentation resulted in reduced lipid deposition in the aorta in animals with induced inflammation from a HFD[38]and the reduced infiltration of inflammatory cells, loss of alveolar bone, preserving the periodontium in animals with periodontitis[42].

Five studies (21.7%) were identified that included immune assessment[23,26,28,32,44]. Kefir has been shown to act by reducing DNA fragmentation in pretreatment and co-treatment in a model using deltamethrin.[26] It has also been shown to influence the increase in the number of apoptotic cells and $TCD4^{+}$ and $TCD8^{+}$ lym-

phocytes, and the reduction of metastatic 4T1 cells in the lung and bone marrow of animals treated with water kefir[44]. Two other studies[28,32] associated the use of kefir with a reduction of TCD4^{+} in Peyer's patches and mast cells[28]. Furthermore, they associated the use of kefir with an increase in the percentage of medium B220-MHC II cells in Peyer's patches and of positive IgA cells in the lamina propria, the latter being only after 2 days of treatment[28]. Furthermore, it is observed that co-treatment with kefir is associated with an increase in phagocytic ability and in the number of aggregated lymphoid nodules (ie, Peyer's patches) in the intestine[32]. Still, in a radiation model, treatment with kefir increased the number of white blood cells and hemoglobin, but when associated with radiation, it was shown to reduce white blood cells and increase red blood cells and platelets[23].

Most studies (n = 22; 95.7%)[22-25,27-44] evaluated inflammatory and molecular markers and their association with treatment or co-treatment with kefir. Pro-inflammatory cytokines such as interleukin (IL)-1, IL-1β, IL-6, IL-11, IL-17, macrophage chemoattractant protein-1 (MCP-1), granulocyte colony-stimulating-factor, granulocyte macrophage colony-stimulating factor, and tumor necrosis factor (TNF-α) were evaluated in some of these studies. Regarding the reduction of pro-inflammatory cytokines, 8 studies (34.8%)[22,27,34,39-40,42-44] associated treatment or co-treatment with kefir with the reduction of hepatic TNF-α mRNA expression and serum concentration in a high fructose diet[22]; masseter muscle and gingival tissue TNF-α mRNA expression and protein expression in a high-fructose corn syrup diet;[27] periodontium immunoexpression;[42] hypothalamus expression and serum concentration of TNF-α in an LPS-induced model;[34] reduction in serum concentration in colon of animals with colitis[39] and rats with ischemia-reperfusion injury;[43] and in hypothalamic paraventricular nucleus and the rostral ventrolateral medulla of spontaneously hypertensive animals.[40] In 5 of these studies (21.7%),[27,34,36,42,44] a reduction in IL-1*β* in masseter muscle and gingival tissue mRNA expression was observed in animals fed a high-fructose corn syrup diet[27], prefrontal cortex mRNA expression in animals with LPS-induced inflammation,[34] adipose tissue mRNA expression in a metabolic syndrome model[36], periodontium immunoexpression[42], and serum concentration and lung protein expression in tumors.[44] In 8 studies (34.8%)[27,29,31,34,38,40-42] a reduction was observed in IL-6 in masseter muscle and gingival tissue[27], in serum of streptozotocin-induced animals[29], those receiving a hyperlidic diet,[31,38] in hypothalamus and

hippocampus tissues,[34] in paraventricular nucleus and rostral ventrolateral medulla in spontaneously hypertensive animals,[40] in diabetic rats,[41] and periodontium immunoexpression.[42] Two (8.7%)[23,31] other studies associated kefir with downregulation of MCP-1 in hepatic[23] and adipose tissue[31]. Thus, kefir is capable of reducing the production of acute phase proteins, as well as reducing the synthesis of pyrogenic and secondary mediators and pro-inflammatory cytokines by macrophages and mesenchymal cells. Kefir was also associated with a reduction in cytokines related to the growth and differentiation of monocytes, eosinophils and production of granulocytes, such as granulocyte colony-stimulating-factor and granulocyte macrophage colony-stimulating factor in animals treated with water kefir[44]. Induction of acute phase proteins and of contributors to inflammatory cascade, such as IL-11[27] and IL-1[29], cytokines involved in the differentiation process, also reduced in animals fed a high fructose diet and hyperglycemic diet and treated with kefir.

On the other hand, some of these studies associated treatment or co-treatment with kefir with an increase in pro-inflammatory cytokines. Four studies (17.4%) reported an increase in TNF-α[28,32,34,37] in *G. intestinalis* infection (mRNA expression),[28] culture of peritoneal macrophages after 24h of LPS stimulation in diabetes rats,[32] in the hypothalamus of female LPS-treated animals,[34] and in colon of animals with syndrome metabolic;[37] another 3 (13.0%)[32,34,37] reported an increased in for IL-1β, suggesting the increase of this cytokine in diabetic animals could be due to improvement of the immunological barrier increasing phagocytic ability of macrophages[32], and stimulation of the immune system to reduce inflammation in the colon in metabolic syndrome.[37] And 1 study reported the cytokines acting in hypothalamus and hippocampus of female animals treated with probiotics[34]. Only 1 study associated water kefir treatment with an increase in IL-2[44] in a lung tumor model, 1 study associated milk-kefir treatment with increased IL-6[36] in animals with metabolic syndrome, and another study reported an increase in IL-17[32] in serum concentration of diabetic animals. This happens because some cytokines can act, in both functions, producing inflammatory or anti-inflammatory effects.

The use of kefir was also related to the increase or reduction of anti-inflammatory cytokines such as IFN-γ, IL-4, IL-10, and IL-12. Thus, we observed in 5 of the 23 articles evaluated (21.7%)[29,32,36,38,42] that treatment or co-treatment with kefir was associated with an increase in IL-10 and, thus, that the beverage can help

in the inflammation control in diabetes, periodontitis, hyperlipidic diet, and metabolic syndrome. Two studies (8.7%) with parasitic infections demonstrated an increase in IFN-γ[28,33] suggesting that this cytokine is important in host defense against *E. histolytica* and *G. intestinalis*, and also that IL-10 was associated with activation of host neutrophils and macrophages to kill trophozoites. One study (4.3%) suggested that the increase of IFN-γ is associated antiproliferative, pro-apoptotic and antitumor mechanisms[44]; however, another study found an association with the downregulation of IL-4[44] mRNA expression that could be associated with obstruction of the production of Th1 cytokines by CD4+ T-helper lymphocytes in a water kefir–treated group with 4T1 breast cancer cells[44], and still another study (4.3%)[34] found a reduction in peripheral IL-12 8h after following LPS exposure, suggesting that probiotic (namely, kefir) exposure reduced central and peripheral LPS-induced inflammation in male mice.

On the other hand, 2 other studies showed that when treating male animals with water kefir[44] and milk kefir[34], both groups had reduced IL-10 levels. The IL-10 reduction in the breast cancer model[44] is involved in angiogenesis and inflammation; here, the low expression of tumor pro-growth-related cytokines is important to control tumor environment by not suppressing CD4 helper T cells and CD8 cytotoxic T cells responsible in immune response, producing an antitumor response and improving the poor prognosis of the disease. However, a reduction in IL-10 levels in mice treated with LPS[34] could be not interesting, because IL-10 contributes to reduce inflammation in this model. Also, the use of milk kefir was related in 3 studies (13.0%) with a reduction in the TNF-α to IL-10 ratio[24,38,42] in models of hypertension, a hyperlipidemic diet, and periodontitis. One of those studies[42] also showed a reduction in the IL-1β to IL-10 and IL-6 to IL-10 ratios in treated animals compared with those with periodontitis. Both IL-10 ratios indicate that kefir contributes to an increase in levels of this cytokine that is important to reduce inflammation.

Some molecular markers involved in metabolism and inflammation modulation in different experimental models associated with kefir treatment were investigated. Kefir treatment was associated with no alteration in auto-antibodies against oxidized low density lipoprotein in an atherosclerosis model treated with water kefir[30]; a reduction in serum lipopolysaccharide (LPS)[40] and microglial activation reducing neuroinflammation[40] ; the reduction of the transcription factor kβ (NF- kβ)[22-23,27,44] , which is

involved in controlling the expression of several genes linked to the inflammatory response in different organs; reduction of platelet glycoprotein 4 (CD36)[25], insulin-like growth factor-1[25], monoacylglycerol acyltransferase[25], diacylglycerol O-acyltransferase 1[25], and peroxisome proliferator activated receptor-γ coactivator-1[25], which are genes related to adipogenesis and inflammation in adipose tissue in animals fed high- fructose and Western diets (WDs). In an experimental cancer model, water kefir promoted the reduction of intracellular adhesion molecules[44] and matrix metalloproteinase-9[44] used in tumor and metastasis regulation and as cancer biomarkers. Kefir treatment promoted a reduction in myeloperoxidase activity in animals with colitis[39] and iNOS[39,44] both in colitis and in animals with lung cancer. In diabetic animals treated with kefir, it was also associated with reduction of iNOS[35] expression but no alteration of endothelial nitric-oxide synthase[35] expression in renal cortex as well as reduction in plasma C-reactive protein[35,41], which indicates that the blood glucose level was controlled in the animals, reducing the inflammation, oxidative stress, and the development of diabetes complications. Altogether, these markers reveal an anti-inflammatory effect of kefir on different metabolic pathways. In contrast, kefir was associated with an increase in the peroxisome proliferator-activated receptor α[31] in the liver and adipose tissue of animals fed an HFD, suggesting a positive effect.

It is important to note that 6 studies (26.1%) demonstrated microbial activity mainly related to the modulation of the intestinal microbiota[22,25,31,34] and reduction of parasites[28,33]. In rats fed a high-fructose diet[22], kefir increased the enrichment of fecal microbiota. The authors found a reduction in the fecal ratio of *Firmicutes* to *Bacteroidetes*, but an increase in *Actinobacteria* suggesting that altogether, kefir treatment promoted an alteration of microbiota composition and restoration of barrier integrity, as well as inhibition of inflammatory pathway in the intestine improving the metabolic profile of rats.

Another study with C57BL-6 mice treated with HFD and a WD[25] showed that kefir upregulated the richness of microbiota in HFD-fed mice, but not in WD-fed mice. The microbiota of HFD and WD mice fed kefir was characterized by the increase in both diets of the *Firmicutes* phylum; however, *Streptococcaceae* and *Lachnospiraceae* were predominant only in HFD-fed mice, and in the WD-fed mice by an increase in 2 other dominant families, namely *Tannerellaceae* and *Ruminococcaceae*. These

gut microbiota alterations were associated by the authors with an improvement in metabolic syndrome because some of the bacteria found to produce beneficial metabolites, such as short-chain fatty acids, and maintain gut health[25]. Similar to the study of Chen et al.,[25] another study[31] included in this review found that in hyperlipidic mice fed kefir had an increase in members of the phyla *Firmicutes* and *Bacteroidetes*, indicating that the kefir group had a significantly lower *Firmicutes* to *Bacteroidetes* ratio than the control group. The proportions of members of the *Lactobacillaceae* and Streptococcaceae, comprising the genera *Lactobacillus* and *Lactococcus*, respectively, were also higher in the kefir group. In addition, the phylum *Actinobacteria*, which includes the genus *Bifidobacterium*, was more prevalent in the kefir group. In contrast, the abundance of the family *Clostridiaceae* in the phylum *Firmicutes* was lower in the kefir group than in the control group. By using targeted analysis, kefir-treated mice had a higher *Lactobacillus* to *Lactococcus* ratio, total yeast, and *Candida,* also significantly lower numbers of *Bacillus fragilis* than the control mice.

Treatment with LPS and kefir, resulted in an increase in the percentage of *Bacteroidetes* comparing male and female animals[34]. In this model, it was also observed that male animals treated with LPS and kefir had reduced relative abundance of *Proteobacteria* and an increase in *Verrucomicrobia*[34]. Kefir treatment was also associated with an antimicrobial effect in a model of *G. intestinalis* infection in female mouse gut by reducing the number of viable trophozoites in the intestinal lumen of mice at 7 days after infection[28] and in another study with the elimination of fecal *E. histolytica* trophozoites after the sixth day of kefir absolute use and the eighth day when kefir was diluted in 50%[33]. It is suggested, therefore, that kefir can act in the modulation of the intestinal microbiota of animals, reducing parasites and inflammation, and providing an increase in bacteria that produce essential organic acids to improve the function of gut.

Other outcomes and conclusions about the use of treatment or co-treatment with kefir are related to the reduction of parasitic infection[28,33] promoting activation of different mechanisms of the humoral and cellular immune system that is deregulated by parasitic infections, thus contributing to protection; reduction in mean arterial pressure[24,40], cardiac hypertrophy and heart rate improving the baroreflex (data from Brazil)[24]; normalization and reduction of serum biochemical markers, mainly glucose[25,27,29,32,35-36,41], and modulation of the lipid profile [23,26-27,30-31,36,38]; reduced syn-

thesis of lipolytic genes[22], synthesis of fatty acids[22], insulin receptor[22], glucose[22], and fructose transporter[22] in the liver; improvement in oxidative stress[26,30,32,35,43-44] and modulation of antioxidant enzymes[23,26-27,30-31,36-37,43]; reduction in body weight[25,27,31-32,41]; improvement in obesity and non-alcoholic fatty liver disease[31], diabetes[32,35,41], and insulin resistance[22,36] and reduced risk of atherosclerosis[30,38]; improvement in emotional behavior at puberty with reduction of depression in female animals and anxiety and stress in males[34]; and gut-related outcomes such as reduced colitis activity index[39] and in relation to cancer and colon, such as decreased aberrant crypt foci[37], intestinal permeability (lactulose and mannitol)[37], and increased short-chain fatty acids[37].

Risk of bias

After evaluating the selected studies, it was observed that all had adequate titles and abstracts and had sufficient scientific context as well as primary and secondary objectives (see Table S2 in the Supporting Information online). Only 1 study[31] did not explain the experimental approach and justification in the introductory section. Information about the ethical statement was presented in 95.7% of the studies (n = 22)[22-32,34-44]; however, only 82.6% (n = 19)[22-28,30-31,35-44] of them declared the nature of ethics review permissions, relevant licenses and national or institutional laws, and guidelines for the care and use of animals.

All studies (n = 23)[22-44] reported the number of animals used per experimental group, the dosage of treatment used, the route of administration and the duration of the intervention. Only 4 studies (17.4%)[26,31,35,42] specified the time of day when the administration was performed and the place of administration (17.4%)[35,38,40,42], and 11 (47.8%)[23,26-27,32-33,37,40-44] justified the choice of dose used. No studies reported how the route of treatment administration was chosen and only 1 study[34] provided information about the study being blind controlled.

All studies reported information regarding species, strain, and sex of the experimental animals; however, only 60.9% (n = 14)[23,26-27,29-30,32-33,35,38-39,41-44] and 82.6% (n = 19)[22,24-25,27-41,44] provided information about weight and age of the animals, respectively. In 34.8% of the studies (n = 8)[24,30,33,35,37-38,40-41] the status and genetic modification were described. Ten studies (43.5%)[22-23,25-27,30,40-43] reported previous procedures applied to animals. For housing and animal husbandry, 56.5% of the

studies (n = 13)[22-23,30,32,34-39,41-42,44] indicated the type of installation or type of cage used, material or housing and 82.6% (n = 19)[22-26,28,30,32-42,44] mentioned rearing conditions. However, 82.6% (n=19)[22-28,30,33,35-44] described evaluations and interventions related to animal welfare before, during or after treatment.

Only 1 study (4.3%)[24] described how the sample size calculation was performed. Information on how the animals were allocated to the experimental groups was described in 47.8% of the studies (n = 11)[22-24,26,29-30,32,36,39-40,42] and the statistical methods used for each analysis, as well as information on the unit of analysis of each study, on the mean and standard deviation, as well as on the methods to assess whether the data met the premises of the statistical approach were described in all 23 reports[22-44]. The health of the animals before the experimental period was reported in 8.7% of the studies (n = 2)[35,41].

DISCUSSION

Probiotics have gained popularity and acceptance among consumers mainly because of their various health-promoting effects, some of which are already recognized in the literature and others related to popular wisdom. Thus, probiotics have long been used as natural components in supplements and functional foods, especially in fermented dairy products. Among these products, we highlight kefir in this study as a promising food, acting in the reduction of inflammation related mainly to inflammatory intestinal, infectious and chronic diseases[45].

This study, therefore, allowed us to compile data from studies with murine models on the beneficial effects of kefir against inflammation and other indirect outcomes. Despite the variability among the results of the studies included in this review, mainly due to the heterogeneity of the ways of inducing inflammation, the main mechanisms involved in the anti-inflammatory responses in the consumption of kefir were verified. In this context, we can say that kefir has an influence on: modulation of the immune response[23,26,28,32,44]; reduction of inflammatory and molecular markers[22-25,27-44]; modulation of serum biomarkers[23,25-27,29-32,35-36,38,41,43]; reduction of parasitic infections[28,33]; improvement in chronic non-communicable diseases[22,24,30-32,35-38,40-41]; changes in mycobiota and intestinal microbiota; improving permeability and production of short-chain fatty acids[22,25,31,34,37]; and improvement in oxidative

stress[26,30,32,35,37,43-44], DNA damage[26], and tissue architecture and inflammation[22-23,25-26,30-31,33,35,38-40,42-44].

The studies included in this review were those in a murine model using *in natura* kefir. This criterion was chosen because of the lower cost benefit, greater practicality of home production and the opportunity to group results that are not associated only with a specific microorganism isolated from the product. This type of experimental model also provides low research cost and control over experimental design and treatments, providing reliable results that are eligible and very similar for further comparison and inference for humans.

Advances in the science of inflammation lead to the clarification and identification of different classes of inflammatory mediators as well as the pathways that control their production and their mechanisms of action. We currently know that inflammation arises in different ways and by different modalities, which are guided by different mechanisms of induction, regulation and resolution. Thus, the inflammatory response has 4 phases of activity: first that of inflammatory inducers (infection or tissue damage), then inflammatory sensors (mast cells and macrophages), inflammatory mediators (eg, cytokines, chemokines), and the affected tissues[46]. Each phase is responsible for activating signaling cascades that are triggered on the basis of the type of pathogen or inducer introduced. For example, bacterial pathogens trigger Toll-like receptors and viral infections trigger type I interferons[46].

Considering the method of inducing inflammation, we can say that the main methods used in the studies included in this review were those that used chronic non communicable diseases or their risk factors as inducers[24,30,37-38,40,42-44] and those that induced inflammation via drugs or substances commonly used to induce intestinal diseases in animals[29,32-36,39,41]. They also highlight the induction of inflammation by consumption of HFD,[25,31,38] consumption of diets rich in fructose[22,27], parasitic infections[28,33], and ingestion of toxic substances[23,26]. In this context, the literature has shown, in recent decades, the change in lifestyle favoring a sedentary lifestyle, greater consumption of ultra-processed and processed foods and the emergence of new risk factors has altered the spectrum of prevalent inflammatory conditions, shifting from acute inflammatory diseases to response to wounds and infections, to low-grade chronic inflammatory states accompanying, for example, type 2 diabetes, hypertension, metabolic syndrome, atherosclerosis, asthma, neurodegenerative dis-

eases and cancer, thus influencing the most commonly used research and inflammation induction methods[47].

Kefir differs from other fermented products because it is produced from kefir grains that includes a specific and complex mixture of lactic acid and acetic acid producing bacteria as well as lactose fermenting and non-fermenting yeasts, which live in a symbiotic association[48]. Different animal milks, such as cow, sheep and goat, and soluble plant-based extracts such as those obtained from soy, coconut and rice can be used for production[49-50]. The type of milk, the type and amount of starter culture, fermentation conditions and time related to temperature and storage conditions affect the microbiological, chemical, and sensory characteristics of kefir[45,51-52].

Despite being studied in recent years, the mechanisms of action of probiotics still require specific investigations regarding the mechanisms of action and interactions with other compounds. There are cases in which important treatment points are also unknown, such as appropriate dose for the application, the time indicated to reach the end of therapy and effects of the interaction of probiotics with food in the intestine and of the interaction between mixtures of probiotic microorganisms[53]. As a general rule, a minimum limit of 10^9 CFU/dose is often used[34,43], although this may differ depending on the strain, expected health effect and possibly even the inoculation matrix used. The National Health Surveillance Agency of Brazil recommends a daily dose of 10^8 -10^9 CFU/day, with 10^7 for BAL for kefir and 10^4 for yeast[54]. More than half of the studies presented in our review presented a daily dose in CFU with different times of experimental period (3-20 wk), the doses varied between 10^5 and 10^{10} CFU/day for BAL and 10^4 - 10^7 CFU/day for yeasts. Studies with lower doses[28] and higher doses[26,32] obtained results on the activation of the humoral and cellular immune response[28], and improvement in the immunocompetence of the animals[32], demonstrating that it is impractical to specify a single dose.

Our findings in this review reveal that kefir acted in the modulation of the immune system, among other beneficial health outcomes, in different experimental models[22-44]. These studies demonstrate the positive influence of the consumption of foods such as kefir with probiotic microorganisms on the immune response[22-44]; however, the mechanisms of action are being elucidated and their immunomodulatory effect is highlighted[22-23,26,28,32-33,42-44]. The preclinical studies evaluated in this work suggest that this effect is associated with an increase in phagocytic ability and in the

number of Peyer's patches in the intestine[32], an increase in lymphocytes and apoptotic cancer cells, and a reduction in metastatic 4T1 cells in the lung and bone marrow[44], and a decrease in inflammatory cell infiltration,[22,26,28,42-43] as well as an association of the use of kefir with an increase in B cells and IgA, preserving the architecture, and consequently, organ function[28,33]. An important aspect is that once established in the gastrointestinal tract, the probiotic promotes proliferation of regulatory T cells and immunoglobulins, mainly secretory IgA, allowing a normal development of the immune system through the induction of tolerance to luminal antigens, helping to fight enteric pathogens, preventing autoimmune diseases and food allergies, contributing to the proper functioning of organs[55].

In this sense, most studies evaluated inflammatory markers and their association with treatment or co-treatment with kefir[22-25,27-44]. It is known that cytokines play an important role in the modulation of the immune system, and kefir, in general, acted in the reduction of pro-inflammatory cytokines such as IL-11[29], IL-1β[27,34,36,42,44], and IL- 6[27,29,31,34,38,40-42], and IL-11,[27] as well as the reduction of TNF-α[22,27,34,39-40,42-44] in more than half of the analyzed studies. Associated with this result is that treatment with kefir also increased anti-inflammatory cytokines such as IL-10,[29,31,33,35,38] and IFN-γ[28,33] and reduced the TNF-α to IL-10[24,38,42] ratio. This behavior is due to the interaction of the probiotic microorganism with dendritic cells that promote the production of cytokines, major histocompatibility complex molecules for antigen presentation, and co-stimulatory molecules that polarize T cells into regulatory and helper T-cell types 1 and 2. In addition, probiotic bacteria can reach the gut-associated lymphoid tissue through special intestinal cells (transcytosis), called M cells and interact directly with dendritic cells, modulating the immune response[56].

The use of kefir has been shown to regulate the innate response and the Th1 to Th2 shift of immune responses and other studies included in this review mentioned that kefir increased pro-inflammatory cytokines such as TNF-α or IL-12 as an initial reaction of the immune system to the TLR agonists present, resulting in the attenuation of the inflammatory response after subsequent interaction with the cells of the immune system[57].There is a wide variety of medicinal applications of kefir, some already with scientific evidence. In fact, as demonstrated by the indirect outcomes we found under inflammation, it is noteworthy that kefir seems to act on the intestinal microbiota[22,25,31,34], providing balance and the establishment of beneficial bacteria,

thus eliminating pathogens (antimicrobial and bactericidal activity)[28,33], as well improving the mediation of immunomodulatory and protective effects through the numerous metabolites[22-25,27-44] and organic acids produced and secreted by kefir,[22,37] in addition to its antihypertensive[24,40], antioxidant[23,26-27,31-32,36-37,43-44], antitumor[37], hypocholesterolemic[31,38], and hypoglycemic action[17,25,27,29,32,36,45,58-60]. Bioactive peptides, produced during milk fermentation by the microbiota present in kefir, are capable of activating macrophages, increasing phagocytosis, suppressing the Th2 immune response, increasing the production of nitric oxide and cytokines, restoration of barrier integrity, and inhibition of the inflammatory pathway, as well as stimulating the secretion of IgG and IgA by B-lymphocytes in the intestinal lumen[61]. According to Vinderola et al.[10], these bioactive compounds can promote a cell-mediated immune response against infections and intracellular pathogens, providing maintenance of the physiological state.

It is important to emphasize that systematic reviews have high levels of evidence, because they assess the variability of numerous studies in order to group information, reaching a consensus among authors on a subject. In this review, it was possible to verify that kefir acts as a probiotic in different experimental models, playing a protective role, helping to reduce inflammation and other risk factors for non-communicable and infectious chronic diseases, improving animal survival. However, it is important to be careful when interpreting the results and using them in clinical practice because, in some studies, there are still some information biases regarding the methodology used and dose and duration of intervention, among other factors.

In summary, studies have demonstrated the numerous biological activities of kefir, highlighting its potential. However, the information was very heterogeneous with different intervention protocols, so more studies on kefir's long-term effects in animal and human models are needed to elucidate the mechanisms involved and metabolites generated, helping determine the effectiveness and use of fermented foods in reducing of risk factor and in the different treatments of inflammatory, chronic and infectious diseases in the population.

