MARIANA DE MOURA E DIAS

EFEITO DO CANDIDATO A PROBIÓTICO *Lactobacillus gasseri* (LG-G12) E, OU, ANTIMICROBIANO (CEFTRIAXONA) ASSOCIADO À OFERTA DE UMA DIETA-PADRÃO: ESTUDO EXPERIMENTAL EM MODELO DE INDUÇÃO DA OBESIDADE

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

Orientadora: Maria do Carmo Gouveia Peluzio

Coorientadores: Solange Silveira Pereira
Leandro Licursi de Oliveira
Lisiane Lopes da Conceição

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MARIANA DE MOURA E DIAS

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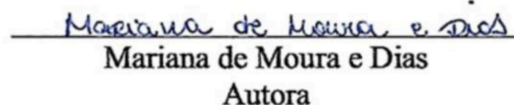

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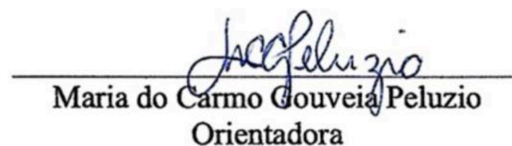

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Orientadora

“O amor é sábio, o ódio é tolo”

Bertrand Russel

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RESUMO

DIAS, Mariana de Moura e, D.Sc., Universidade Federal de Viçosa, agosto de 2021. **Efeito do candidato a probiótico *Lactobacillus gasseri* (LG-G12) e, ou, antimicrobiano (ceftriaxona) associado à oferta de uma dieta-padrão: estudo experimental em modelo de indução da obesidade**. Orientadora: Maria do Carmo Gouveia Peluzio. Coorientadores: Solange Silveira Pereira, Leandro Licursi de Oliveira e Lisiane Lopes da Conceição.

Um organismo saudável apresenta microbiota intestinal equilibrada, e em casos de desequilíbrios, conhecidos como disbiose, há aumento do risco de desenvolvimento de obesidade – relevante problema de saúde pública mundial – e de outras doenças associadas. Logo, a modulação da microbiota intestinal pode ser uma estratégia terapêutica para a obesidade, destacando-se o uso de probióticos e antimicrobianos como moduladores intestinais. No capítulo 1 desta tese, o objetivo foi avaliar os efeitos do consumo do potencial probiótico *Lactobacillus gasseri* LG-G12 e, ou, do antimicrobiano ceftriaxona associado a uma dieta-padrão AIN-93 sobre parâmetros relacionados à obesidade e à permeabilidade intestinal. Para isso, 40 camundongos C57BL/6J foram divididos em cinco grupos experimentais, sendo um grupo controle negativo (sem obesidade induzida pela dieta), um grupo controle positivo (obeso sem intervenção), um grupo potencial probiótico (obeso com intervenção de potencial probiótico), um grupo antimicrobiano (obeso com intervenção de antimicrobiano) e um grupo antimicrobiano seguido de potencial probiótico (obeso com ambas as intervenções). No primeiro momento do experimento houve indução da obesidade, por meio do consumo de uma dieta *high-fat* com 60% de gordura e de uma solução de frutose 10% durante 12 semanas. Em seguida, durante quatro semanas, os animais receberam dieta-padrão e água destilada. Parâmetros relacionados à obesidade, como medidas antropométricas, exames bioquímicos, marcadores de deposição de gordura, glicemia, avaliação da oxidação lipídica; parâmetros relacionados à microbiota intestinal, como sua composição, permeabilidade intestinal e produção de ácidos graxos de cadeia curta; e determinação da função hepática, por meio da dosagem enzimática, foram avaliados de modo a identificar como as intervenções influenciaram a obesidade. O tratamento conjunto foi o mais promissor, em que houve modulação da microbiota intestinal e dos parâmetros relacionados à obesidade, bem como redução de parâmetros biométricos e do perfil lipídico e aumento do tecido adiposo marrom e da produção de butirato. No capítulo 2 foi abordado outro modulador da microbiota intestinal, o kefir. Por meio de uma revisão recente de

literatura, discutiu-se como esse alimento, com alegação probiótica e de fácil acesso à população da América Latina, pode ser utilizado para o tratamento da obesidade, do diabetes mellitus, das doenças hepáticas, das doenças cardiovasculares, das doenças imunes e das desordens neurológicas. Observou-se que a modulação da microbiota intestinal por meio de componentes bioativos é a forma como o kefir diminui a inflamação crônica de baixo grau e a permeabilidade intestinal, gerando benefícios à saúde de quem o consome. Por fim, no capítulo 3 foi apresentado outro estudo com modelo animal. Todos os animais anteriormente utilizados no estudo do capítulo 1 foram novamente examinados, ou seja, aqueles que tiveram obesidade induzida pela dieta e durante a fase de tratamento receberam dieta-padrão AIN-93. Também, quatro novos grupos (n=7) que consumiram dieta *high-fat* com 60% de gordura e solução de frutose 10% durante todo o experimento (controle, obeso tratado com potencial probiótico, obeso tratado com antimicrobiano e obeso tratado com ambas as intervenções) foram estudados. Este trabalho teve como objetivo avaliar como o potencial probiótico *Lactobacillus gasseri* LG-G12, o antimicrobiano ceftriaxona e a dieta *high-fat* interferiram na composição da microbiota intestinal relacionada à produção de ácidos graxos de cadeia curta e à permeabilidade intestinal. Também, buscou-se identificar quais foram as vias metabólicas enriquecidas pela microbiota intestinal mediante as diferentes intervenções. Foram avaliadas a composição da microbiota intestinal, a produção de ácidos graxos de cadeia curta e a excreção de lactulose e manitol. Além disso, procedeu-se à análise metataxonômica da microbiota intestinal, sendo as diferenças de composição microbiana entre os grupos avaliadas por meio da análise discriminante linear do tamanho do efeito (LEfSe). A previsão do potencial funcional foi feita com PICRUSt2. Observou-se que, após o tratamento com as intervenções e o reequilíbrio da microbiota intestinal, a produção de ácidos graxos de cadeia curta contribuiu para o aumento da integridade intestinal e vias metabólicas foram enriquecidas para reverter a disbiose intestinal causada pelo consumo de dieta *high-fat*. Os resultados indicaram que a modulação da microbiota intestinal é promissora para o tratamento da disbiose intestinal e de todas as doenças a ela relacionadas.

Palavras-chave: *Lactobacillus gasseri* LG-G12. Ceftriaxona. Obesidade. Modulação intestinal. Microbiota intestinal. Saúde intestinal.

ABSTRACT

DIAS, Mariana de Moura e, D.Sc., Universidade Federal de Viçosa, August, 2021. **Effect of the candidate probiotic *Lactobacillus gasseri* (LG-G12) and, or, antimicrobial (ceftriaxone) associated with the offer of a standard diet: experimental study on obesity induction model**. Advisor: Maria do Carmo Gouveia Peluzio. Co-Advisors: Solange Silveira Pereira, Leandro Licursi de Oliveira and Lisiane Lopes da Conceição.

A healthy organism has a balanced intestinal microbiota, and in cases of imbalance, known as dysbiosis, there is an increased risk of developing obesity, a relevant global public health problem, and other associated diseases. Therefore, the modulation of the intestinal microbiota can be a therapeutic strategy for obesity, highlighting the use of probiotics and antimicrobials as intestinal modulators. Chapter 1 of this thesis aimed to evaluate the effects of the consumption of the probiotic potential *Lactobacillus gasseri* LG-G12 and/or the antimicrobial ceftriaxone associated with a standard AIN-93 diet on parameters related to obesity and intestinal permeability. For this, 40 C57BL/6J mice were divided into 5 experimental groups: negative control group (no diet-induced obesity), positive control group (obese without intervention), probiotic potential group (obese with probiotic potential intervention), group antimicrobial (obese with antimicrobial intervention) and antimicrobial group followed by potential probiotics (obese with both interventions). In the first stage of the experiment, obesity was induced by consuming a high-fat diet with 60% fat and a 10% fructose solution for 12 weeks, and then for 4 weeks, the animals received the standard diet and distilled water. Parameters related to obesity such as anthropometric measurements, biochemical tests, fat deposition markers, blood glucose, assessment of lipid oxidation; parameters related to the intestinal microbiota such as its composition, intestinal permeability and production of short-chain fatty acids; and determination of liver function through enzyme dosage were evaluated in order to identify how interventions influenced obesity. Joint treatment was the most promising, with modulation of the intestinal microbiota and obesity-related parameters, such as a reduction in biometric parameters and lipid profile, and an increase in brown adipose tissue and butyrate production. Chapter 2 of this thesis has already addressed another modulator of the intestinal microbiota, kefir. A recent literature review discussed how this food, with a probiotic claim and easy access to the population of Latin America, can be used for the treatment of obesity, diabetes mellitus, liver diseases, cardiovascular diseases, immune diseases and neurological disorders. It was observed that the modulation of the intestinal

microbiota through bioactive components is the way in which kefir reduces low-grade chronic inflammation and intestinal permeability, generating benefits to the health of those who consume it. Finally, in chapter 3 of this thesis another study with an animal model is presented. All animals previously used in chapter 1 were re-studied, that is, those that had diet-induced obesity and that during the treatment phase received standard AIN-93 diet. Also, 4 new groups (n=7) that consumed high-fat diet with 60% fat and 10% fructose solution throughout the experiment (control, obese treated with probiotic potential, obese treated with antimicrobial and obese treated with both interventions) were studied. This work aimed to evaluate how the probiotic potential *Lactobacillus gasseri* LG-G12, the antimicrobial ceftriaxone and the high-fat diet interfered in the composition of the intestinal microbiota related to the production of short-chain fatty acids and intestinal permeability. We also sought to identify which metabolic pathways were enriched by the intestinal microbiota through the different interventions performed. The composition of the intestinal microbiota, the production of short-chain fatty acids and the excretion of lactulose and mannitol were evaluated. Furthermore, the metataxonomic analysis of the intestinal microbiota was conducted and the differences in terms of microbial composition between the groups were evaluated using linear discriminant analysis of effect size (LEfSe). The prediction of functional potential was performed with PICRUSt2. It was observed that, after treatment with interventions and the rebalancing of the intestinal microbiota, the production of short-chain fatty acids contributes to the increase of intestinal integrity and that metabolic pathways were enriched to reverse the intestinal dysbiosis caused by the consumption of a high diet -fat. The results found indicate that the modulation of the intestinal microbiota is promising for the treatment of intestinal dysbiosis and all related diseases.

Keywords: *Lactobacillus gasseri* LG-G12. Ceftriaxone. Obesity. Bowel modulation. Gut microbiota. Gut health.

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UFC – Unidades Formadoras de Colônia

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Metodologia

AB – Solução de *Alcian Blue*

ALT – Alanina Aminotransferase

AST – Aspartato Aminotransferase

CAT – Catalase

CDNB – 1-cloro-2,4-dinitrobenzeno

CEA – Coeficiente de Eficácia Alimentar

CEUA/UFV – Comissão de Ética no Uso de Animais da Universidade Federal de Viçosa

CONCEA – Conselho Nacional de Controle de Experimentação Animal

GSH – Glutationa Reduzida

GST – Glutationa S Transferase

HDL-colesterol – Lipoproteína de Alta Densidade

HE – Hematoxilina e Eosina

LDL-colesterol – Lipoproteína de Baixa Densidade

MDA – Malondialdeído

MDS – Dimensionamentos Multidimensionais

PAS – Ácido Periódico de Schiff

SOD – Superóxido Dismutase

SRA – Sequence Read Archive

TBARS = Ácido Tiobarbitúrico

U – Unidades

UFC – Unidades Formadoras de Colônia

VLDL-colesterol – Lipoproteína de Muito Baixa Densidade

Resultados

Artigo 1 – Diet-Induced Obesity in Animal Models: Poinst to Consider and Influence on Metabolic Markers

B-MLCT – Non-enzymatically Interesterified MLCT

carb. – Carbohydrate

CAPES – Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

C-MLCT – Commercially MLCT

DIO – Diet Induced Obesity

E-MLCT – Enzymatically Interesterified MCLT

GLP-1 – Glucagon-Like Peptide-1

HF – High-Fat Diet

HFHC – High-fat High-Cholesterol Diet

HFHS – High-Fat High-Sugar Diet

HFSat – High-Fat Saturated Diet

HFSuc – High-Fat High-Sucrose Diet

HFO – High-Fat Oleic Diet

HS – High-Sugar Diet

LF – Low-Fat Diet

LFLC – Low-Fat Low-Cholesterol Diet

SCFA – Short-Chain Fatty Acids

SD – Sprague-Dawley

W – Wistar

WD – Western Diet

WHO – World Health Organization

Artigo 2 – Antibiotic Followed by a Potential Probiotic Increases Brown Adipose Tissue, Reduces Biometric Measurements and Changes Intestinal Microbiota Phyla in Obesity

AB – Solution of Alcian Blue

ALT – Alanine Aminotransferase

Anvisa – Agência Nacional de Vigilância Sanitária

AP – Abdominal Perimeter

AST – Aspartate Aminotransferase

CAPES – Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

CNPq – Conselho Nacional de Desenvolvimento Científico e Tecnológico

CFU – Colony Forming Units

CT – Total Cholesterol

DNS/UFV – Departamento de Nutrição e Saúde da Universidade Federal de Viçosa

DMB/UFV – Departamento de Microbiologia da Universidade Federal de Viçosa

FAPEMIG – Fundação de Amparo à Pesquisa de Minas Gerais

GPCRs – G Protein-Coupled Receptor

G1 – Control Group

G2 – Obese Group

G3 – Potential Probiotic Group

G4 – Antibiotic Group

G5 – Antibiotic Followed by Potential Probiotic Group

HDL-cholesterol – High-Density Lipoprotein

LDL-cholesterol – Low-Density Lipoprotein

PAS – Schiff's Periodic Acid

PPAR-α – Alpha-Type Peroxisomal Proliferators

SCFA – Short Chain Fatty Acids

SREBP-1 – Sterol Regulatory Element-Binding Tran-Scription Factor 1

TG – Triglycerides

TP – Thoracic Perimeter

UCP-1 – Uncoupling Protein 1

VLDL-cholesterol – Very Low Density Lipoprotein

Resultados suplementares

CAT – Catalase

GST – Glutationa-S-Transferase

G1 – Grupo Controle Negativo

G2 – grupo Obeso sem Tratamento

G3 – Grupo Obeso Tratado com Potencial Probiótico

G4 – Grupo Obeso Tratado com Antimicrobiano

G5 – Grupo Obeso Tratado com Antimicrobiano Seguido de Potencial Probiótico

L/M – Lactulose/Manitol

MDA – Malondialdeído

min – Minuto

ptn – Proteína

SOD – Superóxido Dismutase

TA – Tecido Adiposo

U – Unidade

Capítulo 2 – KEFIR AND INTESTINAL MICROBIOTA MODULATION: IMPLICATIONS IN HUMAN HEALTH

Resultados

Artigo de revisão – Kefir and Intestinal Microbiota Modulation: Implications in Human Health

AST – Aspartate Aminotransferase

ALT – Alanine Aminotransferase

CPT1 – Carnitine Palmitoyltransferase 1

ELANS – American Study of Nutrition and Health

EPM – Elevated Plus Maze

FAS – Fatty Acid Sintase Enzyme

FST – Forced Swim Test

GABA – Gamma Aminobutyric Acid

GLP-1 – Glucagon-Like Peptide

HOMA-IR – Insulin Resistance Index

IFN-γ – Interferon Gama

IL-1β – Interleukin-1 Beta

mL – Mililiter

OFT – Open Field Test

p-AMPK – AMP-Activated Protein Kinase

PPAR α – Peroxisome Proliferator-Activated Receptor Alpha

TGF-β – Transforming Growth Factor Beta

TMAO – Trimethylamine N-Oxide

TMF – Transplantation of Fecal Microbiota

TNF-α – Tumor Necrosis Factor Alpha

Capítulo 3 – AVALIAÇÃO DE MARCADORES DE SAÚDE INTESTINAL EM ANIMAIS TRATADOS COM PROBIÓTICO E, OU, ANTIMICROBIANO

Metodologia

CEUA/UFV – Comissão de Ética no Uso de Animais da Universidade Federal de Viçosa

LDA – Análise Discriminante

LEfSe – Análise Discriminante do Tamanho do Efeito

LG-G12 – *Lactobacillus gasseri* LG-G12

G1 – Grupo Controle Negativo

G2 – Grupo Controle Positivo com Consumo de Dieta *High-fat*

G3 – Grupo Tratado com Potencial Probiótico com Consumo de Dieta High-Fat

G4 – Grupo Tratado com Antimicrobiano com Consumo de Dieta High-Fat

G5 – Grupo Tratado com Antimicrobiano Seguido por Potencial Probiótico com Consumo de Dieta High-Fat

G6 – Grupo Controle Positivo com Consumo de Dieta-Padrão

G7 – Grupo Tratado com Potencial Probiótico e Consumo de Dieta-Padrão

G8 – Grupo Tratado com Antimicrobiano e Consumo de Dieta-Padrão

G9 – Grupo Tratado com Antimicrobiano Seguido por Potencial Probiótico com Consumo de Dieta-Padrão

OTU – Unidades Operacionais Taxonômicas

PCA – Análise de Componentes Principais

PERMANOVA – Análise de Variância Multivariada Permutacional

SEM – Erro-Padrão

STAMP – Statistical Analysis de Metagenomic Profile

Resultados

Artigo original – Modulation of the Gut Bacteriome and Intestinal Health by *Lactobacillus gasseri* LG-G12, Ceftriaxone, and a *High-Fat Diet*

ANOVA – One-Way Analysis of Variance

ASV – Amplicon Sequence Variants

BMI – Body Max Index

CCA – Canonical Correspondence Analysis

CFU – Colony Forming Units

F/B – Firmicutes to Bacteroidetes Ratio

G1 – Negative Control Group

G2 – Positive Control High-Fat Diet Group

G3 – Potential Probiotic High-Fat Diet Group

G4 – Antimicrobial High-Fat Diet Group

G5 – Antimicrobial Combined with Potential Probiotic High-Fat Diet Group

G6 – Positive Control Standard Diet Group

G7 – Potential Probiotic Standard Diet Group

G8 – Antimicrobial Standard Diet Group

G9 – Antimicrobial Combined with Potential Probiotic Standard Diet Group

LDA – Linear Discriminant Analysis

LEfSe – Linear Discriminant Analysis Effect Size

LG-G12 – *Lactobacillus gasseri* LG-G12

L/M – Lactulose to Manitol Ratio

MDS – Multidimensional Scaling Analysis

PCA – Principal Component Analysis

PERMANOVA – Permutational Multivariate Analysis of Variance

SCFA – Short-Chain Fatty Acids

SRA – Sequence Read Archive

STAMP – Statistical Analysis of Metagenomic Profile

UFV – Universidade Federal de Viçosa

Apêndice

$t\alpha/2$ = valor da tabela de distribuição t (two-tailed);

DP = desvio-padrão;

E = diferença que se deseja detectar no estudo

Artigo – Ceftriaxone Causes Dysbiosis and Changes Intestinal Structure in Adjuvant Obesity Treatment

AP – Abdominal Perimeter

CAPES – Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

CNPq – Conselho Nacional de Desenvolvimento Científico e Tecnológico

CONCEA – Conselho Nacional de Controle de Experimentação Animal

CFU – Colony-forming units

DNS – Department of Nutrition and Health

DMB/UFV – Departamento de Microbiologia da Universidade Federal de Viçosa

FAPEMIG – Fundação de Amparo à Pesquisa de Minas Gerais

HPLC – High Performance Chromatography

MDS – Multidimensional Scaling Analysis

PBS – Phosphate Buffered Saline solution

SCFA – Short-Chain Fatty Acids

SE – Standard Error

SRA – Sequence Read Archive

TP – Thoracic Perimeter

UFV – Universidade Federal de Viçosa

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INTRODUÇÃO GERAL

A microbiota intestinal compreende todos os micro-organismos presentes no trato gastrointestinal (DOMINGUEZ-BELLO *et al*., 2019), sendo sua diversidade e equilíbrio essenciais para a saúde humana. Ela é composta, principalmente, pelos filos Bacteroidetes e Firmicutis e pode conter mais de 10^{14} micro-organismos (LEY *et al*., 2006; TREMAROLI; BACKHED, 2012; THURSBY; JUGE, 2017). Esses micro-organismos coexistem em equilíbrio com o organismo humano e nele desempenham importantes funções, como promoção da integridade da mucosa, síntese de vitaminas e produção de bacteriocinas (PUSHPANATHAN *et al*., 2019).

Pode-se dividir a microbiota intestinal entre transiente e autóctone. A transiente coloniza locais onde o trânsito intestinal é mais rápido, como o duodeno e o jejuno, e sofre mais alterações em sua composição (KADOOKA *et al*., 2013; OLIVEIRA; HAMMES, 2016). Já a autócne coloniza o cólon, que tem trânsito intestinal mais lento e não sofre grandes alterações em sua composição (OLIVEIRA; HAMMES, 2016).

Considerada problema de saúde pública mundial devido à sua alta prevalência e ao seu impacto econômico (WHO, 2016), a obesidade é uma doença que gera alta mortalidade e está relacionada a doenças crônicas não transmissíveis e ao aumento da mortalidade precoce (FONTAINE *et al*., 2003; SCHMIDT *et al*., 2011; HRUBY *et al*., 2016; LAVIE *et al*., 2019). Além disso, desde 2004 se discute a relação entre obesidade e microbiota intestinal, visto que Backhed e colaboradores (2004) observaram que camundongos C57BL/6J *germ-free*, ou seja, livres de microbiota intestinal, possuíam 42% menos gordura corporal do que os camundongos convencionais com consumo alimentar 29% inferior. Também, animais *germ-free* que receberam microbiota intestinal de camundongos convencionais tiveram 57% de aumento de gordura corporal e desenvolveram resistência à insulina, mesmo com um consumo alimentar 7% inferior (BACKHED *et al*., 2004).

Em seguida, em 2006, publicou-se o primeiro estudo comparando a microbiota de indivíduos obesos e magros, cujos resultados apontaram que os obesos apresentam menor quantidade de Bacteroidetes e a quantidade deste filo aumenta à medida que o indivíduo consome dieta restrita em energia (LEY *et al*., 2006), ou seja, à medida que ele tenta perder peso. Esse resultado, contudo, deve ser discutido e interpretado com cuidado, uma vez que diferentes fatores influenciam a composição da microbiota intestinal e há diversas metodologias para determinação dessa microbiota, o que pode gerar resultados conflitantes (MAGNE *et al*., 2020).

A literatura também demonstra que a composição da microbiota intestinal pode contribuir para o desenvolvimento da obesidade, visto que aumenta a extração de energia da dieta (BACKHED *et al*., 2004; JUMPERTZ *et al.,* 2011; GOMES *et al*., 2018; CORNEJO-PAREJA *et al*., 2019), amplia a absorção intestinal de macronutrientes (HOOPER *et al*., 2001; STAPPENBECK *et al*., 2002; GOMES *et al*., 2018), altera os hormônios da saciedade (GOMES *et al*., 2018; CORNEJO-PAREJA *et al*., 2019) e impede a atuação dos mecanismos de defesa intestinal (GOMES *et al*., 2018; CORNEJO-PAREJA *et al.,* 2019), gerando um estado de inflamação crônica de baixo grau (CANI *et al*., 2007; CORNEJO-PAREJA *et al*., 2019).

Logo, é crescente o número de estudos que avaliam a modulação da composição da microbiota intestinal como estratégia para o tratamento (HWANG *et al*., 2015; JUNG *et al*., 2015; KONG *et al*., 2019) e prevenção da obesidade (OSTERBERG *et al*., 2015; RAJPAL *et al*., 2015). Assim, probióticos (FAO/WHO, 2001; KIM *et al*., 2017; WGO, 2017), antimicrobianos (CARVALHO *et al*., 2012; DEL FIOL *et al*., 2014; IANIRO *et al.,* 2016) e dieta (KONG *et al*., 2019; MOSZAK *et al*., 2020) são importantes agentes capazes de alterar a microbiota intestinal.

Micro-organismos vivos que, quando consumidos em quantidades adequadas, geram benefícios à saúde do hospedeiro são considerados probióticos (FAO/WHO, 2002). Esses seres microscópicos devem ser preferencialmente de origem humana e reconhecidos como seguro à saúde por meio de evidências científicas, ser viáveis e ativos no veículo em que foram administrados e ser resistentes aos sucos gástricos e intestinais (ANVISA, 2008; MARTINEZ, 2015). Também, no Brasil, para um produto ter alegação de probiótico, ele deve conter no mínimo 10^8 a 10^9 Unidades Formadoras de Colônia (UFC) no produto final pronto para consumo (ANVISA, 2008).

O consumo regular de probióticos gera benefícios, como a modulação de citocinas inflamatórias, a produção de bacteriocinas, o estímulo à integridade da barreira intestinal (WGO, 2017; CERDÓ *et al*., 2019), a competição pela adesão de patógenos e a tolerância a antígenos alimentares, o que fortalece a microbiota intestinal, impedindo a ocorrência da disbiose intestinal (WGO, 2017). Logo, visto que a obesidade também é caracterizada pela disbiose intestinal, os mecanismos anteriormente citados contribuem para o tratamento desta importante doença (CERDÓ *et al*., 2019).

Com capacidade de alterarem a microbiota intestinal, os antimicrobianos são medicamentos que visam inibir o crescimento de micro-organismos específicos (PIDOT *et al*., 2014; LANGE *et al*., 2016). Particularmente em relação ao tratamento da obesidade, sugere-

se que os antimicrobianos com atuação sobre bactérias Gram-negativas sejam mais interessantes. Isso porque o lipopolissacarídeo presente na parede dessas bactérias gera um estado de inflamação crônica de baixo grau, ativando o sistema imune (REIS *et al*., 2018) e estimulando o desenvolvimento da disbiose intestinal e o aumento da gordura corporal (ROSAS-VILLEGAS *et al*., 2017).

Por fim, destaca-se o papel das dietas como moduladoras da microbiota intestinal. Ainda na infância, por exemplo, o tipo de aleitamento e a introdução alimentar são determinantes para a construção de uma microbiota diversa e saudável (MILANI *et al*., 2017; ZHUANG *et al*., 2019). Além disso, na vida adulta se observa que os diferentes macronutrientes e os padrões alimentares geram impacto direto sobre a diversidade microbiana (MOSZAK *et al*., 2020). Consumo excessivo de carboidratos simples, gorduras saturadas e alimentos industrializados, ou seja, o padrão de dieta ocidental, impactam negativamente na microbiota intestinal, estimulando o desenvolvimento da disbiose. Em contrapartida, o consumo de alimentos ricos em fibras, como frutas, vegetais e cereais integrais, bem como padrões de dieta vegetariano e mediterrâneo, contribui para um estado de eubiose intestinal, com diversidade e equilíbrio microbiano (KHALILI *et al*., 2018; MOSZAK *et al*., 2020).

Além da obesidade, a composição da microbiota intestinal também se associa à ocorrência de outras doenças, como síndrome metabólica (BELLIKCI-KOYU *et al*., 2019), diabetes (LIU *et al*., 2020), câncer (HELMINK *et al*., 2019) e alterações na tireoide (KNEZEVIC *et al*., 2020), sabendo-se que a prevenção e tratamento dessas também se associam à composição da microbiota intestinal (BELLIKCI-KOYU *et al*., 2019; HELMINK *et al*., 2019; KNEZEVIC *et al*., 2020; LIU *et al*., 2020). Nesse contexto, o uso de bebidas fermentadas, como o kefir, é um hábito interessante devido ao seu custo acessível, à sua fácil manipulação (ROSA *et al*., 2017) e à ausência de riscos para o consumidor (BELLIKCI-KOYU *et al*., 2019).