CONCLUSION

Inflammation comprises a wide range of processes that affect all aspects of normal physiology and pathology. The field of research has become very large and

there are still some points that still need to be elucidated. The wide range of potential health-promoting effects attributed to kefir used as a probiotic could lead to a further expansion in the popularity of both traditional fermented kefir and future products that may come from manufacturing with kefir fractions or microorganisms.

In this review, kefir has been shown to modulate the immune system in different experimental models, among other secondary outcomes to improve overall health. The fermented beverage has been shown to act in reducing inflammation initially through the alternation between innate Th1 and Th2 responses, reducing pro-inflammatory cytokines while increasing anti-inflammatory ones. In addition, it also mediates immunomodulatory and protective effects through the numerous molecular biomarkers and organic acids produced and secreted by kefir in the intestinal microbiota. In this way, kefir can provide balance and the establishment of beneficial bacteria, thus eliminating pathogenic ones (antimicrobial and bactericidal activity), in addition to acting as an antihypertensive, antioxidant, antitumor, hypocholesterolemic and hypoglycemic, which are factors that contribute to reducing inflammation.

With advances in metagenomics analysis through the development of sequencing technology, an innovative perspective is gained, because with this knowledge, it will be possible to more readily isolate and examine the phenotypic characteristics of individual organisms present in kefir, while providing a bigger view of the evolution of these organisms and how they live in symbiosis on kefir. The additional knowledge gained may also provide crucial information regarding the exact mechanisms and agents responsible for the beneficial effects that have been attributed to kefir in this work.

Acknowledgements

Author contributions. M.d.F.A.P and F.M.A contributed to the conception and design of the study, data collection and analysis; M.d.F.A.P wrote the manuscript abstract; M.d.F.A.P and M.C.G.P. conducted the final reading; M.C.G.P. critically reviewed the manuscript and provided technical knowledge. All authors read and approved the manuscript.

Declaration of interests. The authors have no relevant interests to declare.

Supporting Information

Table S1 **PRISMA 2020 checklist**

Table S2 **Bias analysis (ARRIVE) of experimental studies about anti-inflammatory activity in murine models treated with kefir**

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Tables

Table 1 **PICOS criteria for inclusion and exclusion of studies**

Parameter	Definition
Population	Rodents
Intervention	Supplementation with kefir on treatment in the inflammatory process
Comparison	Placebo; water; saline solution; food products such as milk, fermented milk or yogurt with no supplementation; standard diet for rodents with no supplementation; oils.
Outcomes	Anti-inflammatory activity; immune assessment; histopathological evaluation; assessment of microbiological activity; other solutions and conclusions
Study design	Placebo-controlled rodents' experimental studies were included. *In vitro* studies, humans' studies, reviews, consensus, letters to editor, theses and dissertations were excluded.

PICOS: Parameter, Population, Intervention, Comparison, Outcomes, Study design

Table 2 **Characteristics of preclinical studies on anti-inflammatory activity in a murine model treated with kefir**

References	Country	Animal Model	Number of animals	Sex/Age	Initial weight (g)	Number of groups	Animals/ Group
Akar et al. (2021)[22]	Turkey	*Wistar* Rats	27	M / 3w	NS	3	9
Ali et al. (2020)[23]	Egypt	*Sprague-Dawley Rats*	32	M / 4w	120-150	4	8
Brasil et al. (2018)[24]	Brazil	*Wistar* Kyoto and SHR Rats	24	M/ 21w	NS	3	8
Chen et al. (2021)[25]	Taiwan	C57BL/6 Mice	50	M/ 7w	NS	5	10
El Golli-Bennour et al. (2019)[26]	Tunisia	*Wistar* Albino Rats	25	M/ NS	150 ± 20	5	5
Ekici et al. (2021)[27]	Turkey	*Wistar* rats	32	M / 4w	100	4	8
Franco et al. (2013)[28]	Argentina	C57BL/6 Mice	60	F / 4-5w	NS	4	15
Hadisaputro et al. (2012)[29]	Indonesia	*Wistar* Rats	84	M / 2-3w	150-250	4	21
Jascolka et al. (2013)[30]	Brazil	*ApoE$^{-/-}$ Mice*	23	F/ 10w	18	2	11-12
Kim et al. (2017)[31]	Korea	C57BL/6 Mice	20	M / 4w	NS	2	10
Maciel et al. (2016)[32]	Brazil	*Wistar* Rats	20-40	M / 8w	250	4	5-10
Mohammed (2016)[33]	Iraq	Albino Mice	36	M / 6-8w	20-25	5	6-12
Murray et al. (2019)[34]	Canada	CD1 Mice	160	M and F / 3w	NS	16	10
Punaro et al. (2014)[35]	Brazil	*Wistar* Rats	42	M / 8w	250	4	9-12
Rosa et al. (2016)[36]	Brazil	SHR Rats	30	M/ 2-10d	NS	3	10
Dos Reis et al., 2019[37]	Brazil	*Wistar* Rats	30	M / 8w	NS	3	10
Santanna et al. (2016)[38]	Brazil	LDLr$^{-/-}$ Mice	33	M/ 12w	21-25	4	6-9
Senol et al. (2015)[39]	Turkey	*Wistar* Albino Rats	24	M/ 18w	198-264	4	6
Silva et al. (2019)[40]	Brazil	*Wistar* Kyoto and SHR Rats	27	M / 8w	NS	3	9
Sunarti et al. (2015)[41]	Indonesia	*Wistar R*ats	25	M / 12 w	200-300	5	5
Vieira et al. (2021)[42]	Brazil	*Wistar* Rats	36	M/ NS	200-250	6	6
Yener et al. (2015)[43]	Turkey	*Sprague-Dawley* Rats	24	M/ NS	250-350	3	8
Zamberi et al. (2016)[44]	Malaysia	BALB/c Mice	21	F / 5-6w	20-22	3	7

ApoE: apolipoprotein E; BALB/C: albino mice strain; C57BL/6: C57 black 6 strain; CD1: cluster of differentiation 1 balb/c strain; d: day; F: Female; LDLr: Low-density lipoprotein receptor; M: Male; NS: not specified; SHR: spontaneously hypertensive rats; w: week.

Table 3 **Methodologies used in preclinical studies on anti-inflammatory activity in a murine model treated with kefir**

References	Inflammation Induction	Control Group	Treatment	Administration Via	Dose	Duration of Intervention
Akar et al. (2021)[22]	High-fructose diet	Water	Kefir	Oral/gavage	1 mL/100g bw	6 w
Ali et al. (2020)[23]	*Gamma* irradiation	Water	Kefir	Oral/gavage	0.5 mL/ 100g bw	4 w
Brasil et al. (2018)[24]	Arterial Hypertension	Milk	Kefir	Oral/gavage	0.3 mL/100g bw	8 w
Chen et al. (2021)[25]	High-fat and Western diet	Saline solution	Kefir	Oral/gavage	10^9 CFU	10w
El Golli-Bennour et al. (2019)[26]	Deltamethrin	Corn oil	Kefir	Oral	1 mL	3 d
Ekici et al. (2021)[27]	High-fructose corn syrup diet	Water	Kefir	Oral/gavage	1 mL/100g bw	8w
Franco et al. (2013)[28]	*Giardia Intestinalis* Trophozoites	PBS/Milk	Kefir	Oral	0.01 mL	2 w
Hadisaputro et al. (2012)[29]	STZ injection	Standard diet	Kefir	Oral	3.6 mL/200 g bw	4 w
Jascolka et al. (2013)[30]	Hypercholesterolemia	Water	Water kefir	Oral	over 10^5 CFU/mL	4 w
Kim et al. (2017)[31]	Hyperlipidic diet	Milk	Kefir	Oral	0.4 mL	12 w
Maciel et al. (2016)[32]	STZ injection	Water	Kefir	Oral/ gavage	1.8 mL	8 w
Mohammed (2016)[33]	*Entamoeba histolytica* Trophozoites	Saline solution	Kefir	Oral	3 mL	11 d
Murray et al. (2019)[34]	LPS injection	Milk	Kefir	Oral	5 g/L *ad libitum*	2 w
Punaro et al. (2014)[35]	STZ injection	Water	Kefir	Oral/gavage	1.8 mL	8 w
Rosa et al. (2016)[36]	DMH injection	Water	Kefir	Oral/ gavage	1 mL	8 or 20 w
Dos Reis et al., 2019[37]	Metabolic Syndrome	Saline solution	Kefir	Oral/ gavage	1 mL	10w
Santanna et al. (2016)[38]	Hyperlipidic diet	Standard diet	Kefir	Oral	2.2 mL/100g bw	4w
Senol et al. (2015)[39]	DSS	Milk	Kefir	Oral/ gavage	5 mL	2 w
Silva et al. (2019)[40]	Arterial Hypertension	Milk	Kefir	Oral/ gavage	0.3 mL/100g bw	9w
Sunarti et al. (2015)[41]	Nicotinamide and STZ injection	Water	Kefir	Oral/gavage	2 mL	4 w
Vieira et al. (2021)[42]	Periodontitis	Water	Kefir	Oral/ gavage	0.7 mL	5.5w
Yener et al. (2015)[43]	Ischemia-Reperfusion	Standard diet	Kefir	Oral/gavage	1 mL/ 100g bw	4 w
Zamberi et al. (2016)[44]	Breast Cancer cells (4T1)	Water	Water Kefir	Oral	0.15 mL/100g bw	4 w

Bw: body weight; DMH: 1, 2-dimethylhydrazine; DSS: dextran sodium sulfate; LPS: lipopolysaccharide; PBS: Phosphate-Buffered Saline; STZ: Streptozotocin; CFU: colony forming units.

Table 4 **Main results of preclinical studies on anti-inflammatory activity in a murine model treated with kefir**

Reference	Histopathological evaluation	Immune Evaluation	Inflammatory/ Molecular markers	Microbial Activity	Others
Akar et al. (2021)[22]	↓ Necrotic degeneration of the villi and secretory cells; ↓ macrophages infiltration score (gut); ↓ NF-kβ and iNOS in the illeal tissue; ↑ occludin and claudin-1 expressions; ↓ CD68 in Kupffer cells, fatty degeneration and fat vacuoles (liver).	-	↓ TNF-α and NF-kβ hepatic protein expression; ↓ TNF-α in the liver.	↓*Firmicutes/Bacteroidetes ratio* in feces; ↑ % relative abundance of *Actinobacteria*	↓SREBP-1c and FASN mRNA in the liver; ↓IRS-1, GLUT2 and GLUT5;
Ali et al. (2020)[23]	Normal histological structure of hepatic lobules of Kefir group; IRR+K exhibited good recovery with slight activation of Kupffer cells.	↑ WBC and Hb in kefir group; ↑ RBC, Pt and ↓ WBC in IRR+K group	↓MCP-1, NF-kβ relative gene expression (liver);	-	Kefir group: ↓ TG; IRR+K group: ↓TC, LDL-c., AST, ALT, and ↑ albumin; ↓ NO, LPO, ↑ TAC, GSH and CAT in liver.
Brasil et al. (2018)[24]	-	-	↓TNF-α/IL-10 ratio and ↓ inflammatory status of hypertensive animals after chronic treatment	-	↓ MAP, HR and cardiac hypertrophy in SHRs treated with kefir.
Chen et al. (2021)[25]	Slightly reduced hepatocellular ballooning score on HFDABK in liver; ↓ cross-sectional area of adipocytes in eWAT and rpWAT in HFDABK-mice	-	↓ CD36 and ↑ Pgc-1 Igf1 in HFD-mice treated with kefir. ↓CD36, Dgat1, Mogat1 in WDABK-mice	Kefir ↑ the richness of microbiota in HFDABK- mice *Firmicutes* was abundant in HFDABK and WDABK groups.	↓ Body weight, ↓ blood glucose at 30min and OGTT on HFDABK-mice; ↓ Propionic acid and butyric acid fecal in WDABK-mice.
El Golli-Bennour et al. (2019)[26]	↓Severely damage to hepatocytes and lymphocytic inflammatory infiltrate; ↓ Moderately liver failure in DLM+ Kefir and mild damage for pretreatment with kefir.	↓ DNA fragmentation in pretreatment and co-treatment with kefir	-	-	↓ AST, ALT, total bilirubin and TC. ↓ PC and MDA and restored CAT and SOD on DLM-induced group.
Ekici et al. (2021)[27]	-	-	↓ NF-kβ protein (masseter muscle) and mRNA expression levels (masseter muscle and gingival tissue) of HFCS+ Kefir rats. ↓ TNF-α protein and mRNA expression levels (masseter muscle and gingival tissue) of HFCS+ Kefir rats. ↓ IL-6, IL-1β, IL-11 on masseter muscle and gingival tissue in the HFCS+ kefir rats.	-	↓ Body weight, plasma glucose, fructose, insulin, TG, VLDL, TC, urea, creatinine, uric acid, ALT on HFCS + kefir rats. ↑ CAT, GPx, SOD-1, SOD-2, and Nrf2, but ↓ CYP3A1 mRNA expressions levels (masseter muscle and gingival tissue) of HFCS+ Kefir rats.

Table 4 **continued**

Reference	Histopathological evaluation	Immune Evaluation	Inflammatory/ Molecular markers	Microbial Activity	Others
Franco et al. (2013)[28]	-	↓ Number of CD4+ T cells at 2 days post-infection in PP of *Giardia* infected mice and MCs of K groups. ↑ % B220-MHC II average cells in PP of group K vs. G; K positive IgA cells on the lamina propria only after 2 days and GK after 7 days. Similar values for number of CD4 T cells in MLN; % CD8+ T cells in PP and MLN after 2 and 7 days of infection; MHC II in low and high populations in PP and for B220 positive MLN cells after 2 and 7 days; % of MCs after 7 days of K and GK vs. C.	Similar expression of CXCL10 and CCL20 after 2 hours of infection; The expression of IL-12p40 after 2 hours and 2 days after infection among groups. ↑ Expression of TNF-α in GK, after 2h of infection compared to G; IFN-γ after 2 days of infection in groups K and GK vs. C.	↓ *Giardia* infection (Number of viable trophozoites in intestinal lumen) at 7 days post-infection for GK and G groups.	Mice fed with kefir reduce *G. intestinalis* and promotes the activation of different mechanisms of humoral and cellular immunity that are deregulated by parasitic infection, contributing to protection.
Hadisaputro et al. (2012)[29]	-	-	↓ IL-1 and IL-6 (serum). ↑ IL-10 (serum).	-	↓ Serum glucose
Jascolka et al. (2013)[30]	Kefir vs. Control: No reduction in the area of aorta affected by atherosclerosis and in lesion size in aortic valve. Both groups showed lesions on intermediate stages, with a predominance of macrophage-derived foam cells		Similar blood anti oxLDL antibodies (Kefir vs. Control)		Kefir vs. Control: Similar results in food and liquid intakes; initial body weight and weight; cecal content lipids, hepatic cholesterol, and TG; cholesterol and TG (caecal content); Blood glucose, TC, and LDL-c levels. Hydrogen peroxide, SOD activity and TBARS. ↑ HDL and ↓TG, hydroperoxides and CAT activity
Kim et al. (2017)[31]	Mild steatosis and ↓ liver injury scores in kefir group.	-	↓ IL-6 (serum) ↑ PPAR-α (adipose and hepatic tissues). ↓ MCP-1 in the adipose tissue of kefir-fed mice.	↑ *Lactobacillus* / *Lactococcus*, total yeast; ↓*Firmicutes*/*Bacteroidetes* ratio on kefir group.	↓ TC and LDL-c plasma levels. ↓ AOX expression and Low-grade systemic inflammation; ↓ Final body weightby HFD + kefir.

Table 4 **continued**

Reference	Histopathological evaluation	Immune Evaluation	Inflammatory/ Molecular markers	Microbial Activity	Others
Maciel et al. (2016)[32]	-	↑ Phagocytic ability in the DMK group compared to CTL and CTLK. ↑ number of PP on small intestine in the DMK compared to DM.	↑ IL-10 (culture of peritoneal macrophages) of the DMK group vs. CTLK and vs. DM, after 24 h of incubation, with or without LPS stimulation; ↑ TNF-α in the DMK group vs. DM and CTLK vs. CTL, 24 hours after LPS stimulation; ↑IL-17 in the CTLK and DMK groups, 24 hours after LPS stimulation; ↑IL-1β in the DMK group vs. DM and CTLK after stimulation with LPS.	-	↓ Fasting blood glucose, and body mass, also ↑ insulin compared to DM mice. ↑NO in the DMK group compared to DM;
Mohammed (2016)[33]	Normal structure. Little increase occurs in goblet cells, and no pathological changes were noticed in kefir groups (colon).	-	↑ INF-γ (Serum) (Kefir absolute and kefir diluted vs. Controls);	Feces of mice became clear from the parasite completely for Kefir absolute group at the 6th day and at the 8th for Kefir diluted group post inoculation	-
Murray et al. (2019)[34]	-	-	Males: ↓ IL-12, IL-10 and TNF-α concentration and mRNA expression on LPS-treated-kefir vs. milk group; Females: ↑ IL-1β mRNA expression (hypothalamus and hippocampus) and TNF-α (hypothalamus) in probiotic vs. saline group. ↓ IL-1β mRNA expression in the PFC treated with LPS+ kefir and TNF-α mRNA (hippocampus) vs. non-probiotics groups. ↓ IL-6 (hypothalamus and hippocampus) vs. male in probiotic and milk treated with LPS.	Males: ↑ % of *Bacteroidetes* compared to females. ↓ % *Proteobacteria* post 24 hours and ↑ % of *Verrucomicrobia* (end of treatment) in LPS-treated males compared to their milk-treated counterparts	↓ Sickness behaviour on LPS-treated male and female mice exposed to kefir than the milk control group.
Punaro et al. (2014)[35]	↓ Accumulation of glycogen in the renal tubules in the DMK group when compared to the DM group.	-	Similar expression in eNOS in the DM and DMK rats. ↓ Expression of iNOS in the DMK group vs. DM group (renal cortex). ↓ Plasmatic CRP in DMK vs. DM	-	CTLK vs. DMK: ↑ water and chow intake, diuresis, and ↓ weight gain. The DMK vs. DM: ↓ water and chow intake, diuresis, ↑ weight gain. ↓ The level of NO in the renal cortex, superoxide anion and glycemia

Table 4 **continued**

Reference	Histopathological evaluation	Immune Evaluation	Inflammatory/ Molecular markers	Microbial Activity	Others
Rosa et al. (2016)[36]	-	-	↓ IL-1β and ↑ IL-10 in adipose tissues of MS + kefir compared to PC group. ↑ IL-6 in the Kefir group compared to NC and PC groups.	-	↓ TG, liver lipids, liver TG, CAT, SOD in K group compared to PC group, both with MetS. ↑ Leptin and CRP in the K group. ↓ Fasting glucose, fasting insulin, and HOMA-β when compared to PC group. ↓ OGTT compared to NC.
Dos Reis et al. (2019)[37]	-	-	IL12p70 and IFN-ɣ were similar between groups. ↑ TNF-α and IL-1β in the Kefir group colon.	-	↓ ACF development, intestinal permeability; Kefir ↑ total SCFAs, mainly propionic acid; promoted immunomodulation and improved the local antioxidant response.
Santanna et al. (2016)[38]	↓ Area of lipid deposition in aorta on HD-kefir group compared to others HD groups.	-	The HD-K group prevented the increase in IL-6 in 50%. ↑ IL-10 of HD-Kefir. ↓ TNF-α / IL-10 ratio of HD-Kefir group compared to ND and HD groups.	-	↑ TC and non-HDL cholesterol
Senol et al. (2015)[39]	↓ Histologic colitis scores	-	↓ TNF-α, iNOS expression and colonic MPO activity induced by colitis through kefir treatment.	-	↓ Clinical scores of DAI
Silva et al. (2019)[40]	↑ Paneth cells number/Crypt, ↓ tunica muscularis in SHR-Kefir compared to SHR-animais.	-	↓ Serum LPS, ↓ Microglial activation, TNF-α and IL-6 concentrations in the PVN and RVLM in SHR-Kefir group.	-	↓ MAP in SHR-Kefir
Sunarti et al. (2015)[41]	-	-	↓ IL-6 (diabetic with kefir vs. diabetic control) ↓ Plasmatic CRP level (diabetic rats with goat milk kefir vs. diabetic control rats)	-	↓ Body weight in all diabetic rats vs. normal rats. Plasma glucose in diabetic rats with goat milk kefir, and in diabetic rats with combination of both goat milk and soybean milk kefir.

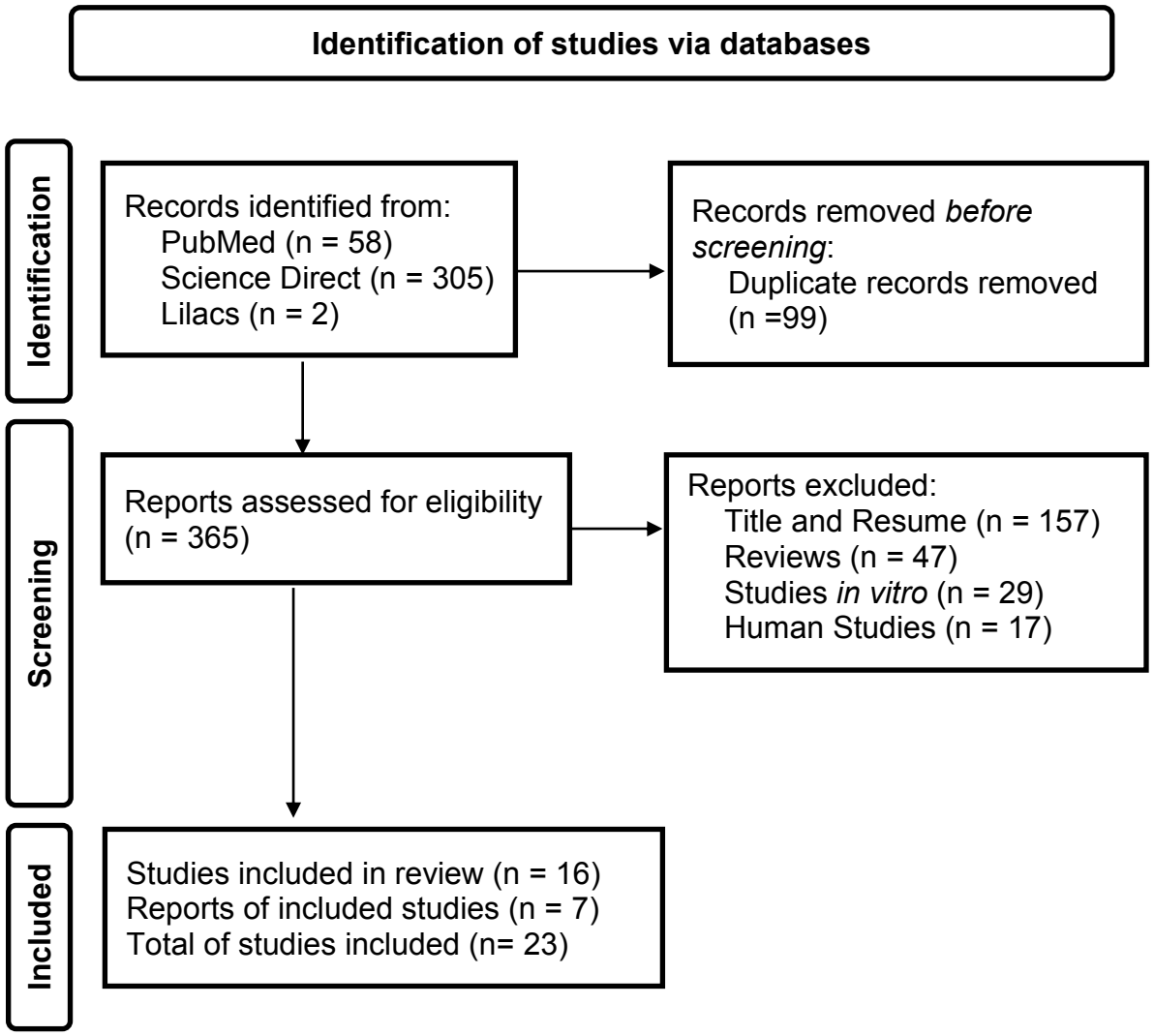
Table 4 **continued**

Reference	Histopathological evaluation	Immune Evaluation	Inflammatory/ Molecular markers	Microbial Activity	Others
Vieira et al. (2021[842]	↓ Inflammatory cell infiltration and a partial preservation of the cementum and of the alveolar bone in the periodontium of animals with periodontitis and treated with MK with 4 days of fermentation (K4 +EP group). ↓ Alveolar bone loss on K4+EP group when compared to EP group.	-	↓ TNF-α, IL-1β and IL-6 immunoexpression in rats with periodontitis. ↑ IL-10 expression. ↓ IL-1β /IL-10, TNF -α/IL-10 and IL-6/IL-10 ratios on MK group, when compared with control and experimental periodontitis groups	-	-
Yener et al. (2015)[43]	↓ Edema, hemorrhage, and leukocyte infiltration in kidney and lungs of K + IR compared to the IR group.	-	↓TNF-α in the K + IR groups compared to the IR group	-	↓ MDA in kidney and lung, serum urea and creatinine. ↑ CAT, SOD, GSH activities in in K + IR group.
Zamberi et al. (2016)[44]	↓ Tumor size and volume in lungs of kefir water-treated group.	↑ Apoptotic cancer cells. ↓ Metastatic 4T1 cells in lungs and bone marrow ↑ T-helper CD4+, cytotoxic T cells CD8+	↑ IFN-γ, IL-2 and ↓ IL-10, IL-1β on kefir water-treated group. ↓ ICAM, iNOS, MMP-9, IL-1β, NF-κB, G-CSF, GM-CSF, IL-4, and TNF-α mRNA expressions.	-	↓ MDA and NO