Formado por bactérias dos ácidos lático e acético, bem como por leveduras, o kefir é um produto fermentado (CODEX, 2003; SANLIER *et al*., 2019) que apresenta potencial para ser considerado alimento probiótico, haja vista que o seu consumo está relacionado a benefícios à saúde (BOURRIE, 2016; WGO, 2017; ANVISA, 2018). Essa capacidade pode ser justificada pela presença de *Lactobacillus* (WGO, 2017), bem como pelo fato de outros micro-organismos constituintes do kefir também apresentarem a capacidade de modular a microbiota intestinal (BOURRIE, 2016).

Diante do exposto, destaca-se que os estudos envolvendo a composição da microbiota intestinal têm sido de grande relevância, uma vez que o padrão de saúde mundial sofreu importantes mudanças nas últimas décadas (COLLEN *et al*., 2016). Assim, cada vez mais se faz necessário entender os mecanismos envolvidos entre a composição da microbiota intestinal e o desenvolvimento de diferentes quadros de saúde e doença. Nesse sentido, neste estudo o objetivo é entender como um potencial probiótico, um antimicrobiano e a dieta influenciam a composição da microbiota intestinal e os parâmetros relacionados à obesidade.

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CAPÍTULO 1

EFEITO DE PROBIÓTICO E, OU, ANTIMICROBIANO ASSOCIADO À OFERTA DE UMA DIETA-PADRÃO: ESTUDO EXPERIMENTAL EM MODELO DE INDUÇÃO DA OBESIDADE

1 JUSTIFICATIVA

Neste capítulo são apresentados os resultados do experimento e das análises realizados na Universidade Federal de Viçosa, entre os anos de 2017 e 2020.

Considerando os impactos gerados pela obesidade, a necessidade de novas formas de tratamento desta doença e a ausência de estudos que avaliem o tratamento sequencial com probióticos e antimicrobianos, objetivou-se avaliar os efeitos do consumo de probiótico e, ou, antimicrobiano associados a uma dieta-padrão, sob parâmetros biométricos, bioquímicos, histológicos e microbianos, em camundongos C57BL/6J induzidos à obesidade.

2 METODOLOGIA GERAL

2.1 Produtos-teste

O potencial probiótico utilizado foi o *Lactobacillus gasseri* LG-G12 (Lemma®, Brasil) e o antimicrobiano, o da classe das ceftazidimas (Triaxton®, Blau Farmacêutica S/A), ambos adquiridos na forma liofilizada.

O *Lactobacillus gasseri* LG-G12 é uma cepa específica comercializada no Brasil. Por esse motivo, ao longo desta tese será denominado como potencial probiótico. Foi ofertado na quantidade de 10^9 Unidades Formadoras de Colônia (UFC)/dia, por ser essa a quantidade necessária para que o produto tenha alegação de probiótico no país (ANVISA, 2008). O cálculo de gramatura suficiente para a oferta dessa quantidade foi realizado de acordo com a informação fornecida pelo fabricante no rótulo do produto.

Esse potencial probiótico foi escolhido por ser de origem humana e, quando administrado em quantidades adequadas, sobreviver à passagem pelo trato gastrointestinal (KIM *et al.*, 2006; RITTER *et al.*, 2009; KAWASE *et al.*, 2011; MIYOSHI *et al.*, 2014), tendo, portanto, características necessárias para ser considerado probiótico. Além disso, estudos têm sugerido que seu consumo regular pode auxiliar na perda de peso ou na diminuição do ganho de peso corporal (KANG *et al.*, 2010; KANG *et al.*, 2013; SHI *et al.*,

2013; MIYOSHI *et al*., 2014; KAWANO *et al*., 2016), sendo interessante para o tratamento da obesidade. Mecanismos que explicam o potencial desse tratamento envolvem modulação da composição da microbiota intestinal, diminuição da inflamação sistêmica e do tecido adiposo e redução da permeabilidade intestinal (KANG *et al*., 2010; KANG *et al*., 2013; SHI *et al*., 2013; MIYOSHI *et al*., 2014; KAWANO *et al*., 2016).

Para o antimicrobiano foram ofertados 500 mg de medicamento/kg de peso corporal dos roedores (RAJPAL *et al*., 2015). Os animais foram pesados semanalmente, sendo a média dos pesos do grupo considerada no cálculo da pesagem do medicamento.

A ceftriaxona dissódica hemieptaidratada (Triaxton®), antimicrobiano de terceira geração da classe das cefalosporinas, foi escolhida por apresentar grande espectro de atuação contra bactérias Gram-negativas, inibindo a síntese da parede celular bacteriana. Além disso, é utilizada no tratamento de diferentes infecções (ANVISA, s. d.; RICHARD, 1984) e não é absorvida pelo organismo, o que permite sua atuação local no intestino e no cólon. Essas características permitem a atuação desse antimicrobiano no combate à inflamação crônica de baixo grau, característica da obesidade, e, portanto, no tratamento adjuvante desta doença (REIS *et al*., 2018).

Ambas as soluções foram ofertadas para os animais por gavagem, na quantidade de 200 μL, sendo este o menor volume possível para dissolver os produtos-teste. Os animais controle receberam água destilada via gavagem e, assim, todos eles foram submetidos ao mesmo tratamento. A produção das soluções dos produtos-teste foi obtida, diariamente, no Laboratório de Bioquímica Nutricional do Departamento de Nutrição e Saúde da Universidade Federal de Viçosa, com água destilada, em utensílios e ambiente destinados exclusivamente para esse fim.

2.2 Cálculo experimental

O tamanho amostral foi calculado conforme proposto por Mera *et al*. (1998), adotando-se como critério uma diferença de 10% no ganho de peso (variável principal) em relação aos tratamentos e um poder estatístico de 98% ($\alpha \leq 0{,}02$). Para os cálculos foram utilizados os valores de peso do estudo de Miyoshi *et al.* (2014). Foram estudados oito animais em cada grupo experimental (Apêndice A).

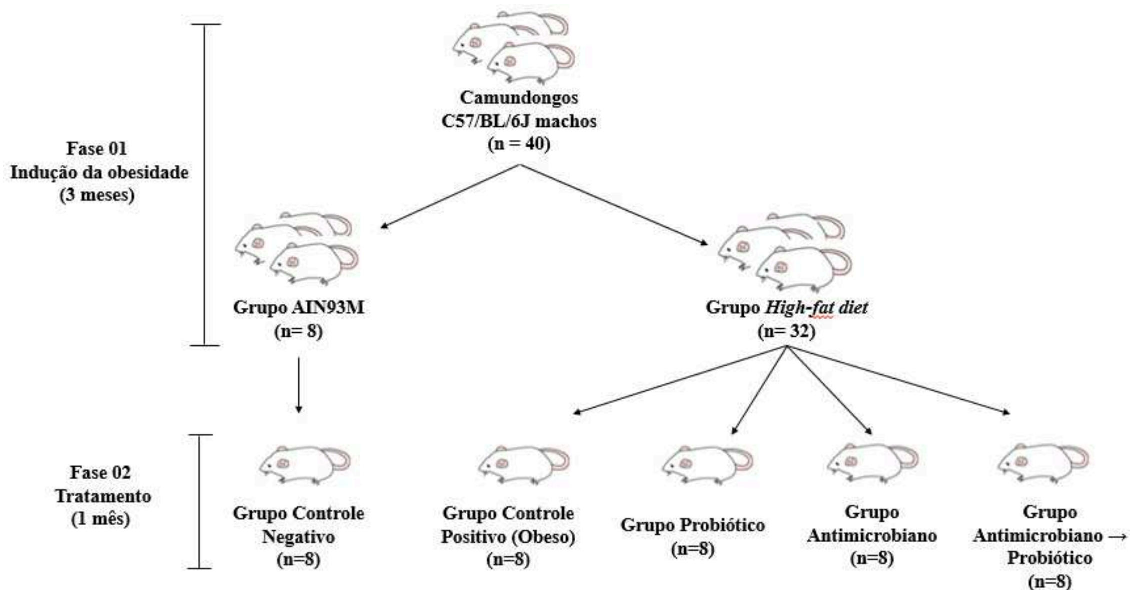
2.3 Desenho experimental

Modelo experimental

Foram utilizados 40 camundongos C57BL/6J, machos, com 30 dias de vida, provenientes do Biotério Central do Centro de Ciências Biológicas e da Saúde da Universidade Federal de Viçosa (UFV).

Primeiramente, os animais permaneceram um mês em período de adaptação. Depois, o experimento foi conduzido durante quatro meses, sendo três meses para a indução da obesidade (fase 1) e um para a realização do tratamento (fase 2). Os animais permaneceram no Laboratório de Nutrição Experimental do Departamento de Nutrição e Saúde da UFV em gaiolas coletivas (oito animais/caixa), em ambiente com temperatura controlada (22 ºC ± 2 ºC) e fotoperíodo de 12 horas. O consumo de água destilada e da dieta foi controlado durante os quatro meses de experimentação (Figura 1).

Figura 1 – Desenho experimental



Camundongos C57BL/6J machos foram randomicamente divididos em dois grupos experimentais, sendo um grupo controle negativo (n=8), que recebeu dieta AIN-93M e água destilada; e um grupo com obesidade induzida pela dieta (n=32), que recebeu dieta *high-fat* 60% e solução de frutose 10%, durante três meses. Após a confirmação da ocorrência de obesidade, os camundongos obesos foram subdivididos, randomicamente, em quatro grupos experimentais, sendo: controle positivo, potencial probiótico, antimicrobiano e antimicrobiano seguido de potencial probiótico, que receberam, via gavagem, como tratamento água, probiótico *Lactobacillus gasseri* LG-G12 (10^9 UFC/dia), antimicrobiano Triaxton® (500 mg/kg de peso) e antimicrobiano Triaxton® (500 mg/kg de peso) seguido de probiótico *Lactobacillus gasseri* LG-G12 (10^9 UFC/dia), respectivamente. A fase de tratamento teve duração de um mês, e todos os grupos receberam dieta AIN93M e água destilada, havendo, portanto, restrição energética em relação ao período anterior.

Fase 1 – Desenvolvimento da obesidade

Após o período de adaptação, os animais foram randomicamente divididos em dois grupos: um grupo-teste (n = 32) e um grupo controle negativo (n = 8). O primeiro recebeu dieta *high-fat* com 60% de gordura (MEMBREZ *et al*., 2008) e solução de frutose 10% (VEDOVA *et al*., 2016), em bebedouro disponível na gaiola, com fornecimento de 0,4 kcal por ml de solução consumida. Já o grupo controle negativo recebeu dieta AIN-93M (REEVES *et al*., 1993) e água destilada, não tendo, portanto, obesidade induzida pela dieta (Figura 1 e Tabela 1).

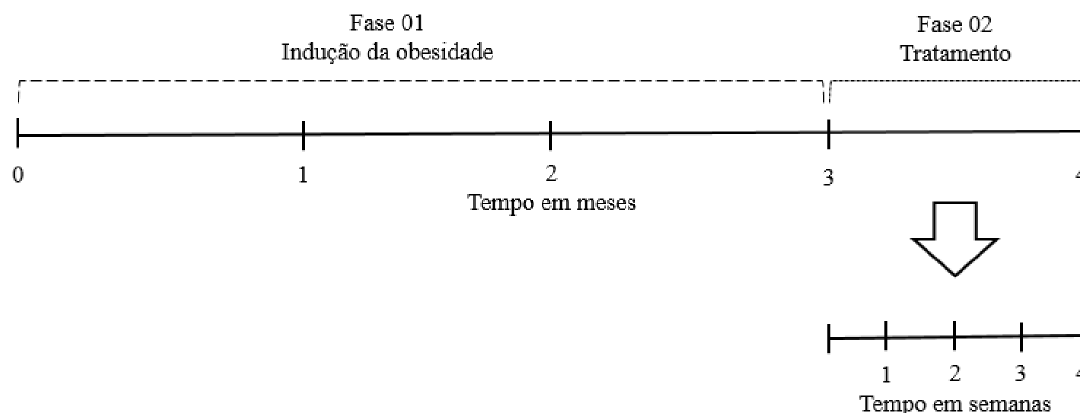
Tabela 1 – Composição das dietas experimentais AIN-93M e *high-fat diet* com 60% de gordura

Ingrediente	**Quantidade (g/100 g de dieta)**	
	AIN-93M	***High-fat diet* 60%**
Amido de milho	46,56	-
Óleo de soja	4,0	3,2
Banha de porco	-	31,7
Caseína	14,0	25,8
Maltodextrina	15,5	16,2
Sacarose	10,0	8,9
Celulose	5,0	6,5
Mix mineral	3,5	1,3
Citrato de potássio	-	2,1
L-cistina	0,18	0,39
Fosfato de cálcio	-	1,7
Mix vitamínico	1,0	1,3
Carbonato de cálcio	-	0,7
Bitartarato de colina	0,25	0,3
% de kcal de gordura	10,0	60,0
% de kcal de carboidrato	75,9	20,0
% de kcal de proteína	14,1	20,0
Densidade calórica (kcal)	265,45	406,7

Fonte: REEVES *et al*., 1993; Research diets (http://www.researchdiets.com/open-source-diets/stock-diets/dio-series-diets); e caseína com > 85% de proteína.

Essa fase experimental teve duração de três meses (MEMBREZ *et al*., 2008) (Figura 2), sendo a dieta administrada pelo esquema de *pair-fed.* Ao final desse período, os animais foram submetidos à aferição e ao cálculo de medidas biométricas (peso corporal, perímetro abdominal, perímetro torácico e índice de Lee), a fim de identificar o desenvolvimento da obesidade.

Figura 2 – Linha do tempo de duração do experimento



Fase 2 – Tratamento da obesidade

Após a confirmação da obesidade pelos maiores valores de peso corporal no grupo que consumiu dieta *high-fat* e solução de frutose, os animais obesos foram novamente divididos, randomicamente, em quatro grupos experimentais. Os grupos, que possuíam oito animais cada, foram: grupo controle positivo (obeso), grupo potencial probiótico, grupo antimicrobiano e grupo potencial probiótico seguido de antimicrobiano (Figura 1).

Durante a fase de tratamento, que teve duração de um mês, os animais consumiram dieta-padrão AIN-93M e água destilada, com o objetivo de promover uma restrição calórica em relação à dieta anteriormente consumida. Além disso, houve a oferta diária, via gavagem, de potencial probiótico, antimicrobiano e antimicrobiano seguido de potencial probiótico.

Os animais dos grupos controle negativo e positivo receberam água destilada, os do grupo potencial probiótico receberam *Lactobacills gasseri* LG-G12 e aqueles do grupo antimicrobiano receberam ceftriaxona durante 15 dias, seguido da oferta de *Lactobacills gasseri* LG-G12 por mais 15 dias, totalizando um mês de tratamento, como os demais grupos (Figura 1)

Fase 3 – Eutanásia e dissecação dos órgãos

Após o período experimental, os animais foram submetidos a jejum de oito horas e anestesiados, de dois a quatro minutos, com isoflurano 3% (Isoflorine®, Cristália). O anestésico inalatório foi utilizado como agente de eutanásia pela necessidade de coleta de sangue e como forma de minimizar os efeitos do estresse na manipulação dos animais.

A eutanásia ocorreu no Laboratório de Nutrição Experimental do Departamento de Nutrição e Saúde da Universidade Federal de Viçosa, em Viçosa, MG, em ambiente de

tranquilidade e adequado, longe do alojamento de outros animais. Tal procedimento ocorreu com a presença de um médico-veterinário e de uma equipe de apoio prévia e devidamente treinada. Todo esse processo foi realizado em consonância com as Normas do Conselho Nacional de Controle de Experimentação Animal (CONCEA, 2008).

As amostras de sangue foram coletadas por incisão na aorta abdominal, em tubos sem heparina, e foram centrifugadas a 12.000 rpm, por 15 minutos. O soro foi separado e armazenado a -80 ºC, para análises futuras.

Fígado, intestino delgado, cólon e tecidos adiposos (visceral, marrom e abdominal) foram lavados com solução salina fisiológica, pesados, identificados e congelados em nitrogênio líquido e, posteriormente, a -80 ºC, para análises posteriores.

Os intestinos delgado e grosso foram acondicionados em recipientes com solução de Carnoy, e o tecido adiposo abdominal o foi em recipientes com solução-tampão de formalina de Carson (CARSON *et al*., 1973) tamponada com 1% de cálcio, para análises histológicas posteriores.

Fezes do ceco e fezes frescas foram armazenadas em *eppendorfs* autoclavados, a -80 ºC, para posteriores análises da composição da microbiota intestinal e do perfil de ácidos graxos de cadeia curta, respectivamente.

2.4 Avaliação do consumo alimentar e das medidas biométricas

O peso corporal dos animais foi verificado semanalmente, em balança digital (Marte®, modelo M 2K, Brasil) com capacidade máxima de 2,1 kg e mínima de 0,5 g. Também, a ingestão alimentar e o consumo hídrico foram verificados a cada três dias, por meio de balança digital (Shimadzu®, modelo BL3200H, Japão) com capacidade máxima de 3,2 kg e mínima de 0,5 g e da utilização de proveta, respectivamente. Os dados de peso corporal e de ingestão alimentar foram utilizados para o cálculo do coeficiente de eficácia alimentar (CEA), dividindo-se o ganho de peso (g) pelo consumo alimentar de ração (g).

As medidas corporais foram aferidas com o auxílio de fita métrica inelástica, determinando-se os perímetros abdominal (medido imediatamente antes das pernas traseiras) e torácico (mensurado imediatamente após as pernas dianteiras). Perímetro abdominal e perímetro torácico são medidas corporais.

O índice de Lee foi calculado a partir da divisão da raiz cúbica do peso corporal (g) pelo comprimento focinho–ânus (cm). Valores mais elevados indicam maior propensão a maiores índices de adiposidade (DEL FIOL *et al*., 2014).

2.5 Teste de tolerância oral à glicose

Ao final da fase 2 de experimentação, foi realizado o teste de tolerância oral à glicose. Para isso, os animais foram submetidos a jejum de 12 horas. Uma primeira coleta de sangue foi realizada com um pique na extremidade da cauda do animal (tempo 0), seguida de leitura com tiras reagentes (Accu-Chek Active, Roche®) em glicosímetro (Accu-Chek Active, Roche®). Para tanto, os animais foram dessensibilizados com isoflurano 3% (Isoflorine®, Cristália), utilizando circuito simples com fluxômetro acoplado a um cilindro de oxigênio.

Posteriormente, uma solução de glicose 20% (2 g/kg de massa corporal) foi administrada aos animais via gavagem, e amostras de sangue foram novamente coletadas, na cauda dos animais, nos tempos de 0, 15, 30, 60 e 90 minutos e analisadas com tiras reagentes (Accu-Chek Active, Roche®) em glicosímetro (Accu-Chek Active, Roche®) (HWANG *et al*., 2015).

A partir dos valores de concentração de glicose aferidos, foi possível obter a área sob a curva da glicose.

2.6 Análises bioquímicas

O colesterol total, a lipoproteína de alta densidade (HDL-colesterol), os triglicerídeos, a alanina aminotransferase (ALT) e a aspartato aminotransferase (AST) foram determinados nas amostras de soro pelo método enzimático-colorimétrico, utilizando *kits* comerciais (Bioclin®, Brasil) (Tabela 2), segundo os procedimentos recomendados pelo fabricante.

As amostras foram analisadas em analisador automático (Mindray® Medical International Limited, modelo BS 200, China), seguindo-se a programação recomendada pela empresa (Bioclin®). As concentrações de lipoproteína de baixa densidade (LDL-colesterol) e da lipoproteína de muito baixa densidade (VLDL-colesterol) foram calculadas de acordo com as equações propostas por Friedewald e colaboradores (1972).

Tabela 2 – *Kits* comerciais (Bioclin®) utilizados nas análises bioquímicas

Parâmetro	Número *Kit*	Nome comercial
Colesterol total	K 083	Colesterol monorreagente
HDL-colesterol	K 071	HDL direto
Triglicerídeos	K 117	Triglicérides monorreagente
ALT	K 049	Transaminase ALT cinética
AST	K 048	Transaminase AST cinética

2.7 Análise histológica

Intestino delgado

Fragmentos do intestino delgado foram removidos e fixados em formalina de Carson (1973), em temperatura ambiente. Após a fixação, esse tecido foi desidratado em gradiente crescente de etanol (70% até álcool absoluto) e incluído em resina à base de hidroxiemetilmetacrilato (Historesin, Leica®). Secções transversais e longitudinais de 5 µm de espessura foram obtidas em micrótomo rotativo RM2265 (Leica®, Suíça), com auxílio de navalhas de vidro e coradas em solução de *alcian blue* (AB) e ácido periódico de Schiff (PAS) (MCMANNUS; MOWRY, 1960).

Para a análise morfométrica, imagens dos fragmentos histológicos foram capturadas com objetiva de 10X diretamente no microscópio de luz LEICA DM750 (Leica®, Suíça), por meio de câmera de vídeo LEICA 170HD (Leica®, Suíça). Com o auxílio do *software* Image Pro-Plus® versão 4.5 (Media Cybernetics), foram tomadas as seguintes medidas, segundo a metodologia proposta por Rosa e colaboradores (2010):

- **Altura das vilosidades**: foram selecionados 10 campos aleatórios por animal, e apenas as vilosidades com epitélio definido e conjuntivo visível foram aferidas.
- **Largura das vilosidades**: nas mesmas vilosidades utilizadas na análise de altura, utilizou-se o ponto médio para medição da largura das vilosidades.
- **Altura das criptas**: foram tomadas as medidas de 10 campos por animal, sendo possível ver a base e o ápice (abertura) da cripta.
- **Largura das criptas**: nas mesmas criptas utilizadas na análise de altura, utilizou-se o ponto médio para medição da largura das criptas.
- **Profundidade da mucosa**: considerou-se como camada mucosa do ápice da cripta até a base da vilosidade.

Tecido adiposo

Fragmentos do tecido adiposo abdominal foram desidratados, incluídos em Paraplast Plus (Sigma®, Suíça) e cortados com 5 µm de espessura. Em seguida, foram corados com hematoxilina e eosina (HE) e as lâminas, montadas com o auxílio de Entellan (Merck®, Alemanha).

Para aferição da área e do número dos adipócitos, 10 campos por animal foram capturados, com a objetiva de 10X, usando um microscópio de luz (Primo Star 2012, Zeiss®, Alemanha), por meio de uma câmera de vídeo Aixo ERc5s (Zeiss®, Alemanha). As imagens

foram analisadas no *software* Image Pro-Plus® versão 4.5 (Media Cybernetics, EUA), e apenas as células com contornos bem definidos e que estavam completas no campo da foto foram analisadas (ROSA *et al*., 2016).

Para a aferição de infiltrados inflamatórios, 10 campos por animal foram capturados, com a objetiva de 20X, diretamente no microscópio de luz LEICA DM750 (Leica®, Suíça), por meio de câmera de vídeo LEICA 170HD (Leica®, Suíça). As imagens também foram analisadas no *software* Image Pro-Plus® versão 4.5 (Media Cybernetics, EUA).

2.8 Análises das enzimas envolvidas no processo oxidativo

Catalase

A atividade da catalase (CAT) foi determinada no sobrenadante do tecido hepático (100 mg) dos animais homogeneizado com PBS 50 mmol/L, macerado com bastão de vidro e centrifugado a 5.000 rpm, a 4 ºC, por 10 minutos. A atividade da catalase foi determinada, em triplicata, pela taxa de decaimento do peróxido de hidrogênio (10 mmol/L), durante 60 segundos, com a leitura em espectrofotômetro (Multiskan Go, Thermo Scientific®, Finlância) a 240 nm, segundo a metodologia de Aebi (1984). Os resultados foram expressos em unidades (U) de CAT/mg de proteína, sendo a dosagem de proteína realizada pelo método de Lowry e colaboradores (1951).

Superóxido Dismutase

Com o mesmo sobrenadante utilizado para a dosagem da CAT foi determinada a atividade da superóxido dismutase (SOD), segundo Dieterich e colaboradores (2000). Essa metodologia é baseada na capacidade da SOD em catalisar a reação do superóxido O_2 ao peróxido de hidrogênio, diminuindo, assim, a razão de auto-oxidação do pirogalol. A análise foi realizada em triplicata, em espectrofotômetro (Multiskan Go, Thermo Scientific®, Finlância) a 570 nm. Os resultados foram expressos em U de SOD/mg de proteína, cuja dosagem foi aferida pelo método de Lowry e colaboradores (1951).

Glutationa S Transferase

A atividade da glutationa S transferase (GST) foi determinada segundo a metodologia adaptada de Habig e colaboradores (2002), com base na habilidade da GST em metabolizar o 1-cloro-2,4-dinitrobenzeno (CDNB), conjugando-o com a glutationa reduzida (GSH). Essa determinação foi realizada, em triplicata, com o sobrenadante anteriormente descrito, para CAT e SOD, acrescido de CDNB e GSH. A leitura foi em espectrofotômetro (Multiskan Go,

Thermo Scientific®, Finlância) a 340 nm, durante 90 segundos. O coeficiente de extinção molar de 9,6 mmol/cm foi usado para calcular a concentração da GST, expressa em nmol/min/mg de proteína da amostra.

2.9 Mensuração da peroxidação lipídica

A mensuração dos metabólitos reativos ao ácido tiobarbitúrico (TBARS) foi realizada de acordo com a metodologia adaptada de Buege e Aust (1978). Este método se baseia na capacidade do malondialdeído (MDA), em condições ácidas e aquecido com o TBARS, de formar um produto de coloração rósea que pode ser mensurado em espectrofotômetro (Multiskan Go, Thermo Scientific®, Finlância) a 535 nm.

Amostras de fígado (100 mg) foram homogeneizadas com PBS 50 mmol/L e centrifugadas a 3.500 rpm por 10 minutos. Ao sobrenadante resultante foi acrescida solução de TBARS, que contém ácidos tricloroacético e tiobarbitúrico. O sobrenadante foi mantido em banho-maria a 100 ºC, por 10 minutos.

O coeficiente de extinção molar 1,56 x 10^5 M/cm foi usado para o cálculo da concentração de TBARS, e a dosagem de proteína foi realizada por método de Lowry e colaboradores (1951), sendo a concentração final expressa em micromol/mg de proteína.

2.10 Avaliação da concentração de lipídios totais, colesterol e triglicerídeos no tecido hepático

Os lipídeos hepáticos foram extraídos com o uso de solventes orgânicos, de acordo com a metodologia adaptada de Folch e colaboradores (1956). Resumidamente, 200 mg de tecido hepático foram macerados com o auxílio de macerador automático (IKA® T10 basic S32), em tubo de vidro, junto com a solução de clorofórmio:metanol, na proporção 1:2, mais a adição de metanol puro.

As amostras foram centrifugadas (3.000 rpm por 10 minutos) (HERMLE® Labnet Z216 MK) e o sobrenadante, transferido para um novo tubo de vidro limpo, previamente pesado. Foram acrescentados às amostras clorofórmio puro e solução de NaCl 0,73%, e uma nova centrifugação foi realizada. O sobrenadante foi, então, descartado e a parede do tubo, lavada três vezes com solução de Folch. O material permaneceu em estufa semiaberta a 37 º C (*overnight*).

A quantidade de lipídeos extraída foi calculada pela diferença de peso entre o tubo após a secagem (com lipídeos) e o tubo vazio. Os lipídios extraídos foram suspensos em 500 µL de Triton X-100 1% para a determinação das concentrações de colesterol total e triglicerídeos, utilizando *kits* comerciais da Bioclin® (Tabela 3), conforme a metodologia descrita por Rosa e colaboradores (2016).