4T1 cell: transplantable mammary tumor cell line; ACF: aberrant crypt focus; ALT: alanine aminotransferase; AOX: alternative oxidase; AST: aspartate aminotransferase; C: control group; CAT: catalase; CCL20: chemokine 20 linker; CD36: platelet glycoprotein 36; CD4+: cluster of differentiation 4 – T help lymphocytes; CD68: platelet glycoprotein 68; CD8+: cluster of differentiation 8 - cytotoxic T lymphocytes; CRP: C-reactive protein; CTL: control; CTLK: kefir control; CXCL10: chemokine linker 10 with C-X-C motif; CYP: cytochrome P450 3A1; DAI: Disease activity index; Dgat-1: diacylglycerol O-acyltransferase 1; DLM: deltamethrin; DM: diabetes; DMK: diabetes kefir; DNA: deoxyribonucleic acid; eNOS: endothelial NO synthase; EP: experimental periodontitis; eWAT: epididymal white adipose tissue; FASN: Fatty Acid Synthase; G: Giardia; G-CSF: granulocyte colony-stimulating-factor; GK: Giardia Kefir; Glut2: glucose transporter 2; Glut2: glucose transporter 5; GM-CSF: granulocyte macrophage colony-stimulating factor; GPx: glutathione peroxidase; GSH: reduced glutathione level; Hb: Hemoglobin; HD: hypercholesterolemic diet; HDK: hypercholesterolemic diet with kefir; HDL: high-density lipoprotein; HFCS: High-fructose corn syrup group; HFD: high-fat diet; HFDABK: high-fat diet with A-B Kefir; HOMA-β: Homeostatic model assessment β; HR: heart rate; ICAM: intracellular adhesion molecules; IFN-γ: interferon gamma; Igf-1: insulin-like growth factor-1; IL: Interleukin; iNOS: nitric oxide synthase; IR: ischemia reperfusion; IRR-K: Irradiated group treated with kefir; Irs-1: Insulin receptor substrate 1; K + IR: kefir + ischemia/reperfusion; K: kefir group; K4 +EP: milk kefir with 4 days of fermentation plus experimental periodontitis; LDL-c: low density lipoprotein cholesterol; LPO: lipid peroxidation; LPS: lipopolysaccharide; MAP: mean arterial pressure; MAP: Mean Arterial Pressure; MC: macrophages; MCP1: macrophage chemoattractant protein-1; MDA: malondialdehyde; MetS: metabolic syndrome; MHC II: major histocompatibility complex II; MK group: milk kefir group; MLN: mesenteric lymph nodes; MMP-9: matrix metalloproteinase 9; Mogat-1: monoacylglycerol acyltransferase; MPO: myeloperoxidases; mRNA: messenger ribonucleic acid; MS + kefir: metabolic syndrome treated with kefir; NC group: negative control group; ND: normal diet; NF-κB: transcript factor kappa B; NO: Nitric oxide; Nrf2: nuclear factor erythroid 2–related factor 2; OGTT: oral glucose tolerance test; oxLDL: anti-oxidized LDL; PC group: positive control group; PC: carbonyl protein; PFC: prefrontal cortex; Pgc-1: peroxisome proliferator-activated receptor-gamma coactivator-1; PP: Peyer Patch; PPAR-α: peroxisome proliferator-activated receptor alpha; Pt: platelet; PVN: hypothalamic paraventricular nucleus; RBC: red blood cells; rpWAT: retroperitoneal white adipose tissue; RVLM: rostral ventrolateral medulla; SCFA: Short chain fatty acid; SHR: spontaneously hypertensive rat; sIgA: IgA-secretion; SOD: superoxide dismutase; SREBP-1: Sterol regulatory element-binding protein 1c; TAC: total anti-oxidant capacity; TBARS: thiobarbituric acid reactive substances; TC: total cholesterol; TG: triacylglycerols; TNF-α: tumor necrosis factor alpha; VLDL: very low density lipoprotein; WBC: White blood cells; WDABK: western diet with A-B Kefir.

Figure

Figure 1 **Flow diagram of the literature search process**



Supplementary material

Table S1 **PRISMA 2020 checklist**

Section and Topic	Item #	Checklist item	Location where item is reported
TITLE			
Title	1	Identify the report as a systematic review.	Lines 2, 30
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	Lines 31-57
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	Lines 87-107
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	Lines 108-114
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	Lines 121-141
Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	Lines 121-141
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	Lines 121-141
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	Lines 133-141, Table 1 and Figure 1

Section and Topic	Item #	Checklist item	Location where item is reported
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g., for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	Lines 143-155
	10b	List and define all other variables for which data were sought (e.g., participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	Lines 143-155
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	Lines 150-155
Effect measures	12	Specify for each outcome the effect measure(s) (e.g., risk ratio, mean difference) used in the synthesis or presentation of results.	Lines 143-148
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g., tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	Lines 143-148
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	Lines 143-148
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	Lines 143-148
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	Lines 143-148
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g., subgroup analysis, meta-regression).	Not applicable
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	Not applicable

Section and Topic	Item #	Checklist item	Location where item is reported
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	Lines 150-155
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	Lines 150-155
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram.	Figure 1 and Lines 158-167
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	Lines 158-167, Figure 1
Study characteristics	17	Cite each included study and present its characteristics.	Lines 169-201
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	Lines 411-449
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimates and its precision (e.g., confidence/credible interval), ideally using structured tables or plots.	Tables 1-4
Results of syntheses	20a	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	Lines 169-449

Section and Topic	Item #	Checklist item	Location where item is reported
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g. confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	Lines 169-449
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	Lines 169-449
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	Not applicable
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	Lines 411-449 Table S2
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	Not applicable
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	Lines 451- 583
	23b	Discuss any limitations of the evidence included in the review.	Lines 569- 577
	23c	Discuss any limitations of the review processes used.	Lines 569-577
	23d	Discuss implications of the results for practice, policy, and future research.	Lines 578-583
OTHER INFORMATION			
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	Not registered
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	Lines 116-119

Section and Topic	Item #	Checklist item	Location where item is reported
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	Not applicable
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	Not applicable
Competing interests	26	Declare any competing interests of review authors.	Line 615
Availability of data, code and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	Lines 116-155

Table S2 **Bias analysis (ARRIVE) of experimental studies about anti-inflammatory activity in murine model treated with kefir**

References	*Akar et al. (2021)*[22]	*Ali et al. (2020)*[23]	*Brasil et al. 2018)*[24]	*Chen et al. (2021)*[25]	*El Golli-Bennour et al. (2019)*[26]	*Ekici et al. (2021)*[27]	*Franco et al. (2013)*[28]	*Hadisaputro et al. (2012)*[29]	*Jascolka et al. (2013)*[30]	*Kim et al. (2017)*[31]	*Maciel et al. (2016)*[32]	*Mohammed (2016)*[33]	*Murray et al. (2019)*[34]	*Punaro et al. (2014)*[35]	*Rosa et al. (2016)*[36]	*Dos Reis et al. (2019)*[37]	*Santanna et al. (2016)*[38]	*Senol et al. (2015)*[39]	*Silva et al. (2019)*[40]	*Sunarti et al. (2015)*[41]	*Vieira et al. (2021)*[42]	*Yener et al. (2015)*[43]	*Zamberi et al. (2016)*[44]	*Percentage (%)*
1. *Context*	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100
2. *Abstract*																								
2.1	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100
3. *Introduction*																								
3.1	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100
3.2	ok	ok	ok	ok	ok	ok	ok	ok	ok	-	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	95.7
4. *Objectives*																								
4.1	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100
5. *Material and methods*																								
5.1	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	-	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	95.7
5.2	ok	ok	ok	ok	ok	ok	ok	-	ok	ok	-	-	-	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	82.6
6. *Study design*																								
6.1	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100
6.2	-	-	-	-	-	-	-	-	-	-	-	-	ok	-	-	-	-	-	-	-	-	-	-	4.3
7. *Experimental Procedures*																								
7.1 *Treatment*																								
7.1.1	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100
7.1.2	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100
7.1.3	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100
7.1.4	-	-	-	-	ok	-	-	-	-	ok	-	-	-	ok	-	-	-	-	-	-	ok	-	-	17.4
7.1.5	-	-	-	-	-	-	-	-	-	-	-	-	-	ok	-	-	ok	-	ok	-	ok	-	-	17.4
7.1.6	-	ok	-	-	ok	ok	-	-	-	-	ok	ok	-	-	-	ok	-	-	ok	ok	ok	ok	ok	47.8

Table S2 **Bias analysis (ARRIVE) of experimental studies about anti-inflammatory activity in murine model treated with kefir**

References	*Akar et al. (2021)*[22]	*Ali et al. (2020)*[23]	*Brasil et al. (2018)*[24]	*Chen et al. (2021)*[25]	*El Golli-Bennour et al. (2019)*[26]	*Ekici et al. 2021*[27]	*Franco et al. (2013)*[28]	*Hadisaputro et al. (2012)*[29]	*Jascolka et al. (2013)*[30]	*Kim et al. (2017)*[31]	*Maciel et al. (2016)*[32]	*Mohammed (2016)*[33]	*Murray et al. 2019*[34]	*Punaro et al. (2014)*[35]	*Rosa et al. 2016*[36]	*Dos Reis et al. 2019*[37]	*Santanna et al. 2016*[38]	*Senol et al. 2015*[39]	*Silva et al. 2019*[40]	*Sunarti et al. (2015)*[41]	*Vieira et al. 2021*[42]	*Yener et al. 2015*[43]	*Zamberi et al. 2016*[44]	*Percentage (%)*
7.1.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
7.2 *Experimental animals*																								
7.2.1	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100
7.2.2	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100
7.2.3	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100
7.2.4	-	ok	-	-	ok	ok	-	ok	ok	-	ok	ok	-	ok	-	-	ok	ok	-	ok	ok	ok	ok	60.9
7.2.5	ok	-	ok	ok	-	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	-	-	ok	82.6
7.2.6	-	-	ok	-	-	-	-	-	ok	-	-	ok	-	ok	-	ok	ok	-	ok	ok	-	-	-	34.8
7.2.7	ok	ok	-	ok	ok	ok	-	-	ok	-	-	-	-	-	-	-	-	-	ok	ok	ok	ok	-	43.5
7.3 *Housing and husbandry*																								
7.3.1	ok	ok	-	-	-	-	-	-	ok	-	ok	-	ok	ok	ok	ok	ok	ok	-	ok	ok	-	ok	56.5
7.3.2	ok	ok	ok	ok	ok	-	ok	-	ok	-	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	-	ok	82.6
7.3.3	ok	ok	ok	ok	ok	ok	ok	-	ok	-	-	ok	-	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	82.6
7.4 *Sample size*																								
7.4.1	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100
7.4.2	-	-	ok	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4.3
7.5 *Allocation of animal into experimental groups*																								
7.5.1	ok	ok	ok	-	ok	-	-	ok	ok	-	ok	-	-	-	ok	-	-	ok	ok	-	ok	-	-	47.8
7.5.2	ok	ok	-	ok	-	ok	-	-	-	-	ok	-	ok	ok	-	-	-	-	ok	ok	ok	-	ok	47.8
7.6 *Experimental outcomes*																								
7.6.1	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100

Table S2 **Bias analysis (ARRIVE) of experimental studies about anti-inflammatory activity in murine model treated with kefir**

References	*Akar et al. (2021)*[22]	*Ali et al. (2020)*[23]	*Brasil et al. (2018)*[24]	*Chen et al. 2021*[25]	*El Golli-Bennour et al. (2019)*[26]	*Ekici et al. (2021)*[27]	*Franco et al. (2013)*[28]	*Hadisaputro et al. (2012)*[29]	*Jascolka et al. (2013)*[30]	*Kim et al. (2017)*[31]	*Maciel et al. (2016)*[32]	*Mohammed (2016)*[33]	*Murray et al. (2019)*[34]	*Punaro et al. (2014)*[35]	*Rosa et al. (2016)*[36]	*Dos Reis et al. (2019)*[37]	*Santanna et al. (2016)*[38]	*Senol et al. (2015)*[39]	*Silva et al. (2019)*[40]	*Sunarti et al. (2015)*[41]	*Vieira et al. (2021)*[42]	*Yener et al. (2015)*[43]	*Zamberi et al. (2016)*[44]	*Percentage (%)*
7.7 *Statistical methods*																								
7.7.1	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100
7.7.2	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100
7.7.3	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100
8. *Results*																								
8.1 *Baseline Data*																								
8.1.1	-	-	-	-	-	-	-	-	-	-	-	-	-	ok	-	-	-	-	-	ok	-	-	-	8.7
8.2 *Number analyzed*																								
8.2.1	ok	ok	ok	ok	ok	ok	-	-	ok	ok	ok	-	ok	ok	ok	ok	ok	-	ok	-	-	-	ok	69.6
8.2.2	-	-	-	-	-	-	-	-	-	ok	-	-	-	-	-	-	-	-	-	-	-	-	-	4.3
8.3 *Outcomes and estimation*																								
8.3.1	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100
8.4. *Adverse events*																								
8.4.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
8.4.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
9. *Discussion*																								
9.1 *Interpretation / scientific implications*																								
9.1.1.	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100

Table S2 **Bias analysis (ARRIVE) of experimental studies about anti-inflammatory activity in murine model treated with kefir**

References	*Akar et al. (2021)*[22]	*Ali et al. (2020)*[23]	*Brasil et al. (2018)*[24]	*Chen et al. 2021*[25]	*El Golli-Bennour et al. (2019)*[26]	*Ekici et al. (2021)*[27]	*Franco et al. (2013)*[28]	*Hadisaputro et al. (2012)*[29]	*Jascolka et al. (2013)*[30]	*Kim et al. (2017)*[31]	*Maciel et al. (2016)*[32]	*Mohammed (2016)*[33]	*Murray et al. (2019)*[34]	*Punaro et al. (2014)*[35]	*Rosa et al. (2016)*[36]	*Dos Reis et al. (2019)*[37]	*Santanna et al. (2016)*[38]	*Senol et al. (2015)*[39]	*Silva et al. (2019)*[40]	*Sunarti et al. (2015)*[41]	*Vieira et al. (2021)*[42]	*Yener et al. (2015)*[43]	*Zamberi et al. (2016)*[44]	*Percentage (%)*
9.1.2	-	-	ok	ok	-	ok	-	-		-	-	-	-	-	-	-	ok	-	-	-	ok	-	ok	26.1
10. ***Generalizability / translation***																								
10.1	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	-ok	ok	ok	ok	ok	ok	ok	ok	-	ok	ok	ok	91.3
11. ***Funding***																								
11.1	ok	ok	ok	ok	ok	ok	-	ok	ok	ok	ok	-	ok	ok	ok	ok	ok	-	ok	ok	ok	ok	ok	87.0
Results	31	32	31	30	31	31	25	25	32	26	30	25	28	34	29	30	32	28	33	32	34	28	32	

Table S2 **The ARRIVE Guidelines for Reporting Animal Research – Questions**

1. Accurate and concise description of the content of the article

2. Abstract

2.1. Summary of the background, research objectives, methods, main findings, and conclusions

3. Introduction

3.1. Sufficient scientific background

3.2. Explanation of the experimental approach and rationale

4. Objectives

4.1. Clear primary and second objectives

5. Materials and methods

5.1. Declaration Ethical

5.2. Nature of the ethical review permissions, relevant licenses and national or institutional guidelines for the care and use of animals

6. Study design

6.1. Number of animals per group experimental

6.2. Information on whether the experimental was performed as a blind controlled study

7. Experimental procedures

7.1. Treatment

7.1.1. Dosage of treatment

7.1.2. Route of administration

7.1.3. Duration of treatment

7.1.4. Time of day for treatment administration

7.1.5. Location used for administration of treatment

7.1.6. Rationale for choice of specific dosage

7.1.7. Rationale for choice of specific route of administration

7.2. Experimental animals

7.2.1. Information regarding on animal species

7.2.2. Strain of the animals

7.2.3. Sex of the animals

7.2.4. Animals weight range

7.2.5. Age of the animals

7.2.6. Description of genetic modification status (Knockout, transgenic, SPF)

7.2.7. Information related to previous procedures performed on the animals

7.3. Housing and husbandry

7.3.1. Housing of experimental animals (type of facility, type of cage or housing, material, number of cage companions)

7.3.2. Husbandry conditions (breeding programme, light/dark cycle, temperature, of water)

7.3.3. Welfare-related assessments and interventions that were carried out before, during, or after the experiment

7.4. Sample size

7.4.1. Total number of animals used in each experimental and the number of animals in each experimental group

7.4.2. Explanation regarding the decision of the number of animals and details of sample size calculation

7.5. Allocation of animals into experimental groups

7.5.1. Full details of how animals were allocated to experimental groups (including randomization or matching)

7.5.2. Order in which the animals in the different experimental groups were treated and assessed

7.6. Experimental outcomes

7.6.1. Clear experimental outcomes assessed

7.7. Statistical methods

7.7.1. Statistical methods used for each analysis

7.7.2. Specification of the unit of analysis for each dataset

7.7.3. Methods used to assess whether the data met the assumptions of the statistical approach

8. Results

8.1. Baseline data

8.1.1. Description of the animal's health status of animals, for each experimental group, before treatment

8.2. Number analyzed

8.2.1. Number or animals in each group included in each analysis (absolute numbers)

8.2.2. Animals or data not included in the analysis (and explanation for the exclusion)

8.3. Outcomes and estimation

8.3.1. Information (Mean/Standard Deviation)

8.4. Adverse events

8.4.1. Information on mortality of experimental animals

8.4.2. Modifications to the experimental protocols made to reduce adverse events

9. Discussion

9.1. Interpretation /scientific implications

9.1.1. Interpretation of the results, taking into account the study objectives and hypotheses, current theory and relevant studies

9.1.2. Comments on the study limitations (sources of bias, limitations of the animal model, imprecision associated with the results)

10. Generalizability /translation

10.1. Comments on how the findings are likely to translate to other species or systems, including relevance to human biology

11. Funding

11.1. List of funding sources and the role of the funder(s) in the study

4. ORIGINAL RESEARCH RESULTS

4.1. Manuscript 2

Manuscript published to Applied Microbiology and Biotechnology (Impact Factor: 5.560). Doi: 10.1007/s00253-023-12630-0.

Title: Milk kefir alters fecal microbiota impacting gut and brain health in mice

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Abstract

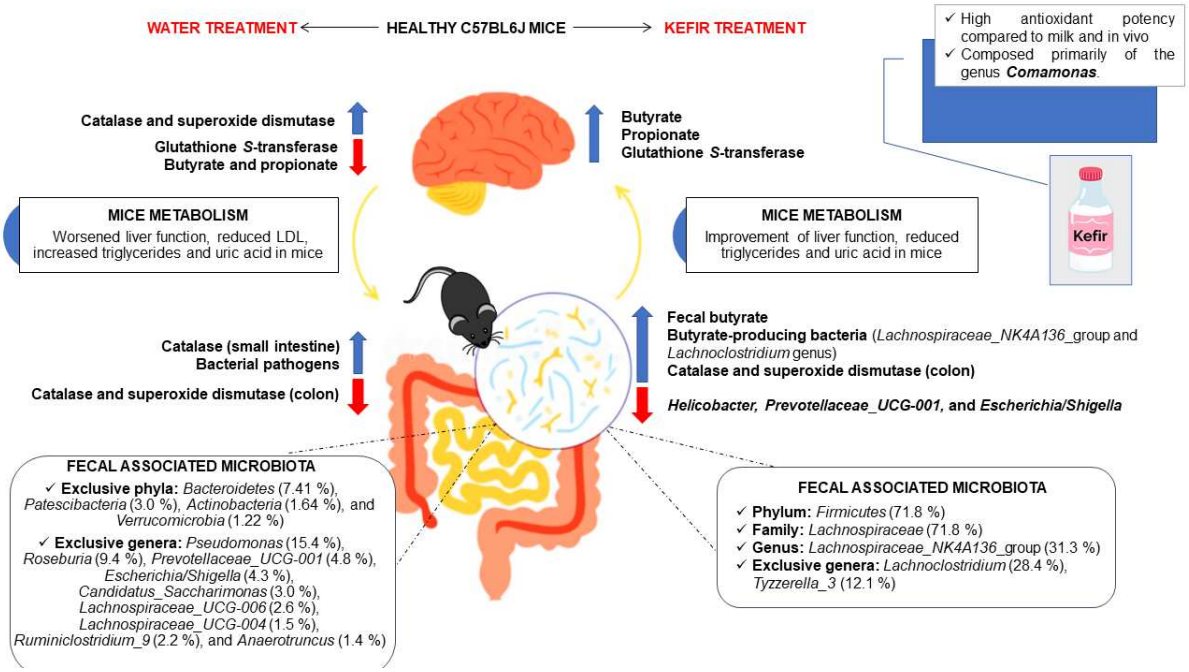
Kefir has been suggested as a possible bacterial prophylaxis against *Salmonella* and IL-10 production seems to be crucial in the pathogenesis of salmonellosis in mice. This study evaluated the role of IL-10 in the inflammation and gut microbiome in mice consuming milk kefir and orally challenged with *Salmonella enterica* serovar Typhimurium. C57BL wild type (WT) (n = 40) and C57BL IL-$10^{-/-}$ (KO) (n = 40) mice were subdivided into eight experimental groups either treated or not with kefir. In the first 15 days, the water groups received filtered water (0.1 mL) while the kefir groups received milk kefir (10% w/v) orally by gavage. Then, two groups of each strain received a single dose (0.1 mL) of the inoculum of *S.* Typhimurium (ATCC 14028, dose: 10^6 CFU mL^{-1}). After four weeks, the animals were euthanized to remove the colon for further analysis. Kefir prevented systemic infections only in IL-$10^{-/-}$ mice, which were able to survive, regulate cytokines, and control colon inflammation. The abundance in *Lachnospiraceae* and *Roseburia*, and also the higher SCFA production in the pre-infection, showed that kefir has a role in intestinal health and protection, colonizing and offering competition for nutrients with the pathogen as well as acting in the regulation of salmonella infectivity only in the absence of IL-10. These results demonstrate the role of IL-10 in the prognosis of salmonellosis and how milk kefir can be used in acute infections.

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

Key Points

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

Graphical Abstract



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*, and *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 lactic acid bacteria (*Lactobacillus*, *Bifidobacterium* and *Clostridium*) and yeasts, such as short-chain fatty acids (SCFAs) (Bengoa et al. 2019; Gao and Zhang 2019).

SCFAs are a result of a complex interplay between diet and gut microbiota. They are mainly produced through bacterial fermentation of complex resistant carbohydrates (e.g., fructooligosaccharides, resistant starch, and inulin) which scape digestion and absorption in the small intestine reaching the colon (Richards et al. 2016). Amino acids can be also fermented to produce SCFAs. Although SCFAs are dependent on diet and bacteria present in the gut, there are specific foods containing SCFAs, for instance some dairy products such kefir that can activate different local beneficial responses (Morrison and Preston 2016). They can also reach brain signaling via the microbiota-gut-brain axis (Silva et al. 2020). This axis is a bi-directional complex communication system between the gastrointestinal tract (GIT), the microorganisms that inhabit it, and the peripheral and central nervous systems (CNS) (Mörkl et 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), and 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.

Materials and Methods

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®, Abre Campo, Minas Gerais, Brazil, batch MG1 AM 5). The fermentation protocol lasts 48 hours. First, the kefir grains were inoculated in milk and the flasks were kept at room temperature, 25 °C ± 2 °C for 24 h, without agitation and 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, for four weeks, via gavage. The same fermentation protocol, using the same kefir grains, was performed daily during all the experimental design period, ensuring that the mice consumed (10^8 CFU/mL for lactic acid bacteria and 10^4 UFC/mL for yeasts) an equivalent common dose for fermented dairy products, such as kefir, and an equal and fresh product as would be made and consumed by consumers in real life. Samples were collected for physicochemical,

microbiological, antioxidant (Supplemental Fig. S1), and metabarcoding analyzes Fig. 1).

Characterization of Milk Kefir Beverage

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 analyzes were made in triplicates. The nutritional composition of kefir is described in Supplemental Table S1.

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®, Lansing, 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). To count coliforms, 25 mL of the sample was mixed with 225 mL of 0.1 % peptone saline solution and homogenized the sample for 60 seconds. From the initial dilution (10^{-1}), the other desired dilutions were made in 0.1 % peptone saline solution. Then, 1 mL of each desired dilution was inoculated into sterilized Petri dishes and 15 mL of violet red bile agar was added to each dish. They were homogenized and after complete solidification of the medium, the plates were incubated at a temperature of 36 °C ± 1 ºC for 24 h. Colonies with typical coliform morphology were counted. The presence of total coliforms was confirmed by gas formation or effervescence when shaken. Confirmation of the presence of thermotolerant coliforms was performed by inoculating the suspected colonies in *Escherichia coli* broth and subsequent incubation at a selective temperature of 45 °C ± 0.2 ºC, in a water bath with agitation. The presence of gas in the Durham tubes shows the fermentation of the lactose present in the medium (Brasil 2003).

For *Salmonella* counting, 25 mL of the sample + 225 mL of 1 % buffered peptone water were used and incubated at 35 °C ± 1 °C for 24 h. Afterwards, a selective enrichment was performed with 1mL of the culture + 10 mL of the tetrathionate broth with incubation at 35 °C ± 1 °C for 24 h. Isolation in selective medium was performed on xylose lysine deoxycholate agar at 35 °C ± 1 °C for 24 h. The presumptive identification was through the triple iron-sugar agar method, in which the production of H_2S was indicated by the black color at the base of the central portion of the tube (Brasil 2003). The counting of LAB, fungi and yeasts, thermotolerant coliforms, and *Salmonella* spp. for fermented beverages is described in Supplemental Table S2.

Antioxidant Analysis of Kefir

Kefir beverage 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 (Yilmaz-Ersan et al. 2018). The extracts were centrifuged at 1420 x g 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, Thermo Scientific, Japan). 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, Thermo Scientific, Japan) at 517 nm after the samples being kept in the dark for 30 minutes. The calibration curve of Trolox was made for both analyzes 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).

Total DNA extraction of milk kefir beverage

Kefir is composed of bacteria and yeast which live in symbiosis and most of this community is composed of bacteria. Thus, when microbiologically characterizing kefir in relation to lactic acid bacteria and yeast it was chosen to focus on bacterial communities to characterize the product and its effect on the abundance of these bacteria in animal feces (10^4 times greater amounts of bacteria than yeast). In that way, DNA from milk kefir beverage was extracted based on the CTAB (cetyltrimethylammonium bromide) method (Chen et al. 2011). Firstly, kefir beverage was centrifuged 10 minutes at 8,000 x g and 4 °C. The precipitate was discarded and the supernatant was used for DNA extraction. The supernatant was mixed in 570 µL tris EDTA buffer. Three microliters of 20 mg proteinase K/mL and 30 µL 10 % sodium dodecyl sulfate solution were added to each sample, and the tubes were incubated at 37 °C for 1 h in a water bath. To the sample was then added 100 µL 5 M NaCl solution and 80 µL CTAB/NaCl, and they were incubated at 65 °C for 10 minutes. After centrifuging for 10 minutes at 12,000 x g and 4 °C, the suspensions were mixed with 1 volume of chloroform-isoamyl alcohol (24:1, v/v) and phenol–chloroform-isoamyl alcohol (25:24:1, v/v/v) respectively, and centrifuged as described above. DNA was then precipitated with 0.6 mL of isopropanol. The quality and quantification of the DNA were analyzed by Nanodrop with calculation of the ration 260/280 and 260/230. In addition, the quality was evaluated by the absence of degradation smear in the 0.8 % agarose gel electrophoresis and positive amplification by polymerase chain reaction (PCR) using 337F and 518R 16S rRNA primers for V3 hypervariable region to detect 16S rRNA (Park et al. 2021). After that, the samples were sent to the company Neoprospecta (Florianópolis, Santa Catarina, Brazil) responsible for the sequencing, on an Illumina HiSeq 2500 platform (Illumina, San Diego, CA, USA) as it were done for fecal samples.

Experimental design

Animals and ethical aspects

Twenty healthy male C57BL-6 mice at eight weeks old, weighing approximately 22 g, 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 (0.1 mL) and the kefir group that received a commercial diet and orogastric gavage with milk kefir (0.1 mL, 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 ºC ± 2 ºC), humidity (60 - 70 %), and a 12 h light/dark cycle. Filtered water and a commercial diet (Presence®, São Paulo, Brazil) were provided ad libitum during the experimental design period of four weeks. The animals were weighed weekly in the morning, and feces were collected in the fourth week.

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 -80 ºC. 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 -80 ºC 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 35/2020, 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, the European Community Guidelines (Directive 2010/63/EU), 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.

Analysis of growth parameters, somatic indices, and food intake

The animals were weighed on a digital scale (Marte Slim®, model M 2K, São Paulo, Brazil), 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, São Paulo, Brazil), and stored at -80 ºC. 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, São Paulo, Brazil).

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®, Shenzhen, China) using commercial kits (Bioclin®, Belo Horizonte, Minas Gerais, Brazil).

Analysis of oxidative stress and antioxidant enzymes

Colon, small intestine, and brain samples were homogenized in phosphate buffer (0.1 M, pH 7) and centrifuged at 10000 x 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).

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. Individual freshly excreted feces (n = 10/group), in the fourth week, were stored at -80 ºC until processing. Stool samples of approximately 50 mg were weighted and homogenized in 950 µL of Milli-Q® (Merck® KGaA, Darmstadt, Germany) water and kept for 30 minutes on ice, being homogenized every 5 minutes. Afterward, the samples were centrifuged at 10000 x g, for 30 minutes, at 4 ºC, three times, collecting the supernatants each time between centrifugations. The final supernatants were filtered through a syringe filter (0.45 µm) 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 Milli-Q® (Merck® KGaA, Darmstadt, Germany) 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) (Bio-Rad®, Hercules, CA, USA), 300 x 7.8 mm, 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 32 ºC and injected volume of 10 µL. The mobile phase was acidified water (0.005 M sulfuric acid).

Metataxonomic analysis of the fecal microbiota

Fecal DNA extraction was performed with 30 ± 2 mg of stool samples collected in the fourth experimental week and performed according to a protocol from Zhang et al. (2006). Individual isogenic mice feces (n = 9/ each group) were distributed in 3 pools with 3 mice per pool. The quality and quantity of the extracted nucleic acids were verified by NanoDrop with calculation of the ration 260/230 and 260/280, 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 Neoprospecta (Florianópolis, Santa Catarina, Brazil) responsible for the sequencing, on an Illumina HiSeq 2500 platform (Caporaso et al. 2012). 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) (Wang and Qian 2009; Caporaso et al. 2012). 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 (Quast et al. 2013). Illumina Hiseq raw sequences generated and analyzed during the current study are available in the Sequence Read Archive (SRA) database (https://www.ncbi.nlm.nih.gov/sra/PRJNA925110) under the BioProject PRJNA925110 (Leinonen et al. 2011).

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. 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 as well as the rarefaction curves were constructed on the R platform (version 1.2.1335). The metataxonomic analysis of kefir and the fecal microbiota were represented by relative abundance (%). The abundance and comparison of specific taxa in fecal samples were carried out by unpaired *T*-test analysis with Welch's correction. The software used to perform statistical analyzes and constructed the graphs was GraphPad® Prism, version 8.3.0. (San Diego, California, USA). Statistical differences were considered significant at $p < 0.05$ (*) and $p < 0.01$(**).

Results

Microbial profiling and antioxidant activity of milk kefir beverage

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 and with an average of 883,086,33 ± 28,404.55 sequences for each sample (n = 3). Low-quality and chimeric sequences were removed. For kefir beverage samples, 2,647,956 high-quality reads were obtained with an average of 882,652 ± 28390,96 sequences for each sample (n = 3). 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 (Supplemental Fig. S2a).

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).

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 ± 0.42 % of free DPPH radical scavenging.

Effect of kefir in the C57BL-6 mice

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).

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

Oral administration of kefir reduced the liver enzymes ALT (p = 0.0334) (Fig. 3a) and AST (p = 0.0230) (Fig. 3b), total proteins (p = 0.0189) (Fig. 3c), and globulins (p = 0.0024) (Fig. 3d.) compared to the water group. Although the oral administration of milk kefir reduced triglyceride (p = 0.0013) (Fig. 4f) and uric acid concentration (p = 0.0062) (Fig. 3g), LDL level was increased (p = 0.0069) (Fig. 3e). Parameters such as alkaline phosphatase (Supplemental Fig. S3c), urea (Supplemental Fig. S3d), creatinine (Supplemental Fig. S3e), albumin (Supplemental Fig. S3h), total cholesterol (Supplemental Fig. S3a), HDL (Supplemental Fig. S3b), glucose (Supplemental Fig. S3f), and calcium (Supplemental Fig. S3g) were not altered in mice fed with kefir.

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).

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 concentrations 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 = 0.0847) and acetic acid (p = 0.5106) between the water and kefir groups (Fig. 5f-g), and butyric acid was not found in the samples.

Diversity and metataxonomic 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 reads were obtained, with an average of 229,521.5 ± 233,033.50 sequences for each sample (n = 9/ group; distributed in 3 pools with 3 mice per pool). 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 (Supplemental Fig. S2b).

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 animals treated with water or kefir ($p > 0.05$).

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. 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 fifty 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 %), and *Puniceicoccaceae* (1.2 %) were found exclusively in the group that received water (Fig. 7b).

Thirteen genera were found among experimental groups (top 50 genera most abundant) (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 %).

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 and food matrices. It was observed in our study that the fecal microbiota of mice 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 colonize to high levels. This fact has parallels with the fact that probiotics most frequently do not colonize 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 dementia (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, such as of the *Enterococcus* and the *Carnobacterium* genus in kefir beverage increases the proportion of microorganisms belonging to the genus *Comamonas*, as well as it results in a substantial decrease in the proportion of enterobacteria such as *Shigella*/*Escherichia* coli and *Helicobacter* (Kim et al. 2015; Bengoa et al. 2020), which corroborate for the results in the mice 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 the *Lachnoclostridium* genus were found in the kefir-treated mice. While the presence of *Helicobacter pylori* in the gastric microbiome has been linked with peptic ulcer disease and gastritis, the low pH promoted by milk kefir intake may limit these types of microbes that live in the gastric lumen (*Shigella/Escherichia coli* and *Helicobacter*) and select for acid-resistant bacterial populations (*Lachnospiraceae* family, *Lachnoclostridium* and *Tyzzerella_3* genera), reducing pathogens (Hollister et al. 2014; Rosa et al. 2017; Tenorio-Salgado et al. 2021; Liu et al. 2022). *Lachnospiraceae* is known to colonize 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). The fermentation that occurs in the beverage favors the presence of some bacteria as found in this study. The diet offered to animals has 5% of fiber, besides this ingestion, there are other factors that can influence the expansion of bacterial communities and consequently the production of SCFAs, the most product produced by these families found. So, SCFAs are a result of a complex interplay between diet and gut microbiota. They are mainly produced from through bacterial fermentation of

complex resistant carbohydrates, however amino acids can also be fermented to produce SCFAs (Richards et al. 2016). Although SCFAs are dependent on diet and bacteria present in the gut, there are specific foods containing SCFAs, for instance some dairy products such as kefir that can and activate different local beneficial responses (Morrison and Preston, 2016).

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 (regulatory T) 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 produced by some genera such as *Enterococcus* and *Carnobacterium*, 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 they 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 behavior, as butyrate and the increase abundance in *Lachnospiraceae* have 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 the 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 beneficial bacteria 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 (Yilmaz-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 DPPH) analyzes 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 antioxidant 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 was 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 analyzes, 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.

Altogether, our results show that kefir has overall health benefits and modulate aspects of the gut-microbiota-brain axis in health mice. 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 fecal microbiota composition were distinct but composed primarily by the genus *Comamonas* in the beverage and of the SCFA-producing bacteria, such as *Lachnospiraceae and Lachnoclostridium* genera 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 mice's growth and adequate food intake. It improved liver function, reduced triglycerides and uric acid reinforcing the general health benefits. The next step for further studies using kefir should be focused in the investigation of 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.

Acknowledgments The authors thank the National Council for Scientific and Technological Development (CNPq), Coordination for the Improvement of Higher Education Personnel (CAPES) – Financing Code 001, and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) for the support to the researches. We thank the Diretoria de Tecnologia de Informação (DTI) at Universidade Federal de Viçosa for the availability of the computational cluster and software used in this work, also thank the Rede Minas Microbiomas for the metagenomic analysis funding.

Author Contribution statement:

AOBR, TAOM and MCGP, Conceptualization; MFAP, LGMA, GCAA, TAOM, and MCGP, Data curation; MFAP, LGMA, GCAA, TAOM, and MCGP, Formal analysis; AOBR, TAOM and MCGP Funding acquisition; MFAP, LGMA, GCAA, BCSC, LFA, JMS, ALB, IXC, AOBR, TAOM, and MCGP Investigation; MFAP, LGMA, GCAA, BCSC, LFA, JMS, ALB, IXC, AOBR, TAOM and MCGP, Methodology; TAOM and MCGP, Project administration; AOBR, TAOM and MCGP, Resources; MFAP, LGMA, and TAOM, Software; AOBR, TAOM and MCGP, Supervision; MFAP, LGMA, GCAA, BCSC, LFA, JMS, ALB, IXC, AOBR, TAOM, and MCGP, Validation; MFAP, LGMA, GCAA, TAOM, and MCGP, Visualization; MFAP, LGMA, GCAA, Writing - original draft; MFAP, LGMA, GCAA, BCSC, LFA, JMS, ALB, IXC, AOBR, TAOM, and MCGP, Writing - review & editing. All authors have read and agreed to the published version of the manuscript.

Compliance with Ethical Standards

Conflict of Interest All authors declare that they have no conflict of interest.

Ethical Approval 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 35/2020, 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, the European Community Guidelines (Directive 2010/63/EU), 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.

Data availability Statement

Illumina Hiseq raw sequences generated and analyzed during the current study are available in the Sequence Read Archive (SRA) database (https://www.ncbi.nlm.nih.gov/sra/PRJNA925110) under the BioProject PRJNA925110.

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Figures

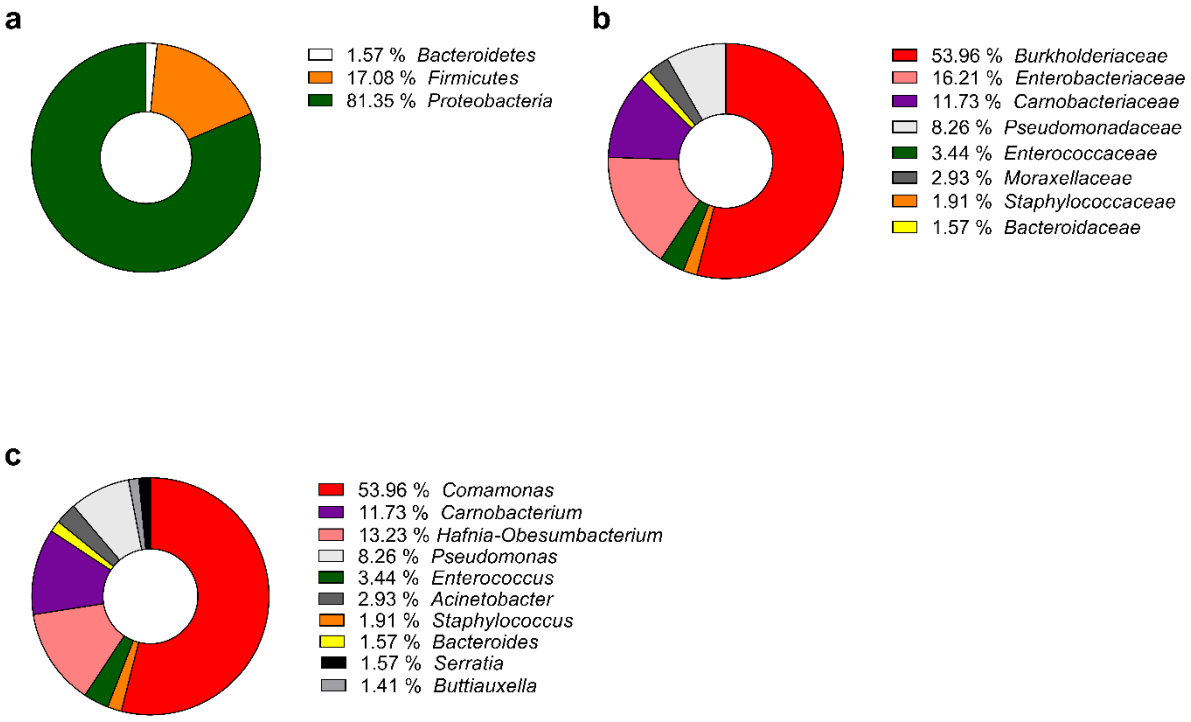


Fig. 1 Relative abundance distribution (%) of major phyla (a), families (b), and genera (c) found in the milk kefir beverage (10 % w/v) (top 50 bacteria). The analysis was made in triplicate (n = 3 kefir samples).

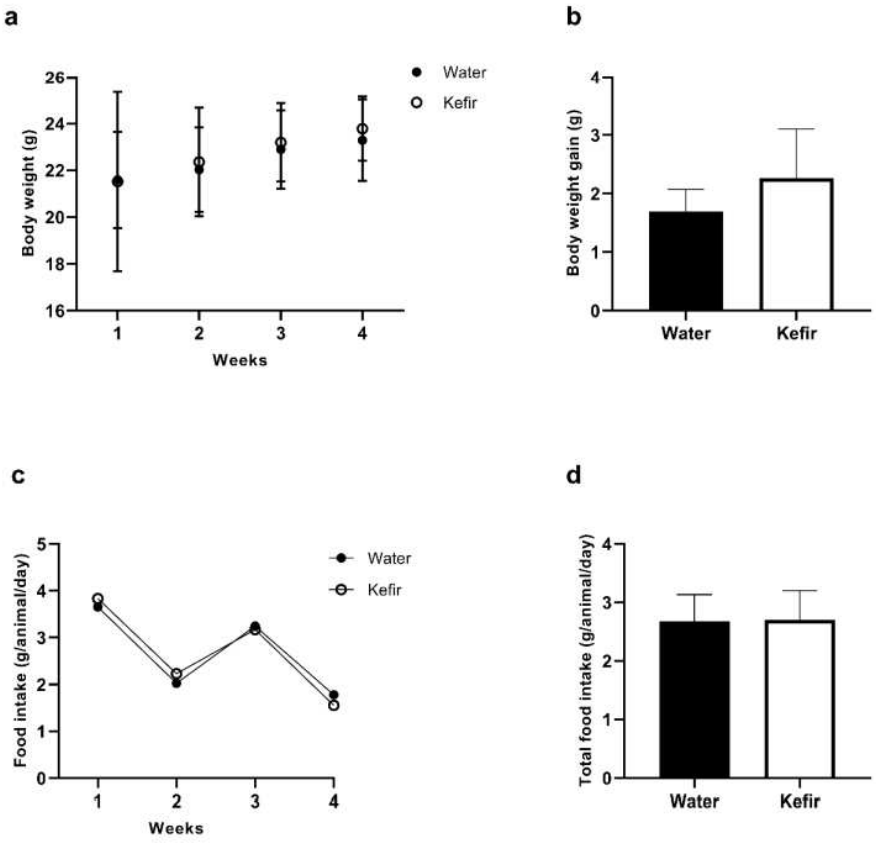


Fig. 2 The effect of treatments on growth parameters and food intake of C57BL6 mice. (a) Body weight (g); (b) Body weight gain (g); (c) food intake (g/mice/day); (d) total food intake. Weight data are expressed as mean ± standard error media (n = 10 mice/group). The results of weekly food consumption 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 ($p < 0.05$)

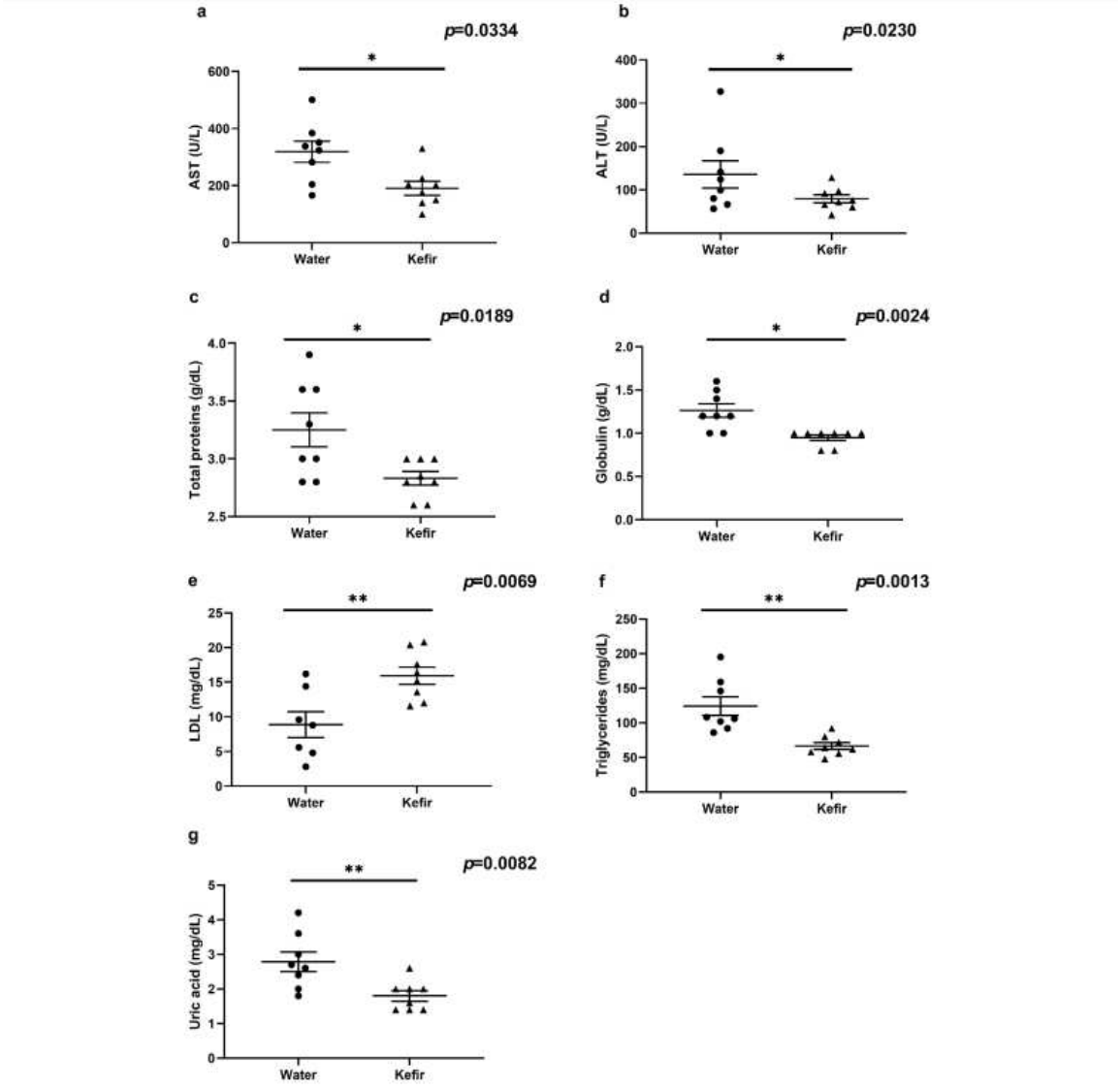


Fig. 3 The effect of treatments on the concentration of serum biomarkers in C57BL6 mice (a) AST (U/L); (b) ALT (U/L); (c) Total proteins (g/dL); (d) globulin (g/dL); (e) LDL (mg/dL); (f) triglycerides (mg/dL); (g) uric acid (mg/dL). Data are expressed as mean ± mean standard error (n = 8 mice/group). Statistical differences between groups were analyzed using the unpaired *T*-test with Welch's correction, in which (*) represents significant differences with $p < 0.05$, (**) $p < 0.01$