Tabela 3 – *Kits* comerciais (Bioclin®) utilizados nas análises de lipídios hepáticos

Parâmetro	**Número *Kit***	**Nome comercial**
Colesterol total	K 083	Colesterol monorreagente
Triglicerídeos	K 117	Triglicérides monorreagente

2.11 Determinação da permeabilidade intestinal

Após o término da fase 2, os animais receberam, via gavagem, 200 µL de uma solução contendo 13,3 mg de lactulose (Daiichi Sankyo®, Brasil) e 10,1 mg de manitol (Synth®, Brasil). Toda a urina excretada nas 24 horas seguintes foi coletada (JIN *et al*., 2008) com o auxílio de gaiolas metabólicas. Durante esse período, os animais permaneceram em jejum.

A quantificação dos açúcares foi realizada por meio de cromatografia líquida de alta eficiência (Shimadzu®, detector modelo: RID 10A, Japão), em comprimento de onda de 210 nm. Para a separação cromatográfica, 20 µL de amostra foram injetados em uma coluna móvel (Aminex®, modelo HPX-87H, EUA), com 300 mm de comprimento x 7,8 mm de diâmetro, fluxo de 1 mL/minuto e pressão de 54 kgf. A água foi utilizada como fase móvel.

2.12 Determinação de ácidos graxos de cadeia curta

A extração dos ácidos graxos de cadeia curta (acético, propiônico e butírico) do conteúdo cecal foi realizada segundo a metodologia proposta por Siegfried e colaboradores (1984).

A leitura foi realizada em cromatógrafo líquido de alta eficiência (Ultimate 3000, Dionex®). Para separação cromatográfica, as amostras foram injetadas em coluna (RezexROA-Organic Acid H+ (8%), Phenomenex®), com comprimento de 300 mm e diâmetro de 7,8 mm. A fase móvel utilizada foi ácido sulfúrico 5-mmolar, fluxo de injeção 0,7 mL/min, volume de injeção de 20 µL e temperatura do forno de 45 ºC. O detector utilizado foi o Rid RH01 (Shodex®).

2.13 Determinação da composição da microbiota intestinal

Após o término da fase 2 foram coletadas amostras de fezes de todos os grupos experimentais, sendo formado um *pool* de fezes de cada grupo, o qual foi utilizado na análise da composição da microbiota intestinal.

Inicialmente, extraiu-se o DNA metagenômico das amostras segundo a metodologia adaptada de Zhang e colaboradores (2006). Após a extração, quantificou-se a concentração em Qubit e verificou a sua qualidade por meio da eletroforese em gel de agarose 1,8%. Amostras que exibiram razão de absorvância nos comprimentos de onda 260/230 superiores a 1,8 foram consideradas aptas para a etapa de sequenciamento.

O sequenciamento do DNA da região 16S RNAr foi realizado pela empresa Macrogen (Macrogen Inc®, Seul, Coreia de do Sul), usando-se o sequenciador Illumina MiSeq (Illumina, USA). Os "amplicons" foram obtidos por PCR usando *primers* específicos (Bakt 341F e Bakt 805R) para as regiões V3 e V4 do gene 16S. Os dados brutos do sequenciamento (arquivos no formato FASTQ) foram trimados pelo programa Trimmomatic v 0.36 (BOLGER *et al*., 2014) com "cut off" de Phred Quality de 30.

Os dados trimados foram processados usando o pacote DADA2 versão 1.8 (CALLAHAN *et al*., 2016) na plataforma R, versão 3.6.1 (https://cran.r-project.org). O processamento dos dados seguiu todos os passos recomendados pelos desenvolvedores do DADA2, incluindo as seguintes etapas: (1) Carregamento dos dados no software; (2) Trimagem dos dados para remoção das bases de baixa qualidade; (3) Filtragem para eliminar sequências que ficaram menores que 160 nucleotídeos; (4) Remoção das redundâncias e identificação das sequências únicas; (5) Eliminação de quimeras e estimação dos erros nos amplicons sequenciados; e (6) Análise da frequência das sequências não redundantes e sua classificação taxonômica, baseando-se em alinhamentos com o banco de dados "Silva release 138" (QUAST *et al*., 2012).

Os arquivos de saída do pacote DADA2 foram utilizados como entrada do pacote phyloseq (MCMURDIE; HOLMES, 2012) para análise dos resultados. Utilizando esse pacote, os gráficos de dimensionamento multidimensionais (MDS) foram gerados para caracterizar a variabilidade entre as replicatas e entre os grupos experimentais. Nesses gráficos, cada ponto representa uma amostra da microbiota nas cinco condições estudadas (G1 a G5). Utilizando o pacote phyloseq, foram também calculados os índices de Chao1, Shannon e Simpson, para comparar a biodiversidade da microbiota intestinal (diversidade alpha). A composição das comunidades bacterianas foi analisada em níveis de filo, família e gênero.

Os dados fastq brutos foram submetidos ao Sequence Read Archive (SRA) do NCBI, sob o número de acesso PRJNA745938.

2.14 Análises estatísticas

A normalidade das variáveis foi determinada pelo teste de Kolmogorov-Smirnov. Para comparação entre dois grupos, na fase de indução da obesidade, foram utilizados o teste T para amostras independentes e amostras com distribuição paramétrica e o teste de Mann-Whitney para amostras com distribuição não paramétrica.

Para comparação entre os cinco grupos experimentais na fase de tratamento, em caso de distribuição paramétrica, foi utilizada a análise de variância (*One Way* ANOVA), seguida do teste complementar de comparações múltiplas de Tukey. Em caso de distribuição não paramétrica, foi realizado o teste de Kruskal Wallis, seguido do teste complementar de comparações múltiplas de Dunn's.

As análises estatísticas foram executadas no software *Package Statistical System 20.0 for Windows Evaluation Version* (SPSS, 2010) e no software *Scientific Data Analysis and Graphing Software* (SIGMAPLOT, 2008), assumindo-se um $p < 0,05$.

Os resultados foram expressos em média ± desvio-padrão, para os dados paramétricos; e em mediana ± intervalo interquartílico, para os dados não paramétricos.

2.15 Aspectos éticos e registro

Este projeto foi aprovado após ser submetido à Comissão de Ética sobre o Uso de Animais instituída pela Universidade Federal de Viçosa (CEUA/UFV), Protocolo Nº 33/2018 (Anexo 1). Todas as Normas do Conselho Nacional de Controle de Experimentação Animal (CONCEA, 2008) foram seguidas.

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3 RESULTADOS

3.1 Artigo de revisão

Título: "Diet-Induced Obesity in Animal Models: Poinst to Consider and Influence on Metabolic Markers".

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Diabetology & Metabolic Syndrome

REVIEW

Open Access

Diet-induced obesity in animal models: points to consider and influence on metabolic markers

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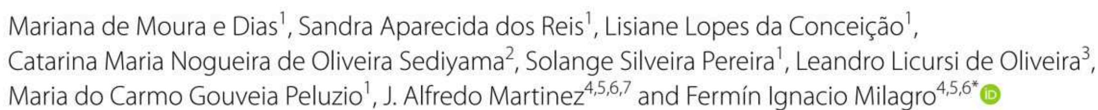

Abstract

Overweight and obesity are a worldwide public health problem. Obesity prevalence has increased considerably, which indicates the need for more studies to better understand these diseases and related complications. Diet induced-obesity (DIO) animal models can reproduce human overweight and obesity, and there are many protocols used to lead to excess fat deposition. So, the purpose of this review was to identify the key points for the induction of obesity through diet, as well as identifying which are the necessary endpoints to be achieved when inducing fat gain. For this, we reviewed the literature in the last 6 years, looking for original articles that aimed to induce obesity through the diet. All articles evaluated should have a control group, in order to verify the results found, and had worked with Sprague–Dawley and Wistar rats, or with C57BL-/-6 mice strain. Articles that induced obesity by other methods, such as genetic manipulation, surgery, or drugs were excluded, since our main objective was to identify key points for the induction of obesity through diet. Articles in humans, in cell culture, in non-rodent animals, as well as review articles, articles that did not have obesity induction and book chapters were also excluded. Body weight and fat gain, as well as determinants related to inflammation, hormonal concentration, blood glycemia, lipid profile, and liver health, must be evaluated together to better determination of the development of obesity. In addition, to select the best model in each circumstance, it should be considered that each breed and sex respond differently to diet-induced obesity. The composition of the diet and calorie overconsumption are also relevant to the development of obesity. Finally, it is important that a non-obese control group is included in the experimental design.

Keywords: Obesity, High-fat diet, Obesogenic diet, Animal model, Inflammation

Introduction

Obesity is a global public health issue with high prevalence in all age groups [1, 2]. It generates a considerable social and economic impact, since it affects people's health and quality of life [2]. Classically, obesity is defined as a visceral and subcutaneous lipid accumulation and body weight gain that may impair health [3, 4]. However, it is frequent to be accompanied by the deposition of lipids (ectopic fat) in non-adipose tissues, such as the liver [5].

The treatment and prevention of obesity involves the control of body weight and adiposity through a negative energy balance in which both, diet and physical activity, are important. But, due to changes in people's lifestyles, with less physical activity and shifts in eating behavior, the study of alternatives for the treatment of obesity, such

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as functional foods, and bioactive compounds, is gaining increasing relevance [2].

Obesity rates are increasingly higher [1, 2], which indicates that the strategies currently used are insufficient to control this disease, and that preclinical studies with this disease are still necessary [6]. To study the development of obesity and its risk factors, researchers use diet-induced obesity animal models, since these models reproduce with greater reliability human obesity in comparison with genetic models [7]. In addition, studies with animal models are carried out under controlled conditions, which facilitates the understanding of the results.

This article aims to evaluate diets-induced obesity models in mouse and rat published in the last 6 years. It seeks to identify which are the main methodological strategies to induce obesity through diet, as well as identifying which are the main parameters to be taken into account to achieve a successful model.

Methodology

The search for articles was carried out manually on PubMed database by a single researcher in February 2020. The combination of the descriptors used was "*diet*" + "*obesity*" and "*high fat diet*" + "*obesity*".

In the PubMed database the following filters were selected: "species/other animals", "case reports", "clinical trial", "clinical trial veterinary", "comparative study", "controlled clinical trial", "evaluation study", "newspaper article", "observational study", "observational study veterinary", "periodical index", "programmatic clinical trial", "randomized controlled trial", "twin study" e "validation study". Duplicated articles were excluded and the rest were evaluated according to the inclusion and exclusion criteria (Table 1). We want to highlight that the aim of this study was to evaluate the induction of obesity through the diet. Therefore, any study that used other ways to induce obesity was excluded. We also highlight that we evaluated only original articles, published between 2015 and 2020, in English, and that worked with Sprague–Dawley and Wistar rats, or with C57BL-/-6 mice strain.

Results and discussion

Selection of articles

Initially, 90474 articles were found: 70658 by using the terms "*diet*" + "*obesity*" and 19816 by the terms "*high fat diet*" + "*obesity*". After using the filters on PubMed database, 1625 articles were found for the "*diet*" + "*obesity*" search and 819 articles for the "*high fat diet*" + "*obesity*" search, totaling 2444 articles. After the exclusion of 812 articles that were duplicated, 1632 articles were considered eligible for reading titles and abstracts. According to the inclusion and exclusion criteria (Table 1), 165 articles were selected for full reading, 1447 articles were excluded and 20 articles were not available for reading, due to restricted access to their abstracts (Fig. 1).

After reading the complete articles, 99 were considered ineligible by some of the exclusion criteria established (Table 1). Another 14 articles were excluded because they did not include a control group and 18 articles were excluded because they did not provide enough information to conclude that the treatment led to obesity. After reading the selected articles, one additional article was included in the study, totaling 35 articles (Fig. 1).

Table 1 Inclusion and exclusion criteria used to evaluate the pre-selected articles

Inclusion	Exclusion
Diet-induced obesity	Genetic manipulation
The main objective was the induction and evaluation of obesity	Drug-induced obesity
Study must be done with Sprague–Dawley or Wistar rats, or with C57BL-/-6 mice strain	Surgically induced obesity
Original articles	Main objective was the induction and/or evaluation of other diseases affect for obesity (diabetes, metabolic syndrome, heart disease, liver disease, dyslipidemia and surgery for weight loss)
Presence of a control group	Main objective was the induction and/or evaluation of other diseases (neurological diseases, cancer, rheumatological diseases, endocrine diseases, gynecological diseases and kidney diseases)
Used commercial diets or produced them from standard ingredients	The objective was to evaluate weight loss
Published in the last 6 years (2015–2020)	Studies in which there was no induction of obesity
Articles in English	Studies in which there was an intervention before the obesity induction period
	Study on pregnancy and/or lactation model
	Study of a smoking model
	Study with humans
	Study with cell culture
	Study with non-rodent animals (ex: dogs, cats, birds, monkeys, rabbits …)
	Review articles, letters to the reviewer and book chapters
	Studies published in languages other than English

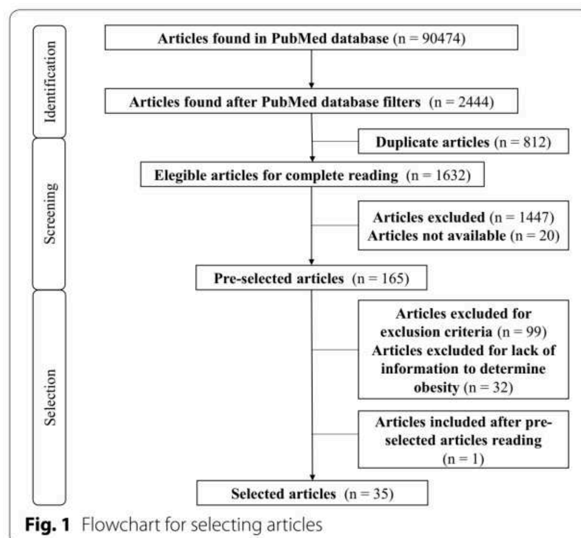

Fig. 1 Flowchart for selecting articles

Studies that used non-commercial diets, such as cafeteria diets, were excluded, since the nutritional composition varied widely compared to diets produced from standardized ingredients and commercial diets.

Processed foods can contain food additives and be low in vitamins and minerals, which can influence the composition of the intestinal microbiota and, consequently, the occurrence of obesity and other metabolic changes. So, in these cases, it is difficult to determine whether a metabolic outcome is only due to the high content of lipids or whether the high amount of food additives or low content of micronutrientes may influence it. In addition, diets produced from food may contain food additives, which make it difficult to assess the real effect of nutrients on the development of obesity [8].

Diet composition

High-fat diets are commonly used to induce obesity in animals [8–10] since they generate adverse metabolic effects, meaning that diet is one of the major contributors to the obesity epidemic [1, 11].

All 35 studies evaluated used a high-fat diet to induce obesity; however, the amount of calories from lipids ranged from 41 to 60% (Table 2). Despite looking like a wide margin, according to Research Diets Inc [14], diet induced-obesity (DIO) animal models usually provides between 45 to 60% of calories from fats; therefore, all selected studies follow this recommendation. Nine studies [15–23] did not provide the composition of the macronutrients directly, which made it difficult to calculate the amount of calories from fat.

The consumption of diets rich in fat can result in the development of human-like obesity, since it increases body adiposity and leptin, and stimulates the development of hypertension and glucose intolerance. Matias et al. [3] observed that offering a diet rich in sugar did not lead to the development of metabolic changes that characterize obesity. On the other hand, offering a diet with an excessive amount of fat leads to an increase in the adiposity index and visceral and body fat gain in comparison with sugar or control diets [3].

In addition, some studies highlighted that in their high-fat diets the main lipid source was saturated fatty acids [2, 3, 8–10, 16, 17, 21–30], while others did not discuss the type of fatty acids used. This information should be available in the articles, since quantity and quality of fatty acids can interfere in the success of obesity induction [8, 10]. Depending on the amount consumed, saturated or long-chain fatty acids can lead to a greater accumulation of body fat through the resynthesis of new triglycerides [27], as well as an increase in the production of inflammatory cytokines, which is a classical change observed in human obesity [27, 31].

The degree of response to the diet depends on its nutritional composition [11]. Additionally, the determination of nutritional composition is important to assess the occurrence of obesity and to evaluate the results considered control/standard. Therefore, control diets must have a nutritional basis similar to obesogenic diets, which helps to interpret the results without bias [8]. That is, the test diet and the control diet should differ only in relation to the specific macronutrient (carbohydrate or fat) used to induce obesity. The micronutrients, fiber and other ingredients must remain the same in order to observe how a specific macronutrient influences or not the outcome of obesity.

The palatability of diets interferes in the amount consumed. Therefore, the consumption of palatable diets (as cafeteria diets) is relevant to the increase in food consumption, including compulsive behavior, and consequently weight gain [8, 15]. Diets rich in salt, sugar and fat are known to have good palatability. The exposure to this type of diet interrupts the expression of clock-genes, modifying the day-night pattern of food intake, as well as changing dopamine signaling [15], which contributes to weight gain.

Caloric excess is essential for the development of obesity [19]. In this sense, although high-fat diets have a high sacietogenic potential, which reduces food consumption, the consumption of a small amount is able to efficiently increase weight and body fat due to the high caloric intake [7].

The diet-offering method directly affects consumption and the ability to induce obesity. Thus, it is likely that when the diet is offered according to the ad libitum or free access methods, food intake is stimulated [19].

Table 2 Characterization of the experimental design of the evaluated studies

Article ID	Obesogenic diet			Control diet			Diet offering method	Intervention time	Animals		
	Type	%	kcal/g	Type	%	kcal/g			Type	Sex	Weeks-old
Asokan et al. 2018 [56]	High-fat◊	60.0 fat	–	Standard	–	–	Ad libitum	10 weeks	C57BL/6J mice	Male	6
Bortolin et al. 2018 [8]	High fat◊	60.0 fat	–	Control	–	–	Ad libitum	18 weeks	Wistar rats	Male	8
	Western◊	42.5 fat	–								
Heo et al. 2018 [2]	High-fat◊	60.0 fat, 20.0 carb., 20.0 protein	–	Low-fat◊	10.0 fat, 70.0 carb., 20.0 protein	–	Free access	10 weeks	Sprague–Dawley rats	Male	5
Hira et al. 2018 [24]	High-fat high-sucrose ■	30.0 fat, 40.0 sucrose	5.1	Control (AIN-93G)	–	4.0	Free access	8 days 8 weeks	Sprague–Dawley rats	Male	5
Lee et al. 2018 [27]	High-fat B-MLCT ■	30.0 fat	5.5	Low-fat B-MLCT ■	7.0 fat	4.0	Ad libitum	16 weeks	C57BL/6J mice	Male	6
	High-fat E-MLCT ■			Low-fat E-MLCT ■							
	High-fat C-MLCT ■			Low-fat C-MLCT ■							
Iñiguez et al. 2018 [25]	High-fat◊	60.0 fat	–	Control	–	–	Pair-feeding	10 weeks	C57BL/6J mice	Male	8
Kazeminasab et al. 2018 [12]	High-fat◊	45.0 fat, 35.0 carb., 20.0 protein	–	Low-fat◊	10.0 fat, 70.0 carb., 20.0 protein	–	Ad libitum	12 weeks	C57BL/6J mice	Male	6
Matias et al. 2018 [3]	High-sugar□	25.9 fat, 52.3 carb., 24.8 protein	3.6	Control□	25.6 fat, 52.3 carb., 21.8 protein	3.5	Free access	20 weeks	Wistar rats	Male	4
	High-fat□	37.6 fat, 44.6 carb., 17.8 protein	4.6								
	High-fat/ high-sugar□	37.4 fat, 43.4 carb., 19.2 protein	4.5								
Miranda et al. 2018 [19]	High-fat	–	4.6	Control	-	3.7	Free access	6 weeks	Sprague–Dawley rats	Male	-
									Wistar rats	Male	-
Rocha Rodrigues et al. 2018 [57]	High-fat◊	71.0 fat, 11.0 carb., 18.0 protein	–	Standard◊	35.0 fat, 47.0 carb., 18.0 protein	-	Pair-fed	17 weeks	Sprague–Dawley rats	Male	6
Son et al. 2018 [21]	High-fat	–	–	Normal (AIN-93)	–	–	Ad libitum	8 weeks	Sprague–Dawley rats	Male	5
Virto et al. 2018 [62]	High-fat●	35.2 fat, 35.5 carb., 20.4 protein	5.4	Control●	3.0 fat, 60.0 carb., 16.0 protein	2.9	Ad libitum	26 weeks	Wistar rats	–	–
Wu et al. 2018 [30]	High-fat◊	23.7 fat, 53.3 carb., 23.0 protein	5.1	Normal○	13.3 saturated fat, 60.5 carb., 26.2 protein	3.6	Ad libitum	6 weeks	C57BL/6 mice	Male	6
Yuan et al. 2018 [35]	High fat◊	45.0 fat, 35.0 carb., 20.0 protein	–	Normal◊	4.0 fat, 62.0 carb., 18.0 protein	-	Ad libitum	18 weeks 24 weeks	C57BL/6J mice	Male	7

Table 2 (continued)

Article ID	Obesogenic diet			Control diet			Diet offering method	Intervention time	Animals		
	Type	%	kcal/g	Type	%	kcal/g			Type	Sex	Weeks-old
Aslani et al. 2017 [63]	High-fat◊	42.0 fat, 39.0 carb., 19.0 protein	–	Normal◊	11.0 fat, 61.0 carb., 28.0 protein	–	Ad libitum	12 weeks	Wistar rats	Male	8
Blancas-Velazquez et al.2017 [15]	High-fat high-sugar	–	–	Control◊	12.0 fat, 65.0 carb., 23.0 protein	2.8	Ad libitum	6 weeks	C57BL/6J mice	Male	6–8
Karimi et al. 2017 [18]	High-fat (AIN-76A)	–	5.5	Standard	–	3.8	Ad libitum	12 weeks 27 weeks	Sprague–Dawley rats	Male	6
Kim et al. 2017 [33]	High-fat◊	60.0 fat	–	Standard◊	12.0 fat, 64.0 carb., 24.0 protein	–	Ad libitum	6 weeks	C57BL/6 mice	Male	4
Kus et al. 2017 [68]	High-fat◊	60.0 fat	–	Control (AIN-93G)	–	–	–	15 weeks	C57BL/6 mice	Male	–
La Frano et al. 2017 [26]	High-fat high-cholesterol◊	60.0 fat	5.2	Low-fat low-cholesterol◊	10.0 fat	3.8	Ad libitum	18 weeks	C57BL/6J mice	Male	12
Pan et al. 2017 [61]	High-fat◊	45.0 fat	–	Normal◊	15.0 fat	–	Free access	16 weeks	C57BL/6J mice	Male	4
Picklo et al. 2017 [10]	High-fat oleic◊ High-fat saturated◊	50.0 fat, 30.0 carb., 20.0 protein	5.5	Low fat◊	16.0 fat, 64.0 carb., 20.0 protein	4.3	–	8 weeks	C57BL/6 mice	Male	12
Yang et al. 2017 [22]	High-fat	–	–	Control	–	–	–	6 weeks	Sprague–Dawley rats	–	–
Choi et al. 2016 [9]	High-fat◊	40.0 fat	–	Normal◊ (AIN-76)	11.5 fat	–	–	12 weeks	C57BL/6J mice	Male	8
Jambocus et al. 2016 [17]	High-saturated fat	–	–	Normal	–	–	Ad libitum	12 weeks	Sprague–Dawley rats	Male	3
Krishna et al. 2016 [1]	High-fat◊	41.0 fat, 40.0 carb., 19.0 protein	–	Standard◊	11.0 fat, 65.0 carb., 19.0 protein	–	Ad libitum	14 weeks	C57BL/6J mice	Male	3
Naidu et al. 2016 [20]	High-fat	–	–	Normal (AIN-93)	–	–	–	30 days	Wistar rats	Male	-
Zhao et al. 2016 [64]	High-fat◊	45.0 fat, 36.0 carb., 19.0 protein	–	Control◊	12.0 fat, 60.5 carb., 27.5 protein	–	–	8 weeks	Sprague–Dawley rats	Male	-
Huang et al. 2015 [16]	High-fat	–	–	Normal	–	–	Ad libitum	12 weeks	Sprague–Dawley rats	Male	–
Li et al. 2015 [51]	High-fat●	45.0 fat	–	Control●	10.0 fat	–	Ad libitum	22 weeks	C57BL/6J mice	Female	6–8
Nam et al. 2015 [28]	High-fat◊	45.2 fat, 38.0 carb., 16.8 protein	4.7	Low-fat◊	11.7 fat, 67.5 carb., 20.8 protein	3.8	Ad libitum	11 weeks	C57BL/6J mice	Male	4
Rodríguez- Rodríguez et al. 2015 [29]	High-fat◊	45.2 fat, 34.2 carb., 20.6 protein	–	Control◊	12.0 fat, 67.4 carb., 20.6 protein	–	Free access	12 weeks	C57BL/6NCrl mice	Male	8

Table 2 (continued)

Article ID	Obesogenic diet			Control diet			Diet offering method	Intervention time	Animals		
	Type	%	kcal/g	Type	%	kcal/g			Type	Sex	Weeks-old
Savetsky et al. 2015 [34]	High-fat◊	60.0 fat	–	Normal◊	13.0 fat	–	Ad libitum	10–12 weeks	C57BL/6J mice	Male	6
Wyatt et al. 2015 [13]	High-fat◊	60.0 fat	–	Control◊	10.0 fat	–	–	5 weeks 8 weeks	C57BL/6J mice	Male	6
Zhang et al. 2015 [23]	High-fat	–	–	Normal (AIN-93 M)	–	–	Ad libitum	16 weeks	C57BL/6 mice	Male	5

□, % g/kg; ■, % w/w; ◊, % kcal; ○, % g/100 g; ●, Not determine the unit of %; B-MLCT, Non-enzymatically interesterified MLCT; E-MLCT, Enzymatically interesterified MCLT; C-MLCT, Commercially MLCT; carb., carbohydrate

On the other side, although the pair-feeding method limits the amount of diet to which animals will have access to, there are cases in which pair-fed diets can achieve different weight gain outcomes, something that has been attributed to the differences in macronutrient composition [32]. In addition, the amount of calories available may be different between groups, which allow high-calorie (obesogenic) diets to have the expected effect when compared to normocaloric diets, regardless of whether they have different palatability and satiety.

The intervention time required for the development of obesity varies widely, ranging from 8 days to 27 weeks (Table 2). Obesity phenotype [3, 30, 33–35], as well as metabolic changes typical of obesity–such as increased glucose intolerance–, [30] becomes more apparent after a longer exposure to an obesogenic diet. According to Blancas-Velasquez et al. [15], after 3 weeks of intervention there is a change in the pattern of fat consumption, which indicates that interventions lasting over 3 weeks may generate better results for the induction of obesity. Matias et al. [3] highlights that the seventh week was a turning point for the increase in weight gain; and Savetsky et al. [34] discussed the importance of a long intervention period, from 10 to 12 weeks, for the consolidation of the phenotypic and metabolic characteristics of obesity.

The consumption of a high-fat diet leads to changes in the composition of the intestinal microbiota [8], which is a classic parameter that usually accompanies the development of obesity [33, 36, 37]. The Western pattern diet, rich in sugar, fat and ultra-processed foods leads to changes in intestinal permeability, which results in an increase in endotoxemia, insulin resistance, steatosis and inflammation of the adipose tissue [38, 39], which results in obesity development [36, 38, 39].

Furthermore, obesity associated intestinal dysbiosis is characterized by a low microbial diversity and an imbalance between the different microorganisms of the intestinal microbiota, with a large number of pathogenic bacteria [8, 36, 39]. In this scenario there is lower production of short-chain fatty acids (SCFA, like acetate, propionate and butyrate) which leads to less protection of the intestinal epithelium, since SCFA are related to occludin and zonulin, and also leads to a drop in the production of glucagon-like peptide-1 (GLP-1) resulting in decreased satiety and increased insulin resistance, inflammation and lipid accumulation [24, 38, 40, 41].

Dysbiosis can also stimulate an excessive production of acetate, which can also contribute to the occurrence of obesity. This scenario occurs since the increase in acetate stimulates the activation of the parasympathetic pathway, which increases the secretion of ghrelin stimulating both an increase in food consumption and a greater secretion of insulin [42].