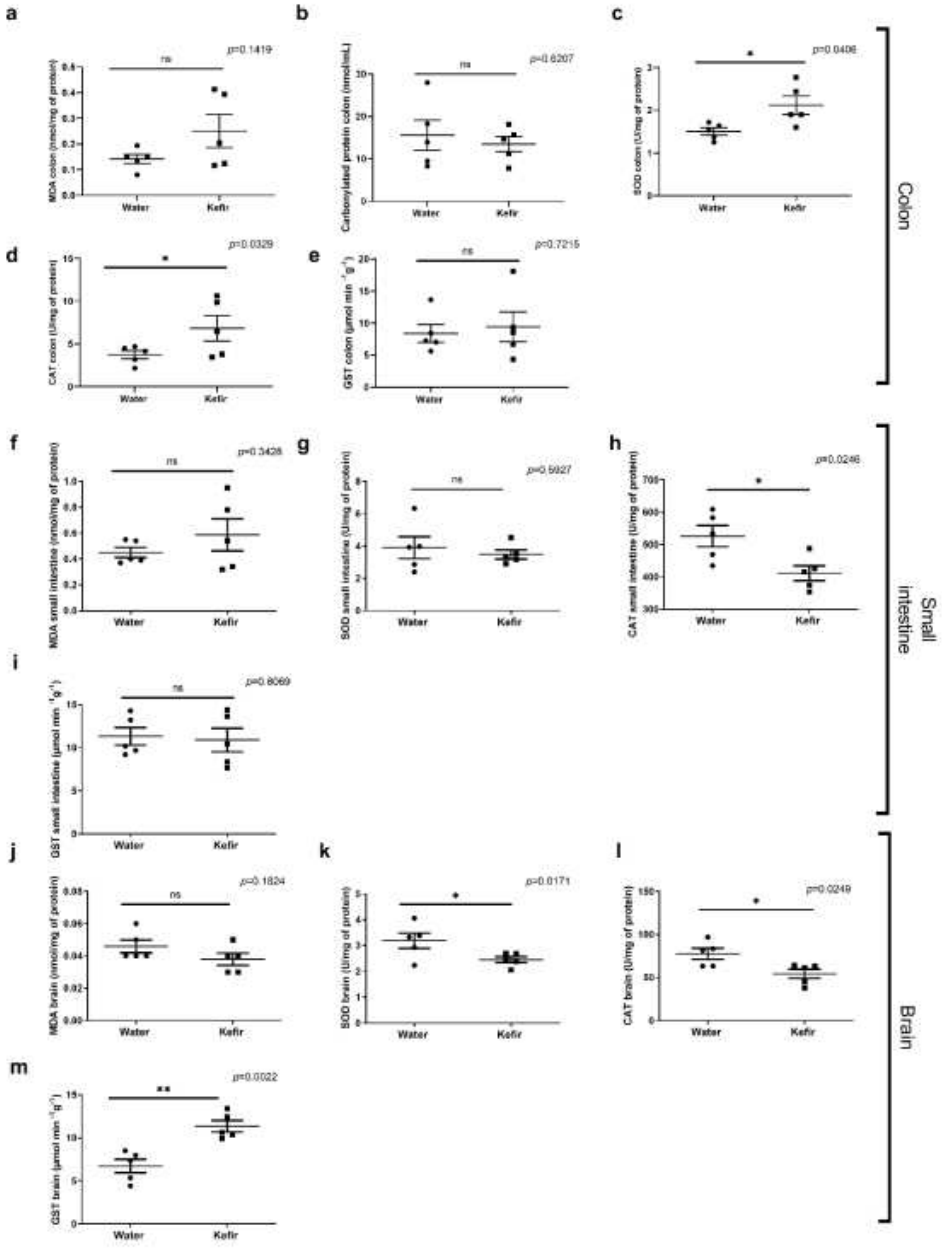


Fig. 4 The effect of treatments the biomarkers of oxidative stress and antioxidant enzymes on the colon, small intestine, and brain in C57BL6 mice. Data were expressed as mean ± mean standard error (n = 5 mice/group). (a) MDA (malondialdehyde) colon; (b) carbonylated protein colon; (c) superoxide dismutase (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 ($p < 0.05$), (**) $p < 0.01$

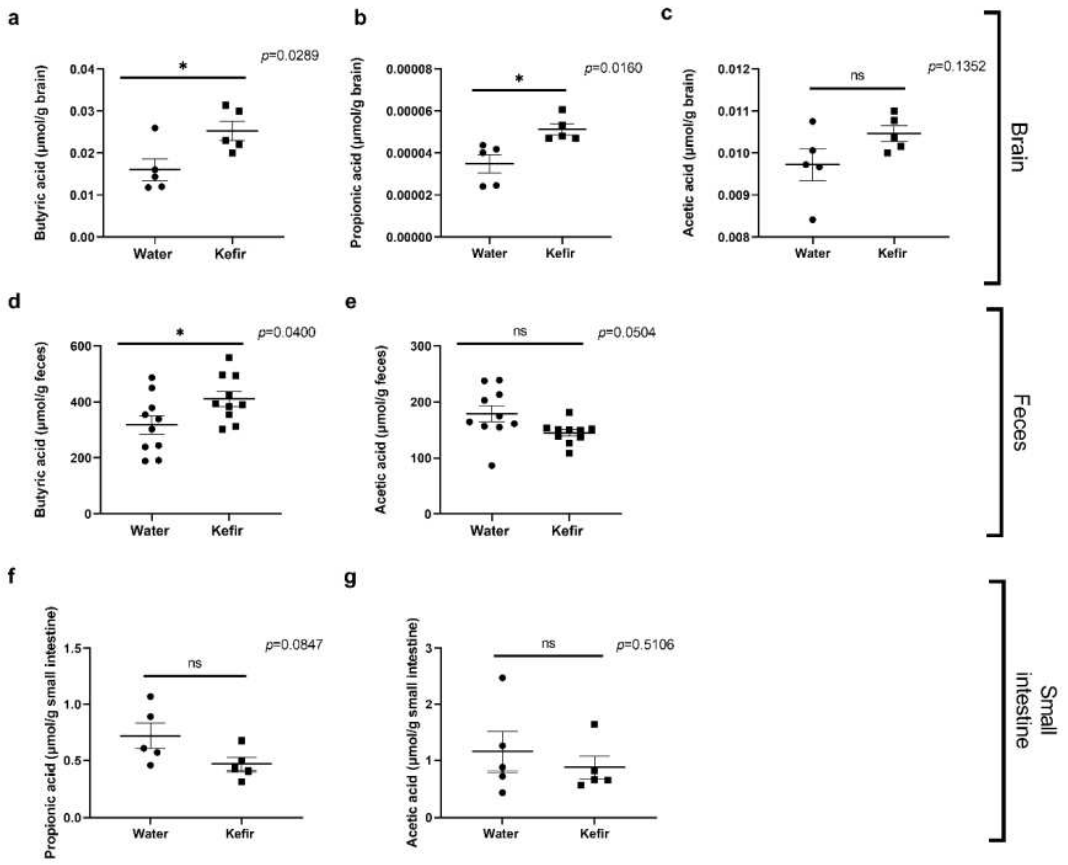


Fig. 5 The effect of treatments on SCFAs concentration on the brain, feces, and small intestine in C57BL6 mice. (a) Butyric acid (µmol/g of the brain); (b) propionic acid (µmol/g of the brain); (c) acetic acid (µmol/g of the brain). (d) Butyric acid (µmol/g of feces); (e) acetic acid concentrations (µmol/g 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 ($p < 0.05$)

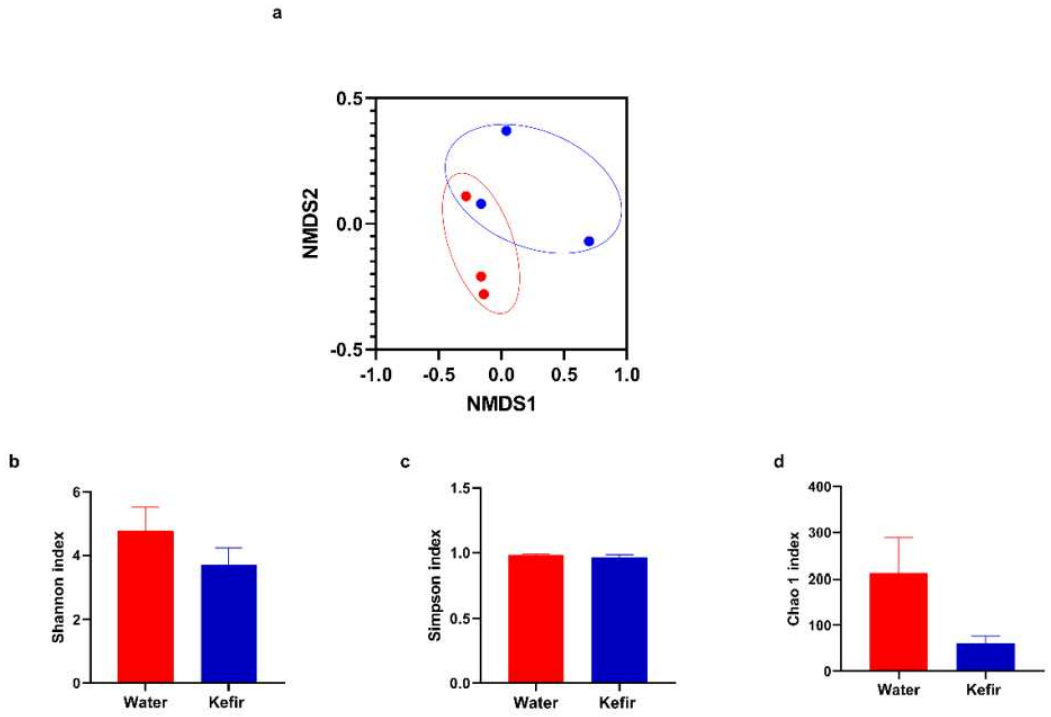


Fig. 6 Non-metric multidimensional scaling (NMDS) plot (a) of Metagenomics sequencing data in C57BL6 mice treated or not with kefir. Red dots represent water treatment; Blue dots represent kefir treatment. (b, c) Alpha bacterial diversity; (d) bacterial richness (n= 9/group, distributed in 3 pools with 3 mice per pool). Statistical differences between groups were analyzed using the unpaired *T*-test, in which (*) represents significant differences ($p < 0.05$)

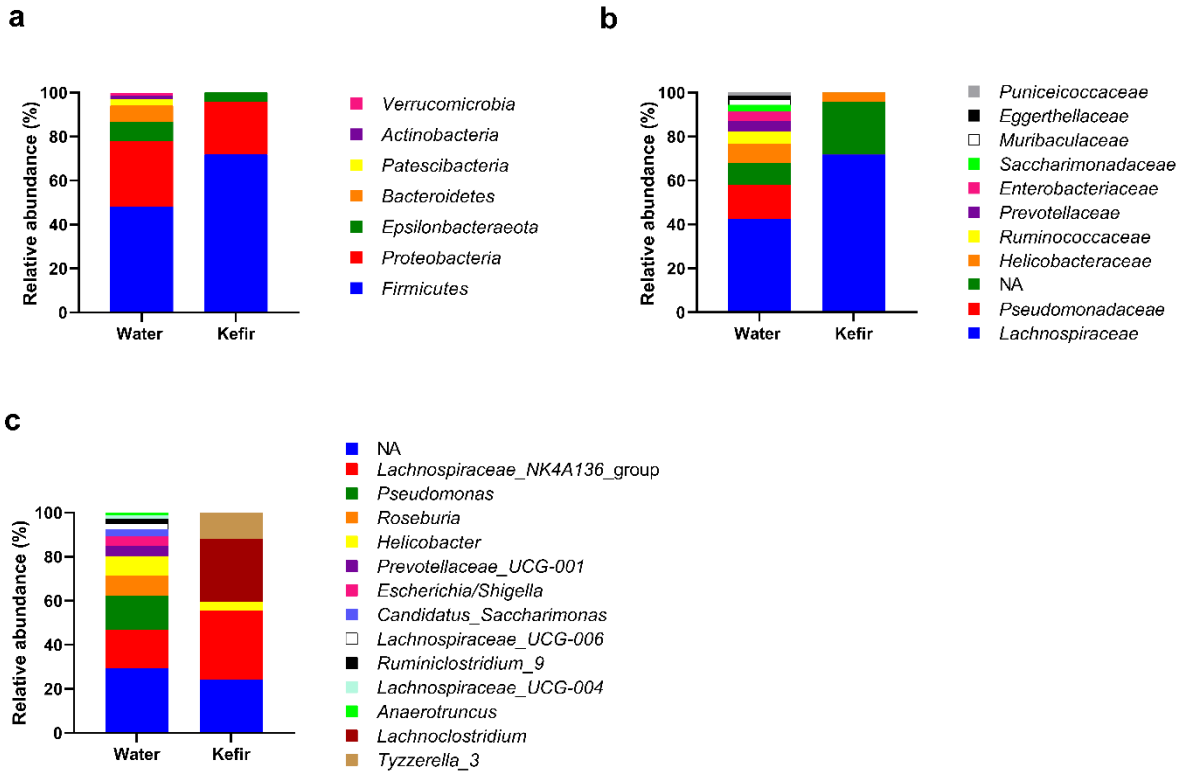


Fig. 7 Relative abundance (%) distribution of phyla (a), families (b), and genera (c) across the water and kefir experimental groups (10 % w/v) (top 50 bacteria) (n = 9/group, distributed in 3 pools with 3 mice per pool). The taxa were sorted by the decreasing order of average relative abundance. NA means not available.

Supplemental Table S1

Supplemental 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

Supplemental Table S2

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

Analysis	**Kefir**
Lactic acid bacteria (CFU*/g)	1.3×10^8
Molds and yeasts (CFU/g)	0.34×10^4
Thermotolerant coliforms (MPN*/g)	< 3.0
Salmonella spp.	Absent /25g

*CFU/g: colony forming unit/ per gram, MPN/g: most probable number/ per gram

Supplemental Table S3

Supplemental 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 unpaired *T*-test with Welch's correction, in which (**) represent significant differences p <0.01.

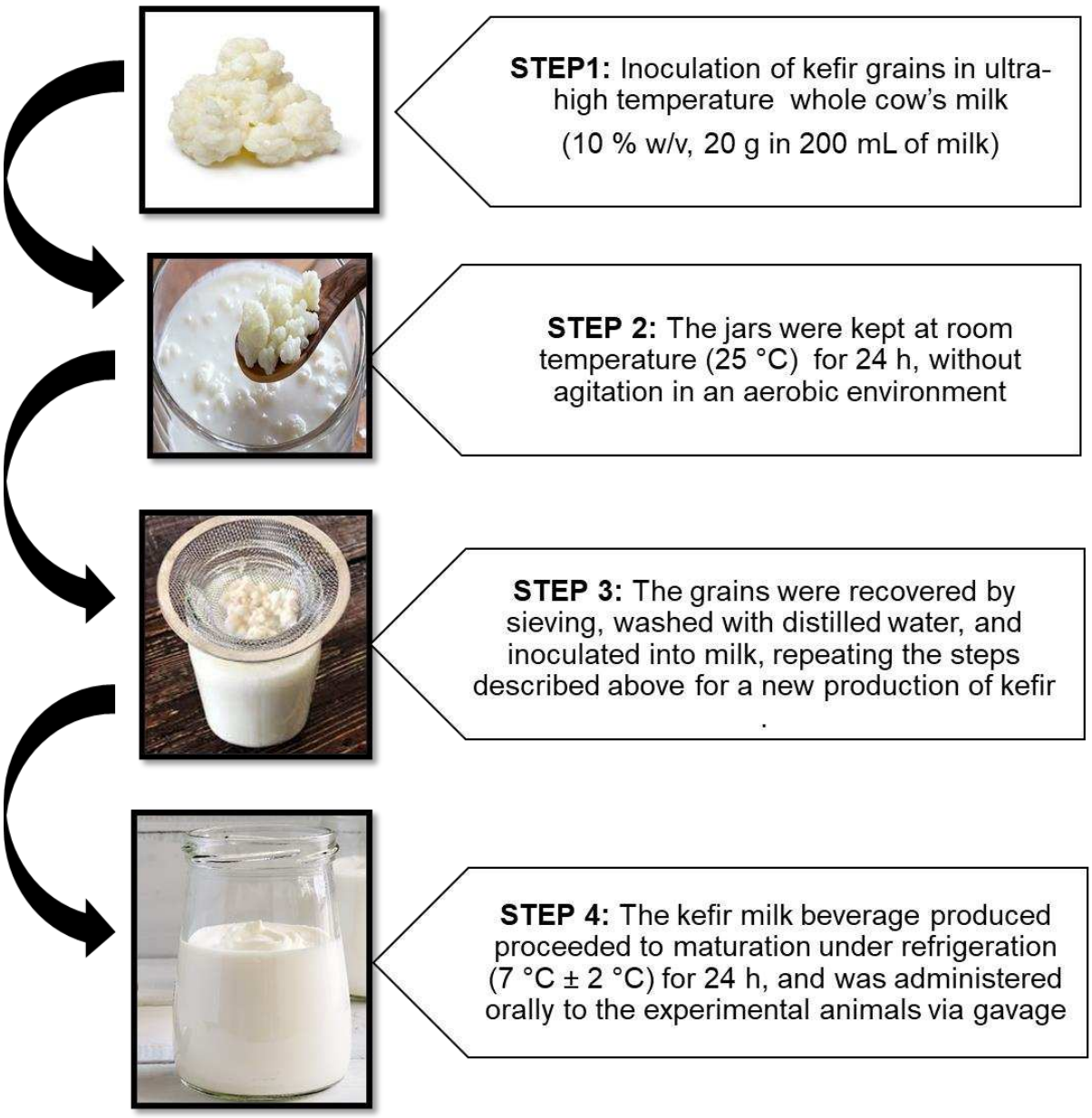
Supplemental Table S4

Supplemental Table S4 Somatic index of the organs of C57BL6 mice treated or not with kefir.

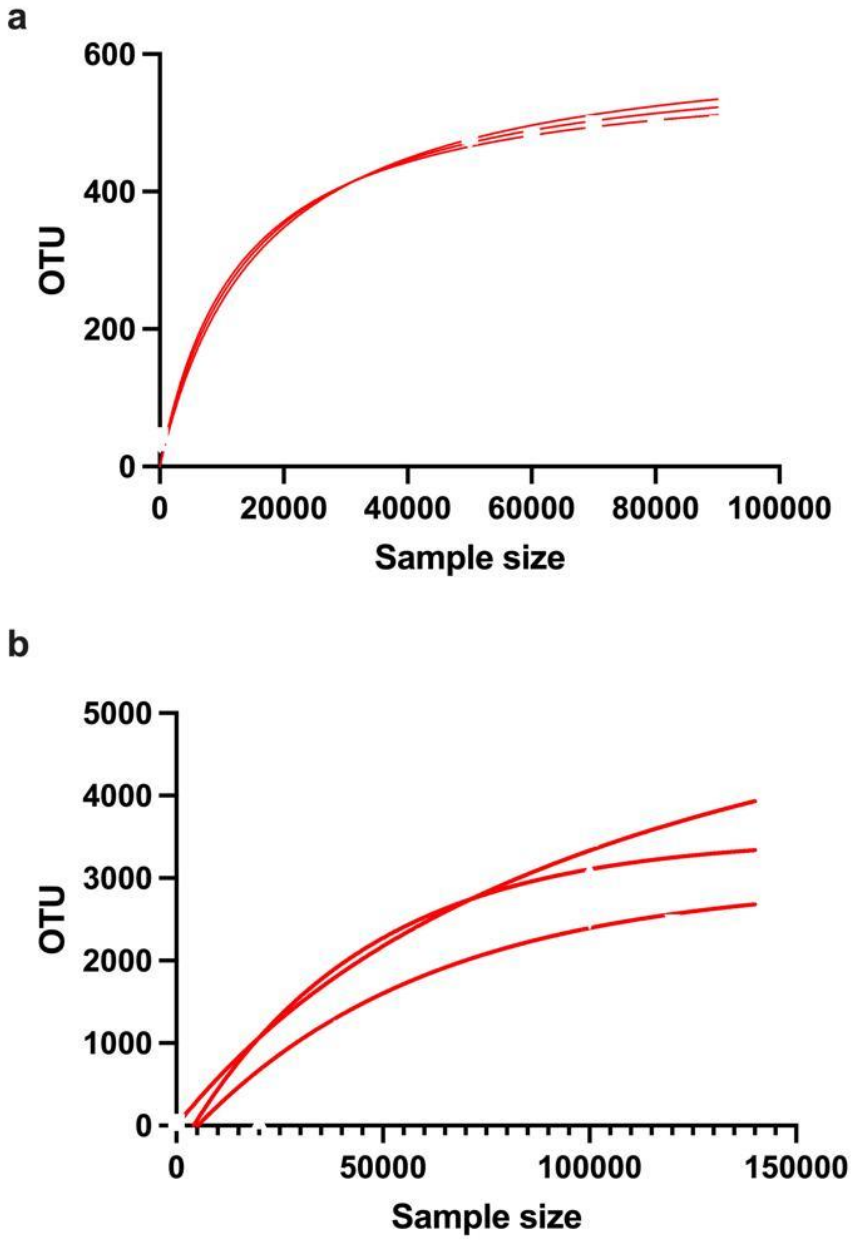
Organs	Water	SD†	Kefir	SD†
Brain (g)	1.80	0.14	1.78	0.10
Colon (g)	1.19	0.34	1.17	0.21
Small intestine (g)	5.71	0.53	4.96	0.70

†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 unpaired *T*-test with Welch's correction, in which (*) represent significant differences $p < 0.05$.

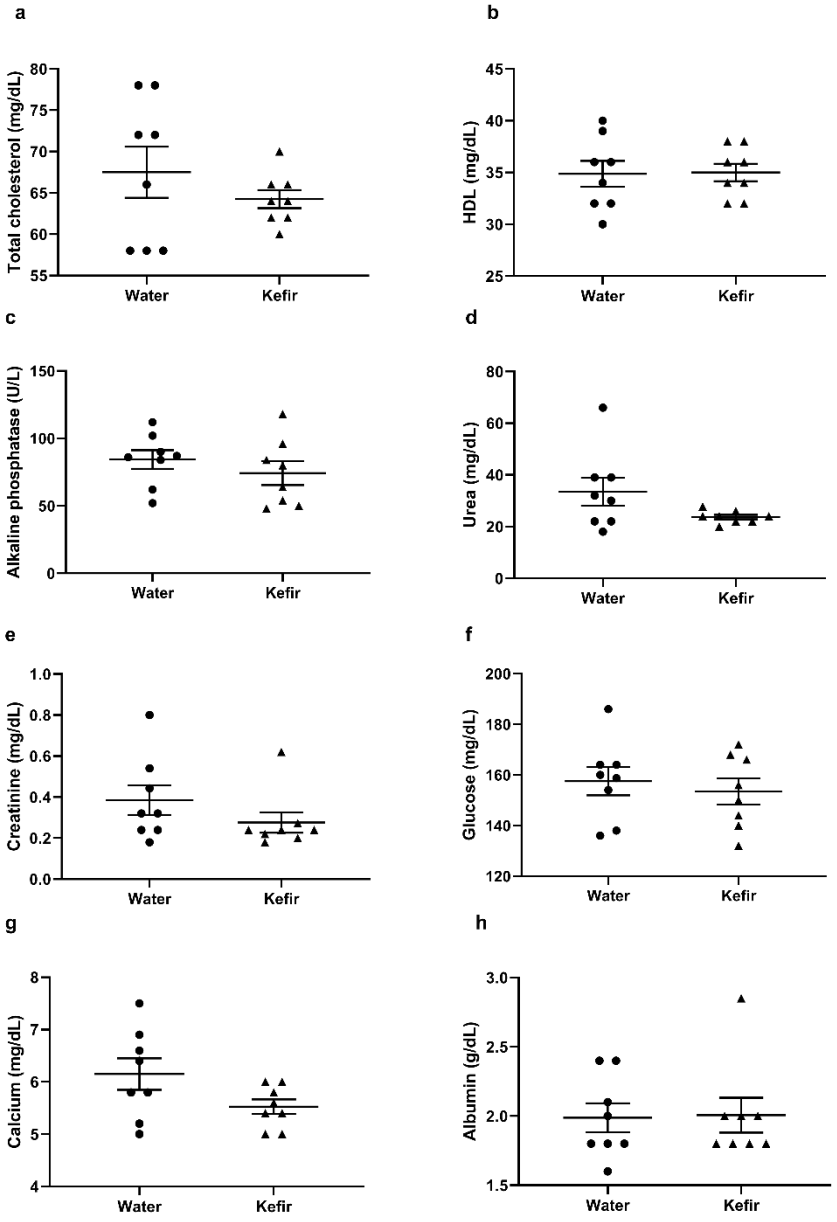
Supplemental Fig. S1 Flowchart of the production of milk kefir (10% w/v)



Supplemental Fig. S2 Rarefaction curve. (a) kefir samples; (b) fecal samples. Operational taxonomic unit (OTU).



Supplemental Fig. S3 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 $p < 0.05$.



4.2. Manuscript 3

Manuscript published in Food & Function (Impact Factor: 6.317), https://doi.org/10.1039/D2FO04063H.