Therefore, we understand that one of the key factors for the development of obesity through the consumption of a high-fat diet is the alteration of the intestinal microbiota, always aiming for a state of eubiosis, that is, a balance in gut microbiota composition [33, 43].

Experimental animals

The animal species most commonly used for obesity induction through diet is mouse, with isogenic or inbred strains, such as C57BL/6, C57BL/6J, AKR/J, and A/J [7]. In the present study, most of the analyzed studies used C57BL/6J mice (Table 2). Those animals are more susceptible to fat accumulation, gaining body weight and disruptions in glucose metabolism when fed an obesogenic diet [44]. Other strain used was the C57BL/6NCrl, which was developed for the study of lipoprotein and cholesterol metabolism (The Jackson Laboratory©) [45, 46].

Rats are used in DIO studies (Table 2). Sprague–Dawley rats are considered a good model for inducing obesity through diet, since they have a behavior similar to humans with regard to excessive food consumption, which can cause weight gain and changes in lipid metabolism [22]; however, Wistar rats are more susceptible to the development of obesity through diet, since they usually consume a higher amount of high-fat diet than the Sprague–Dawley. Also, differences in lipid metabolism, as fatty acid uptake and lipogenesis, as well as the interaction between genes and diet, make Wistar rats more susceptible to DIO [19].

The age [1] and the sex [47] of the animals can interfere in the development of obesity. Krishna et al. [1] observed that DIO develops better in younger animals. Additionally, when young, speed in weight gain is also greater because elderly animals can adapt metabolically to the increase in adiposity; also, less inflammation is observed in these animals, which causes less glycemic and hepatic alterations [1]. Mice aged 6 to 8 weeks can be considered young adult mice [15].

Young male mice have bigger weight gain than females; however, when they are middle-aged the opposite occurs, and female mice have bigger weight gain than males. Inflammatory genes—upregulated in juvenile males—and hormonal parameters—estrogens can regulate inflammatory pathways in female—may justify the results found [47]. Gene expression in the arcuate nucleus—low in males—[48] and differences in metabolic programming between males and females—related to the expression of genes in the endoplasmic reticulum and hepatic energy metabolism– also contribute to sex-specific weight gain [49].

Male animals are commonly used in the study of obesity; however, if the study aims to evaluate the brown adipose tissue, females should be used, since this tissue is more easily observed in this sex [50, 51]. However, as a limitation that currently exists, it is important to find a model that achieve a similar obesity degree in both, males and females, in order to study both sexes in the same experiment.

Main parameters used to assess the development of obesity

Food intake

Calorie overconsumption leads to an increase in body weight gain and abdominal fat accumulation [2, 10, 15, 19]. These body alterations may lead to deactivation of liver and mesenteric genes responsible for beta-oxidation [25]. Besides, it can deregulate AMPK [2, 35] and SIRT1 proteins in the mesenteric white adipose tissue and skeletal muscle [2]. Additionally, the increase in abdominal fat accumulation can raise blood leptin concentrations [3, 15], leading to leptin resistance. Thus, both the increase in weight and body fat mass generate a cycle that feeds back on itself.

The taste and texture of diets influence the amount of food consumed. Diets rich in processed foods, with high levels of sodium, sugar and saturated fatty acids, are more palatable, which can lead to a higher weight gain in comparison with purified diets even when saturated fatty acids are added to them [52]. Additionally, DIO must contain a low concentration of fibers [25], since these nutrients are capable to induce satiety and increase the production of GLP-1 and SCFA, which stimulate a lower energy consumption [24].

Considering that a spontaneous caloric increase is difficult to achieve in rodents, even when flavored diets are offered [6], DIO must have a high caloric density [16, 30].

In an attempt to induce a voluntary hyperphagia, Blancas-Velazquez et al. [15] provided extra calories to C57BL/6J mice through a 10% water-sugar solution (0.4 kcal/ml). With the sugar solution, the animals had free access to regular chow food, fat-rich pellets, and a bottle of tap water. In this way, the mice could eat what pleased them most, allowing hyperphagia and obesity induction [15]. The problem is that it is difficult to calculate the amount of calories that each animal consumes.

Markers related to body weight and adiposity

Because of the absence of a specific marker and a consensus, for both mice and rats, that defines the presence or absence of obesity, some studies have established their own parameters: the difference of 15% [53] or 20 g in body weight between test and control groups [54]; adiposity index determination [3]; creation of cutoff points [1]; and calculation of body mass index [55].

No article considered the distribution of body composition (calculated by DXA or EchoMRI) as a parameter for detecting obesity. However, the majority of studies consider the differences in total body weight gain as the main parameter to assess the outcome of the development of obesity (Table 3).

When there is no significant difference in body weight gain, other parameters can be considered [3, 56, 57]. In this way, Matias et al. [3] did not observe differences in weight gain after the animals consumed a high-fat/high-sugar diet, but there was a gain in total white adipose tissue, which indicates the occurrence of obesity. Also, Rocha-Rodrigues et al. [57] reported an increase in visceral adipose fat compared to weight gain, as well as an increase in leptin concentration in animals fed a high-fat diet.

Visceral fat is the depot that surrounds the abdominal organs. It is more vascularized, innervated, inflammatory, metabolically active and sensitive to lipolysis, which results in greater release of cytokines, fatty acids and triglycerides. Therefore, in humans it is related to a higher mortality prediction when compared to subcutaneous adipose fat [58, 59]. In Wistar rats the consumption of a high-fat diet appears to lead to an increase in the number of fat cells (hyperplasia) in the subcutaneous adipose fat, whereas in the visceral adipose fat greater hypertrophy of the adipose tissue is observed [60].

Body fat accumulation depends on the connection between different metabolic pathways, as well as the interaction between genes and diet. Wistar rats, for example, have a differential expression of genes in the subcutaneous adipose tissue in comparison with Sprague–Dawley rats, which justifies the higher fat depots found in this breed [19]. The consumption of a high-fat diet leads to an increase in the uptake of fatty acids and lipogenesis [19, 33], resulting in adipocytes hyperplasia [2, 20, 61] and hypertrophy [2, 20, 28, 35, 61]. This increase in adipose tissue can cause tissue hypoxia, which can impair the production and release of obesity regulatory hormones, such as leptin, adiponectin and ghrelin, and exacerbate inflammation [57]. Additionally, body fat increase can cause muscular cell damage, since it enhances cell susceptibility to protein degradation and apoptosis [56]; therefore, DIO is able to cause the metabolic and morphological changes that characterize human obesity.

A low-grade inflammatory condition is often observed in obese animals [1, 2, 27, 28, 34, 35, 56]. This inflammatory status can be triggered by a high consumption of saturated fatty acids, which can be found in high concentrations in obesogenic diets [27]. Diets

Table 3 Changes in markers related to body weight and adipose tissue depots

Article ID	Weight gain	Final body weight	Total adipose tissue/ Body fat	White adipose tissue	Mesenteric fat	Epididymal fat	Perirenal fat	Brown adipose tissue	Lee index
Asokan et al. 2018 [56]	–	HF = Standard	HF = Standard	–	–	–	–	–	–
Bortolin et al. 2018 [8]	HF = Control	HF = Control	–	HF = Control	–	–	–	–	–
	WD > Control	WD > Control	–	WD > Control	–	–	–	–	–
Heo et al. 2018 [2]	HF > LF	HF > LF	–	HF > LF	HF > LF	HF > LF	HF > LF ○	HF = LF	–
Hira et al. 2018 [24] (For 8 days and/or 8 weeks)	HFSuc > Control	HFSuc > Control	–	–	–	–	–	–	–
Iñiguez et al. 2018 [25]	HF > Control	–	HF > Control	–	HF > Control	HF > Control	–	–	–
Kazeminasab et al. 2018 [12]	HF > LF	–	–	–	–	–	–	–	–
Lee et al. 2018 [27]	All HF > All LF	All HF > All LF	All HF > All LF	–	All HF > All LF	All HF > All LF	All HF > All LF	–	–
Matias et al. 2018 [3]	–	HF > Control	HF > Control	–	–	–	–	–	–
	–	HS = Control	HS = Control	–	–	–	–	–	–
	–	HFHS = Control	HFHS > Control	–	–	–	–	–	–
Miranda et al. 2018 [19]	–	HF SD = Control SD	HF SD = Control SD	–	HF SD = Control SD	HF SD > Control SD	HF SD = Control SD	–	–
	–	HF W > Control W	HF W > Control W	–	HF W > Control W	HF W > Control W	HF W > Control W	–	–
	–	HF > Control	HF > Control	–	HF > Control	HF > Control	HF > Control	–	–
Rocha Rodrigues et al. 2018 [57]	–	HF = Standard	–	–	–	–	–	–	HF = Standard
Son et al. 2018 [21]	HF > Normal	–	HF > Normal	–	HF > Normal	HF > Normal	HF > Normal	–	–
Virto et al. 2018 [62]	–	HF > Control	–	–	–	–	–	–	–
Wu et al. 2018 [30]	–	HF > Normal	–	–	–	–	–	–	–
Yuan et al. 2018 [35] 18 semanas	–	HF = Normal	–	–	–	–	–	–	–
Yuan et al. 2018 [35] 24 semanas	–	HF > Normal	HF > Normal	HF > Normal	–	HF > Normal	HF > Normal	–	–
Aslani et al. 2017 [63]	–	HF > Normal	HF > Normal	–	–	–	–	–	HF > Normal
Blancas-Velazquez et al. 2017 [15]	–	HFHS > Control	–	–	–	–	–	–	–
Karimi et al. 2017 [18] 12 semanas	–	HF > Standard	–	–	–	–	–	–	–

Table 3 (continued)

Article ID	Weight gain	Final body weight	Total adipose tissue/ Body fat	White adipose tissue	Mesenteric fat	Epididymal fat	Perirenal fat	Brown adipose tissue	Lee index
Karimi et al. 2017 [18] 27 semanas	HF = Standard	HF > Standard	HF > Standard	–	–	–	–	–	–
Kim et al. 2017 [33]	–	HF > Standard	–	–	–	–	–	–	–
Kus et al. 2017 [68]	–	HF > Control	–	–	–	–	–	–	–
La Frano et al. 2017 [26]	–	HFHC > LFLC	–	–	–	HFHC > LFLC	–	–	–
Pan et al. 2017 [61]	HF > Normal	HF > Normal	–	–	HF > Normal	–	–	–	–
Picklo et al. 2017 [10]	–	HFO > LF	HFO > LF	–	–	HFO > LF	–	–	–
	–	HFSat > LF	HFSat > LF	–	–	HFSat > LF	–	–	–
Yang et al. 2017 [22]	–	HF > Control	–	–	–	–	–	–	HF > Control
Choi et al. 2016 [9]	HF > Normal	HF > Normal	HF > Normal	–	HF > Normal	HF > Normal	HF > Normal	–	–
Jambocus et al. 2016 [17]	HSF > Normal	–	–	–	–	–	–	–	–
Krishna et al. 2016 [1]	–	HF > Standard	HF > Standard	–	–	–	–	–	–
Naidu et al. 2016 [20]	–	HF > Normal	–	–	–	–	–	–	–
Zhao et al. 2016 [64]	HF > Control	HF > Control	–	–	–	HF > Control	HF > Control	–	–
Huang et al. 2015 [16]	HF > Normal	HF > Normal	–	–	–	HF > Normal	HF > Normal	–	–
Li et al. 2015 [51]	–	HF > Control	–	HF > Control	–	–	–	HF > Control	–
Nam et al. 2015 [28]	–	HF > LF	–	–	–	HF > LF	HF > LF	–	–
Rodríguez-Rodríguez et al. 2015 [29]	–	HF > Control	–	–	–	–	–	–	–
Savetsky et al. 2015 [34]	–	HF > Normal	–	–	–	–	–	–	–
Wyatt et al. 2015 [13] (For 5 and 7 weeks)	–	HF > Control	HF > Control	–	–	–	–	–	–
Zhang et al. 2015 [23]	–	HF > Normal	–	HF > Normal	HF = Normal	HF > Normal	–	–	–

HF, High-fat diet; HFHC, High-fat high-cholesterol diet; HFHS, High-fat high-sugar diet; HFSat, High-fat saturated diet; HFSuc, High-fat high-sucrose diet; HFO, High-fat oleic diet; HS, High-sugar diet; LF, Low-fat diet; LFLC, Low-fat low-cholesterol diet; SD, Sprague–Dawley; W, Wistar; WD, Western diet; ○, Perirenal + Retroperitonial

rich in saturated fats can elevate the production of inflammatory cytokines, such as TNF-α [1, 2, 28, 35, 56] and IL-6 [56], as a consequence of the hypertrophy of the adipocytes [2, 35], leading to an infiltration of macrophages and dendritic cells in adipose tissue [1].

This inflammatory condition contributes to the development of metabolic disorders [1, 35], such as diabetes [27]; to a decrease in lymphatic function and cutaneous hypersensitivity [34]; and to the occurrence of other diseases, such as periodontitis [62] and respiratory allergies [63]. Furthermore, the immune system can also be altered, with an improvement in this system when there is a modulation of the production of inflammatory cytokines [64].

The consumption of a high-fat diet can also reduce the brown adipose tissue, since it may inhibit the biosynthesis of fatty acids and increase oxidative stress and cell apoptosis [51]; therefore, a high-fat diet can stimulate the development of white adipose tissue [2, 8–10, 16, 19, 21, 23, 25–28, 35, 51, 61, 64] and suppress the development of the brown adipose tissue [51]. This is relevant since the brown adipose tissue, in humans, is negatively correlated with the body mass index and with central obesity markers which suggests that low levels of brown adipose tissue may be indicator of obesity and obesity-related diseases [65].

In addition, the increase in brown adipose tissue may be a strategy to fight obesity, since higher levels of brown adipose tissue indicate greater energy expenditure, with consequent weight loss [65, 66]. Therefore, low levels of brown adipose tissue can contribute to the perpetuation of obesity.

Glycemic markers

Higher values in blood glucose [8, 9, 16, 17, 20, 23, 27, 29, 51] and insulin concentrations [1, 2, 8, 9, 16, 17, 20, 23, 25–27, 29] as well as in HOMA (homeostatic model assessment) index [2, 8, 23–25, 27, 29, 57] have been observed in the groups that consumed an obesogenic diet. Similarly to obesity, for an effective induction of insulin resistance, a long period of intervention with DIO must occur [25]. In this sense, the absence of changes in these parameters in some studies can be justified by the short intervention period (Table 2).

Changes in the glycemic parameters occur because the insulin metabolism is unable to adapt to the damage caused by the chronic excess of calories offered by the obesogenic diet, which gradually deteriorates insulin activity, leading to insulin resistance and subsequent type 2 diabetes development [67]. Also, the obesogenic diet can induce fat (ectopic) accumulation in the pancreas, which stresses greatly beta cells, disrupting insulin production [23, 27]. This accumulation in organs other than the adipose tissue, such as the liver, can also lead to insulin resistance and hyperglycemia, since saturated fatty acids interfere in the activity of insulin receptor and glucose transporters [23]. Additionally, the mitochondria of the brown adipose tissue are also affect by DIO impairing glucose metabolism [51].

Serum lipid profile

Triglycerides are the main component of adipose tissue; therefore, high serum concentrations of this lipid may indicate the presence of metabolic changes [21]. Likewise, serum concentration of cholesterol is also an important parameter for the assessment of obesity, since the greater the availability of serum cholesterol, the greater the deposition of fatty acids into the adipose tissue and the liver [33].

In this way, some of the evaluated studies noticed an increase in the serum concentration of triglycerides [1, 2, 9, 16, 17, 21–23, 25, 35, 51, 61, 63] and cholesterol [2, 9, 16, 23, 27, 29, 35, 51, 61, 63] in the groups fed with DIO. This change seems to be especially related to saturated fatty acid-rich [27] obesogenic diets (Table 2). Additionally, diets with high concentration of long-chain fatty acids can also alter the serum lipid profile, since, after hydrolysis, these fatty acids can be used for the synthesis of new triacylglycerol molecules [27].

Liver health

Liver health, measured through hepatic triglycerides, can be impaired by the development of obesity. Hepatic steatosis happens because the excess of fat present in the body is stored in this organ causing intracytoplasmatic accumulation of triglycerides. This ectopic accumulation occurs as a consequence of the downregulation of AMPK [35, 61] and upregulation of SREBP-1c [61], which generates lipogenesis, and increases the synthesis of fatty acids by the liver [23]. Furthermore, beta-oxidation is downregulated, which increases the hepatic lipid stocks [16, 23, 25]. Thus, the reduction in this parameter is positive for the treatment of obesity, with the expression of lipogenic genes conditioned [21].

As the amount of stored fat increases, the liver starts to suffer oxidative damage [16] and, as a result of hepatocyte lysis, the serum concentrations of the enzymes alanine aminotransferase and aspartate aminotransferase increase [16, 21, 68]. Thus, the increase in the concentrations of both aminotransferases can be associated with the increase in liver weight as well as to hepatic steatosis [25]. In this context, it has been described that high-fat diet (71% of kcal fat) induces similar degree and pattern of steatosis and liver triglyceride content in male Wistar and Sprague–Dawley rats, being males more susceptible than females [69].

Conclusions

In the present study, we included studies that used Sprague–Dawley and Wistar rats, as well as C57BL-/-6 mice, because these are the main models used for DIO. This strategy can be considered a limitation of the study, since other rodent models may also be prone to diet-induced obesity; however, they are not widely used. In fact, the biggest difficulty to find an effective model for DIO is the lack of standardization among obesity-inducing protocols. Different times of intervention, diets, types of fat and carbohydrates, animal strains, and sex, among others, are used in the studies, which makes it difficult to compare the results and to better evaluate and determine the best way to induce obesity in an animal model.

Among the animal obesity models, those that develop a phenotype more similar to human physiopathology are those induced by dietary challenge; in this context, better results are obtained through high-fat diets with high concentrations of saturated fatty acids, since these diets directly affects the metabolism, are palatable and have a high caloric density, which stimulates weight and body fat gain.

To choose an animal model for a study of diet-induced obesity, it should be considered that rats and mice respond differently to this type of diet; in addition, strain, sex and age, affect the response to the obesogenic diet, with young animals and males being more sensitive to obesity-related comorbidities.

The markers used to assess the development of obesity include body weight and fat (total, subcutaneous and visceral) gain, but other parameters related to inflammation, hormone concentration, blood glycemia, lipid profile, and liver health are often desired. It is suggested that these markers should be used together, since the presence of more than one of these markers reinforces the determination of obesity. Changes in the release of inflammatory cytokines are used to justify the symptoms found, not being a determining parameter for the induction of obesity. As there are no cutoff points for any of these parameters in animals, researchers should always conduct their studies with a non-obese control group so that the results can be compared.

Abbreviations

DIO: Diet induced-obesity; DXA: Dual energy X-ray absorptiometry; GLP-1: Glucagon-like peptide-1; SCFA: Short chain fatty acids.

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Authors' contributions

MMD has been responsible for conception and design and for aquisition, analysis and interpretation of data. LLC, CMNOS, SSP, LLO and MCGP have been involved in drafting the manuscript and revising it critically. SAR, JAM and FIM have revised the manuscript critically, giving importante intelectual content. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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3.2 Artigo original

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Autores: Mariana de Moura e Dias, Sandra Aparecida dos Reis Louzano, Lisiane Lopes da Conceição, Rayssa da Conceição Fernandes, Tiago Antônio de Oliveira Mendes, Solange Silveira Pereira, Leandro Licursi de Oliveira e Maria do Carmo Gouveia Peluzio.

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Antibiotic Followed by a Potential Probiotic Increases Brown Adipose Tissue, Reduces Biometric Measurements, and Changes Intestinal Microbiota Phyla in Obesity

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Abstract
The development of adjuvant therapies for obesity treatment is justified by the high prevalence of this disease worldwide, and the relationship between obesity and intestinal microbiota is a promising target for obesity treatment. Therefore, this study aimed at investigating the adjuvant treatment of obesity through the use of potential probiotics and antibiotics, either separately or sequentially. In the first phase of the experiment, animals had diet-induced obesity with consumption of a high saturated fat diet and a fructose solution. After this period, there was a reduction in caloric supply, that is the conventional treatment of obesity, and the animals were divided into 5 experimental groups: control group (G1), obese group (G2), potential probiotic group (G3), antibiotic group (G4), and antibiotic followed by potential probiotic group (G5). The adjuvant treatments lasted 4 weeks and were administered daily, via gavage: Animals in G1 and G2 received distilled water, the G3 obtained *Lactobacillus gasseri* LG-G12, and the G4 received ceftriaxone. The G5 received ceftriaxone for 2 weeks, followed by the offer of *Lactobacillus gasseri* LG-G12 for another 2 weeks. Parameters related to obesity, such as biometric measurements, food consumption, biochemical tests, histological assessments, short-chain fatty acids concentration, and composition of the intestinal microbiota, were analyzed. The treatment with caloric restriction and sequential supply of antibiotics and potential probiotics was able to reduce biometric measures, increase brown adipose tissue, and alter the intestinal microbiota phyla, standing out as a promising treatment for obesity.

Keywords Obesity · Probiotics · Antibiotics · Intestinal microbiota

Introduction

The standard nutritional treatment for obesity is based on the change of eating and living habits, decreasing caloric consumption and increasing energy expenditure [1–4].

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Since 2004, a relationship has been observed between the risk for the obesity development and the intestinal microbiota composition [5]. In this scenario, the modulation of this microbiota is suggested to be functional as an adjuvant treatment for obesity [6, 7].

This intestinal microbiota is formed by archaea, fungi, helminths, viruses, and other microorganisms [8]. In healthy adult individuals, this microbiota can encompass more than 100 trillion microorganisms, which are predominantly anaerobic bacteria, belonging mainly to *Firmicutis* and *Bacteroidetes* phyla [9, 10]. This preeminent diversity of intestinal microbiota leads to a lower propensity for alterations within this microbiota by external events [7], i.e., lesser constant imbalances related to the increased risk for obesity development [6, 11–16], as well as another diseases like diabetes [17], non-alcoholic fatty liver disease [18], or polycystic ovary syndrome [19].

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Probiotics are considered potential modulators for the intestinal microbiota composition, and their use in the adjuvant treatment of obesity has been investigated by the scientific community [6, 20–22]. These are live microorganisms which, when consumed in adequate quantities, confer a beneficial upshot in the host health [23]. Synthesized antibiotics are other potential modulators [6, 24–27] which mainly act on inhibiting the growth of specific microorganisms, depending on their mode of action [28] and, in this way, can substantially effect on the gut microbiota composition [7].

Among the different probiotic microorganisms, the genus *Lactobacillus* stands out with a large number of species considered probiotic [21], such as *Lactobacillus gasseri*. It is of human origin and, when administered in adequate amounts, is able to survives to the passage through the intestine [29], having necessary characteristics to be considered a probiotic [30]. In addition your regular consumption can help with weight loss [29, 31–33], being interesting for the treatment of obesity. Mechanisms that explain the capacity to treat the obesity involve modulation of the intestinal microbiota, decreased of adipose tissue inflammation, of systemic inflammation and of intestinal permeability [29, 31–33].

Obesity is a worldwide public health issue, which has elevated prevalence and socioeconomic impact [34]. This medical condition triggers an increase in early mortality, augmenting the mortality cases as a whole, since obesity is linked to the occurrence of chronic non-communicable diseases, such as cardiovascular disease and diabetes [2, 35].

Considering the complexity of obesity and the urge to improve the treatments already available about this medical condition, this study aimed at investigating innovative alternatives to enhance treatments for obesity and its secondary metabolic changes in an animal model by modifying the composition of the diet, associating it with adjuvant treatment as well as with a potential probiotic and/or antibiotic.

Materials and Methods

Test Products

Lactobacillus gasseri LG-G12 was used as a potential probiotic (Lemma®, São Paulo, Brazil), in the dosage of 10^9 colony forming units (CFU)/day, which is the minimum dose to be offered to have a probiotic claim according to Agência Nacional de Vigilância Sanitária (Anvisa) [30]. The calculation to acquire this quantity was carried out according to information provided by the manufacturer. This potential probiotic was chosen due to the positive effects of different strains of *Lactobacillus gasseri* on controlling excessive weight and body adiposity [29, 31–33, 36] and due to the fact that it has characteristics to be considered a probiotic [30], such as its human origin and its ability to survive intestinal transit [29]. In addition, it is observed that different strains of *Lactobacillus gasseri* decreases systemic inflammation and intestinal permeability [29, 31–33] which are recognized mechanisms made by probiotics in the control of obesity [4, 6, 37].

The selected antibiotic was ceftriaxone disodium heptahydrate (Triaxton, Blau Famacêutica S/A®, Cotia, Brazil) in the dosage of 500 mg/kg of each mice body weight [14]. This antibiotic acts mainly against gram-negative bacteria, which have been linked to an increased risk for obesity development [38]. In addition, ceftriaxone is not absorbed by the body, acting only locally in the small intestine and colon [39, 40].

Experimental Design

The study was approved by the Ethics Committee on Animals Use of Universidade Federal de Viçosa, under protocol 33/2018. The principles recommended by the Nacional Council for the Control of Animal Experimentation were followed [41].

Forty C57BL/6 J mice from the Central Vivarium of the Biological and Health Sciences Center, Universidade Federal de Viçosa, Minas Gerais, Brazil, were used in this experiment.

Throughout the experiment, the animals remained in collective cages (8 animals/box), in an environment with controlled temperature (22 °C ± 2 °C) and a 12-h photo period. The animals had free access to water/fructose (Synth®, Diadema, Brazil) solution and to food, administered according to the pair-feeding scheme.

Obesity induction (phase 01) was achieved by randomly dividing the animals into two groups: obese group ($n = 32$) and control group ($n = 8$). During this 12-week phase, the animals in the obese group received a high-fat diet with 60% of calories from saturated fat [42] and 10% fructose solution (Synth®) [43], which replaced drinking water (0.4 kcal per mL of solution consumed). The control group was fed with AIN-93M [44] diet, and distilled water was provided.

After phase 01, a significant difference ($p < 0.005$) in body mass between the groups was noticed indicating obesity induction. Consequently, the animals in the obese group were redistributed into 4 experimental groups ($n = 8$) for the treatment commencement (phase 02). Phase 02 lasted for 4 weeks, and all animals received a standard AIN-93M diet and distilled water during that period, as well as supplementary adjuvant treatments with potential probiotic and/or antibiotic. This diet modification represents a change in the composition of the animals' diet in order to offer lower caloric input, which represents the standard nutritional treatment for obesity.

The adjuvant treatments were administered daily, via gavage. Animals in the control (G1) and obese (G2) groups

received distilled water, while the animals in the potential probiotic group (G3) obtained 10^9 CFU of *Lactobacillus gasseri* LG-G12 (Lemma®)/day and the animals in the antibiotic group (G4) received 500 mg of ceftriaxone (Blau Famacêutica S/A®)/kg. The group supplied with antibiotics and potential probiotics (G5) received 500 mg of ceftriaxone (Blau Famacêutica S/A®) /kg for 2 weeks, followed by the offer of 10^9 CFU of *Lactobacillus gasseri* LG-G12 (Lemma®) for another 2 weeks.

Throughout the experimental period, animal's body weight was measured weekly and food consumption was recorded every 3 days. Body measurements, including abdominal perimeter (measured immediately before the hind legs) and thoracic perimeter (deliberated immediately after the front legs) were assessed at the end of phases 01 and 02, with the aid of an inelastic measuring tape. The abdominal perimeter/thoracic perimeter ratio, the Lee index, and the body fat percentage were calculated.

After 4 months of experiment, the animals were submitted to 12 h of fasting, anesthetized with isoflurane 3% (Cristália®, Itapira, Brazil) and submitted to total exsanguination. Samples of blood, feces, intestines, and adipose tissue were collected and stored for further analysis.

Evaluation of Biochemical Parameters

Total cholesterol, high-density lipoprotein (HDL-cholesterol), triglycerides, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were determined in serum samples, by a colorimetric enzymatic method, using commercial kits (Bioclin®, Belo Horizonte, Brazil) according to procedures described by the manufacturer. The samples were analyzed in an automatic analyzer (Mindray Medical International Limited, BS 200 model, Shenzhen, China).

The concentrations of low-density lipoprotein (LDL-cholesterol) and very low density lipoprotein (VLDL-cholesterol) were calculated according to equations proposed by Friedwald and collaborators [45].

Histological Analysis

Fragments of abdominal adipose tissue were dehydrated, in increasing gradients of ethanol, and included in Paraplast Plus (Sigma®, Buchs, Switzerland). Subsequently, 5 µm histological fragments were obtained in a rotating microtome (model CUT 4055, Olympus®, Miami, Florida, EUA) and stained with both hematoxylin and eosin.

Intending to measure the area and the number of adipocytes, 10 fields per animal were captured through the 10× objective, directly from the light microscope (Primo Star 2012, Zeiss®, Oberkochen, Germany), using the Aixo ERc5s video camera (Zeiss®). The images were analyzed using Image Pro-Plus® software version 4.5 (Media Cybernetics, Rockville, Maryland, USA). All cells that were complete in the photo field portraying well-defined contours were counted [46], and the diameter of twenty cells per field was measured. Moreover, the cell diameter was measured in replicate in different positions.

Inflammatory infiltrate counting was similarly performed. In this assessment, 10 fields per animal were captured through the 20× objective [47]. The images were also analyzed using Image Pro-Plus® software version 4.5 (Media Cybernetics), seeking to identify the presence of inflammatory infiltrates at the intersections of a grid with 315 points.

Tissue samples were removed, fixed in Carson's formalin [48], dehydrated in an increasing ethanol gradient, and included in hydroxyemethylmethacrylate resin (Historesin, Leica®, Zürich, Switzerland) to carry out the morphometric analysis of the small intestine. Slices with 5 µm were obtained on a rotating microtome RM2265 (Leica®) and stained in a solution of Alcian blue (AB) and Schiff's periodic acid (PAS) [49].

The photomicrographs were captured with a 10× objective directly from the LEICA DM750 light microscope (Leica®) using a LEICA 170HD video camera (Leica®). Analyzes were performed with the aid of Image Pro-Plus® software, version 4.5 (Media Cybernetics) morphometric, with the villus height being measured merely in the villi with well-defined epithelium and visible connective tissue.

Villus width was measured at 3 different points (apical, medial, and basal) in the same villus; the mean of these points was considered as the final outcome. Crypt depth was estimated through the images where the base and the apex (opening) of the crypt were discernable [50].

Determination of Intestinal Permeability

After the end of phase 02, the animals received via gavage 200 µL of a solution containing 13.3 mg of lactulose (Daiichi Sankyo®, Barueri, Brazil) and 10.1 mg of mannitol (Synth®, Diadema, Brazil). All urine excreted in the next 24 h was collected [51] with the aid of metabolic cages. During this period, the animals remained fasting.

The quantification of sugars was performed using high-performance liquid chromatography (detector model RID 10A, Shimadzu®, Tokyo, Japan) at a wave length of 210 nm. For chromatographic separation, 20 µL of sample was injected into a mobile column (Aminex model HPX-87H, Bio-Rad®, São Paulo, Brazil), 300 mm long × 7.8 mm in diameter, with a flow of 1 mL/min and pressure of 54 Kgf. Water was used as a mobile phase.

Determination of Short Chain Fatty Acids

The extraction of short chain fatty acids (acetic, propionic, and butyric), from the caecal content, was carried out

according to methodology proposed by Siegfried and collaborators [52].

Readings were performed on a high-performance liquid chromatograph (Ultimate 3000, Dionex, Thermo Fisher Scientific®, Waltham, Massachusetts, USA). For chromatographic separation, samples were injected in a column (RezexROA-Organic Acid H+ (8%), Phenomenex®, Torrance, California, USA) with a length of 300 mm and a diameter of 7.8 mm. The mobile phase used was 5 mmolar of sulfuric acid, the injection flow was 0.7 mL/min, the injection volume equaled to 20 μL, and 45 °C was set as oven temperature. The detector used was Rid RH01 (Shodex®, Tokyo, Japan).

Determination of Intestinal Microbiota Composition

After phase 02, fecal samples were collected from all experimental groups, forming a pool of feces from each group, which was used in the analysis of the intestinal microbiota composition. The samples were analyzed in triplicate, and the pool of feces was used because the animals are isogenic and live in a controlled environment, which means that they are biological replicates.

Initially, DNA from the 16S RNAr region was extracted from samples in accordance with the methodology adapted from Zhang and collaborators [53]. After extraction, the concentration in Qubit was quantified and the quality was verified through electrophoresis in 1.8% agarose gel. Samples denoting an absorbance ratio at 260/230 wave lengths greater than 1.8 were considered suitable for the sequencing step.

DNA sequencing was performed by Macrogen Company (Macrogen Inc®, Seoul, South Korea) using the Illumina MiSeq sequencer (Illumina, San Diego, CA, USA). The amplicons were obtained by PCR, utilizing specific primers (Bakt 341F and Bakt 805R) for V3 and V4 regions of 16S gene. The raw sequencing data (files in the FASTQ format) were trimmed by the Trimmomatic v 0.36 program [54] with a cut off of Phred Quality by 30.

Trimmed data were processed using the DADA2 package version 1.8 [55] on R platform, version 3.6.1 (https://cran.r-project.org/). The data processing followed all the steps recommended by the DADA2 developers, including the following: (1) loading data into software, (2) trimming data to remove low quality bases, (3) filtering to eliminate sequences that were smaller than 160 nucleotides, (4) removing redundancies and identifying unique sequences, (5) eliminating chimeras and estimating errors in sequenced amplicons, and (6) analyzing the frequency of non-redundant sequences and their taxonomic classification, based on alignments performed with the Silva release 138 database [56].

The output files from DADA2 package were used as input to phyloseq package [57] to analyze the results. Using the phyloseq package Chao1, Shannon and Simpson indices were calculated to compare the biodiversity of the intestinal microbiota. Bacterial communities' composition was analyzed at the phylum, family and gender level.

Statistical Analysis

Data normality was determined using the Kolmogorov-Smirnov test. Comparison in phase 01 was performed by T test for independent samples. Comparisons between the experimental groups, in phase 2, were performed by the variance test (one-way ANOVA), followed by Tukey's multiple comparisons, or employing the Kruskal-Wallis test followed by Dunn's multiple comparison test. Statistical analyses were performed with the Package Statistical System 20.0 for Windows Evaluation Version software (StataCorp LLC®, College Station, Texas, USA, 2010) and Scientific Data Analysis and Graphing Software 11.0 (IBM®, Armonk, New York, USA, 2008), assuming $p < 0.05$. The results were expressed according to the normality of the variables, i.e., mean ± standard deviation and median ± interquartile range. The figures were made in the GraphPad Prism 5 for Windows Software (GraphPad®, San Diego, CA, USA, 2007).

Results

Biometric Parameters

During the treatment phase, no difference was perceived in body mass gain between the experimental groups ($p = 0.073$; Fig. 1), although lower body mass in G1 was observed when

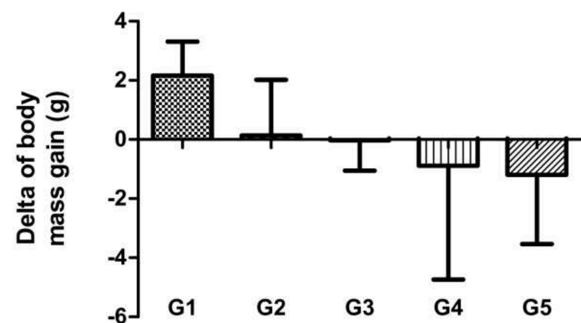

Fig. 1 Difference in mass gain during treatment. **G1** control group, **G2** obese group, **G3** potential probiotic group, **G4** antibiotic group, and **G5** antibiotic followed by potential probiotic group. Results expressed as mean and standard deviation. One way ANOVA

Table 1 Antibiotic followed by potential probiotic group has differences in biometric measurements and somatic indexes at the end of treatment phase

Measurements	G1	G2	G3	G4	G5	p
Abdominal perimeter (AP) (cm)	7.00 ± 0.50^{ab}	7.50 ± 0.50^{a}	7.50 ± 0.63^{a}	7.00 ± 0.00^{ab}	6.50 ± 1.00^{b}	0.002§
Thoracic perimeter (AT) (cm)	7.00 ± 0.50^{a}	7.00 ± 0.50^{ab}	7.00 ± 0.50^{a}	6.50 ± 0.50^{ab}	6.00 ± 0.50^{b}	0.000§
AP/TP	1.00 ± 0.04	1.07 ± 0.23	1.07 ± 0.07	1.08 ± 0.01	1.00 ± 0.08	0.050§
Lee index	0.34 ± 0.02^{a}	0.33 ± 0.01^{ab}	0.33 ± 0.01^{ab}	0.33 ± 0.01^{ab}	0.32 ± 0.01^{b}	0.046
Body fat (%)	0.77 ± 0.26	0.70 ± 0.11	0.92 ± 0.10	0.64 ± 0.23	0.81 ± 0.28	0.179
Total adipose tissue (g)	0.99 ± 0.33	0.93 ± 0.12	1.22 ± 0.05	0.88 ± 0.22	1.18 ± 0.32	0.050
Brown adipose tissue (g)	0.27 ± 0.09^{abc}	0.24 ± 0.04^{a}	0.34 ± 0.03^{bc}	0.28 ± 0.07^{abc}	0.37 ± 0.07^{bc}	0.002
Epididymal adipose tissue (g)	0.70 ± 0.20	0.56 ± 0.10	0.71 ± 0.07	0.53 ± 0.23	0.71 ± 0.27	0.243
Abdominal adipose tissue (g)	0.14 ± 0.01^{a}	0.12 ± 0.01^{ab}	0.17 ± 0.04^{ab}	0.08 ± 0.03^{b}	0.10 ± 0.04^{b}	0.000

G1 = control group, G2 = obese group, G3 = potential probiotic group, G4 = antibiotic group; G5 = antibiotic followed by potential probiotic group. Different letters indicate significant differences ($p < 0.05$). Results expressed as mean and standard deviation for samples with parametric distribution. One-way ANOVA followed by Tukey's post hoc test. § = Results expressed as median ± interquartile range for samples with non-parametric distribution. Kruskal-Wallis test followed by Dunn's post hoc test

Different superscripts letters (a, b, c) on the same line indicate differences between groups ($p < 0.05$)

compared with the other experimental groups (data not shown, $p = 0.005$) at the beginning of that phase.

G5 exhibited lower values of abdominal and thoracic perimeters in comparison to G3, and inferior values of Lee's index when compared with G1 (Table 1). In addition, G5 displayed higher amounts of brown adipose tissue than G1 ($p = 0.046$) and G2 ($p = 0.003$) and lower amounts of abdominal adipose tissue than G1 ($p = 0.034$).

Food and Caloric Consumption

After 4-week treatment, no significant differences were noticed between food consumption ($p = 0.155$) and caloric consumption ($p = 0.154$).

Biochemical Parameters

Lower values of triglycerides and VLDL-cholesterol were observed in G5 when compared with G2 and G4 (Table 2).

Histological Assessment

Differences in abdominal adipose tissue per area ($p = 0.441$), number of adipocytes ($p = 0.098$), and the presence of inflammatory infiltrates ($p = 0.256$) were not found between the different experimental groups.

In the small intestine, G4 showed more elevated villus height ($p = 0.004$) and greater mucosa depth ($p = 0.001$) when compared with G1.

Table 2 Antibiotic followed by potential probiotic group has differences in biochemical parameters

Parameter	G1	G2	G3	G4	G5	p
CT (mg/dL)	120.12 ± 9.59	127.42 ± 21.51	136.26 ± 12.34	144.53 ± 55.04	114.07 ± 10.08	0.167
HDL (mg/dL)	64.42 ± 6.02	73.68 ± 5.69	77.46 ± 7.71	81.22 ± 31.27	63.20 ± 5.29	0.153
TG (mg/dL)	76.66 ± 11.20^{ab}	110.56 ± 14.55^{a}	98.13 ± 16.73^{ab}	94.80 ± 44.46^{a}	56.58 ± 5.96^{b}	0.007
LDL (mg/dL)	40.36 ± 7.22	31.62 ± 7.76	39.17 ± 3.37	44.35 ± 16.63	39.56 ± 14.22	0.249
VLDL (mg/dL)	15.33 ± 2.24^{ab}	22.11 ± 2.91^{a}	19.63 ± 3.35^{ab}	18.96 ± 8.89^{a}	11.32 ± 1.19^{b}	0.007
AST (U/L)	185.17 ± 39.61	138.48 ± 10.71	151.01 ± 17.97	156.80 ± 60.08	190.00 ± 41.50	0.059
ALT (U/L)	44.96 ± 4.00	41.80 ± 2.50	46.88 ± 2.00	48.00 ± 32.00	45.33 ± 17.33	0.068§

CT = total cholesterol, HDL = HDL-cholesterol, TG = triglycerides, LDL = LDL-cholesterol, VLDL = VLDL-cholesterol, AST = aspartate aminotransferase, ALT = alanine aminotransferase, G1 = control group, G2 = obese group, G3 = probiotic group, G4 = antibiotic group, G5 = antibiotic followed by potential probiotic group. Different letters on the same line indicate differences between groups. Results expressed as mean and standard deviation. One-way ANOVA followed by Tukey's post hoc test. § = Results expressed as median ± interquartile range for samples with nonparametric distribution. Kruskal-Wallis test followed by Dunn's post hoc test

Different superscripts letters (a, b) on the same line indicate differences between groups

Determination of Intestinal Permeability

Intestinal permeability presented no alterations when exposed to different treatments. This could be proved by the absence of difference in the lactulose/mannitol ratio among all treatments. Nevertheless, G4 excreted higher quantities of lactulose than G1 ($p = 0.014$) and inferior amounts of mannitol to G3 ($p = 0.001$) and G5 ($p = 0.005$).

Concentration of Short-Chain Fatty Acids

G4 portrayed a lower cecal concentration of acetic acid than the other groups ($p = 0.000$) while G5 had a higher concentration of butyric acid than G2 and G4 ($p = 0.002$). No difference was observed among groups for propionic acid ($p = 0.073$).

Analysis of Intestinal Microbiota

Intestinal microbiota composition did not differ between experimental groups according to the diversity indexes ($p > 0.05$; Fig. 2). However, despite of the apparent microbiota homogeneity, certain taxa exhibited variations in their frequency: The *Bacteroidetes* phylum was smaller in G2 than other groups ($p < 0.05$), and G4 presented higher frequency within this phylum than G5 ($p = 0.012$). For the *Firmicutis* phylum, G2 showed values inferior to G1 ($p < 0.001$), G4 ($p < 0.001$), and G5 ($p = 0.033$), while G4 had more elevated frequency than G3 ($p = 0.005$). Regarding the *Clostridiales* order, minor values were observed in G2 in comparison with the other groups ($p < 0.05$) and higher values in G4 compared with G5 ($p = 0.012$). Moreover, G2 presented lower levels of *Bacteroidetes* genus than G1, G3, and G4 ($p < 0.001$), while G5 had less *Bacteroidetes* than G2 ($p = 0.005$) and G4 ($p = 0.012$; Fig. 2).

Discussion

Obesity is a complex and multifactorial disease which has a treatment based on caloric deficit for loss of body mass. Thus, additional strategies may be required to satisfactorily enhance this treatment. Therefore, the present study provided an adjuvant treatment with *Lactobacillus gasseri* LG-G12 (Lemma®) and ceftriaxone (Blau Famacêutica S/A®) separately (potential probiotic and antibiotic groups, respectively) and sequentially (antibiotic followed by potential probiotic group) in addition to the standard nutritional treatments with caloric restriction.

Regarding body mass, other studies have also found no significant change in weight gain during four weeks treatment, which may indicate that body weight is a parameter that needs a longer period of intervention time to be altered. Kong and collaborators [22] pointed out a lack of difference in body mass, between groups fed a normal diet or a high-fat diet, after offering probiotics for 4 weeks. In addition, Vaughn and collaborators [16] noticed accumulation of body fat when samples were exposed to a high-fat diet for 7 days, but no alterations were observed regarding weight gain.

Contrary to expectations, modifying the rodents diet [44] to standard nourishment with lower caloric supply in phase 2 [1–3] may also have contributed to the absence of differences in relation to body mass. High sugar diets, when compared with high fat diets, present more noticeable impacting changes on both obesity-related intestinal microbiota [22] and AIN93 diet, presenting 75.9% of calories from carbohydrates.

The abdominal perimeter reflects the visceral fat content and is associated with total body fat, standing out as an important parameter for the assessment of central obesity [1]. In this context, minor levels of abdominal perimeter value found in G5 may be resulted from the action of ceftriaxone against Gram-negative bacteria [38, 58, 59], which indicates that changes in the intestinal microbiota were responsible for the reduction of adiposity [60]. A similar mechanism was described by Choi and collaborators [61], who discussed that the reduction of fat liver and triglyceride levels after a treatment with *Lactobacillus plantarum* LMT1-48, with a consequent diminution of body mass and adipose tissue, is justified by the modulation levels of lipopolysaccharides [61]. Since these are molecules present in Gram-negative bacteria, the modulation of those can trigger a chronic low-grade inflammation [19, 61], highlighting that this process is a classic way of developing obesity [4, 37, 38, 61].

Greater amounts of brown adipose tissue were found in G5, which is a positive outcome since this tissue maintains body temperature above room temperature, produces heat from thermogenesis, increases metabolic rates, and regulates metabolic risk factors [62]. Similar to these results, a study performed by Park and collaborators [63] with the probiotic *Lactobacillus amylovorus* KU4 demonstrated certain protection against obesity by stimulating the production of brown adipose tissue and enhancing the metabolic effects resulted from this process. The increase in terms of body temperature, mitochondrial function, rate of oxygen consumption, expression of uncoupling protein 1 (UCP-1), and in expression of the receptor activated by alpha-type peroxisomal proliferators (PPAR-γ) [63] was also evidenced, reinforcing the importance of the brown adipose tissue in the resistance against obesity.

Obesity leads to changes in lipid metabolism, including increased levels of triglycerides and LDL-cholesterol and reduced HDL-cholesterol [64]. Thus, the lower triglyceride values in G5 suggested that this treatment positively modulates the lipid metabolism and, consequently, obesity [65].

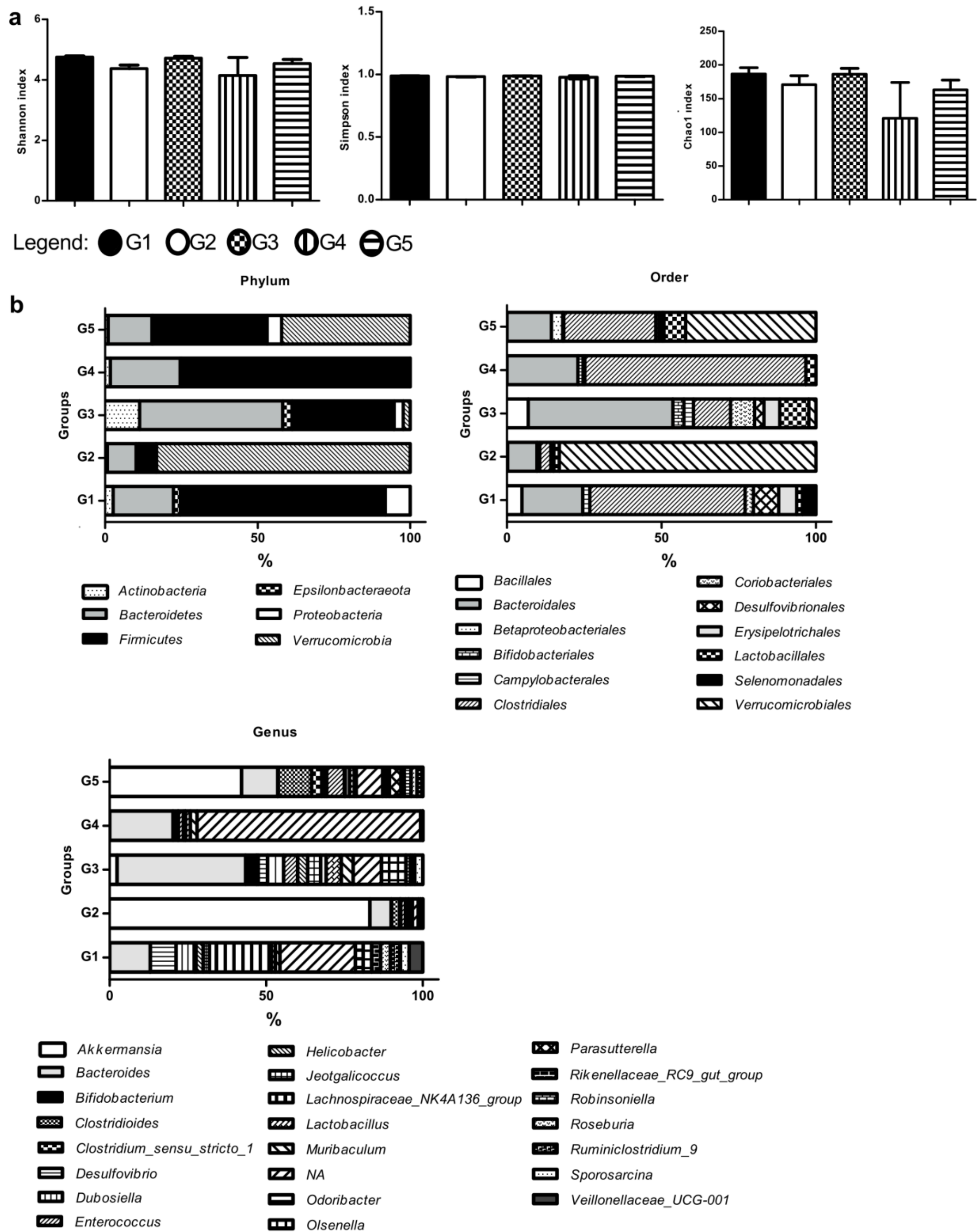

Fig. 2 Composition of intestinal microbiota: Homogeneity in diversity indexes but differences in frequencies. **a** indices of diversity of the intestinal microbiota in the different study groups; **b** structure of theintestinal microbiota at the level of phylum, order, and gender; **G1** control group, **G2** obese group, **G3** potential probiotic group, **G4** antibiotic group and **G5** antibiotic followed by potential probiotic group

In addition, VLDL is a lipoprotein capable of transporting lipids from the liver to the circulation. After VLDL hydrolysis, free fatty acids are released and can be used by tissues or stored in the form of adipose tissue [64]. Therefore, the lower levels of triglycerides in G5 may indicate less availability of free fatty acids to be stored, which is also a remarkable outcome for obesity treatment.

Elevated concentrations of butyric acid found in the current study suggested promotion of intestinal health, since short chain fatty acids (SCFA) are a source of energy for the colonic epithelium, especially butyrate, which has an important role in cell differentiation and, therefore, in intestinal health. SCFA increased production also leads to a decrease in intestinal pH, which prevents the growth of pathogenic bacteria. Moreover, SCFA regulate the adaptive immune system through the activation of regulatory T cells [66], provide a rich energy source for the epithelial cells in the colon, protecting intestinal integrity [37], and induce lipogenesis through activation of sterol regulatory element-binding transcription factor 1 (SREBP-1) [4], which are positive in obesity cases. Thus, there is an urge for regulation of metabolic pathways and health promotion [67].

SCFA also assist in weight control as these fatty acids act as signaling molecules, binding to the G protein-coupled receptor (GPCRs). This link stimulates the production of leptin, which is a hormone related to both satiety and energy metabolism. This link also stimulates the production of hormones that reduce intestinal motility and enhance the absorption of SCFA, which will be used for lipogenesis in the liver. Therefore, the inhibition of this association is a potential therapeutic target to modulate obesity, since this linkage decreases energy extraction from the diet [37, 68].

Caloric restriction and augmented energy expenditure are the first preferences for the treatment of obesity [1–4]. However, in cases of non-responsiveness to this treatment, i.e., in scenarios where weight loss of 5 to 10% is not achieved within 6 months, drugs combined with the use of standard therapy are recommended as alternatives [3, 69].

Drugs for obesity treatment are scarce and not always efficient [3, 37]. Therefore, by consolidating the relationship between obesity and intestinal microbiota [6, 37, 59, 60], as well as the role of antibiotics in modulating this microbiota [26, 27] for the treatment of obesity, especially those that act against Gram-negative bacteria and are not absorbed by body, the focus only on intestinal action is justified [39, 40]. Cephalosporins, which are a third-generation antibiotic, act by decelerating the growth of pathogenic bacteria which results in the restoration of the intestinal microbiome and protection against diseases [58]. Thus, cephalosporins stand out as an interesting option for the treatment of obesity through intestinal modulation.

Contrary to what was expected [20–22, 24–27], the treatments in this study did not lead to changes in the diversity of the intestinal microbiota. However, it is noteworthy that the intestinal microbiota has the ability to rebalance itself after external disturbances [7], which assists in understanding the aforementioned outcomes.

In addition, the treatment with antibiotics followed by potential probiotics was effective in reducing the *Bacteroidetes* phylum and increasing the *Firmicutis* phylum. This indicates a modulation of the intestinal microbiota, altering the classic understanding of obesity, which was usually based on a greater proportion of *Bacteroidetes* in relation to the *Firmicutis* phylum [11, 42, 70].

Using antibiotics followed by potential probiotics for the treatment of obesity is innovative, and the results found in the present study evidenced this treatment as promising. In addition, the results found with this type of treatment have economic potential since an effective treatment of obesity leads to harm reduction with other associated diseases, which reduces the economic costs with drugs or associated treatments, as well as avoids the use of drastic and invasive interventions procedures such as bariatric surgery, which lead to economic and social impact [4, 37].

Hence, further and innovative designs involving the use of antibiotics and potential probiotics sequentially are suggested. Furthermore, studies encompassing a diverse range of antibiotics associated with potential probiotics are interesting whether aiming at different targets and mechanisms of action, as well as at other periods of intervention and distinctive nutritional strategies for weight loss.

The use of antibiotics followed by potential probiotics was positive for the obesity treatment, leading to a reduction in biometric measures and lipid profile, increase in the amount of brown adipose tissue and butyric acid, and resulting in the alteration of the intestinal microbiota phyla. It is understood that these outcomes were acquired resulted from the treatment efficiency in bringing together the characteristics of antibiotic and potential probiotic treatments that targeted Gram negative bacteria and intestinal colonization, respectively. This led to a modulation of intestinal microbiota, which resulted in the improvement of other parameters related to obesity.

We emphasize that due to the potential of the results we found, it is necessary to study this type of sequential treatment in humans, which can clinically confirm the results found by us. So, this article is promising due to its innovative nature, which dwells within the urge for adjuvant treatments for obesity, a worldwide public health issue with critical socioeconomic impacts.

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Author Contribution MMD participated in all production stages of this article (handling the animals, execution of analysis, analysis of the results and writing of the article); SARL and RCF assisted in the handling of animals, in the execution of analyzes, and in writing of this article; LLC, TAOM, SSP, LLO, and MCGP guided in the choice of the analyzes performed, participated in the analysis of the results, and assisted in the writing of this article. All authors reviewed the manuscript.

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Declarations

Ethics Approval The study was approved by the Ethics Committee on Animals Use of Universidade Federal de Viçosa, under protocol 33/2018. The principles recommended by the Nacional Council for the Control of Animal Experimentation were followed [41].

Consent for Publication All authors consent with the publication of this article.