The role of IL-10 in regulating inflammation and gut microbiome in mice consuming milk kefir and orally challenged with S. Typhimurium

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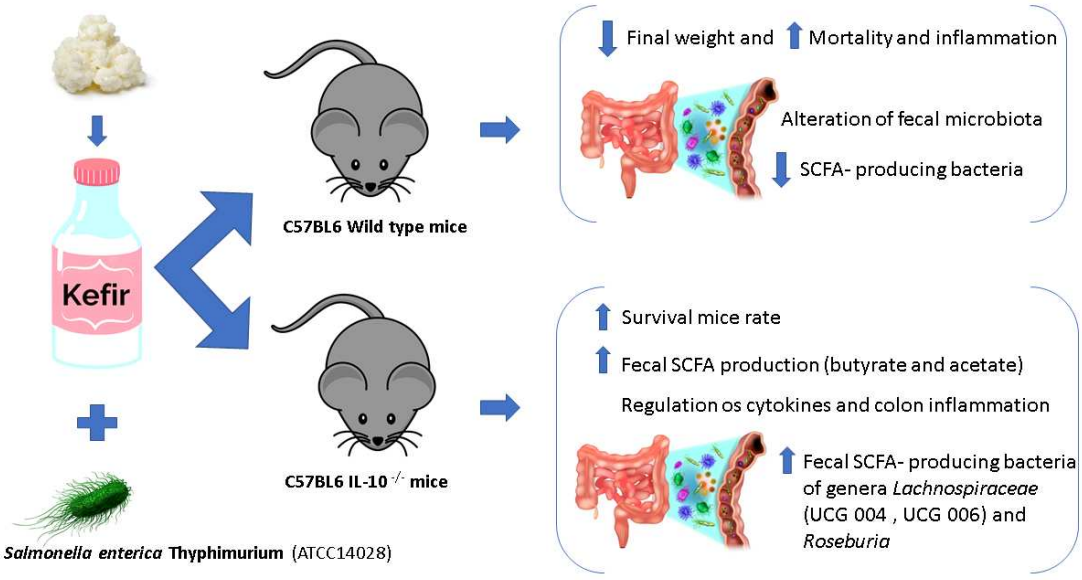
Kefir has been suggested as possible bacterial prophylaxis against *Salmonella* and IL-10 production seems to be crucial in the pathogenesis of salmonellosis in mice. This study aimed to evaluate the role of IL-10 in the inflammation and gut microbiome in mice consuming milk kefir and orally challenged with *Salmonella enterica* serovar Typhimurium. C57BL wild type (WT) (n=40) and C57BL IL-$10^{-/-}$ (KO) (n=40) mice were subdivided into eight experimental groups either treated or not with kefir. In the first 15 days, water groups received filtered water (0.1 mL) while kefir groups received milk kefir (10% w./vol.) orally by gavage. Then, two groups of each strain received a single dose (0.1 mL) of the inoculum of *S*. Typhimurium (ATCC 14028, dose: 10^6 CFU/mL). After four weeks, the animals were euthanized to remove the colon for further analysis. Kefir prevented systemic infections only in IL-$10^{-/-}$ mice, which were able to survive, regulate cytokines, and control of colon inflammation. The abundance in *Lachnospiraceae* and *Roseburia* also the higher SCFA production in the pre-infection showed that kefir has a role in intestinal health and protection, colonizing and offering competition for nutrients with the pathogen as well as acting in the regulation of *Salmonella* infectivity only in the lack of IL-10. These results demonstrate the role of the IL-10 in the prognosis of salmonellosis and how milk kefir can be used in acute infections.

Graphical Abstract



Kefir has been suggested as a possible bacterial prophylaxis against *Salmonella* and IL-10 production seems to be crucial in the mice pathogenesis disease

Introduction

Salmonellosis, a foodborne disease caused by the enteric Gram-negative bacterium *Salmonella*, is considered the most widespread zoonosis in the world.[1] Every year, about 1.3 billion cases of *Salmonella* gastroenteritis cause about 3 million deaths worldwide.[2] In the United States (US) alone, there are approximately 1.2 million infections, 23,000 hospitalizations, and 50 deaths each year.[3] In contrast, salmonellosis is the second most common gastrointestinal infection in European Union (EU) countries.[3,4] The transmission of *Salmonella* is mainly associated with the ingestion of contaminated food.[1] Its control represents a challenge for public health, considering the emergence of new serovars and the increase in antibiotic resistance, both in developing and developed countries, indicating that this microorganism is the most common and relevant etiological agent of enteric infections.[5]

Salmonella initially settles down in the liver and spleen, where it replicates into phagocytic cells, promoting hepatomegaly and splenomegaly.[6] Subsequently, there is recognition of the microorganism, through phagocytic cells, by the innate immune system through pathogen-associated molecular patterns (PAMPs), which results in

the production of pro-inflammatory cytokines and infiltration of monocytes and neutrophils at the sites of inflammation.[7] In the last phase of the infectious process, effector mechanisms of adaptive immunity are generated, causing B and T cells to intervene, and anti-*Salmonella* antibody titers to increases.[8] Thus, T cells transformed into Tregs, when induced, secrete interleukin-10 (IL-10), and suppress host immune responses, which could help *Salmonella* evade host immune responses in the gut causing systemic infection.[8]

The use of probiotics has been pointed out as possible bacterial prophylaxis against *Salmonella* infection, due to the protective effect conferred by some probiotic-isolated strains against the challenge in mice with *Salmonella.*[9] Among the microorganisms used in the production of probiotic-fermented beverages, kefir stands out and its consumption is increasing in many countries due to scientific evidence associated with beneficial effects on human health.[10] Kefir grains are a symbiotic association between yeasts, lactic acid bacteria, and acetic acid bacteria, surrounded by a polysaccharide matrix.[11-12] Previous studies on kefir reported its antimicrobial action, mainly related to the production of organic acids, peptides (bacteriocins), carbon dioxide, hydrogen peroxide, ethanol, and di-acetyl; immunomodulation through the formation of bioactive peptides during fermentation or digestion processes (short-chain fatty acids), as well as the production of cytokines and stimulation of different signalling pathways.[12-15]

Thus, the experimental challenge of *S.* Typhimurium in mice constitutes an excellent study model for understanding the infectivity and pathogenicity of this enteropathogen, enabling possible mechanisms of prevention and treatment of salmonellosis.[16] A growing number of studies have evaluated the benefits of probiotics on gut health as well as the effect on regular consumption of kefir; however, there are still open questions about their role in the mechanisms of action in acute infections and safety.[12] Therefore, this study becomes necessary to verify whether regular consumption of milk kefir and the absence of IL-10 can alter the immunological system, colon inflammation, and microbiota composition of wild-type C57BL and IL-$10^{-/-}$ mice orally challenged with *Salmonella enterica* serovar Typhimurium.

Material and Methods

Milk Kefir Beverage Preparation

The kefir grains were obtained from a household in the city of Viçosa, located in the Zona da Mata Mineira, region of the state of Minas Gerais, Brazil. The grains were inoculated at a concentration of 10% w/v, 20 g with 200 mL of ultra-high temperature (UHT) whole cow's milk (Cotochés, batch MG1 AM 5). The beverage was 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 filtered water, and inoculated again into milk, repeating the steps described above for a new production. Then, the milk kefir beverage produced proceeded to maturation under refrigeration at 7 °C ± 2 °C for 24 h, and it was orally administered to the experimental animals, *via* gavage.

Experimental design

Salmonella enterica serovar Typhimurium inoculation

The experimental challenge was performed using *Salmonella enterica* subspecies *enterica* serovar Typhimurium-ATCC14028. The pathogen was isolated at Fundação Ezequiel Dias (FUNED, Belo Horizonte, Brazil) and kindly provided by Prof. Dr Jacques Robert Nicoli. The pathogenic microorganisms were preserved in Brain Heart Infusion broth (BHI, Difco, Sparks, USA) added with sterilized glycerol (30%) at -80 °C in an ultra-low temperature freezer (Bio Freezer, Forma Scientific, Marietta, USA). Before the experiment, the bacteria were activated by a passage in BHI broth, being kept in aerobic conditions for 18 h at 37 °C for growth.

Animals

Eighty healthy male, eight-week-old C57BL6 (n = 40) and C57BL6 IL-$10^{-/-}$ (*n* = 40) mice, weighing approximately 22 g, were obtained from the Central Animal House of the Health and Biological Sciences Center of the Universidade Federal de Viçosa (UFV). One day before the experimental period, the mice were randomized by weight using the online Research Randomizer® software available at <https://www.randomizer.org>. Both strains, wild type (WT) and IL-10 knockout (KO), were divided into eight experimental groups (*n* = 10 per group), namely: wild-type

uninfected water (WNI), wild-type infected water (WI), wild-type infected kefir (KI), wild-type uninfected kefir (KNI), IL-$10^{-/-}$ uninfected water (WNIKO), IL-$10^{-/-}$ infected water (WIKO), IL-$10^{-/-}$ infected kefir (KIKO), and IL-$10^{-/-}$ uninfected kefir (KNIKO). In the first fifteen days, the groups' WI, WNI, WNIKO, and WIKO received commercial diet and filtered water (0.1 mL) and the groups' KI, KNI, KNIKO, and KIKO received commercial diet and milk kefir (10% w/v, approximately 4.5 mL kg^{-1} for humans) orally by gavage. On the fifteenth day of the experiment, the animals of groups WI, KI, WIKO, and KIKO received 0.1 mL of the *Salmonella enterica* serovar Typhimurium - ATCC14028 inoculum (dose: 10^6 CFU/mL^{-1}).[17]

All animals were housed collectively, separated by lineage, in polyethylene cages (five animals per cage) in the Experimental Nutrition Laboratory during the entire experiment. However, after the infection, the infected animals were housed at the Experimental Laboratory of Immunology and Infectiology. All mice were kept in a temperature-controlled (22 ± 2 °C) and humidity (60-70%), as well as a 12-hour 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, increasing the expected effects of sedation and deep anesthesia, and physiological parameters and reflexes were evaluated to determine the degree of anesthesia sensitivity. During the euthanasia procedure, the colon was removed, washed in physiological saline, weighed, and stored in an ultra-freezer at -80 °C for further analysis.

Ethical aspects

The experimental protocol was approved by the National Technical Commission on Biosafety (CTNBio) and by the Ethics Committee on Animal Experimentation (CEUA) of the Universidade Federal de Viçosa, Minas Gerais, Brazil, under process number 35/2020, in compliance with current legislation (Law No. 11,794, of October 8, 2008). The experiment was also 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.

Analysis of animal parameters, survival, and food intake

The animals were weighed individually, weekly, on a fixed day (Monday), on a digital scale (Marte Slim, model M 2K), and the survival was monitored by daily observation and presented in graphs of the percentage of survival. 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 using the same electronic scale.

Metataxonomic analysis of the fecal microbiota

Fecal DNA extraction was performed according to a protocol adapted from Zhang *et al.*[18] The quality and quantification of the DNA were analyzed by Nanodrop with calculation of the ration 260/280 and 260/230, absence of degradation smear in the 0.8% agarose gel electrophoresis and positive amplification by Polymerase Chain Reaction (PCR) using 337F and 518R16S rRNA primers for V3 hypervariable region.[19] 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).[20-21] 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 gender. Illumina Hiseq raw sequences generated and analyzed during the current study are available in the Sequence Read Archive (SRA) database (https://www.ncbi.nlm.nih.gov/sra/ PRJNA932214) under the BioProject PRJNA932214.

Quantification of fecal short-chain fatty acids (SCFA)

SCFA extraction was based on the method of Smiricky-Tjardes *et al.*[22] with modifications. Approximately 50 mg of stool samples were weighed and homogenized in 950 µl of deionized water and kept for 30 min on ice, being

homogenized every 5 min. Then, the samples were centrifuged at 10000 x g for 30 min at 4 °C, three times, and the final supernatant was filtered through a syringe filter (0.45 µm) and placed in a vial. The analysis was performed using high-performance liquid chromatography (HPLC) on a chromatograph (Shimadzu of Brazil, São Paulo-SP) coupled to an ultraviolet (UV) detector (model SPD-20A VP). The concentration of fatty acids was evaluated according to the standard curve of acetic and butyric acids (SUPELCO®). Data were obtained in mg mL^{-1} (ppm) and transformed into a percentage of fecal mass (mg of FA per mg of feces). The result was expressed in mmol of FA per g of feces.

Assessment of the immune response profile in the colon

The concentrations of cytokines were determined using the Cytometric Bead Array (CBA) (BD™) kit according to the recommendations of the manufacturer with modifications described below. Colon fragments (50 mg) were ground with the aid of a tissue homogenizer (IKA®, model T10 basic) in phosphate buffer pH 7, and kept on ice. Subsequently, the samples were centrifuged (10000 x g, 10 min, 4 °C) and the supernatant was collected. A diluent solution was used to dilute the beads and then the samples were distributed in microtubes with 25 µL of sample.

Then, the detector solution was added to samples and proceeded to 2 h of incubation. Then, 1 mL of the washing solution was added, centrifuged (1800 x g for 5 min at 4 °C) and the supernatant was discarded. The remaining volume in the microtubes was used on the flow cytometer. A ten-point standard curve was constructed and the data were processed in the FCAP Array Software v3.0. All results were expressed in pg per g of tissue.

Histopathological analysis of the colon

The mice's colon fragments were collected and kept in Carnoy's solution. These fragments were cut transversally, processed in an increasing series of alcohols, and later embedded in paraffin. Then, the tissues were sectioned using a semiautomatic microtome, fixed on glass slides and the stained using the Hematoxylin & Eosin (H&E) technique. A microscope coupled to a digital camera (Zeiss®, Inc.) was used to capture the photomicrographs. These photographs were taken for each of the tissue slides with 40x magnification, and later the number of fields with the presence

of inflammatory infiltrate in the intestinal wall was counted using the Image J software.

Statistical Analysis

Initially, the data were submitted to the Kolmogorov-Smirnov normality test. The results were presented as mean and standard or error median deviation, then treated by the One-Way ANOVA, and a post-test of Tukey was performed to verify differences between the experimental groups. For the survival rate, the data were expressed as a percentage of survival and the Long-rank test was performed. The histopathological analysis was represented by inflammatory infiltrates (%).

The metataxonomic analysis of the fecal microbiota was represented by relative abundance (%) and the statistical differences between groups were performed using the ANOVA One-Way and Bonferroni post-test. Correlations between continuous variables were determined by Pearson's (parametric data) or Spearman (non-parametric data) correlation. The significance level adopted was 5%, in which the *p*-value considered statistically significant was $p < 0.05$. The GraphPad Prism software, version 8.00 (San Diego, California, USA), was used to perform the statistical analysis and graphs.

Results

Survival, weight, and food intake of mice

The survival rate of the animals was compared to verify the role of IL-10 among the strains of mice and to obtain information about the safety and protection capacity of the treatments used (water and kefir) against *S.* Typhimurium (Fig. 1a, Fig. 1b). Thus, we observed that the oral administration of both treatments, in control groups (WNI, WNIKO, KNI, and KNIKO), was able to maintain the survival of the animals at 100% throughout the experimental period. However, after the sixth day of infection, the survival rates of infected animals decreased, and the WI group maintained 50% of animal survival ($n = 5$) and 90% in the WIKO group ($n = 9$) at the end of the experimental period. Interestingly, the oral administration of kefir in wild animals (KI) kept only 10% of animals surviving when compared to the KIKO group (100%). It can be noted that the absence of IL-10 exerted a differential effect on the survival of the animals over the weeks in the acute infection with *S.* Typhimurium.

At the beginning of the experiment, the mean body weight among the experimental groups treated with kefir was homogeneous (Fig. 1d); however, the knockout animals treated with water were heavier than the wild animals (Fig. 1c). On the other hand, after infection with *S.* Typhimurium, we observed a reduction in the final weight of the animals in the WI and KI groups compared those in WIKO and KIKO groups (Fig. 1e and 1f). No changes were found in food intake between the experimental groups during the intervention period (Fig. 1g and 1h). These results agree with what was found in the post-infection period for survival, thus demonstrating the influence of IL-10 on infection control that mainly affects the growth of the animals but not the food intake among the experimental groups.

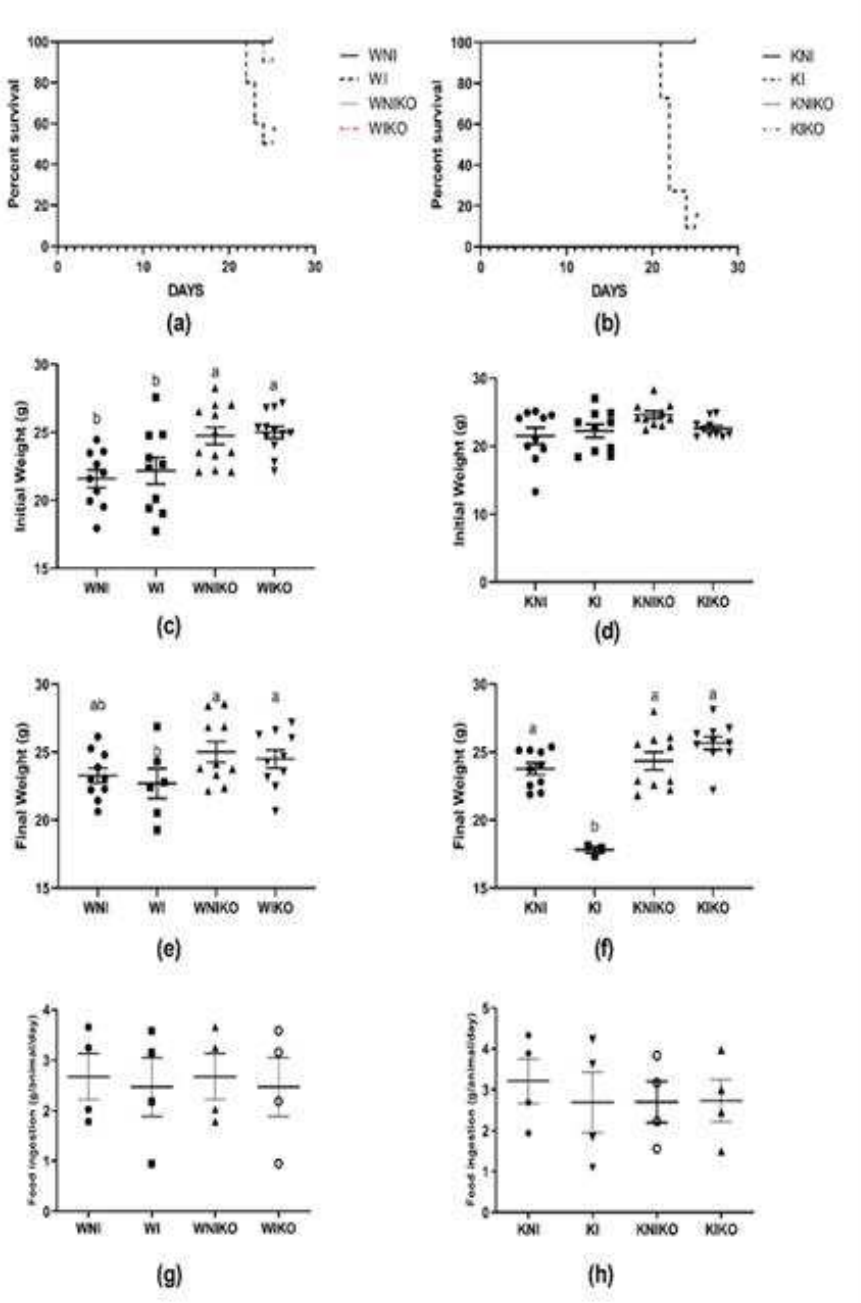


Fig. 1 Survival, growth parameters, and food intake of mice treated or not with kefir and orally challenge with S. Typhimurium. (a, b) Survival rate (%); (c, d) Average weekly body weight (g); (e, f) Final weight gain(g); (g, h) Mean food consumption (g/mice/day). Wild type uninfected water (WNI), wild type infected water (WI), wild type infected kefir (KI), wild type uninfected kefir (KNI), IL-10 knockout uninfected water (WNIKO), IL-10 knockout infected water (WIKO), IL-10 knockout infected kefir (KIKO), and IL-10 knockout uninfected kefir (KNIKO). 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 analysed using ANOVA One-Way and Tukey`s post-test, in which (*) represents significant differences ($p < 0.05$).

Metataxonomic analysis of the fecal microbiota

A non-metric multidimensional scale analysis (NMDS) was performed to analyse the observed variability. The NMDS plot (Fig. 1Sa and 1Sb†) showed that the distances between samples from the water group (WNI, WI) and wild-type kefir (KNI, KI) are smaller than the samples from the water group and IL-10$^{-/-}$ kefir (WIKO and KNIKO), except for the WIKO and KIKO groups, which resemble wild ones. Thus, we noticed that the wild groups, regardless of treatment and infection, present greater microbial homogeneity, but only after infection, did the animals in the IL-10$^{-/-}$ group become more similar to each other. The α diversities were compared between groups using the Shannon (Fig. 1Sg and 1Sh†) and Simpson (Fig. 1Se and 1Sf†) indices, while the Chao 1 index was used to assess bacterial richness (Fig. 1Sc and 1Sd†). Thus, there was no statistically significant difference in terms of bacterial diversity or bacterial richness ($p > 0.05$) between animals treated with water or kefir.

At the phylum level, *Proteobacteria*, *Bacteroidetes*, *Epsilonbacteraeota*, and *Firmicutes* were the most predominant among the fecal samples recovered between the experimental groups (Fig. 2a and 2b). In the absence of infection, we can observe that, except for the *Proteobacteria* phylum, wild animals have a greater relative abundance when compared to IL-10$^{-/-}$ animals. Furthermore, the absence of IL-10 associated with *Salmonella* infection, in both treatments [water (WI, WIKO) and kefir (KI, KIKO)], promoted a reduction in the relative abundance of phylum *Bacteroidetes* (78.6% vs. 20%, $p = 0.051$; 45.1% vs. 26.6%, $p = 0,8857$) and *Epsilonbacteraeota* (2.3% vs. 0%, $p = 0,3778$; 9.6% vs. 1.5%, $p = 0.3780$), and an increase in *Firmicutes* (5.2% vs. 26.9%, $p = 0.2153$; 31.2% vs. 45.1%, $p = 0.0790$) and *Proteobacteria* (13.8% vs. 53.1%, $p = 0.0905$; 14.1% vs. 26.8%, $p = 0.2849$) compared to wild type animals (Fig. 2a-2b).

In the absence of infection and IL-10, in both treatments (WNIKO vs. WNI, KNIKO vs. KNI, respectively), there was an increase in the relative abundance of the families *Burkholderiaceae* (26.7% vs. 3.9%, $p = 0.1317$; 30.6% vs. 0%, $p = 0.2912$) and *Desulfovibrionaceae* (27.8% vs. 0%, $p = 0.0409$; 14.7% vs. 0%, $p = 0.5160$), as well

as exclusively in the IL-10$^{-/-}$ group that received water (WNIKO) there was an increase in the families *Bacillaceae* (10,5% vs. 0%, *p* = 0.2275) and *Muribaculaceae* (12.9% vs. 7.6%, *p* = 0.2987) compared to wild type animals (WNI). Furthermore, the families *Bacillaceae* (10.5%) and *Pseudomonadaceae* (20.1%) were exclusive to the groups treated with water, WNIKO and WNI, respectively.

Among infected animals, IL-10 interfered with the profile of bacterial families (Fig. 2c and 2d). For animals with salmonellosis treated with water, there was an increase in relative abundance of *Burkholderiaceae* (32.4% vs. 13.8%, *p* = 0.3000), *Desulfovibrionaceae* (13.5% vs. 0%, *p* = 0.0641), *Lachnospiraceae* (25.4% vs. 0%, *p*=0.0646), and a reduction in *Helicobacteraceae* (0% vs. 2.3%, *p* = 0.3777), *Lactobacillaceae* (1.5% vs. 5.2%, *p* = 0.3784), and *Prevotellaceae* (0% vs. 22.2%, *p* = 0.0156) in IL-10$^{-/-}$ (WIKO) animals compared to wild type animals (WI). For those treated with kefir, we observed that there was an increase in *Desulfovibrionaceae* (7.7% vs. 0%, *p* = 0.2559), *Lachnospiraceae* (40.7% vs. 28.1%, *p* = 0.0268), and *Lactobacillaceae* (4.4% vs. 3.1%, *p* = 0.6157) at the same time as there was a reduction in the relative abundance of *Burkholderiaceae* (8.6% vs. 14.1%, *p* = 0.8838), *Helicobacteraceae* (1.5% vs. 9.6%, *p* = 0.3780), *Muribaculaceae* (26.6% vs. 39.1%, *p* = 0.9630), and *Prevotellaceae* (0% vs. 6.0%, *p* = 0.7515) families in IL-10$^{-/-}$ (KIKO) animals compared to wild type animals (KI).

Regarding the genera found, twelve appeared in the fecal microbiota among the experimental groups (Fig. 2e and 2f). Thus, in the absence of infection in IL-10$^{-/-}$ in animals treated with water, we observed an increase in relative abundance in the genera *Bacillus* (10.5% vs. 0%, *p* = 0.1950), *Desulfovibrio* (27.8% vs. 0%, *p* = 0.0410) and *Parasutterella* (26.7% vs. 3.9%, *p* = 0.1317) compared to the wild group (WNIKO vs. WNI). However, among those who received kefir, we can say that the genera *Alloprevotella* (2.9% vs. 0%, *p* = 0.2567), *Desulfovibrio* (14.7% vs. 0%, *p* = 0.5158), and *Parasutterella* (30.6% vs. 0%, *p* = 0.2913) were more abundant compared to the wild group (KNIKO vs KNI).

The absence of IL-10 associated with *Salmonella* infection allowed an increase in the relative abundance of the *Desulfovibrio* (14.7% vs. 0%, *p* = 0.4286; 13.5% vs. 0%, *p* = 0.3215) genus and a reduction in *Alloprevotella* (0% vs. 4.8%, *p* = 0.4612; 0% vs. 6.0%, *p* = 0.1350) and *Helicobacter* (1.5% vs. 9.5%, *p* = 0.3780; 0% vs. 2.3%, *p* = 0.3777), in both treatments (KIKO vs. KNIKO; WIKO vs. WI), respectively). In the water-treated groups, the IL-10$^{-/-}$ infected animals had a greater abundance of the

genus *Lachnospiraceae_NK4A136_group* (25.4 % vs. 0%, *p* = 0.1641) and *Parasutterella* (32.4% vs. 13.8%, *p* = 0.3000), as well as a reduction of the genera *Lactobacillus* (1.5% vs. 5.2%, *p* = 0.3784) and *Prevotellaceae_UCG-001* (0% vs. 17.4%, *p* = 0.1046) compared to the wild (WIKO vs. WI). In the infected groups that received kefir, we can still say that the absence of IL-10 reduced the relative abundance of genera *Lachnospiraceae_NK4A136_group* (10.3% vs. 28.1%, *p*=0.8494) and *Parasutterella* (8.6% vs. 14.1%, *p*=0.8835); however, it increased *Lachnospiraceae_UCG-004* (12.7% vs. 0%, *p*=0.0018), *Lachnospiraceae_UCG-006* (12.0% vs. 0%, *p*=0.0224), and *Roseburia* (5.7% vs. 0%, *p*=0.0058) compared to wild animals (KIKO vs. KI).