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

Availability of Data and Material All data and materials are available from the corresponding authors on reasonable request.

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3.3 Resultados suplementares ao artigo original

Tabela 1 – Peso corporal semanal dos grupos experimentais durante a fase de tratamento

Semana	**Grupo**					**p**
	G1	**G2**	**G3**	**G4**	**G5**	
0	21,84 ± 1,04[a]	25,12 ± 2,5[b]	24,96 ± 0,80[b]	24,97 ± 2,07[b]	24,56 ± 2,39[b]	0,005
1	24,07 ± 1,08	25,04 ± 1,62	24,66 ± 0,72	24,70 ± 1,93	25,77 ± 2,96	0,588
2	22,20 ± 1,11	22,20 ± 1,03	22,26 ± 0,89	22,50 ± 2,05	22,94 ± 2,61	0,508
3	23,10 ± 1,17	22,72 ± 0,91	23,67 ± 1,11	22,77 ± 2,27	22,92 ± 2,28	0,888
4	24,0 ± 0,76	24,34 ± 1,32	24,93 ± 0,88	24,08 ± 2,16	23,37 ± 1,45	0,546

Em que G1 = grupo controle negativo, G2 = grupo obeso sem tratamento, G3 = grupo obeso tratado com potencial probiótico, G4 = grupo obeso tratado com antimicrobiano e G5 = grupo obeso tratado com antimicrobiano seguido de potencial probiótico. Resultados expressos em média e desvio-padrão. ANOVA *One Way* seguido do teste *Post Hoc* de *Tukey*. Letras iguais na mesma linha indicam ausência de diferenças significativas.

Tabela 2 – Parâmetros dietéticos durante a fase de tratamento

Medida	**G1**	**G2**	**G3**	**G4**	**G5**	**p**
Consumo (g) 1ª semana	9,36 ± 0,16	8,07 ± 0,80	7,62 ± 0,26	7,01 ± 2,55	8,35 ± 0,50	0,475
Consumo (g) 2ª semana	9,25 ± 0,11	8,23 ± 0,18	8,37 ± 0,79	8,36 ± 0,26	8,12 ± 0,13	0,158
Consumo (g) 3ª semana	13,91 ± 0,38[a]	12,19 ± 0,30[b]	11,97 ± 0,18[b]	12,07 ± 0,50[b]	12,31 ± 0,23[b]	0,003
Consumo (g) 4ª semana	9,33 ± 0,04	8,15 ± 0,16	7,48 ± 1,21	8,12 ± 0,11	8,16 ± 0,43	0,155
Δ Consumo (g)	-0,03 ± 0,19	0,08 ± 0,65	-0,14 ± 1,47	1,11 ± 2,66	-0,19 ± 0,07	0,868
Consumo (kcal) 1ª semana	35,6 ± 0,59	30,68 ± 3,08	28,95 ± 0,99	26,68 ± 9,68	31,74 ± 1,90	0,477
Consumo (kcal) 2ª semana	35,15 ± 0,38	31,29 ± 0,69	31,82 ± 3,04	31,76 ± 0,99	30,87 ± 0,47	0,159
Consumo (kcal) 3ª semana	52,90 ± 1,44[a]	46,32 ± 1,15[b]	45,53 ± 0,69[b]	45,87 ± 0,18[b]	46,82 ± 0,87[b]	0,003
Consumo (kcal) 4ª semana	35,48 ± 0,15	30,99 ± 0,59	28,41 ± 4,60	30,86 ± 0,40	31,01 ± 1,65	0,154
Δ Consumo (kcal)	-0,11 ± 0,74	0,31 ± 2,48	-0,54 ± 5,59	4,20 ± 10,08	-0,73 ± 0,25	0,869
Consumo hídrico (mL)	23,32 ± 1,30[a]	23,29 ± 3,49[a]	18,47 ± 2,33[ab]	23,96 ± 1,83[ac]	22,25 ± 2,18[a]	0,031
CEA	0,26 ± 0,02[a]	- 0,02 ± 0,04[b]	- 0,07 ± 0,05[b]	- 0,12 ± 0,10[b]	- 0,25 ± 0,05[c]	0,000

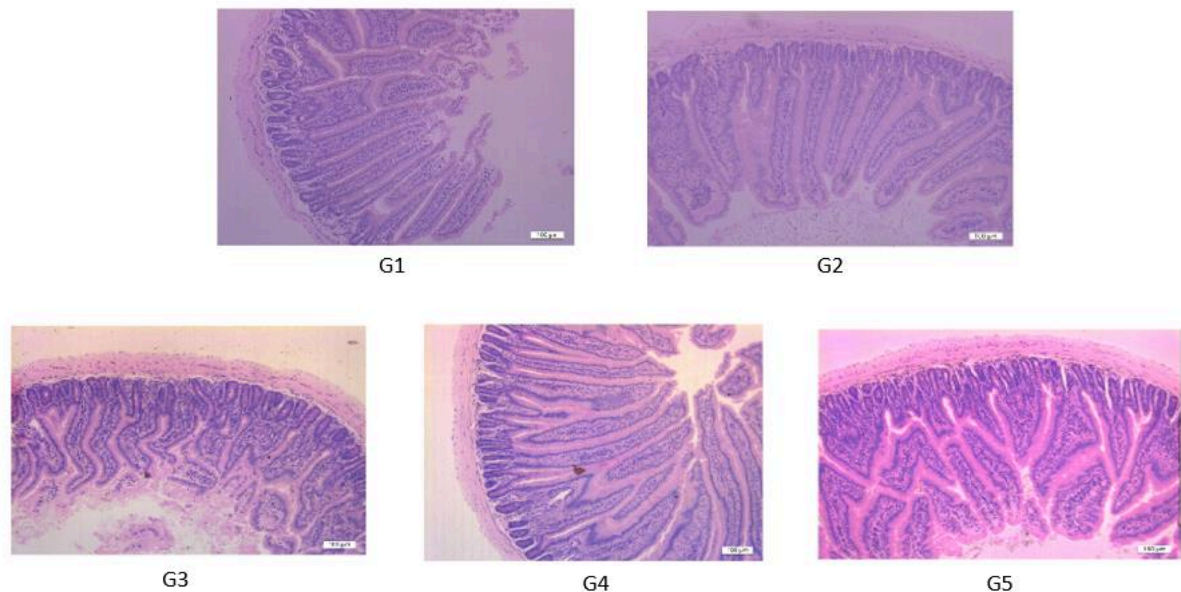
Em que G1 = grupo controle negativo, G2 = grupo obeso sem tratamento, G3 = grupo obeso tratado com potencial probiótico, G4 = grupo obeso tratado com antimicrobiano, G5 = grupo obeso tratado com antimicrobiano seguido de potencial probiótico e Δ = diferença entre a 4ª e a 1ª semana de tratamento. Letras diferentes indicam diferenças significativas ($p<0,05$). Resultados expressos em média e desvio-padrão para amostras com distribuição paramétrica. ANOVA *One Way* seguida do teste *Post Hoc* de Tukey.

Tabela 3 – Teste de tolerância oral à glicose dos grupos experimentais ao final da fase de tratamento

Tempo (min)	G1 (mg/dL)	G2 (mg/dL)	G3 (mg/dL)	G4 (mg/dL)	G5 (mg/dL)	p
0	$171,33 \pm 8,57^{a}$	$151,0 \pm 8,88^{a}$	$141,66 \pm 13,74^{a}$	$72,80 \pm 9,80^{b}$	$148,29 \pm 24,98^{a}$	0,000
15	$158,66 \pm 27,08$	$172,33 \pm 6,66$	$174,16 \pm 32,57$	$150,60 \pm 10,89$	$178,14 \pm 38,96$	0,259
30	$172,16 \pm 19,66^{a}$	$127,00 \pm 5,56^{ab}$	$149,66 \pm 40,83^{ab}$	$116,80 \pm 9,04^{b}$	$154,43 \pm 33,35^{ab}$	0,020
60	$152,66 \pm 20,16$	$157,66 \pm 27,06$	$170,00 \pm 23,08$	$146,40 \pm 6,98$	$170,43 \pm 43,68$	0,715
90	$131,66 \pm 25,00^{a}$	$122,33 \pm 24,17^{a}$	$165,33 \pm 23,61^{ab}$	$119,80 \pm 8,17^{a}$	$223,00 \pm 60,69^{b}$	0,000

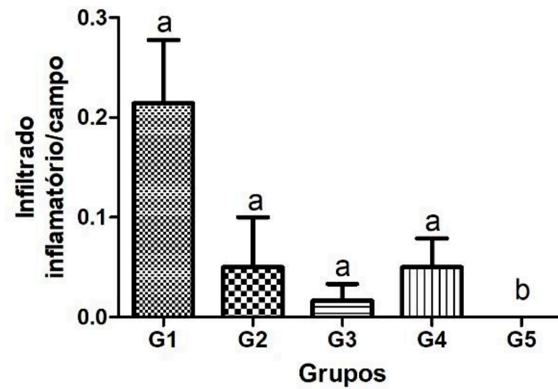
Em que G1 = grupo controle negativo, G2 = grupo obeso sem tratamento, G3 = grupo obeso tratado com potencial probiótico, G4 = grupo obeso tratado com antimicrobiano e G5 = grupo obeso tratado com antimicrobiano seguido de potencial probiótico. Letras diferentes indicam diferenças significativas ($p<0,05$). Resultados expressos em média e desvio-padrão para amostras com distribuição paramétrica. ANOVA *One Way* seguida do teste *Post Hoc* de Tukey.

Figura 1 – Fotomicrografia do intestino delgado dos grupos experimentais



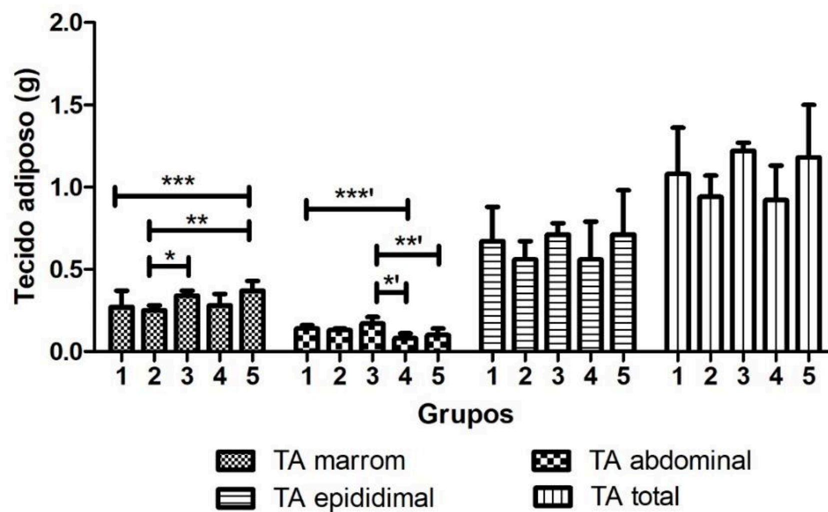
Coloração por hematoxilina e eosina. Ampliação de 100X, em que G1 = grupo controle negativo, G2 = grupo obeso sem tratamento, G3 = grupo obeso tratado com potencial probiótico, G4 = grupo obeso tratado com antimicrobiano e G5 = grupo obeso tratado com antimicrobiano seguido de potencial probiótico.

Figura 2 – Infiltrados inflamatórios do tecido adiposo



Em que G1 = grupo controle negativo, G2 = grupo obeso sem tratamento, G3 = grupo obeso tratado com potencial probiótico, G4 = grupo obeso tratado com antimicrobiano e G5 = grupo obeso tratado com antimicrobiano seguido de potencial probiótico. Letras iguais indicam ausência de diferenças significativas.

Figura 3 – Distribuição de tecidos adiposos entre os diferentes grupos



Em que *: p = 0,031; **: p = 0,003; ***: p = 0,046; *′: p = 0,000; **′: p = 0,002; ***′: p = 0,009; TA = tecido adiposo; 1 = grupo controle negativo; 2 = grupo obeso sem tratamento; 3 = grupo obeso tratado com potencial probiótico; 4 = grupo obeso tratado com antimicrobiano; e 5 = grupo obeso tratado com antimicrobiano seguido de potencial probiótico. Resultados expressos em média e desvio-padrão para amostras com distribuição paramétrica. ANOVA *One Way* seguida do teste *Post Hoc* de Tukey.

Tabela 4 – Enzimas envolvidas no processo oxidativo e de peroxidação lipídica

Enzima	G1	G2	G3	G4	G5	p
SOD (U/mg ptn)	36,11 ± 3,20[a]	33,48 ± 1,33[abc]	34,55 ± 2,57[ac]	30,87 ± 3,91[bc]	30,03 ± 0,53[b]	0,001
CAT (U/mg ptn)	12,57 ± 2,53[a]	15,80 ± 0,01[ab]	16,55 ± 3,46[ab]	14,09 ± 7,55[ab]	19,49 ± 2,68[b]	0,009
GST (nmol/min/ mg ptn)	2,44 ± 0,18	2,37 ± 0,33	2,29 ± 0,81	2,76 ± 0,31	3,03 ± 0,69	0,057
MDA (nmol/min/ mg ptn)	1,42 ± 0,43	1,91 ± 0,12	1,66 ± 0,39	1,49 ± 0,11	1,63 ± 0,49	0,363

Em que SOD = superóxido dismutase, CAT = catalase, GST = glutationa-S-transferase, MDA = malondialdeído, G1 = grupo controle negativo, G2 = grupo obeso sem tratamento, G3 = grupo obeso tratado com potencial probiótico, G4 = grupo obeso tratado com antimicrobiano, G5 = grupo obeso tratado com antimicrobiano seguido de potencial probiótico, U = unidade, mg = miligrama, ptn = proteína, nmol = nanomol e min = minuto. Letras diferentes na mesma linha indicam diferenças entres os grupos. Resultados expressos em média e desvio-padrão. ANOVA *One Way* seguida do teste *Post Hoc* de Tukey.

Tabela 5 – Lipídeos hepáticos nos diferentes grupos experimentais

Lipídeo hepático	Grupo					p
	G1	G2	G3	G4	G5	
Total (mg)	3,43 ± 1,21[a]	4,75 ± 3,05[ab]	3,20 ± 1,68[a]	0,83 ± 0,28[a]	7,42 ± 2,22[b]	0,001
Triglicerídeo (mg/g)	2,85 ± 0,82[ab]	1,77 ± 0,91[a]	3,33 ± 0,23[ab]	1,82 ± 0,23[ab]	3,78 ± 1,08[b]	0,004
Colesterol (mg/g)	0,78 ± 0,11[a]	0,64 ± 0,06[b]	0,69 ± 0,05[ab]	0,64 ± 0,01[ab]	0,76 ± 0,07[a]	0,002

Em que G1 = grupo controle negativo, G2 = grupo obeso sem tratamento, G3 = grupo obeso tratado com potencial probiótico, G4 = grupo obeso tratado com antimicrobiano e G5 = grupo obeso tratado com antimicrobiano seguido de potencial probiótico. Resultados expressos em média e desvio-padrão. ANOVA *One Way* seguida do teste *Post Hoc* de Tukey.

Tabela 6 – Permeabilidade intestinal nos diferentes grupos experimentais

Açúcar (%)	Grupo					p
	G1	G2	G3	G4	G5	
Lactulose	0,03 ± 0,00[a]	5,02 ± 5,85[ab]	0,7 ± 0,06[ab]	0,75 ± 0,02[b]	0,13 ± 0,56[ab]	0,005
Manitol	0,22 ± 0,00[abc]	0,26 ± 0,00[abc]	0,30 ± 0,00[ac]	0,18 ± 0,08[b]	0,28 ± 0,04[ac]	0,001
Razão L/M	0,12 ± 0,00	19,36 ± 24,18	2,32 ± 0,18	5,1 ± 5,08	0,42 ± 2,72	0,051

Em que L/M = lactulose/manitol, G1 = grupo controle negativo, G2 = grupo obeso sem tratamento, G3 = grupo obeso tratado com potencial probiótico, G4 = grupo obeso tratado com antimicrobiano e G5 = grupo obeso tratado com antimicrobiano seguido de potencial probiótico. Letras diferentes na mesma linha indicam diferenças entres os grupos. Resultados expressos em média ± desvio-padrão para amostras com distribuição paramétrica e mediana ± intervalo interquartílico para amostras com distribuição não paramétrica. ANOVA *One Way* seguida do teste *Post Hoc* de Tukey para amostras com distribuição paramétrica e Kruskal Wallis seguido do teste de Dunn's para amostras com distribuição não paramétrica.

CAPÍTULO 2

KEFIR AND INTESTINAL MICROBIOTA MODULATION: IMPLICATIONS IN HUMAN HEALTH

1 JUSTIFICATIVA

Neste capítulo são apresentados os resultados de atividades desenvolvidas em colaboração com a Universidad de Navarra (UNAV), Espanha, sob a supervisão dos Professores Fermín Ignacio Milagro e J. Alfredo Martinez, durante o ano de 2020.

2 RESULTADOS

2.1 Artigo de revisão

Título: "Kefir and Intestinal Microbiota Modulation: implications in human health".
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REVIEW
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Kefir and Intestinal Microbiota Modulation: Implications in Human Health

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In the last decades changes in the pattern of health and disease in Latin America and in the world has been observed, with an increase in cases of chronic non-communicable diseases. Changes in intestinal microbiota composition can contribute to the development of these diseases and be useful in their management. In this context, the consumption of fermented foods with probiotic properties, such as kefir, stands out due to its gut microbiota-modulating capacity. There is an increasing interest in the commercial use of kefir since it can be marketed as a natural beverage containing health-promoting bacteria and has been gaining international popularity in Latin America. Also the consumption of these drinks in Latin America seems to be even more relevant, given the socioeconomic situation of this population, which highlights the need for disease prevention at the expense of its treatment. In this narrative review, we discuss how kefir may work against obesity, diabetes *mellitus*, liver disease, cardiovascular disorders, immunity, and neurological disorders. Peptides, bioactive compounds and strains occurring in kefir, can modulate gut microbiota composition, low-grade inflammation and intestinal permeability, which consequently may generate health benefits. Kefir can also impact on the regulation of organism homeostasis, with a direct effect on the gut-brain axis, being a possible strategy for the prevention of metabolic diseases. Further studies are needed to standardize these bioactive compounds and better elucidate the mechanisms linking kefir and intestinal microbiota modulation. However, due to the benefits reported, low cost and ease of preparation, kefir seems to be a promising approach to prevent and manage microbiota-related diseases in Latin America and the rest of the world.

Keywords: kefir, probiotics, health, fermented foods, gut microbiota

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INTRODUCTION

In the last decades, a major change in global health has been observed (1), with changes in the intestinal microbiota. The formation of intestinal microbiota during childhood is believed to be a key factor in the development of human diseases, such as allergies, neurological disorders and obesity (2–4), which indicates that the intestinal microbiota has a crucial role in the progress of new strategies for maintaining health.

The intestinal microbiota is a set of microorganisms that inhabit the human intestinal tract, and is composed by archaea, bacteria, fungi, helminths, and others (5). In a healthy adult individual, it is believed to be composed of more than 10^{14} microorganisms (6). According to the World Gastroenterology Organization stomach and duodenum have very low number of microorganisms, mainly lactobacilli and streptococci. Jejunum and ileum have an intermediate amount (10^4-10^7 cells per gram) (7) while large intestine has the largest amount with more than 10^{12} cells per gram, especially anaerobic microorganisms (7, 8). This microbial diversity also suggests the importance of the role that the intestinal microbiota plays in human health, indicating that—unlike what was thought in the past—microorganisms are not necessarily negative or the cause of diseases. On the contrary, it is increasingly clear that they co-evolved with human hosts and the presence of microorganisms can be important for maintaining health (1–4).

Knowledge on the importance of bacteria for human health dates back over 150 years (9), and fermented foods are important in this context. Through fermentation, food has contributed to the evolution of humanity due to its increased conservation capacity and shelf life, development of flavors, and health benefits (10, 11).

Able to resist to the digestive system, beneficial microorganisms occurring in fermented foods are able to act in the intestine. Fermented foods are able to modify the composition of the intestinal microbiota, improve the control of intestinal permeability, increase its barrier function (10–12), activate digestive enzymes, and assist in the production of short-chain fatty acids and vitamins. In addition, fermented foods have bioactive compounds and peptides with prebiotic, antimicrobial, anti-inflammatory, and anti-oxidant activities. Thus, the consumption of fermented foods has been reported to reduce the risk of incidence of certain diseases, such as metabolic syndrome, cardiovascular diseases, diabetes, and cancer; relieves lactose intolerance symptoms; and increases immunity and health in general (10, 11).

Probiotics stand out among fermented foods (10). According to the definition, probiotics are living microorganisms that provide health benefits to those who consume them (13, 14). From the beginning of Elie Metchnikoff's studies (1907) to the present day, *Lactobacillus* and *Bifidobacteria* are important species of lactic acid bacteria used as probiotic with evidence of their importance in human health (9, 10). In addition, in the scenario of diseases caused by changes in the intestinal microbiota, the use of probiotics stands out since the probiotics are capable of promoting homeostasis of the intestinal microbiota through mucin production, competition for pathogen adherence, inflammation control, pH change, cytokine production, as well as having immunomodulatory and anti-inflammatory properties, which results in a better healthy condition (7, 15).

Kefir is a fermented product (11, 16) formed by a single culture composed of lactic and acetic acid bacteria and yeast (17). It is a low-cost food, accessible to the general population, easy to handle and with great functional potential (18) due to its bioactive compounds (11, 18) like exopolysaccharides, conjugated fatty acids and peptidases (11, 16). Kefir consumption does not seem to result in negative effects (18) for adult humans (19) or animals (20). In kefir produced with whole milk, high cholesterol content can be observed, and in individuals with intolerance to lacteal proteins, allergic reactions may be observed, that can be avoided by replacing the milk matrices for water and sugar, which reinforces the absence of adverse effects of kefir (21).

Kefir peptides are able to improve parameters related to obesity in Sprague Dawley rats with diet-induced obesity. They act to inhibit lipogenesis by downregulating the fatty acid synthase (FAS) enzyme and increasing the phosphorylated acetyl coenzyme A carboxylase (p-ACC) protein; rising lipid oxidation and the expression of AMP-activated protein kinase (p-AMPK), peroxisome proliferator-activated receptor alpha (PPAR-α) and carnitine palmitoyltransferase 1 (CPT1); and decreasing the inflammatory response and oxidative modulation—with a reduction in tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β) and transforming growth factor beta (TGF-β) cytokines (22) (**Table 1**). In addition, the strains found in kefir, such as *Lactobacillus harbinensis, Lactobacillus paracasei,* and *Lactobacillus plantarum*, have a role in the tolerance to bile acids and salts, in the adhesion of the intestinal mucosa and in antimicrobial resistance, indicating that bacteria occurring in kefir are capable of standing the gastrointestinal tract, as well as having probiotic and antioxidant activities (43).

Given the above, we will discuss studies with animals and humans that reported the role of kefir and its bioactive compounds on the treatment of diseases characterized by shifts in intestinal microbiota.

KEFIR IN LATIN AMERICA

Non-communicable diseases are those diseases with a long duration and multifactorial causes which include genetic, psychological, environmental and behavioral factors. Also known as chronic diseases, some examples are cardiovascular disease, cancer and diabetes (44).

The consumption of milk-based fermented drinks is related to the prevention of chronic non-communicable diseases, with an

Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; CPT1, carnitine palmitoyltransferase 1; ELANS, American study of nutrition and health; EPM, elevated plus maze; FAS, fatty acid sintase enzyme; FST, forced swim test; GABA, gamma aminobutyric acid; GLP-1, glucagon-like peptide; HOMA-IR, insulin resistance index; IFN-γ, interferon-gama; IL-1β, interleukin-1 beta; mL, mililiter; OFT, open field test; p-AMPK, AMP-activated protein kinase; PPAR α, peroxisome proliferator-activated receptor alpha; TGF-β, transforming growth factor beta; TMAO, trimethylamine N-oxide; TMF, transplantation of fecal microbiota; TNF-α, tumor necrosis factor alpha.

TABLE 1 | Main results of kefir intervention studies.

References	Population	Disease/condition	Kefir consumption	Main outcomes in kefir groups
Human studies				
Bellikci-Koyu et al. (19)	- Humans	- Metabolic syndrome	- 180 mL kefir/day; - 12 weeks of intervention time;	- ↑ Actinobacteria, fasting insulin and HOMA-IR; - ↓ pro-inflammatory cytokines, such as TNF-α and IFN-γ, and in systolic and diastolic pressure.
Fathi et al. (23)	- Women aged 25–45 years	- Obesity	- 2 servings/day; - 8 weeks of intervention time.	- ↓ serum levels/ratios of lipoproteins (TC, LDL, Non-HDL, TC/HDL, and LDL/HDL).
Ostadrahimi et al. (24)	- Humans aged 35–65 years	- Type 2 diabetes mellitus	- 600 mL/day of probiotic fermented milk containing *Lactobacillus casei*, *Lactobacillus acidophilus* and *Bifidobacteria*; - 8 weeks of intervention times.	- ↓ HbA1C
Ozcan et al. (25)	- Post-menopausal women	- Sleep quality, quality of life and depression	- 500 mL kefir daily (instructed to drink 250 mL in the morning and in the evening); - 30 days of intervention time.	- MENQOL, BDI, and WHIIRS scores showed significant changes
Praznikar et al. (26)	- Humans	- Obesity/intestinal integrity	- 300 mL of kefir/day; - 3 weeks of intervention time.	- ↓ serum zonulin levels, glucose and HDL cholesterol, and self-reported appetite perceptions; - ↑ the positive affect or mood.
St-Onge et al. (27)	- Humans (men)	- Hypercholesterolemy	- 500 mL kefir/day; - 2 periods of 4 weeks of intervention time.	- ↑ fecal isobutyric, isovaleric and propionic, the total amount of fecal short chain fatty acids, and fecal bacterial content.
Animal studies				
Bourrie et al. (28)	- C57BL/6 female mice	- Obesity induced by diet	- 100 μL kefir by gavage/day; - 12 weeks of intervention time.	- ↓ weight gain, plasma cholesterol, and hepatic triglyceride deposit.
Chen et al. (29)	- Sprague Dawley rats	- Hepatic steatosis induced by high-fat diet	- 10^7 to 10^{10} CFU of *Lactobacillus mali* APS1 by gavage/day; - 12 weeks of intervention time.	- ↓ HOMA index, hepatic lipid accumulation; - ↑ GLP-1, hepatic antioxidant activity, butyrate and Bacteroidetes/Firmicutes.
Choi et al. (30)	- C57BL/6J mice	- Obesity induced by high-fat diet	- 0.1 to 0.2% kefir powder-supplemented high-fat diet - 8 weeks of intervention time.	- ↓ body weight, epididymal fat pad weight, adipocyte diameters, genes related to adipogenesis and lipogenesis, proinflammatory marker levels in epididymal fat, hepatic triacylglycerol concentrations, serum alanine transaminase, aspartate transaminase activities, serum triacylglycerol, total cholesterol, low-density lipoprotein-cholesterol.
Golli-Bennour (31)	- Wistar rats	- Hepatotoxicity	- Not informed	- Normalized the elevated serum levels of AST, ALT, total bilirubin, and cholesterol;
Kim et al. (32)	- Female BALB/c mice	- Obesity	- 0.2 mL kefir milk orally/day; - 3 weeks of intervention time.	- ↓ Firmicutes, Proteobacteria, Enterobacteriaceae and Firmicutes/Bacteroidetes ratio; - ↑ Bacteroidetes, Lactobacillus, and Lactococcus, and total yeast; - Suppressed proliferation of the opportunistic pathogen Enterobacteriaceae.
Kim et al. (33)	- Male C57BL/6 mice	- Obesity and non-alcoholic fatty liver disease induced by 60% high-fat diet	- 0.2 mL of saline with 2 × 10^8 CFU of *L. kefiri* DH5; - 0.2 mL of saline with 2 × 10^8 CFU of *Leuconostoc. mesenteroides* DH4 - 0.2 mL of saline with 2 × 10^8 CFU of *L. kefiri* DH7 - 6 weeks of intervention time.	-↓ body weight, epididymal adipose tissue weight, blood triglyceride, LDL-cholesterol levels, hepatic steatosis, adipocyte diameter; - Modulated gut microbiota; - Upregulated PPARα, FABP4, and CPT1 expression in the epididymal adipose tissues

(Continued)

TABLE 1 | Continued

References	Population	Disease/condition	Kefir consumption	Main outcomes in kefir groups
Kim et al. (20)	- Dogs	- Quality of life	- 200 mL of kefir once a day (*ad libitum*); - 2 weeks of intervention time.	- ↑ lactic acid bacteria and lactic acid bacteria: Enterobacteriaceae ratio; - ↓ Firmicutes: Bacteroidetes ratio.
Le Barz et al. (34)	- C57BL/6 mice	- Obesity induced by diet	- 1 of 3 Lactobacillus strains (Lb38, *L. plantarum*; L79, *L. paracasei/casei*; Lb102, L. *rhamnosus*) or *Bifidobacterium* strains (Bf26, Bf141, 2 different strains of B. *animalis* ssp. *lactis*) administered with diet at 10^9 CFU/day; - 8 weeks of intervention time.	- ↓ diet-induced obesity, visceral fat and inflammation; - ↑ glucose tolerance, insulin sensitivity and intestinal integrity.
Lim et al. (35)	- C57BL/6J mice	- Obesity induced by high-fat diet	- 5% water-soluble EPS and 8% resides after EPS removal from the probiotic kefir in diet; - 4 weeks of intervention time.	- ↓ body weight gain, adipose tissue and plasma very low-density lipoprotein cholesterol; - ↑ *Akkermansia spp*. in feces.
Noori et al. (36)	- Rats	- Nicotine cessation-induced anxiety, depression and cognition impairment	- 5 mg/kg/day of cow milk kefir or soy milk kefir; - 7 days of intervention time.	- Improved anxiety, learning and memory impairment; - ↓ the severity of depression.
Rosa et al. (37)	- Spontaneously Hypertensive Rats	- Metabolic syndrome	- 1 mL kefir/day; - 10 weeks of intervention time.	- ↓ plasma triglycerides, liver lipids, liver triglycerides, insulin resistance, fasting glucose, fasting insulin, thoracic circumference, abdominal circumference, products of lipid oxidation, pro-inflammatory cytokine expression (IL-1β); - ↑ anti-inflammatory cytokine expression (IL-10).
Sun et al. (38)	- Kunming male mice	- Depression	- *L. kefiranofaciens* ZW3 a dose of 10^7 CFU, 10^8 CFU and 109 CFU/mouse/day; - 6 weeks of intervention time.	- Improved depression-like behavior and independent exploration ability; - Regulated biochemical disorders in the hypothalamic–pituitary–adrenal axis, immune system and tryptophan metabolism caused by stress; - Modulated the composition of the gut microbiota, and alleviate constipation.
Tiss et al. (39)	- Wistar rats	- Obesity, type 2 diabetes, hyperlipidemia and liver-kidney toxicities in high-fat-high-fructose diet	- 10 mL/kg of body weight of a fermented soymilk by kefir; - 90 days of intervention time.	- ↓ pancreas lipase and alpha-amylase; - Reverted back all these histological toxicities.
Tung et al. (22)	- Sprague Dawley rats	- Obesity induced by high-fat diet	- 164 mg/kg of body weight of kefir peptides; - 8 weeks of intervention time.	- ↓ FAS enzyme, inflammatory response and oxidative modulation (TNF-α, IL-1β and TGF-β cytokines); -↑ p-ACC protein, fatty acid oxidation and expression of p-AMPK, PPAR-α and CPT1.
Tung et al. (40)	- ApoE knockout mice	- High fat diet-induced atherosclerosis	- 100 mg/kg low-dose kefir peptides powder; - 400 mg/kg high-dose kefir peptides powder; - 12 weeks of intervention time.	- Improved atherosclerotic lesion development by protecting against endothelial dysfunction; - ↓ oxidative stress, aortic lipid deposition, inflammatory immune response, fibrosis; - Attenuating macrophage accumulation.
Vinderola et al. (41)	- BALB/c mice	- Immunomodulation	- 3.1 ± 0.3 ml/kefir daily; - 2, 5, or 7 consecutive days of intervention time.	- Modulated the mucosal immune system in a dose-dependent manner
Wouw et al. (42)	- C57BL/6J mice	- Brain physiology and behavior	- 0.2 mL/kefir daily; - 3 weeks of intervention time.	- Ameliorated the stress-induced; - ↓ serotonergic signaling; - ↑ fear-dependent contextual memory; - Stimulate the production of GABA neurotransmitter.

Where: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BDI, beck depression inventory; CFU, colony forming unit; CPT 1, carnitine palmitoyltransferase 1; EPS, exopolysaccharides; FABP4, fatty acid-binding protein 4; FAS, fatty acid synthase enzyme; GABA, gamma aminobutyric acid; GLP-1, glucagon-like peptide; kg, kilogram; HbA1C, glycated hemoglobin; HDL, high-density lipoprotein cholesterol; HFD, high fat diet; HOMA-IR, insulin resistance index; IFN-γ, Interferon-gamma; IL-1β, interleukin-1 beta; IL-10, interleukin−10; IT, intervention time; LDL, low-density lipoprotein cholesterol; MENQOL, menopause-specific quality of life questionnaire; mg, milligram; mL, milliliters; p-AMPK, AMP-activated protein kinase; PPAR α, peroxisome proliferator-activated receptor alpha; TC, total cholesterol; TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor alpha; WHIIRS, the women's health insomnia rating scale; μL, microliters; ↑, increase; ↓, decrease.

association between the consumption of yogurt and a reduction in weight gain, and lower risk of obesity and cardiovascular diseases (45). Fermented milks also aid in the digestion of lactose and are a source of calcium and protein, therefore presenting interesting nutritional value (18, 46).

The consumption of yogurt and fermented drinks varies widely among countries: Brazil has a consumption similar to the United States (46) being, Argentina and Chile the countries with the highest consumption in Latin America (45).

According to data from the American Study of Nutrition and Health (ELANS), which aims to identify the consumption of food directly related to the occurrence of chronic non-communicable diseases, $< 3.5\%$ of the Latin American population has a good consumption of yogurt (45). This consumption is considered low and indicates that there is a consumption gap to be filled, since fermented drinks can contribute to improving the health of those who consume it (46), contributing to the prevention and treatment of chronic non-communicable diseases.

In Brazil, the consumption of yogurt has been increasing among populations with higher income, being typically consumed outside home (47) and, in Latin America, a similar movement is observed, with consumption mainly among the youngest and with highest socioeconomic level (45). Therefore, as an alternative to the consumption of yogurt, we highlight the use of milk kefir, which is also a fermented product, with health benefits due to its probiotic content and the presence of bioactive compounds (18).

Medicines and supplements for the treatment of chronic non-communicable diseases, such as probiotics, can have significant costs for the Latin American population, which may turn their consumption impossible (48). Kefir, on the other hand, can be produced by the consumer himself, using baits acquired by donation, which significantly reduces production and consumption costs (18, 49–51). This allows the use of kefir by a larger portion of the Latin American population. In addition, among the high-income population, kefir consumption also stands out, as there is a tendency, in this audience, to seek healthier and more nutritious foods, such as kefir (52).

The benefits of kefir have not yet been widely publicized among the Latin American population. However, scientific interest in this fermented product is gaining more researchers in Latin America. The antifungal property of kefir, for example, has been studied both in the production of cereals and in the preservation of food. In a study by Gamba et al. (53) there is significant resistance to natural and artificial fungal contamination in arepas (typical dish from Venezuela, Colombia, Bolivia, and Panama) cooked in kefir. In addition, the arepas maintained the organoleptic characteristics of the traditional product after adding kefir, with an improvement in the product's useful life.

The use of kefir to treat diseases has also been studied by Latin American researchers, observing reductions in pre-neoplastic lesions in the intestinal colon (54) and improvement of parameters related to obesity after consumption of this fermented drink (37) in animal models. Better quality of life for individuals with lactose intolerance and osteoporosis (55) has been also observed.

Studies analyzing the effects of kefir on the modulation of the intestinal microbiota (56) and the immune system (57) have also been carried out. In addition, the microbial non-kefir fraction, known as post-biotics (metabolites produced in the fermentation process of dietary components as well as the endogenous components generated by bacteria-host interactions that influence human health) have been widely studied due to their antagonism against pathogenic bacteria such as *Escherichia coli*, *Salmonella* spp. and *Bacillus cereus* (58, 59). This indicates that kefir produced in Latin America is indeed a food with health potential, being interesting its use by the Latin American population.

MODULATION OF THE INTESTINAL MICROBIOTA

The components of intestinal microbiota, that is, archaea, fungi, helminths, bacteria and other microorganisms (5), can take two forms: in balance or not. In the first case, known as eubiosis, microbiota tolerates small changes, which may come from the environment, the diet or the water consumed, presenting flexibility to maintain its balance. Cases of major changes, however, such as translocation or growth of a specific bacterial groups, colonization by pathogenic bacteria, use of antibiotics, and changes in lifestyle, lead to imbalance, that is, dysbiosis (60, 61).

Regardless of balance, intestinal microbiota affects the functionality of various organs such as brain, liver, pancreas, intestine, and heart (60, 61). Furthermore, the intestinal microbiota participates in the development and maturation of organs and physiological processes (60), which suggests that modulation of gut microbiota may be a key event for the treatment of diseases and maintenance of health.

Fortunately, modulation of the intestinal microbiota is a reality. Through technological advances, such as interventions with bariatric surgery (62) or fecal transplant (63), or via food (19, 20, 32), the obtained results are promising, showing, in fact, changes in the composition of the intestinal microbiota.

In a longitudinal study that evaluated obese patients undergoing bariatric surgery, body weight reduction and metabolic improvements occurred via modulation of the intestinal microbiota. Compared to individuals of normal weight, patients who underwent bariatric surgery had variation in the *Firmicutes*, *Fusobacteria,* and *Verrucomicrobia* phyla. In addition, after bariatric surgery, the presence of *Akkermansia muciniphila* was observed. It is a species positively associated with lipid metabolism and negatively associated with adipose tissue inflammation and with circulating levels of glucose, insulin, leptin, and triglycerides, which suggests that its presence indicates improvement in the expression of healthy metabolism markers (62).

Fecal microbiota transplantation also shows its potential modulating role. In a double-blind study with 22 obese patients [body mass index (BMI) $\geq$ 35 kg/m^2] without associated metabolic changes, such as type 2 diabetes, non-alcoholic hepatitis, and metabolic syndrome, the researches worked by

offering the transplantation of fecal microbiota (TMF) of a thin donor using capsules (63).

The consumption of TMF capsules was considered safe. A positive modulation of the intestinal microbiota was observed. At the beginning of the experiment, obese individuals had an intestinal microbiota with a specific profile; however, after TMF intake, it was observed a similarity between the microbiota profile of obese patients and that of the thin donor, as well as changes in beta diversity, that is, changes in the species composition of the microbiota (63).

Reduction of taurocholic acid was also observed in the feces of individuals who received TMF (63), and this change is related to the alterations seen in the composition of the intestinal microbiota. Secondary bile acids, for example, are produced in the colon and are influenced by the microbiota present on it. This production varies according to the colonic microbiota and may contribute to a state of health or disease (64).

In addition, bile acids have antibacterial capacity. They also participate in the progression of diseases associated with changes in intestinal microbiota, such as obesity and gastrointestinal diseases, contributing to the modulation of inflammatory processes and signaling energy metabolism events and through their performance as a biological detergent (64). Hence, there is a two-way path where both gut microbiota influences the production of bile acids, and bile acids contribute to intestinal modulation performance.

In a study that sought to evaluate the role of kefir in the modulation of the intestinal microbiota of mice, it was observed that the consumption of this drink was not able to change the total number of bacteria in intestinal microbiota; however, when evaluating the phyla in the group that consumed kefir, a reduction in Enterobacteriaceae and an increase in *Lactobacillus* and *Lactococcus* contents were observed during the 3-week intervention period; also, a decrease in Firmicutes and Proteobacteria and an increase in Bacteroidetes, *Lactobacillus* and *Lactococcus* at the end of the experiment was observed. On the other hand, there was a significant increase in fecal yeasts after the consumption of kefir (32) (**Table 1**).

Such results indicate that kefir was able to improve the intestinal microbiota of mice that consumed it, with an emphasis on Enterobacteriaceae reduction. This is considered a pathogenic family, usually dysregulated in situations of behavioral and metabolic changes—such as the consumption of diets which are high in fat and low in fiber, during aging, and in cases of inflammation—(32) emphasizing that such eating habits and inflammation are characteristic of obesity (62, 65–67).

Similarly, a study with dogs confirmed that kefir was able to modulate intestinal microbiota. It decreased the Firmicutes:Bacteroidetes ratio and increased the lactic acid bacteria:Enterobacteriaceae ratio, which suggests an improvement in the animal's health. There were also changes in phylum, family and species level, which indicates that kefir was able to modulate the canine intestinal microbiota (20) (**Table 1**).

In humans, modulation of intestinal microbiota also occurs after the consumption of kefir, as discussed by Bellikci-Koyu et al. (19) who investigated individuals with metabolic syndrome that were supplemented with kefir for 12 weeks (**Table 1**). After the intervention, there was a significant increase in *Actinobacteria*, as well as changes in the genera of the phyla Bacteriodetes and Firmicutes, in the group that consumed kefir (19).

This intestinal modulation interferes with metabolic parameters, characteristic of metabolic syndrome, showing improvement in fasting insulin and insulin resistance index (HOMA-IR), and a decrease in pro-inflammatory cytokines, such as TNF-α and interferon-gama (IFN-γ), and in systolic and diastolic pressure. Moreover, a correlation was observed between these parameters and the intestinal microbiota: body weight gain and BMI were positively correlated with the relative abundance of Firmicutes and Proteobacteria and negatively correlated with the relative abundance of Clostridia. Correlations between the composition of the intestinal microbiota and the amount of fat mass, waist circumference, LDL-cholesterol, homocysteine, insulin, and blood pressure were also found (19).

There are many factors that can alter the composition of the intestinal microbiota in humans, such as age, sex, initial formation of intestinal microbiota, food consumption—with emphasis on fiber consumption–, lifestyle, and use of medications—especially antibiotics (1–4). Therefore, the results observed by Bellikci-Koyu et al. (19) are relevant, paving the way for further studies that prove the modulation of the human intestinal microbiota through the use of kefir in individuals with metabolic syndrome.

KEFIR AND OBESITY

Dysbiosis and other determinants of intestinal microbiota formation in childhood, such as birth via cesarean section or formula feeding, are associated with a higher risk of developing obesity, both in child and adulthood (3, 68). Children treated with antibiotics, specially macrolides, amoxicillin, cefdinir, vancomycin, and tetracyclines (69) had a higher incidence of obesity (3), since antibiotics can alter the composition of the intestinal microbiota, leading to a dysbiotic state (3, 70). In addition, the intestinal microbiota is demonstrably different in obese and eutrophic conditions (62, 71), which reinforces the relationship between intestinal microbiota and obesity. Thus, obesity and overweight, characterized by the excess or accumulation of body fat with a consequent increase in health risk (72), can also be influenced by the intestinal microbiota.

Kefir could act against obesity by inhibiting enzymes related to the digestion of carbohydrates and lipids, which will result in less energy release. For example, Tiss et al. (39) using a fermented drink with kefir produced from soymilk, evaluated the activity of lipase and α-amylase *in vitro* and in the intestine and pancreas of rats under high-calorie diet-induced obesity (**Table 1**).

In the *in vitro* analysis, the study showed the ability of kefir to inhibit α-amylase and pancreatic lipase. Authors assumed that this ability is related to the presence of isoflavone aglycones, such as genistein, daidzein and glycitein, present in the drink after the fermentation process. In the *in vivo* section, the obese animals treated with the fermented drink were more stimulated to perform physical activity. Intestinal and pancreatic lipase activity decreased in the groups receiving kefir, leading to a reduction

in total cholesterol and LDL-cholesterol, and an increase in HDL-cholesterol rates, as well as body weight loss. Inhibition of intestinal and pancreatic α-amylase activity and, consequently, decreased blood glucose and protection of liver and kidney tissues from toxicity were also observed after kefir consumption; that is, this fermented drink was able to reverse parameters related to obesity (39).

Bourrie et al. (28) also evaluated kefir regarding the reduction of weight gain and plasma cholesterol in C57BL/6 female mice with obesity induced with a diet of 40% calories from fat and 1.25% cholesterol. The animals received 100 μL of kefir or milk over a period of 12 weeks, with 4 different drinks of traditional kefir and 1 of commercial kefir (28) (**Table 1**).

Among traditional kefir, two types decreased weight gain and plasma cholesterol and one type reduced hepatic triglyceride deposit, which indicates its potential in controlling obesity with improved metabolic function. The difference between the results is due to the different microbiological compositions, viscosity, and pH of fermented drinks (28).

Kim et al. (33) evaluated the anti-obesity effects of kefir in C57BL/6 mice with high-fat diet-induced obesity and non-alcoholic fatty liver disease (**Table 1**). The *Lactobacillus kefiri* DH5 strain was able to decrease body weight, adipose tissue and plasma lipid parameters, acting through the reduction of cholesterol in the intestinal lumen and the upregulation of PPARα in adipose tissue. PPARα is a transcription factor involved in the process of lipid oxidation and consequent metabolism of carbohydrates and lipids, noting that its activation is related to an increase in hepatic steatosis and inflammation (73).

Moreover, the animals that consumed this strain presented a variation in the composition of their intestinal microbiota, with a lower number of Proteobacteria and Enterobacteriaceae, when compared to the non-supplemented animals. These results indicate that *Lactobacillus kefiri* DH5 is a potential probiotic strain for the treatment of obesity (33).

Lim et al. (35) evaluated the effect of exopolysaccharides derived from kefir grains, showing that the beneficial effects found could be related to the viscosity of exopolysaccharides produced by the bacteria present in kefir (**Table 1**). Authors observed that exopolysaccharides were able to suppress obesity *in vitro*, through the supply of adipogenesis. Also, reductions in body weight gain, adipose tissue weight and plasma very low-density lipoprotein cholesterol concentration (VLDL) occurred *in vivo* (35).

Such *in vivo* results were explained by both the presence of bacterial metabolites and by the product's viscosity, which generates appetite suppression and reduces energy consumption as well as glucose and lipid absorption. Furthermore, the supply of kefir exopolysaccharides was able to increase the abundance of *Akkermansia* (35). *Akkermansia muciniphila* undergoes changes according to the diet consumed, modulates the intestinal microbiota, changes inflammatory conditions in adipose tissue, and improves metabolic parameters, like body weight, adiposity, inflammation markers and biochemical parameters, which suggests a great potential for the treatment of obesity (74).

KEFIR AND DIABETES MELLITUS

The development of diabetes mellitus is associated with low-grade chronic inflammation. Changes in intestinal permeability, which are favored by an imbalance in the intestinal microbiota, encourage the occurrence of this inflammation, which leads to resistance to systemic insulin, with consequent development of diabetes (3, 75). In addition, factors that affect the formation of the intestinal microbiota—such as maternal health during pregnancy, birth via cesarean section, the use of antibiotics during childhood, and the presence of intestinal dysbiosis in childhood—are also related to the development of diabetes (3).

In a study with Wistar rats with monosodium glutamate-induced metabolic syndrome, it was observed that whole milk kefir (via gavage, for 10 weeks) was able to reduce insulin resistance. Such results were attributed to the calcium content consumed by the animals, as well as the bioactive compounds produced during the fermentation of kefir. Moreover, kefir stimulated uptake of glucose by muscle cells, which contributed to the reduction of insulin resistance (37) (**Table 1**).

Lactobacillus mali APS1 is a strain isolated from kefir grain that may be useful in the treatment of diabetes. In a study (**Table 1**) with mice consuming a high-fat diet, the administration of this strain was able to reduce serum glucose and HOMA index, increasing the levels of glucagon-like peptide (GLP-1) and butyrate (29). The reduction in the HOMA index indicates glycemic control (76) and the increase in GLP-1 indicates control of hunger and possible protection of pancreatic beta cells, which are insulin-producing cells, essential for maintaining glycidic homeostasis (77). In addition, a recent review discusses the decrease in butyrate content as a characteristic of intestinal dysbiosis in diabetes (78). Therefore, these results are positive.

In humans, a beneficial role of kefir in the treatment of diabetes mellitus has been also observed, as discussed in a work with 60 diabetic patients, aged 35–65 years. The patients were divided into 2 groups: the kefir probiotic group and the conventional fermented milk group, and both received 600 mL/day of the treatment drink for 8 weeks. After the intervention, patients supplemented with kefir presented lower values of fasting glucose and glycated hemoglobin than those that received the other fermented drink (24) (**Table 1**).

The healthy outcomes generated by kefir were attributed to its probiotic composition, mainly *Lactobacillus* and *Bifidobacterium.* These bacteria present a hypoglycemic effect since they stimulate the production of insulinotropic peptides and glucagon-like peptides, leading to an increase in the uptake of glucose by muscle cells, as well as stimulating the production of hepatic glycogen, which uses the glucose available in the bloodstream (24).

KEFIR AND LIVER DISEASES

Toxins produced by intestinal microbiota and a picture of metabolic endotoxemia—that is, altered intestinal permeability—allow the development of a low-grade chronic inflammation. This inflammatory condition stimulates the activation of toll-like receptors and macrophages, which generates hepatic and

systemic inflammation, explaining the relationship between the intestinal microbiota and the occurrence of liver diseases (75).

In addition to its effect on obesity, the consumption of *Lactobacillus kefiri* DH5 presented a hepatoprotective effect. The visual aspect of the liver of the animals that consumed this strain was similar to the animals that did not consume a high-fat diet, as well as presenting, microscopically, less lipid accumulation and smaller fat cells (33).

The hepatoprotective capacity of kefir was also assessed by Golli-Bennour et al. (31) who studied the effect of kefir on hepatotoxicity caused by a pesticide: deltamethrine (**Table 1**). In Wistar rats, the authors observed that deltamethrine altered liver parameters, such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin, and cholesterol, when compared to control groups without the pesticide; however, with kefir intake, these parameters were lower. Furthermore, the supply of pesticide and kefir was able to decrease the levels of carbonylated protein and malondialdehyde, as well as increasing the levels of catalase and superoxide dismutase when compared to the group that received only deltamethrine. These protein and lipid peroxidation disorders indicate oxidative stress and toxicity development by the pesticide. On the other hand, kefir was not able to induce oxidative stress by itself, and was able to revert the inflammatory condition (31).

Less histological and DNA damage was also observed in groups treated with pesticide and kefir when compared to the groups that received only deltamethrine, with this histological and genetic disarchitecture resulting from the inflammatory process previously described. It was concluded that kefir was not only able to revert the inflammatory condition but did not generate any damage, since the group treated with kefir alone obtained similar results to the control group. This finding indicates that kefir has a good antioxidant capacity (31), being a potential tool in the prevention and treatment of liver damage caused or mediated by oxidative stress.

The effect of *Lactobacillus mali* APS1, isolated from sugary kefir grains, was also tested in the fatty liver of rat fed with a high-fat diet, showing a significant reduction in weight, weight gain, hepatic lipid accumulation, and serum levels of AST and ALT. This strain acted through changes in intestinal microbiota composition, reducing the proportion of bacteria associated with non-alcoholic liver diseases and regulating lipid metabolism and oxidative stress response, which leads to suppression of hepatic steatosis progression (29).

KEFIR AND CARDIOVASCULAR CHANGES

Cardiovascular diseases are also related to obesity's intestinal dysbiosis (4). In addition, changes in the intestinal microbiota can lead to the production of compounds such as trimethylamine N-oxide (TMAO), which increase the risk of developing cardiovascular diseases (79, 80).

Tung et al. (40) evaluated the effect of kefir peptides in Apo E –/– mice with high-fat diet-induced atherosclerosis (**Table 1**). After a 12-week intervention, the consumption of kefir led to a decrease in the evolution of atherosclerotic lesions, with less lipid deposition at the root of the aorta and suppression of the inflammatory immune response, through a reduction in oxidative stress, the accumulation of macrophages and the release of IL-1β and TNF-α cytokines. Moreover, kefir prevented the endothelial adhesion of monocytes, decreasing the evolution of the atherosclerotic lesion. These results indicate that the consumption of kefir could be useful in the prevention and treatment of atherosclerosis (40).

One of the risk factors for the occurrence of cardiovascular diseases is dyslipidemia. In this sense, in the host, there is less diversity of intestinal microbiota and a greater chance of dysbiosis. That is, a greater chance of inflammation and changes in intestinal permeability is observed, with negative consequences for the health of the host (81).

In dyslipidemia, changes in the production of short-chain fatty acids and bile acids have been observed. Short-chain fatty acids are metabolite substrates that participate in energy production, lipogenesis, gluconeogenesis, and cholesterol synthesis. On the other hand, primary bile acids can bind to the farnesoid X receptor, and this molecule is also related to the development of obesity. Both factors participate in lipid metabolism and intestinal microbiota change (81), indicating that modulation of intestinal microbiota may be a therapeutic alternative to prevent dyslipidemia.

Kefir may be an option in the treatment of dyslipidemia. Choi et al. (30) observed that kefir prevented the increase of lipid parameters in mice fed with an obesogenic diet, proposing that kefir acted by preventing lipid intestinal absorption (**Table 1**). In humans, the consumption of kefir drink (250 ml) for 8 weeks also improved the lipid profile, which was similar to the control group that consumed low-fat milk. This improvement in the lipid profile was related to the loss of body weight, achieved with the consumption of kefir, as well as to the changes generated in the intestinal microbiota, which led to an increase in the production of short-chain fatty acids and bile acids (23) (**Table 1**); however, it is noteworthy that the amount of kefir offered and the intervention time are fundamental to achieve the desired result for improving dyslipidemia (27) (**Table 1**).

KEFIR AND IMMUNITY

The correct formation of intestinal microbiota during childhood is fundamental for the complete development of the baby, since the intestinal microbiota is part of the immune system, being important especially in the first months of life, when the rest of the immune system is still forming. In this context, premature babies may have immaturity in the immune, respiratory and neurological systems, suggesting a possible relationship between them (3). In addition, the immune system and the intestinal microbiota play a symbiotic function, maintaining a picture of non-inflammatory homeostasis: in cases of intestinal dysbiosis, there is an activation of the immune system, which leads to changes in the host's immunity. These changes can impair the maturation of the innate immune system or lead to autoimmune diseases, such as type 1 diabetes (61).

The immunomodulatory capacity of kefir has been tested in BALB/c mice by using different concentrations of commercial kefir (diluted at 1/10, 1/50, 1/100, or 1/200 proportions) and pasteurized kefir (diluted at 1/6, 1/10, 1/50, 1/100 proportions). The results show that kefir modulates the immune response in a dose-dependent manner by stimulating intestinal IgA production and inhibiting the Th1-type immune response (41) (**Table 1**).

Le Barz et al. (34) also evaluated kefir for probiotic strains with immunometabolic properties (**Table 1**). *Lactobacillus rhamnosus* Lb102 and *Bifidobacterium* Bf141 showed good results in the treatment of obesity and in metabolic syndrome, with a reduction in visceral fat and inflammation, and an improvement in glucose tolerance and insulin sensitivity. These results were explained by a modulation of the intestinal microbiota and the maintenance of intestinal integrity by the probiotics (34).