Of all identified genera, regardless of the lineage of the animals and the treatment used, the genera *Bacillus* and *Pseudomonas* were not identified in the infected groups and the genus *Roseburia* exclusively appeared in the KIKO group. *Pseudomonas* was observed only in the WNI group as well as *Bacillus* only in the WNIKO group. Thus, we can say that *Salmonella* infection associated or not with the absence of IL-10 is capable of altering the profile of the fecal microbiota of the animals.

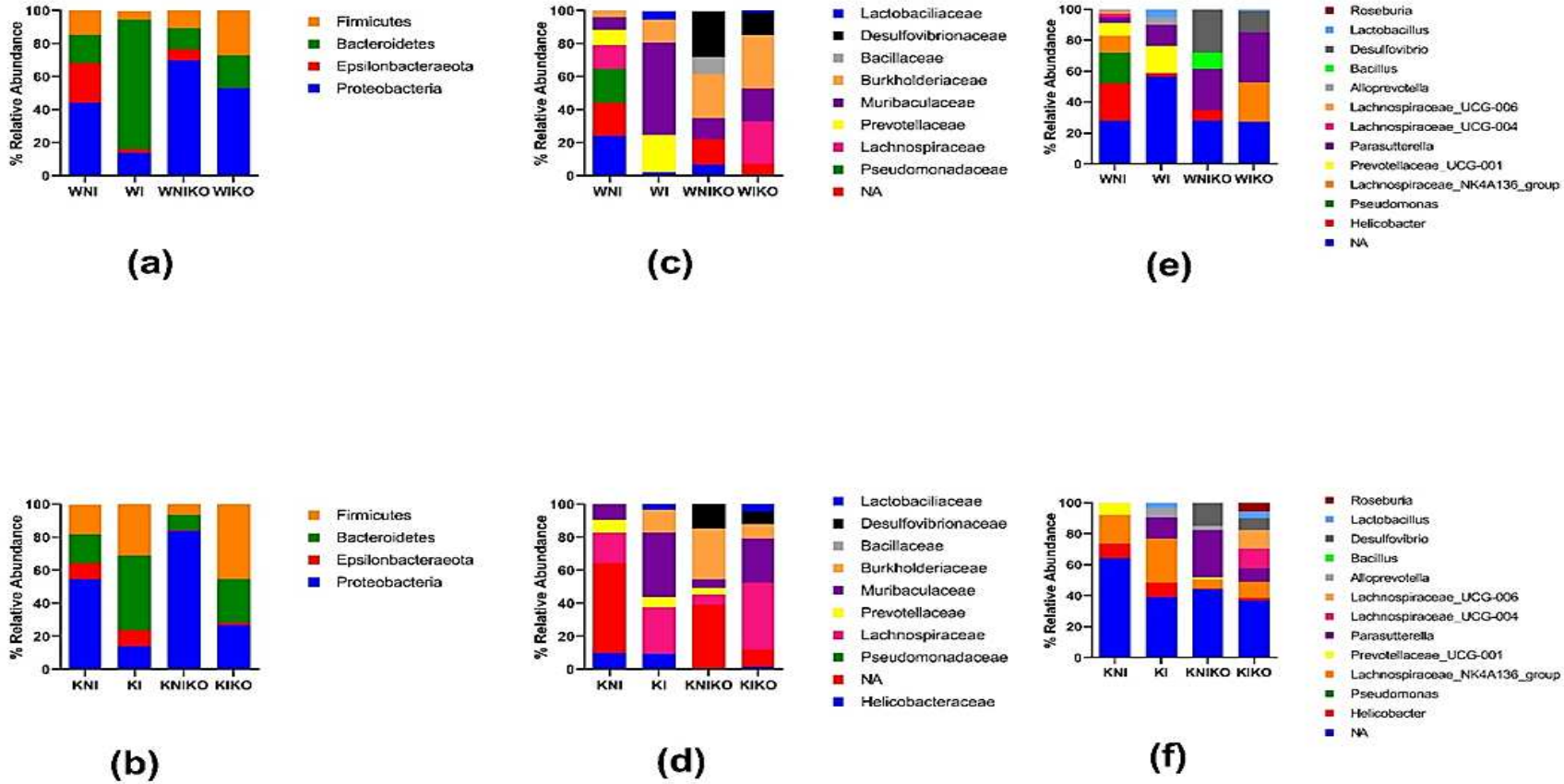


Fig. 2 Relative abundance (%) of phylum (a, b), families (c, d), and genera (e, f) (top 50 bacteria) in the mice treated or not with kefir and orally challenge with S. Typhimurium. Wild type uninfected water (WNI), wild type infected water (WI), wild type infected kefir (KI), wild type uninfected kefir (KNI), IL-10 knockout uninfected water (WNIKO), IL-10 knockout infected water (WIKO), IL-10 knockout infected kefir (KIKO), and IL-10 knockout uninfected kefir (KNIKO). Data were expressed in descriptive statistic represented by relative abundance (%). Statistical differences between groups were made using the ANOVA One-Way and Bonferroni post-test ($p < 0.05$).NA means not available.

Quantification of fecal short-chain fatty acids (SCFA)

In the first week of experiment, it was observed that in the feces of animals in the knockout groups the concentrations of acetic and butyric acid were higher compared to wild type animals treated with water (WNIKO and WIKO vs. WNI) (Fig. 3a and e). For the groups treated with kefir, there was an increase in acetic acid in the IL-10$^{-/-}$ animals (KNIKO and KIKO vs. KI) compared to the wild type (Fig. 3b and d) and for butyric acid, we observed a reduction in the KI group compared to KNIKO (Fig. 3f).

After 15 days of treatment, we observed differences for acetic acid only between WNIKO vs. WI (Fig. 3c) and KIKO vs. KNI (Fig. 3d); however, no differences were observed between the experimental groups for butyric acid (Fig. 3g, 3h).

Thus, the lack of IL-10 production influenced the production of SCFA, since the knockout animals before infection had, in at least one of the groups, regardless of treatment, higher concentrations of butyric acid in the first week and an increase in acetic acid during the first 15 days (pre-infection), contributing to intestinal health in these animals.

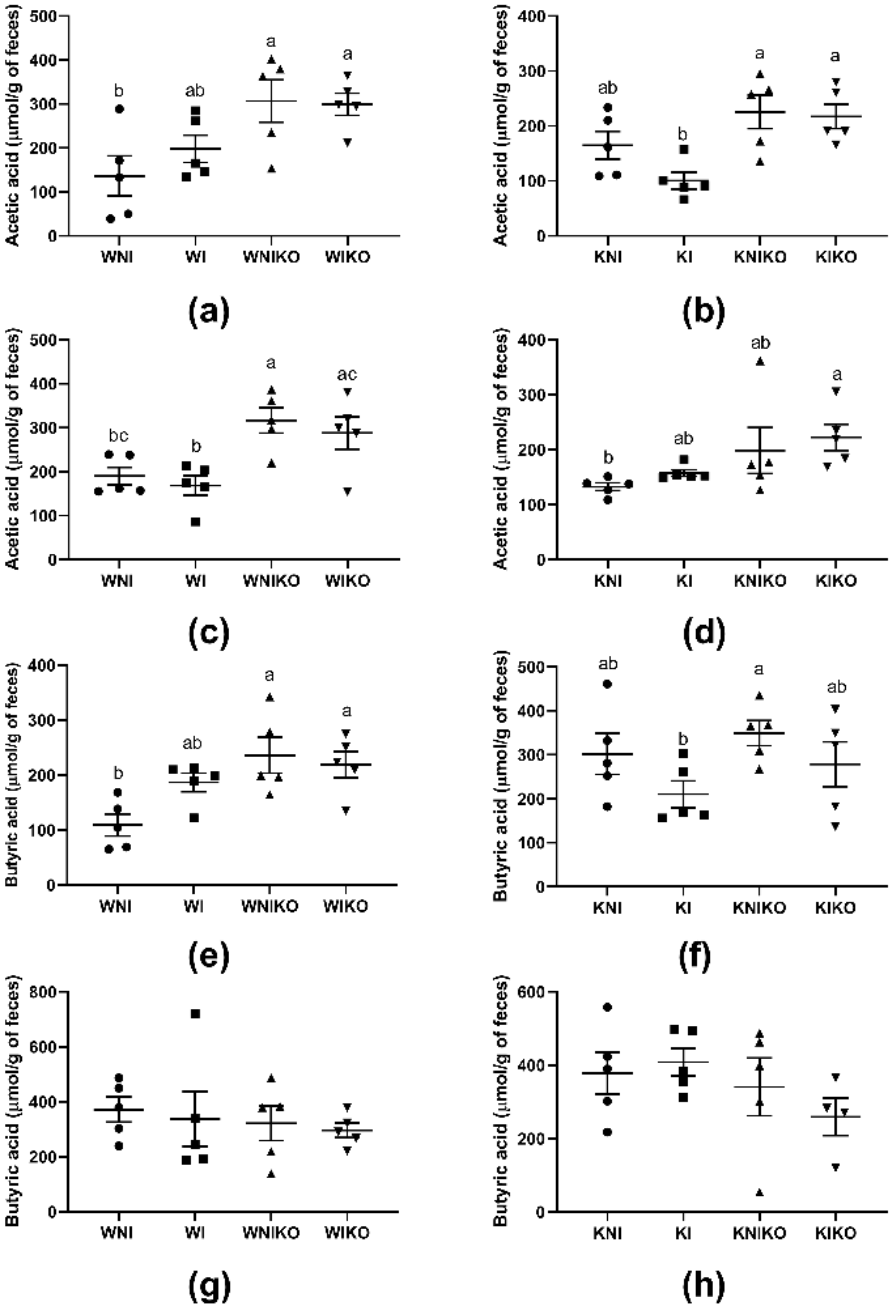


Fig. 3 Fecal SCFAs concentration in mice treated or not with kefir and orally challenge with *S.* Typhimurium. (a-b) Acetic acid (µmol/g of feces) in the first experimental week; (c-d) Acetic acid (µmol/g of feces) in the second experimental week; (e-f) Butyric acid concentrations (µmol/g of feces) in the first experimental week; (g-h) Butyric acid concentrations (µmol/g of feces) in the second experimental week. Wild type uninfected water (WNI), Wild type infected water (WI), Wild type infected kefir (KI), Wild type uninfected kefir (KNI), IL-10 knockout uninfected water (WNIKO), IL-10 knockout infected water (WIKO), IL-10 knockout infected kefir (KIKO), and IL-10 knockout uninfected kefir (KNIKO). Data are expressed as mean ± mean standard

error (n = 5 mice/group). *Statistical differences between groups were made using the ANOVA One-Way and Tukey`s post-test, where (*) represents significant differences ($p < 0.05$).

Assessment of the immune response profile in the colon

It is observed that IL-17 concentrations in the colon were higher in the infected IL-10 knockout animals that received water (WIKO) compared to the groups without infection (WNI or WNIKO) (Fig. 2Se†). Oral administration of kefir associated with the absence of IL-10, regardless of infection, (KNIKO and KIKO) promoted a reduction in INF-γ concentrations in the colon when compared to the wild type (KNI) group (Fig. 2Sj†). No significant differences were found between the experimental groups for the concentrations of IL-2, IL-4, and TNF-α in both treatments (Fig. 2Sa-2Sd† and Fig. 2Sg-2Si†, respectively). Among the infected groups (WI vs. WIKO), IL-10 did not affect any of the cytokines analysed for the groups treated with water at infection and it was not possible to compare the groups treated with kefir because the wild type (KI) group that received kefir mostly died before euthanasia.

Histopathological analysis of the colon

The photomicrographs and the percentage of inflammatory infiltrate in the colon are shown in Fig.3S†. In the presence of infection, we can observe that wild animal treated with water had higher inflammatory infiltrate compared to non-infected animals (WNI vs. WI). It was observed that in the absence of IL-10 associated with infection with *S.* Typhimurium, animals treated with water showed a decrease in the percentage of inflammatory foci in the colon when compared to wild animals (WIKO vs. WI). No significant differences were found for infected animals treated with kefir compared to controls (KIKO vs. KNIKO and KNI). Thus, we can say that IL-10, in *Salmonella* infection, allows for a greater inflammatory focus in mice, consistent with the percentage of survival of the experimental groups.

Correlation Analysis of gut microbiota and inflammation

Correlation analysis was used to investigate relationships between bacterial community structure at the phylum, family and genus levels; the inflammatory infiltrate in the colon; and cytokines (Fig. 4S†). At the phylum level of animals uninfected treated with water (WNIKO), a positive correlation was observed between *Firmicutes* with IL-17 ($r = 1$; $p = 0.0032$), *Bacteroidetes (*$r = 0.94$; $p = 0.0165$) and

Epsilonbacteraeota with IFN-γ (*r* = 0.94; *p* = 0.016). In wild type infected water-treated mice (WI), a positive correlation between *Proteobacteria* (*r* = 0.99; *p* = 0.0078) and *Epsilonbacteraeota* with TNF-α (*r* = 0.99; *p* = 0.0080); and *Firmicutes* with IFN-γ (*r* = 0.99; *p* = 0.0380). On contrary, in infected water-treated mice IL-10$^{-/-}$ (WIKO), *Proteobacteria* was negative correlated with TNF-α (*r* = -0.92; *p* = 0.0223) and IL-4 (*r* = -0.89; *p* = 0.0411). Regarding kefir treatment, a positive correlation, in KNI group, was observed between *Bacteroidetes* with inflammatory infiltrate in the colon (*r* = 0.99; *p* = 0.040) and a negative correlation between *Proteobacteria* with IL-17 were found in KNIKO (*r* = -0.99; *p* = 0.01). In IL-10$^{-/-}$ infected mice fed with kefir, *Bacteroidetes* were negative correlated with TNF-α (*r* = -0.99; *p* = 0.040) and *Epsilonbacteraeota* positive correlated with inflammatory infiltrate cells (*r* = 1; *p* = 0.0061).

Among families, in water treated mice without infection (WNIKO), *Bacillaceae* was positive correlated with IL-17 (r = 0.99; p = 0.032); however, *Desulfovibrionaceae* and *Helicobacteraceae* were negative correlated with TNF-α (*r* = -0.99; *p* = 0.038) and with IFN-γ, respectively (*r* = -0.94; *p* = 0.016). In addition, a positive correlation in water-fed infected animals (WI and WIKO), was observed in *Burkholderiaceae* with TNF-α (WI: *r* = 0.99; *p* = 0.008), *Helicobacteraceae* (WI: *r* = 0.99; *p* = 0.0078) with TNF-α; *Lactobacillaceae* with IFN-γ (WI: *r* = 0.99; *p* = 0.0380), IL-2 (WIKO: *r* = 0.91; *p* = 0.032), IL-4 (WIKO: *r* = 0.99; *p* = 0.0001), and TNF-α (WIKO: *r* = 0.99; *p* = 0.0009). On contrary, in WIKO group, *Burkholderiaceae* was negative correlated with IL- 4 (*r* = -0.97; *p* = 0.0066) and TNF-α (*r* =-0.98; *p*= 0.0026). In kefir treatment without infection (KNI and KNIKO), *Murilobacteriaceae* was negative correlated with IL-2 (KNI: *r* = -0.99; *p* = 0.0231) and IL-4 (KNI: *r* = -0.99; *p* = 0.0263); however, *Desulfovibrionaceae* (KNIKO: *r* = 0.99; *p* = 0.0010) and *Burkholderiaceae* (KNIKO: *r* = 0.99; *p* = 0.0003) were positive correlated with TNF-α. In IL-10$^{-/-}$ infected mice fed with kefir (KIKO), *Lactobacillaceae* was positive correlated with inflammatory infiltrate cells (*r* = 1; *p* = 0.0061) and negative correlation was observed between *Lachnospiraceae_NK4A136_group* with IL-4 (*r* = -0.93; *p* = 0.0198), TNF-α (*r* = -0.97; *p* = 0.0052), and IFN-γ (*r* = -0.95; *p* = 0.0121); and *Muribaculaceae* with TNF-α (*r* = -0.99; *p* = 0.0442).

Focusing on genus level, regarding animals treated with water, a positive correlation was observed, among uninfected mice, in *Alloprevotella* with inflammatory infiltrate cells (WNI: r = 0.99; p = 0.0098); *Bacillus* with IL-17 (WNIKO: *r* = 1; *p* = 0.0032); and

among infected mice, *Helicobacter* (WI: r = 0.99; p = 0.0078), *Lactobacillus* (WI: r = 0.99; p = 0.0080), *Prevotellaceae_UCG-001* (WI: r = 1; p = 0.0059), *Parasutterella* (WI: r = 0.99; p = 0.0078), *Alloprevotella* (WI: r = 0.99; p = 0.0078) with TNF-α. However, the genus *Desulfovibrio* (WNIKO: r = -0.99; p = 0.0380) and *Parasutterella* was negative correlated with TNF-α (WIKO: r = -0.98; p = 0.0026) and IL-4 (WIKO: r = -0.97; p = 0.0066). Furthermore, in kefir fed mice without infection (KNI and KNIKO), we observed a positive correlation in the genus *Prevotellaceae_UCG-001* with IL-4 (KNI: r = 0.99; p = 0.0242) and IL-17 (KNI: r = 1; p = 0.0041); *Desulfovibrio* with IL-2 (KNIKO: r = 0.99; p = 0.0070) and TNF-α (KNIKO: r = 0.99; p = 0.0111); however, a negative correlation was observed between *Lachnospiracea_NK4A136_group* with IL-4 (KNI: r = -0.97; p = 0.0053), IL-17 (KNI: r = -0.94; p = 0.0166); *Helicobacter* with IL-2 (KNI: r = -0.99; p = 0.0094) and IL-4 (KNI: r = -0.99; p = 0.0399). In infected animals fed with kefir (KIKO), *Lachnospiraceae_UCG-004* were negative correlated with TNF-α (r = -0.75; p = 0.0302) and *Roseburia* were positive correlated with inflammatory infiltrate cells (r = 0.96; p = 0.0072).

Discussion

In developing countries, bacterial and parasitic diseases still account for most deaths, while in other societies the decline of these diseases is accompanied by an increase in inflammatory, allergic, autoimmune, and by an increase of metabolic disorders which affect intestinal immunity and microbiota.[23] Despite the wide range of dietary antigens and commensal microorganisms, the intestine has a complex and differentiated immune system that is able to tolerate them.[23] It mainly exerts the function of allowing permeability leading to the efficient reabsorption of nutrients from the intestinal lumen; on the other hand, it must function as a resistant defensive barrier, preventing the invasion of intestinal pathogens.[24]

The infection caused by *S.* Typhimurium in mice is similar to that caused by *S.* Typhi in humans. Hence, this murine model can be used to assess the infectious cycle and immune response in the context of systemic *Salmonella* infection.[16] C57BL6 animals are vulnerable to *S.* Typhimurium infection and cause a high mortality 10–14 days after an orally induced infection, as observed in our study (90% in KI). Furthermore, in this strain, no specific activation of T cells could be detected after infection according to Bueno *et al.*[25]. Silva[26] observed, however, that oral administration of *P.*

pentosaceus 40 and *W. confusa 1* in mice was not able to increase the survival of the animals after infection with *S.* Typhimurium. These variations in survival may occur due to the experimental model, as well as the animal strains, the time, the dosage of the bacteria used in the challenge, and the proposed treatment.[25] As a result, *S.* Typhimurium can activate anti-inflammatory and tolerogenic mechanisms in these mice, facilitating systemic infection without activating the immune response.

S. Typhimurium requires the innate production of IL-10 by B cells to cause systemic infections in mice, according to Neves *et al.*[27], because the transfer of B cells from mice incapable of producing IL-10 increases its resistance to infection. This study also suggested that stimulation of B cells by *Salmonella* lipopolysaccharides (LPS) via toll-like receptor-4 (TLR4) is a major stimulus for these cells to produce IL-10, which promotes systemic infection.[27] Our findings support this effect, as IL-10$^{-/-}$ animals infected with *S.* Typhimurium survived after ten days. Other studies have shown that co-infection of *S.* Typhimurium with *Plasmodium falciparum* rises the bacteria's capacity to induce systemic infection due to increased IL-10 production by the host caused by this parasite.[28-29] In agreement with the role of IL-10 in *S.* Typhimurium infection, mice treated with a neutralizing antibody that blocks IL-10 function had a reduced ability to cause systemic infection.[16] Curiously, studies on persistent pathogens such as *Leishmania major*, human cytomegalovirus, and *Mycobacterium tuberculosis* have shown that IL-10 deficiency improves the elimination of these pathogens, with varying degrees of immunopathology.[16]

The gut microbiota's homeostasis is determined by host characteristics and environmental conditions[12], but *Firmicutes* and *Bacteroidetes* account for the most of the total community in the gut microbiome.[30] This composition is largely unaffected by acute perturbations because its adaptation allows it to return to its initial state. The altered environment promoted by salmonellosis may be the reason for the reduction in abundance of *Bacteroidetes* and *Epsilonbacteraeota* and higher levels of abundance in *Firmicutes* and *Proteobacteria* phylum in our IL-10$^{-/-}$ infected mice compared to wild type. Commonly, pathogen infection can lead to an imbalance (dysbiosis) in the intestinal environment, where opportunistic growth of Gram-negative members is favoured over probiotics.[30]

In addition, among the fifty main bacteria, at the family level, nine predominant families were identified in the fecal microbiota of our mice *Muribaculaceae* (WI and KI), *Burkholderiaceae* (WIKO, KNIKO), *Desulfovibrionaceae* (WNIKO),

Lachnospiraceae (KNI and KIKO), and *Helicobacteraceae* (WNI). Even though inflammation and treatment with fermented foods like kefir can alter the balance between the pathogen and the protective microbiota in different experimental groups, our findings indicate the presence of common families in the microbiota of mice.

Colonic bacteria, such as those belonging to the genus *Desulfovibrio*, found in abundance in the IL-10$^{-/-}$ mice of our work, can produce hydrogen sulphide (H_2S) that is converted later to thiosulfate by the cecal mucosa.[31] When there is an excess production of H_2S by host microbiota and pathogens (*e.g. Salmonella*), H_2S may be linked to mucus disruption and inflammation contributing to the development of inflammatory gut diseases.[31] Although the action mechanisms have not been fully elucidated, H_2S may be associated with mucus layers integrity reducing the risk of its rupture, favouring the permanence of the microbiota biofilm to the epithelium, modulating inflammation and tissue injury, as well as inhibiting the invasive pathogens, contributing to reduce *S.* Typhimurium.[32] In addition, research findings suggest that dietary H_2S obtained from sulphur phytochemicals or ingestion of amino acids may also modulate the bacterial diversity and function of gut microbiota. Corroborating our results, Wu *et al.*[31] also found different responses to *S.* enteritidis infection, improved intestinal barrier function and structure of the cecal microbiota, especially an increased abundance of the *Desulfovibrio* genus via H_2S production that may help chicks to resist *Salmonella* invasion.

SCFAs are the result of bacterial fermentation of non-digestible carbohydrates from the diet and of endogenous origin.[12,33] Butyrate is the most studied SCFA as it helps to regulate the balance between colonocyte proliferation, differentiation, and apoptosis as well as acetic acid, which exhibits anti-inflammatory activity.[34] Similar to butyric acid, this SCFA can suppress nuclear transcription factor (NF-κβ) activation, in addition to regulating the gene expression of pro-inflammatory cytokines.[35] In our study, kefir associated with a lack of IL-10 increased the production of fecal SCFAs. This increase is due to mainly by the consumption of fermented foods, such as kefir, and by the bacterial that make up their intestinal microbiota, which naturally produces them, such as the genus *Lachnospiraceae* (UCG004, UCG006) and *Roseburia* founded in the KIKO group[36]. However, in some cases, the amount of SCFA produced may not be sufficient to protect and inhibit the worsening of the prognosis of inflammatory diseases, such as *Salmonella* infection, as seen in the WI and KI infected groups, in which they presented low percentages of survival and increased

of the inflammatory focus in the colon. A hypothesis considered is that, even though the wild-type mice increase *Lactobacillus*, *Lachnospiraceae_NK4A136_group*, and *Prevotellaceae_UCG-001*, the SCFA production may not be sufficient and the increase in the production of H_2S produced by some microbiota genera and by the pathogen (*Salmonella*) interfered in the reduction in the oxidation of butyric acid, reducing the energy of the colonocytes.

In the model of murine salmonellosis, the protective effects of probiotics and a healthy gut microbiome include the competitive exclusion of pathogens; the capacity to reside in the intestines; improvement of the intestinal barrier function through the interaction of pathogen-associated molecular patterns with pattern recognition receptors and the expression of mucin and tight junction proteins; production of antimicrobial proteins; and regulation of the immune system.[15,37-39] These assumptions are proven in our study because the absence of IL-10 in infected animals treated with water allowed an increase in IL-17 concentrations while treatment with kefir (KIKO and KNIKO) reduced IFN-γ in the colon compared to WT animals. Thus, IL-17 is a pro-inflammatory cytokine that leads to the formation of IL-6 and IL-8 as well as the intercellular adhesion molecule in fibroblasts.[40-41] This cytokine, during the process of *Salmonella* infection, activates the innate response in the mucosa mainly through the recruitment of neutrophils, which are important to prevent the spread of *Salmonella* through the intestine. Thus, its levels are expected to be high in infected animals and reduced in the non-infected kefir group.[42-43]

Salmonella infection is typically accompanied by intense inflammation[8]. Based on the correlation analysis between the concentration of cytokines, colon inflammation, and the abundance, it is important to notice that all phyla, families, and genera found in fecal microbiota of WI group were positive correlated with TNF-α or IFN-γ, which explains the partial survival (50%) of this type of mice. Hence, in hosts with inflammatory diseases, enteric bacteria are potentially exposed to high levels of TNF-α and IFN-γ.[44-46] Furthermore, *Lactobacillaceae* family of infected IL-$10^{-/-}$ water-fed mice were positive correlated with IL-2 and TNF-α. These both groups (WI and WIKO) survived better than KI group, possibly by not neutralizing TNF-α, increasing the immune defense by stimulating macrophage functions, which critically determine the outcome of infection, and the WIKO group also because of the lack of IL-10. These cytokines reduction is known to be involved in the increase of the severity of *Salmonella* infection and decreases survival of the host.[44-46] In this context, IFN-γ is

probably the most studied cytokine in host defense against *Salmonella*. Although IFN-γ can affect many functions in phagocytic and non-phagocytic cells, the most likely mechanism by which IFN-γ exerts its functions in host defense against *Salmonella* is by activating the ability of macrophages to kill *Salmonella.*[44,46]

Regarding kefir treatment in IL-10$^{-/-}$ infected mice (KIKO), *Bacteroidetes* phylum, *Muribaculaceae* family, and *Lachnospiraceae_UCG-004* were negative correlated with TNF-α. *Lachnospiraceae* family was negative correlated with IL-4, explaining the control of inflammation and survival of mice. Although kefir treatment was negative correlated with other important cytokines of salmonella infection, the neutralization of IL-4 functions could be responsible to reduce the number of bacteria in the peritoneal cavity after infection, which was accompanied by increased serum levels for IFN-γ.[47] In another study using IL-4$^{-/-}$ mice, the lack of IL-4 was associated with delayed death after *Salmonella* infection, then lack of IL-4 is protective against Salmonella infection, suggesting that IL-4 downregulates crucial defenses against this pathogen. A possible underlying mechanism is IL-4 mediated inhibition of IFN-γ production by T cells.[48] Nonetheless, these studies could suggest that IL-10, like IL-4, is anti-protective against Salmonella. Although additional studies are required, using probiotic foods and genetically resistant hosts like our IL-10$^{-/-}$ in salmonellosis model is important to assess the functions of IL-10 in controlling infection with this pathogen.

Conclusions

Milk kefir consumption prevented systemic infections only in IL-10$^{-/-}$ mice, which were able to survive, regulate cytokines, control colon inflammation, and modulate microbiota composition. Kefir had a role in the regulation of *Salmonella* infectivity in the lack of IL-10 production by increasing SCFA-producing beneficial bacteria (*Lachnospiraceae* and *Roseburia*) and by the higher SCFA production, in the pre-infection. Together, these results allowed us to comprehend that IL-10 production is a key factor in the infective cycle of *S.* Typhimurium in mice and how milk kefir acts in salmonellosis progression. Therefore, it is necessary to continue expanding the studies on kefir to evaluate other markers and the action of beneficial microorganisms in the disease mechanism.

Author Contributions

Conceptualization, A.O.B.R., T.A.O.M., and M.C.G.P.; Data curation, M.F.A.P. and L.G.M.A.; Formal analysis, M.F.A.P., L.G.M.A., L.L.O., T.A.O.M and M.C.G.P.; Funding acquisition, A.O.B.R., T.A.O.M., and M.C.G.P.; Investigation, M.F.A.P., L.G.M.A., and T.A.O.M.; Methodology, M.F.A.P., L.G.M.A., B.C.S.C., B.C.S., L.L.O, R.V.G. T.A.O.M., A.O.B.R, and M.C.G.P.; Project administration, M.F.A.P. and L.G.M.A.; Resources, R.V.G., A.O.B.R., T.A.O.M., and M.C.G.P.; Software, M.F.A.P., L.G.M.A., L.L.O., and T.A.O.M.; Supervision, M.F.A.P.,T.A.O.M., and M.C.G.P.; Validation, M.F.A.P, L.G.M.A., L.L.O., A.O.B.R.,T.A.O.M., and M.C.G.P.; Visualization, M.F.A.P., L.G.M.A., B.C.S.C., B.C.S., L.L.O, R.V.G., A.O.B.R., T.A.O.M., and M.C.G.P.; Writing – original draft, M.F.A.P; Writing – review and editing, M.F.A.P., L.G.M.A., B.C.S.C., B.C.S., L.L.O, R.V.G., A.O.B.R., T.A.O.M., and M.C.G.P. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

The authors thank the Diretoria de Tecnologia de Informação (DTI) at Universidade Federal de Viçosa for the availability of the computational cluster and software used in this work. We are grateful to the Núcleo de Análise de Biomoléculas (NuBioMol) of the Universidade Federal de Viçosa (UFV) for providing the facilities for the conduction of the experiments and data analysis. NuBioMol is financially supported by the following Brazilian agencies: Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Coordenacão de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Financiadora de Estudos e Projetos (Finep) and Sistema Nacional de Laboratórios em Nanotecnologias (SisNANO)/Ministério da Ciência, Tecnologia e Informação (MCTI).

This research was funded by National Council for Scientific and Technological Development (CNPq), Coordination for the Improvement of Higher Education Personnel - Brazil (CAPES) – Financing Code 001 and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG). We also thank Rede Minas Microbiomas for their support in the metagenomics analysis.

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Supplementary material

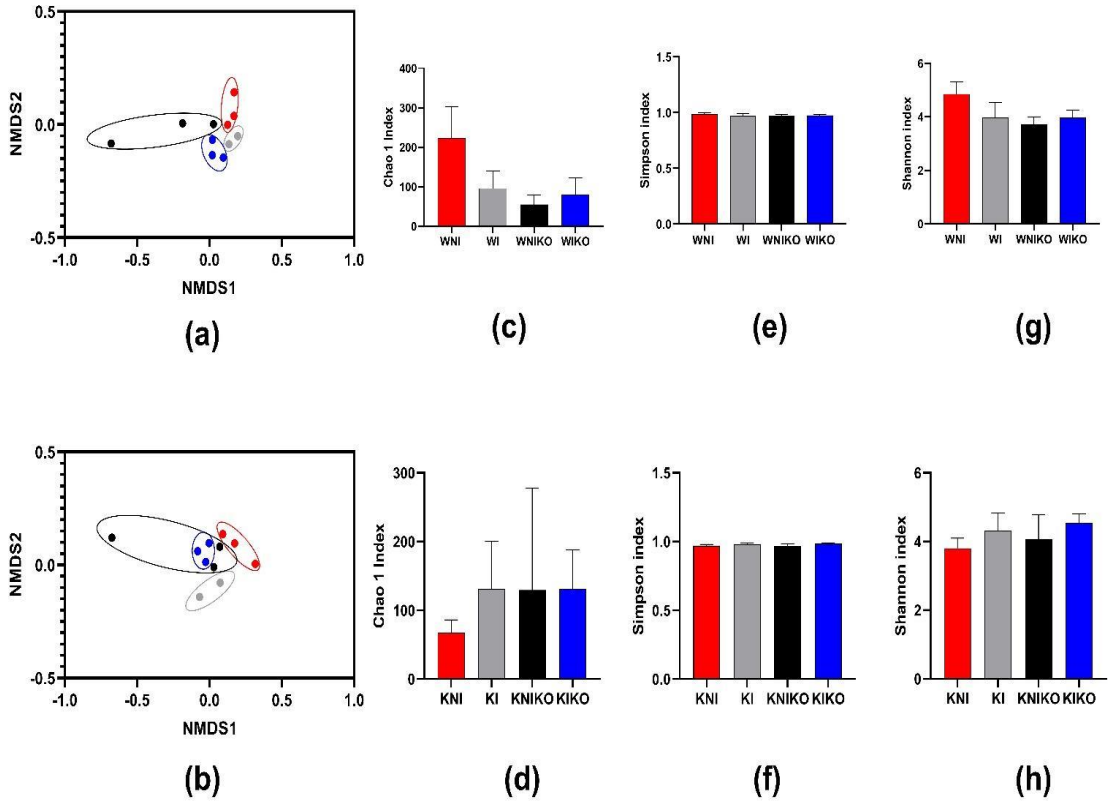

Fig. 1S Non-metric multidimensional scaling (NMDS) plot (a,b) of metagenomic sequencing data in mice treated or not with kefir and orally challenged with *S.* Typhimurium. Red dots represent wild type uninfected water (WNI) (a) and wild type uninfected kefir (KNI) (b), Gray dots represents wild type infected water (WI) (a) and wild type infected kefir (KI) (b), Black dots represents IL-10 knockout uninfected water (WNIKO) (a) and IL-10 knockout uninfected kefir (KNIKO) (b), Blue dots represents IL-10 knockout infected water (WIKO) and IL-10 knockout infected kefir (WIKO)(b). (c,d) Chao 1 Index; (e,f) Simpson Index (g,h) Shannon index .*Statistical differences between groups (n= 9 distributed in 3 pools with 3 mice per group) were analysed using ANOVA One-Way and Tukey's post-test, in

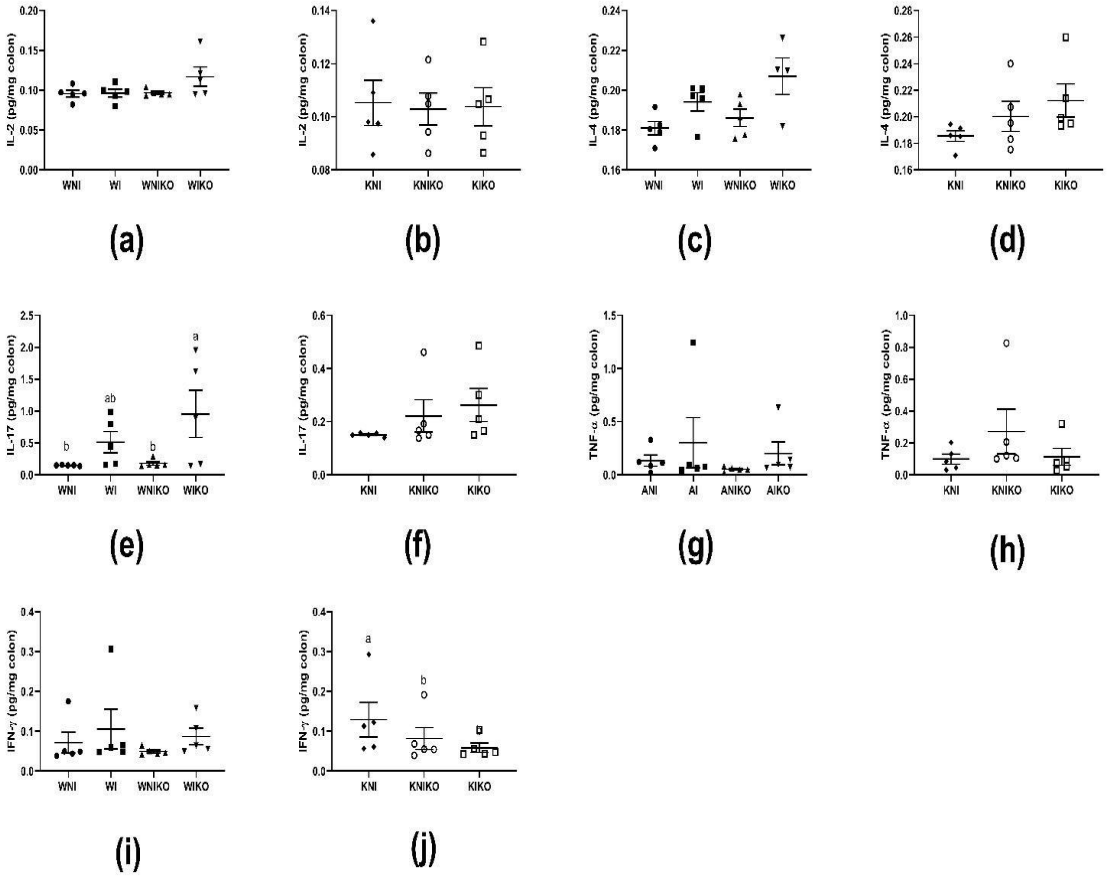

Fig. 2S Cytokines concentration in the colon of mice treated or not with kefir and orally challenged with *S.* Typhimurium. (a,b) IL-2 (pg/mg colon); (c,d) IL-4 (pg/mg colon); (e,f) IL-17A (pg/mg colon); (g,h) TNF-α (pg/mg colon); (i,j) IFN-γ (pg/mg colon). Wild type uninfected water (WNI), wild type infected water (WI), wild type uninfected kefir (KNI), IL-10 knockout uninfected water (WNIKO), IL-10 knockout infected water (WIKO), IL-10 knockout infected kefir (KIKO), and IL-10 knockout uninfected kefir (KNIKO). Data are expressed as mean ± mean standard error (n = 4-5 mice/group). Statistical differences between groups were analyzed using the ANOVA One-Way test followed by Tukey's post test, in which different letters represent significant differences with $p < 0.05$.

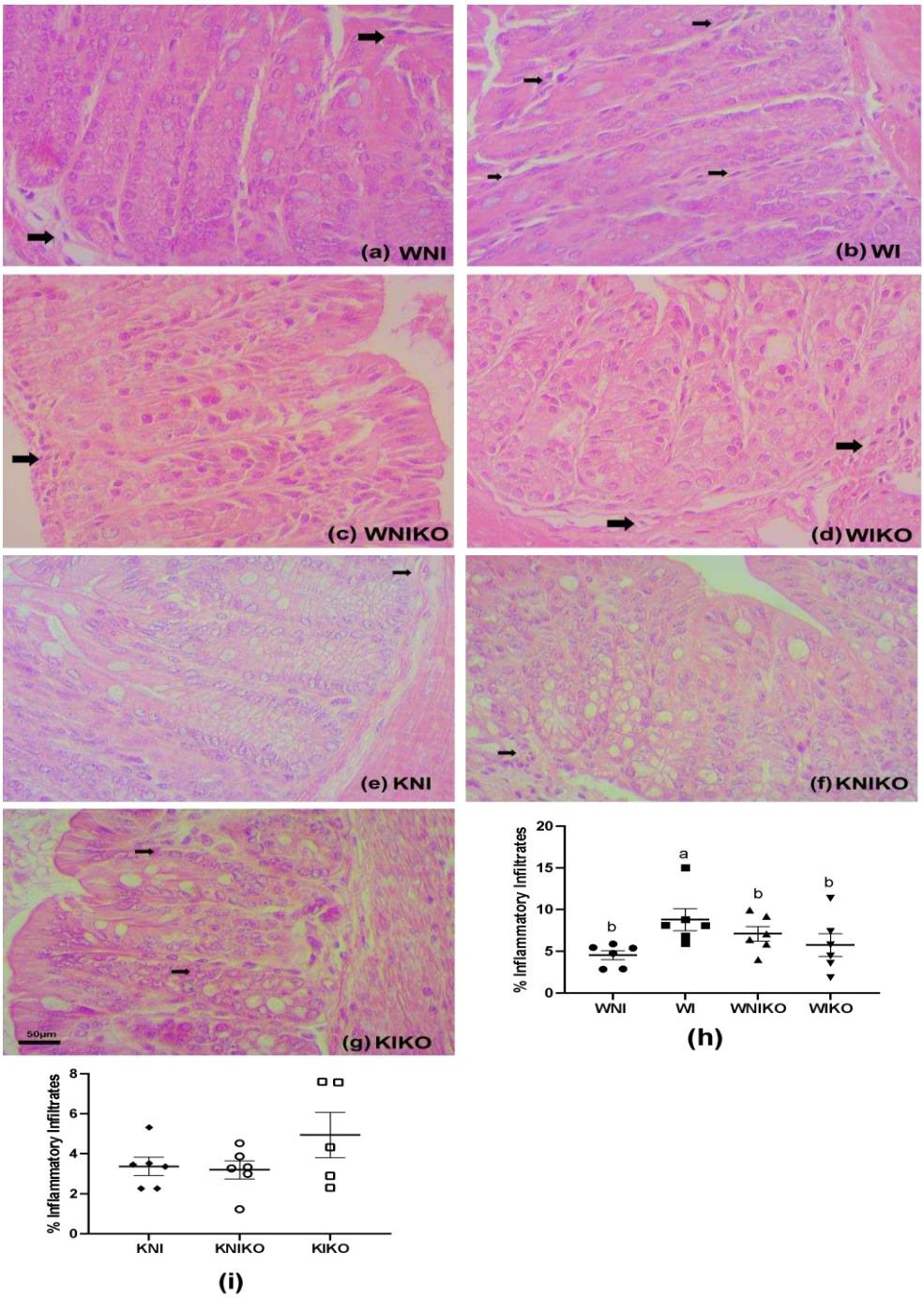

Fig. 3S Representative photomicrographs of colon sections and inflammatory infiltrates (%) of mice treated with water and orally challenged with *S.* Typhimurium. (a) Wild type uninfected water (WNI), (b) wild type infected water (WI), (c) IL-10 knockout uninfected water (WNIKO), (d) IL-10 knockout infected water (WIKO), (e) wild type uninfected kefir (KNI), (f) IL-10 knockout uninfected kefir (KNIKO), and (g) IL-10 knockout infected kefir (KIKO). (h) inflammatory infiltrates (%) of mice treated with water, (i) inflammatory infiltrates (%) of mice treated with kefir. Slides stained with hematoxylin and eosin (H&E) at ×40 magnification. Scale bar = 50 µm. Black

arrows indicate the presence of inflammatory infiltrates in the colon. Data are expressed as mean ± mean standard error (n = 6 mice/group). Statistical differences between groups were analyzed using the ANOVA One-Way test followed by Tukey's post-test, in which different letters represent significant differences with $p < 0.05$.

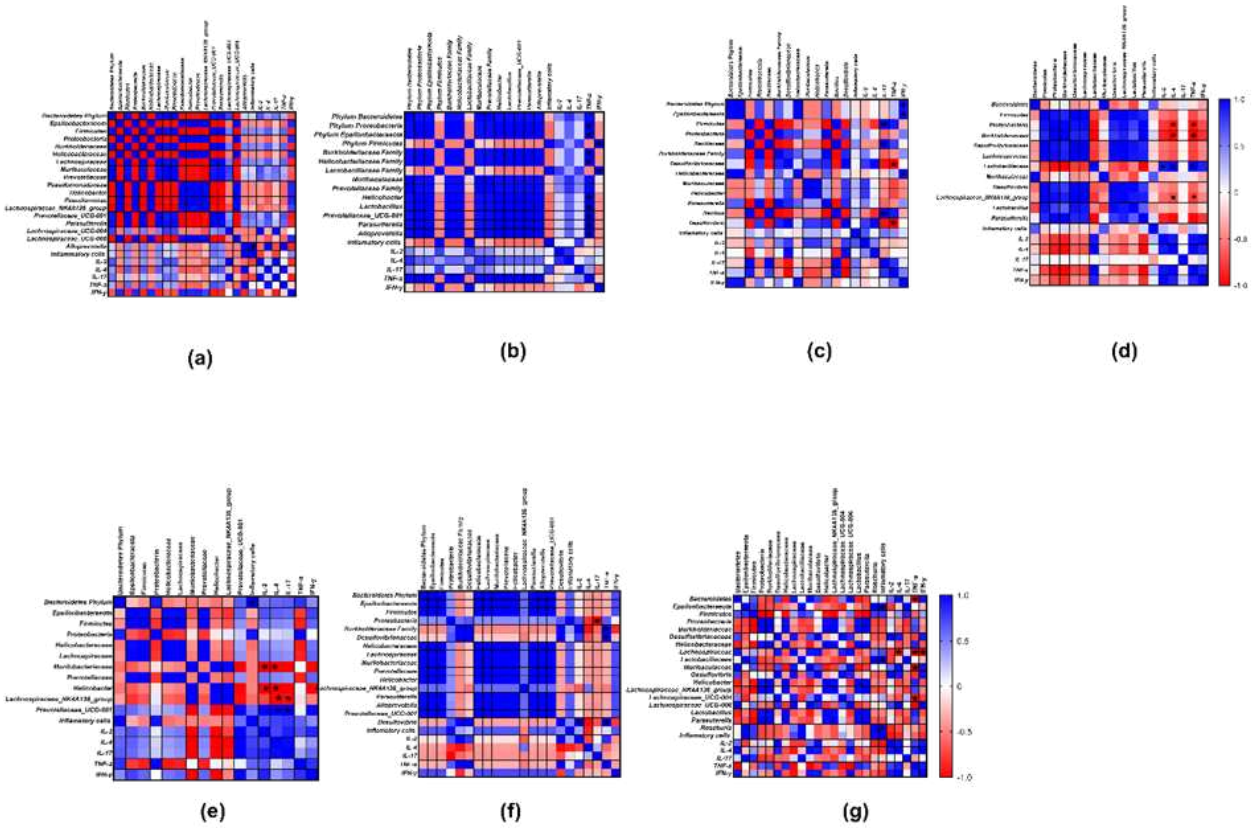

Fig. 4S Correlation analysis of relative abundance of gut microbiota (phylum, family and genus level) and inflammation in mice treated or not with kefir and orally challenged with S. Typhimurium. (a) Wild type uninfected water (WNI), (b) wild type infected water (WI), (c) IL-10 knockout uninfected water (WNIKO), (d) IL-10 knockout infected water (WIKO), (e) wild type uninfected kefir (KNI), (f) IL-10 knockout uninfected kefir (KNIKO), and (g) IL-10 knockout infected kefir (KIKO). Correlations between continuous variables were determined by Pearson's (parametric data) or Spearman (non-parametric data) correlation (n=5). The significance level adopted was 5%, in which the p-value considered statistically significant was $p < 0.05$.

5. CONCLUSION

Kefir has been shown to modulate the immune system in different experimental models, among other secondary outcomes to improve overall health. The beverage mediates immunomodulatory and protective effects through the numerous molecular biomarkers and organic acids produced and secreted by kefir in the intestinal microbiota. The review data indicates that kefir can provide balance and the establishment of beneficial bacteria, thus eliminating pathogenic ones (antimicrobial and bactericidal activity); in addition to acting as an antihypertensive, antioxidant, antitumor, hypocholesterolemic and hypoglycemic, which are factors that contribute to reducing inflammation.

Our results in the manuscript 2 demonstrate that kefir positively influences the gut-microbiota-brain axis, in health mice, contributing to the preservation of gut and brain health. Milk kefir beverage exhibited highly antioxidant capacity compared to milk and in mice by increasing antioxidant enzymes in brain and colon. The beverage and fecal microbiota composition were distinct but composed primarily of SCFA-producing bacteria contributing to increase SCFA in brain and colon.

In manuscript 3, the use of kefir in salmonellosis model was effective in the survival only of IL-10$^{-/-}$ mice. The absence of IL-10 seems to improve the elimination of the pathogen and prevented systemic infections by providing intestinal microbial homeostasis. Kefir consumption in these mice showed antimicrobial and anti-inflammatory activity via an increase in SCFA production and decreased in colon inflammation. Furthermore, it showed an immunomodulatory effect through the regulation of cytokines improving the inflammatory response. Kefir had a role in intestinal health and protection by acting in the regulation of mice infectivity. Together, these results allowed us to comprehend the role of the IL-10 in the prognosis of salmonellosis and how milk kefir can be used in acute infections.

The next step for further studies using kefir should be focused in the investigation of the effects of kefir and kefir-associated bacterial strains as a psychobiotic food focused on neurological diseases. It is also necessary to continue expanding the studies on kefir to evaluate other markers and the action of microorganisms in the progression of salmonellosis. The additional knowledge gained may also provide

crucial information regarding the exact mechanisms and agents responsible for the beneficial effects that have been attributed to kefir in this work.

6. APÊNDICE

6.1. Manuscript 4

This manuscript was written in collaboration with PhD student Larissa Gabriela Morais de Ávila of the Graduate Program in Biotechnology at the Federal University of Minas Gerais (UFMG) under the guidance of Prof. doctor Tiago Antonio de Oliveira Mendes. This article was included in this work as an appendix, since the students have the same level of contribution. However, this article is still under construction and submission. It was included in its entirety only in Larissa's thesis and below is the title page and the abstract for knowledge of the article.

Title: 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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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 its consumption 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 infiltrates in the colon associated to increase of butyric acid. Analysis of microbiome indicated that animals treated with kefir had a higher relative abundance 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 adverse effects.

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