In humans, the action of kefir on intestinal integrity has also been observed. A study encompassing 28 healthy, overweight and asymptomatic adults found that the use of kefir modified serum zonulin concentration (26) (**Table 1**). Zonulin is a protein that participates in the integrity of tight junctions, that is, the maintenance of intestinal integrity. In the presence of tight junctions, there is control of the passage of molecules through the paracellular content, which prevents the development of inflammatory processes and the occurrence of diseases. However, the increase in zonulin production leads to loss of function of the intestinal barrier with consequent passage of antigens through the paracellular content and activation of the innate immune response (82).

The probiotic capacity of kefir was able to modulate intestinal microbiota composition, which prevented excessive intestinal permeability by increasing serum zonulin concentrations. Consequently, there was a control of low grade chronic inflammation generated in cases of altered intestinal permeability, as has been proposed in obesity (26).

KEFIR AND NEUROLOGICAL CHANGES

Neurological changes have multifactorial causes and intestinal microbiota also participates in this process. The composition of intestinal microbiota impacts the health of the microglia and the development of neuronal circuits, which contribute to neurological health (4). In addition, individuals with autism have a characteristic intestinal microbiota, which strengthens the relationship between intestinal microbiota and neurological disorders (1–4).

Noori et al. (36) assessed the role of kefir, fermented in both soy and cow's milk, in the treatment of depression, anxiety and cognitive impairment in an animal model subjected to stress due to the use of nicotine (**Table 1**). For this purpose, the animals were submitted to elevated plus maze (EPM) to assess anxiety, open field test (OFT) to assess locomotor activity and anxiety and forced swim test (FST) to assess depression. Both types of kefir were able to improve anxiety, decrease the severity of depression, and improve cognitive function throughout the treatment. Since kefir is a food rich in tryptophan, which is the precursor of serotonin, it is believed that kefir may be able to act on serotonin metabolism (36). Depression is related to changes in neuroplasticity being serotonin a neuromodulator capable of stimulating the development of neuronal plasticity (83). So, modulation in serotonin is a classic way of treating depression (36, 83).

Kefir has been reported to protect neurons from degradation through its anti-inflammatory capacity. Additionally, kefir may be able to activate receptors in the brain that stimulate learning and memory. So, in view of the cognitive improvement found, kefir can be potentially used for both prevention and treatment of depression and anxiety, especially in cases related to nicotine consumption. The authors believed that the results can be extrapolated to humans; however, further studies are needed to confirm this hypothesis (36).

The ability to modulate the intestinal microbiota and, consequently, anxiety and depression also suggests a positive role of kefir in these diseases (38, 42) (**Table 1**). Animals fed with kefir presented a specific intestinal microbiota composition, which presumably acts positively on the gut-brain axis. In addition, through analysis of the microbiome, authors suggest that kefir was able to stimulate the production of the gamma aminobutyric acid (GABA). It is hypothesized that *Lactobacillus reuteri* possibly converted 2-oxoglutarate to glutamate, which was subsequently converted into GABA, because of modulation of the intestinal microbiota by kefir, that leads to an increase in the production of *Lactobacillus reuteri*, a bacterial strain with benefits to the host's immune and metabolic system (42).

Kefir was also evaluated in an animal model simulating human depression, with stress induced through 7 stressors during 6 weeks. Mice supplemented with *Lactobacillus kefiranofaciens* ZW3 strain, isolated from kefir, had more movement (which indicates an increase in the ability to explore the environment and socialize), a greater preference for sucrose (which indicates that the animals returned to have pleasure) and a higher amount of water in their stools (which indicates a lower probability of having constipation, which is a condition associated with the occurrence of depression). In addition, there was an improvement in tryptophan metabolism, an increase in anti-inflammatory cytokines, a reduction in pro-inflammatory cytokines, and changes in the composition of the intestinal microbiota, with an increase in Actinobacteria, Bacteroides, Lachnospiraceae, Coriobacteriaceae, Bifidobacteriaceae *and Akkermansia,* and reduction in Proteobacteria (38).

All of these benefits indicate that the consumption of kefir was able to alter the metabolic pathways that led to the development of depression; however, further studies are needed to identify the optimal dosage to be consumed, so that the benefits of kefir are acquired by the host that consumes it (38).

The role of kefir on neurological diseases has also been studied in humans. For example, Ozcan et al. (25) assessed the consumption of kefir on sleep quality, quality of life and depression in post-menopausal women (**Table 1**). The consumption of kefir was positively correlated with the quality of life and quality of sleep, which might be a simple strategy, with good cost benefit and an alternative for the treatment of menopause; however, there were no promising results regarding to depression (25).

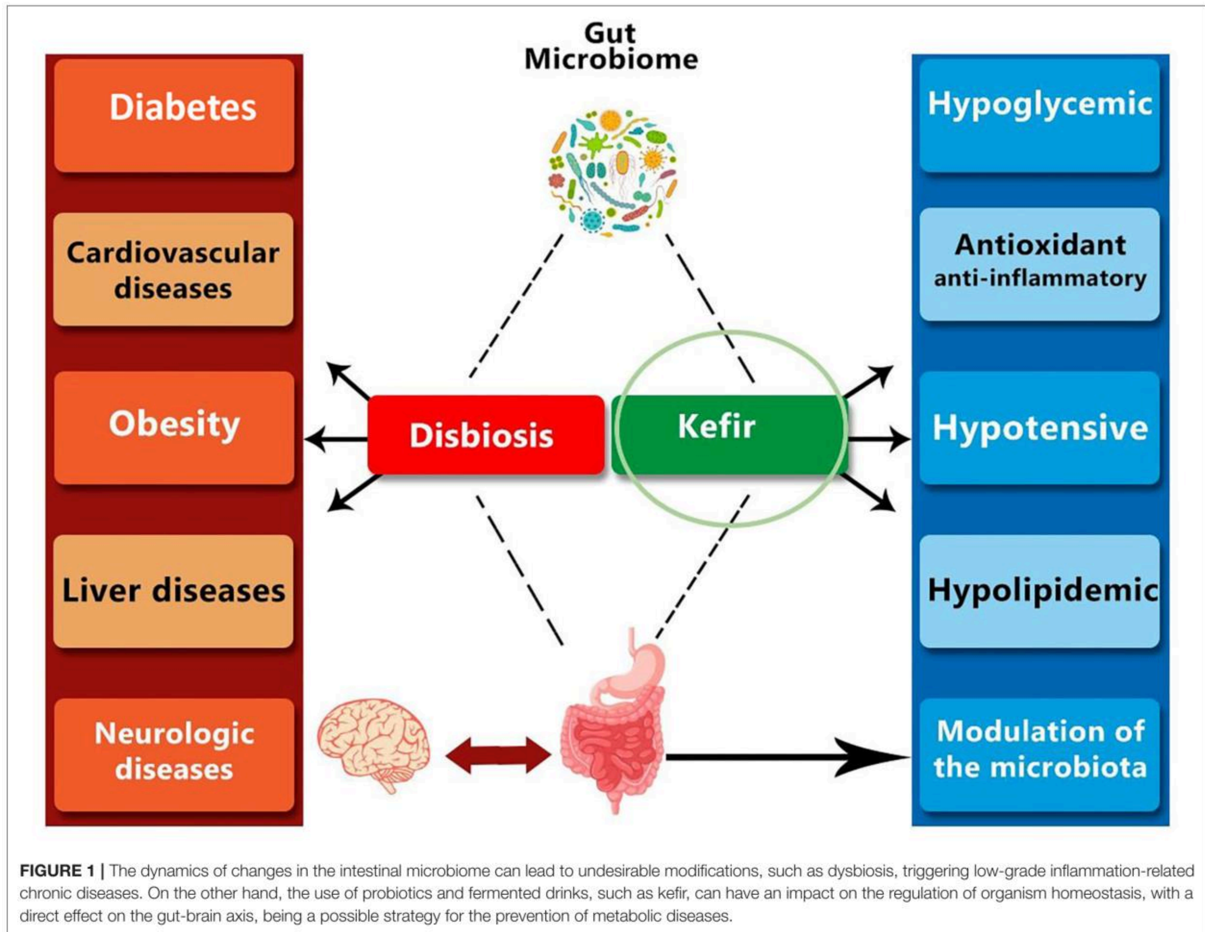

FIGURE 1 | The dynamics of changes in the intestinal microbiome can lead to undesirable modifications, such as dysbiosis, triggering low-grade inflammation-related chronic diseases. On the other hand, the use of probiotics and fermented drinks, such as kefir, can have an impact on the regulation of organism homeostasis, with a direct effect on the gut-brain axis, being a possible strategy for the prevention of metabolic diseases.

Studies that evaluate the psychobiotic activities of kefir on depression may be of great relevance, since the consumption of most antidepressants can generate side effects such as weight gain. In a study performed in Canada, for example, there was an association between obesity and high prevalence of antidepressants' prescription. Moreover, a greater prescription of antidepressants in the most severe grades of obesity (classes II and III) is commonly observed. The use of kefir, in this case, is particularly interesting since the use of medications can worsen obesity and decrease the chance of a positive response to treatment (84). Kefir could also be useful for the care of obese and depressed patients, although more intervention studies should be undertaken to properly evaluate its potential.

CONCLUSIONS

The inclusion of kefir in the Latin American market presents a good alternative as an adjuvant therapy in non-communicable diseases and may display an economic prognosis, since, in 2016, the value of kefir market in Latin America was amounted to 150.8 million U.S. dollars and it is expected to rise to about 204.7 million U.S. dollars by 2021 (85).

Alterations in gut microbiota, in the form of dysbiosis or metabolic endotoxemia shows systemic activity (**Figure 1**), since they allow the occurrence of low-grade chronic inflammations that affect the organism as a whole.

The modulation of the intestinal microbiota, thus, stands out as a good strategy for the prevention and treatment of diseases. The use of fermented foods with probiotic activity is a nutritional alternative to drug treatments, and kefir, due to the absence of harmful effects regarding its consumption—in animals and humans–, low cost, ease of preparation, and microbiological composition—rich in bioactive compounds, metabolites, and peptides—stands out as a potential food with functional benefits. Besides that, promising effects as immunomodulatory, hypocholesterolemic, antihypertensive and glycemic control are expected. However, it is mandatory to deepen into the molecular mechanisms and the microorganisms involved, and more well-controlled human intervention studies are required.

TABLE 2 | Microbiological and nutritional characterization of the kefir used in the different studies and production protocols (when available).

References	Concentration	Microbiological and nutritional composition	Production protocol
Bellikci-Koyu et al. (19)	–	- Culture DC1500I; - *Lactococcus lactis* subsp. *lactis, L. lactis* subsp. *cremoris, L. lactis* subsp. *diacetylactis, Leuconostoc mesenteroides* subsp. *cremoris, Lactobacillus kefiri, Kluyveromyces marxianus, Saccharomyces unisporus*.	- Produced in whole milk 3.5%; - Distribution to volunteers twice a week.
Bourrie et al. (28)	1% (w/v)	For commercial kefir: - *Streptococcus thermophilus*, *Lactobacillus delbrueckii subsp. bulgaricus*, *L. casei*, *L. acidophilus*, *L. delbrueckii subsp. lactis*, *L. rhamnosus*, *Bifidobacterium lactis*, *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*, *L. lactis* subsp. *cremoris*, *Leuconostoc mesenteroides* subsp. *cremoris*; - 8.0×10^6 CFU/ml. For traditional kefir: - No information.	- Produced daily in cow's milk 2%; - Fermentation at 22°C for 18 h.
Chen et al. (29)	–	- *Lactobacillus mali* APS1; - 10^7 to 10^{10} CFU/mg.	- Fermentation at 30°C for 12 h.
Fathi et al. (23)	–	–	- Produced by Fars Pegah Dairy Co., Shiraz, Iran.
Golli-Bennour (31)	–	- Traditional Tunisian culture, containing lactic acid bacteria; - Predominant population are lactic acid bacteria ($9.5 \pm 0.15 \times 10^{10}$ CFU/g) and yeast ($9.2 \pm 0.14 \times 10^6$ CFU/g).	- Daily production.
Kim et al. (32)	10%	- Each mL contains: 9.62 ± 0.19 Log CFU of lactic acid bacteria, 9.52 ± 0.12 Log CFU of acetic acid bacteria and 7.67 ± 0.30 Log CFU of yeast.	- Fermentation at 25°C for 24 h.
Kim et al. (33)	5% (w/v)	- *Lactobacillus kefiranofaciens, L. kefiri, L. lactis, Leuconostoc mesenteroides*.	- Fermentation at 25°C for 24 h.
Kim et al. (20)	10%	- Each mL contains: 9.32 ± 0.23 Log CFU of lactic acid bacteria and 7.12 ± 0.36 Log CFU of yeast.	- Fermentation at 25°C for 24 h.
Lim et al. (35)	1:10 (w/w)	- Grains from the KU Center for Food Safety, College of Veterinary Medicine, Konkuk University.	- Produced in UHT milk; - Fermentation ate 30°C overnight; - Production for 6 continuous weeks followed by lyophilization and storage at - 20°C.
Noori et al. (36)	–	- pH: 4.8	- Cow's milk UHT or soy milk; - Fermentation at 25°C for 24 h followed by storage at 4°C to interrupt the process.
Ostadrahimi et al. (24)	–	- *Streptococcus thermophiles*, *Lactobacillus casei*, *L. acidophilus*, *Bifidobacterium lactis*; - Fat: 0.3%.	- Weekly production.
Ozcan et al. (25)	–	- Kefir produced by Altinkiliç Company, Turquia; - No sugar and no flavor.	- Stored at 4°C and delivered to volunteers weekly.
Praznikar et al. (26)	–	- *Lactobacillus parakefiri*, *L. kefiri*, *L. kefiranofaciens* ssp. *kefirgranum*, *Kluyveromyces marxianus*, *Kazachstania exigua*, *Rhodosporidium kratochvilovae;* - 80% water; - pH 4.03.	- Produced by Ljubljanske mlekarne (Ljubljana, Slovenia)
Rosa et al. (37)	5% (w/w)	- Lactic acid bacteria: 2.78×10^7 CFU/ml; - Yeast: 2.94×10^8 cell/ml; - Lactic acid 0.806 ± 0.04 g/100 g; - Fat: 3.03 ± 0.16 g/100 g; - Protein: 3.03 ± 0.01 g/100 g; - pH 4.10 ± 0.10.	- Produced in pasteurized milk (3.5% protein, 5% carbohydrate and 3% fat); - Fermentation at 25–28°C for 24 h.
St-Onge et al. (27)	–	–	- Produced by Liberty Co, Candiac, Québec.
Sun et al. (38)	–	- Culture Collection Center of the Institute of Microbiology, Chinese Academy of Sciences (accession number CGMCC2809); - *Lactobacillus kefiranofaciens* ZW3.	–

(Continued)

TABLE 2 | Continued

References	Concentration	Microbiological and nutritional composition	Production protocol
Talib et al. (43)	10% (w/v)	- Mainly: *Lactobacillus harbinensis*, *L. paracasei*, *L. plantarum*; - All: *L. harbinensis* B22, HBUAS5305, NBRC 100982, FQ003; *L. brevis* HDRS2; *L. sp* MS6; *L. plantarum* Gt2, ZDY36a, HBUAS52249, NWAFU1558, Akhavan-Q3, Y-2-9, MSD1-4, DSR M2, LQ80; *L. paracasei* HBUAS52231, HBUAS53273; *L. casei* YQ116, H19.9.	- Produced in water solution with brown sugar; - Fermentation at room temperature for 24 h.
Tiss et al. (39)	5% (w/v)	–	- Soy milk; - Fermentation at 24 h; - Storage at 4°C (Produced every 7 days before consumption).
Tung et al. (40)	10% (w/v)	–	- Fermentation at 20°C for 20 h; - Kefir undergoes two fermentation processes: First at 5% w/v concentration and then at 10% w/v concentration.
Vinderola et al. (41)	–	–	- Produced in pasteurized milk with 1.8% fat by Les Produits de - Marque Liberté (Candiac, Québec, Canada)
Wouw et al. (42)	2% (w/v)	–	- Produced in whole milk from Irish cows; - Fermentation at 25°C for 24 h.

Where: CFU, colony forming unit; g, gram; mg, milligram; mL, milliliters; pH, hydrogen potential; UHT, ultra-high temperature; v, volume; w, weight.

FUTURE PERSPECTIVES

Kefir is a low-cost fermented product that is gaining interest due to its potential impact in the prevention and treatment of non-communicable diseases. Yet, the microbiological composition of kefir varies according to the geographical location, fermentation matrix (water solution with sugar, whole cow's milk, skimmed cow's milk, goat's milk, donkey milk, among others), environmental conditions (temperature and fermentation time), and grain (g)/drink (mL) ratio used in the fermentation of the product. Also, the presence of yeasts and their proportion in the drink, as well as the production conditions, among others, can generate drinks with different compositions and characteristics (**Table 2**). Consequently, as a future perspective, it is believed that the next studies will focus on the development of a unified production protocol, as well as on the determination of which microorganisms should be present in the starter culture and the drink, in order to consider that the final product, in fact, can be classified as a kefir.

STUDY LIMITATIONS

As previously discussed, there are many factors involved in the production and dosage of kefir. This variability hinders the reproducibility of the results discussed in this narrative review and impairs future meta-analyses, considering this factor as a limitation.

AUTHOR CONTRIBUTIONS

MCGP has been responsible for conception of this article. MCGP and MMD have been involved in aquisition, analysis and interpretation of data, as well as in drafting the manuscript, and revising it critically. JAM and FIM have revised the manuscript critically, giving important intelectual content. All authors have read and approved the final manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CAPÍTULO 3

AVALIAÇÃO DE MARCADORES DE SAÚDE INTESTINAL EM ANIMAIS TRATADOS COM PROBIÓTICO E, OU, ANTIMICROBIANO

1 JUSTIFICATIVA

Neste capítulo são apresentados os resultados de análises realizadas em parceria com os pesquisadores Dr. Vinícius da Silva Duarte e Dr. Davide Porcellato, da Universidade Norueguesa de Ciências da Vida (Noruega); e Dr. Lúcio Flávio Macedo Mota, da Universidade de Padova (Itália), entre os anos de 2020 e 2021.

Para a elaboração deste capítulo foram utilizados dados dos projetos "Comparação entre a Atuação de Probiótico e, ou, Antimicrobiano Associado à Restrição Energética: um estudo experimental em modelo animal obeso" e "Efeito do Tratamento com Antimicrobiano ou Probiótico sobre Parâmetros Associados com a Obesidade e a Resistência Insulínica em Modelo Animal".

O objetivo foi analisar como a produção de ácidos graxos de cadeia curta (ácidos acético, propiônico e butírico) e os marcadores de permeabilidade intestinal (lactulose e manitol) se relacionavam com a microbiota intestinal.

2 METODOLOGIA GERAL

2.1 Produtos-teste

O potencial probiótico utilizado foi o *Lactobacillus gasseri* LG_G12 (Lemma®, Brasil), enquanto o antimicrobiano foi o da classe das ceftazidimas (Triaxton®, Blau Farmacêutica S/A).

Descrições completas sobre esses produtos encontram-se no item 2.2.1 desta tese.

2.2 Cálculo experimental

O tamanho amostral foi calculado seguindo o proposto por Mera e colaboradores (1998), conforme previamente descrito no item 2.2.2 desta tese (Apêndice A).

2.3 Desenho experimental

Modelo experimental

Foram utilizados 72 camundongos C57BL/6J, machos, com 30 dias de vida, provenientes do Biotério Central do Centro de Ciências Biológicas e da Saúde da Universidade Federal de Viçosa, conforme previamente descrito no item 2.2.3 desta pesquisa.

Fase 1 – Desenvolvimento da obesidade

O desenvolvimento da obesidade aconteceu mediante a oferta da dieta *high-fat* e da solução de frutose, como previamente descrito no item 2.2.3 desta tese.

Fase 2 – Tratamento da obesidade

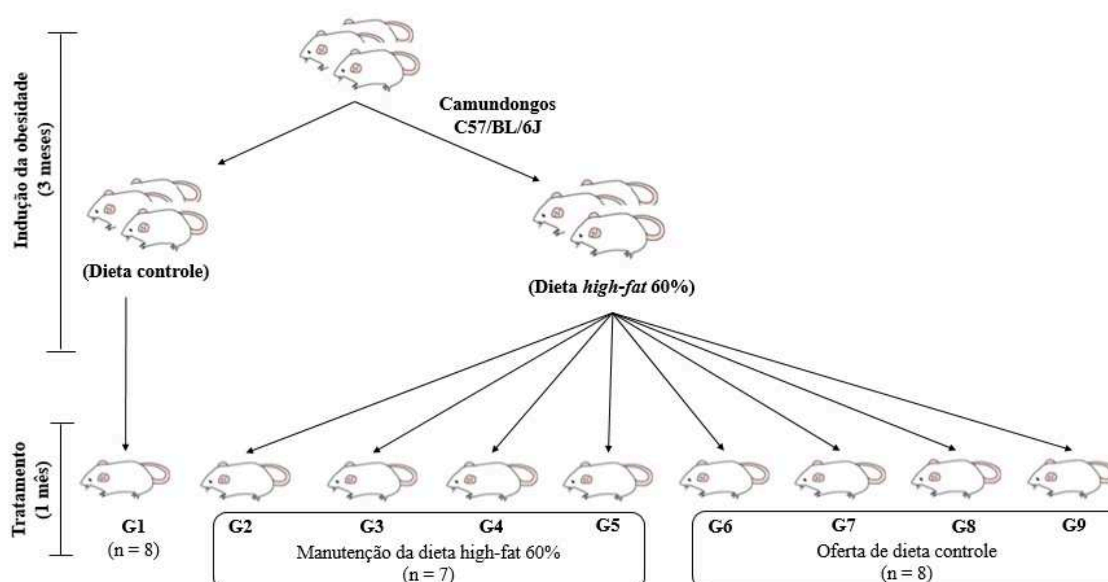
Após a confirmação da obesidade pelos maiores valores de peso corporal no grupo que consumiu dieta *high-fat* e a solução de frutose, os animais obesos foram novamente divididos randomicamente. Assim, obteve-se a presença de nove grupos experimentais, distribuídos entre grupo controle negativo (G1), grupos intervenção A (G2 a G5), que consumiram dieta *high-fat* durante a fase de tratamento; e grupos intervenção B (G6 a G9), que consumiram dieta-padrão e água durante a fase de tratamento (Tabela 1).

Tabela 1 – Descrição dos grupos experimentais

Grupo	**Intervenção**	**Definição**
G1	-	Grupo controle negativo
G2	A	Grupo controle positivo com consumo de dieta *high-fat*
G3	A	Grupo tratado com potencial probiótico com consumo de dieta *high-fat*
G4	A	Grupo tratado com antimicrobiano com consumo de dieta *high-fat*
G5	A	Grupo tratado com antimicrobiano seguido por potencial probiótico com consumo de dieta *high-fat*
G6	B	Grupo controle positivo com consumo de dieta-padrão
G7	B	Grupo tratado com potencial probiótico e consumo de dieta-padrão
G8	B	Grupo tratado com antimicrobiano e consumo de dieta-padrão
G9	B	Grupo tratado com antimicrobiano seguido por potencial probiótico com consumo de dieta-padrão

Durante a fase de tratamento, que teve duração de um mês, os grupos G2 a G5 (n = 7) continuaram recebendo dieta *high-fat* e solução de frutose 10%, assim como na fase 1 do experimento. Já os outros animais (n = 8) consumiram dieta-padrão AIN-93M e água destilada, com o objetivo de promover restrição calórica em relação à dieta anteriormente consumida. Além disso, houve a oferta diária, via gavagem, de potencial probiótico (*Lactobacillus gasseri* LG-G12), antimicrobiano (ceftriaxona) e antimicrobiano seguido de probiótico. Assim como previamente descrito no item 2.2.3 desta tese, os animais receberam os tratamentos por gavagem (Figura 1).

Figura 1 – Desenho do experimento



Em que G1 = grupo controle negativo, G2 = grupo controle positivo com consumo de dieta *high-fat*, G3 = grupo tratado com potencial probiótico com consumo de dieta *high-fat*, G4 = grupo tratado com antimicrobiano com consumo de dieta *high-fat*, G5 = grupo tratado com antimicrobiano seguido por potencial probiótico com consumo de dieta *high-fat*, G6 = grupo controle positivo com consumo de dieta- padrão, G7 = grupo tratado com potencial probiótico e consumo de dieta-padrão, G8 = grupo tratado com antimicrobiano e consumo de dieta-padrão e G9 = grupo tratado com antimicrobiano seguido por potencial probiótico com consumo de dieta-padrão.

2.4 Determinação da permeabilidade intestinal

Após a fase de tratamento, os animais receberam solução de lactulose (Daiichi Sankyo®, Barueri, Brasil) e manitol (Synth®, Diadema, Brasil), por gavagem, sendo-lhes coletada toda a urina das 24 horas subsequentes. A quantificação dos açúcares foi realizada por cromatografia líquida de alta eficiência (detector modelo RID 10A, Shimadzu®, Tóquio, Japão), como previamente descrito no item 2.2.11 desta tese.

2.5 Determinação dos ácidos graxos de cadeia curta

A extração dos ácidos graxos de cadeia curta (acético, propiônico e butírico) do conteúdo cecal foi realizada por cromatografia líquida de alta eficiência (Ultimate 3000, Dionex, Thermo Fisher Scientific®, Waltham, Massachusetts, EUA), como previamente descrito no item 2.2.12 desta tese.

2.6 Determinação da composição da microbiota intestinal

Como previamente descrito no item 2.2.13 desta tese, a extração do DNA metagenômico das amostras de fezes foi realizada segundo a metodologia adaptada de Zhang e colaboradores (2006), e o sequenciamento do DNA da região 16S rRNA foi executado pela empresa Macrogen (Macrogen Inc®, Seul, Coreia de do Sul) com o uso do sequenciador Illumina MiSeq (Illumina, USA). Os dados foram processados pelo pacote DADA2, versão 1.8 (CALLAHAN *et al*., 2016).

Os dados fastq brutos foram submetidos ao Sequence Read Archive (SRA) do NCBI, com os números de acesso PRJNA705760 e PRJNA745938.

2.7 Análises estatísticas

A abundância das unidades operacionais taxonômicas (OTU) foi dimensionada e, em seguida, procedeu-se à análise de componentes principais (PCA) usando a função prcomp do Programa R (R Core tram 2021). A normalização foi realizada para garantir que os resultados do PCA fossem matematicamente independentes da medida de sobreposição. A análise fatorial para informações de OTU foi feita com o objetivo de obter as variáveis que contribuem para a maior diferenciação entre os grupos de tratamento usando o pacote FactoMineR R (LÊ *et al*., 2008).

A normalidade das variáveis foi determinada pelo teste de Shapiro-Wilk. Para dados paramétricos, foi adotada a análise de variância unilateral (*One Way* ANOVA) seguida pelo teste *Post Hoc* de comparação múltipla de Tukey, enquanto para dados não paramétricos o teste de Kruskal-Wallis foi aplicado e complementado pelo teste de comparações múltiplas de Dunn. Os resultados foram expressos como média ± erro-padrão da média (SEM). Táxons com abundância diferente após a fase de tratamento que mais provavelmente explicam as diferenças entre os grupos (ou seja, biomarcadores fecais) foram avaliados usando a

ferramenta de Análise Discriminante Linear (LDA) associada ao tamanho do efeito (LEfSe) (SEGATA *et al*., 2010), definindo, assim, o valor alfa de 0,05 e o limite de Log LDA de 2,0.

A diversidade beta foi avaliada usando as métricas UniFrac não ponderadas e ponderadas para avaliar as dissimilaridades da comunidade bacteriana entre os grupos. A análise de variância multivariada permutacional (PERMANOVA) foi usada para testar se as distâncias entre as amostras dentro de determinado grupo são mais semelhantes entre si ou não. As correlações entre as variáveis contínuas foram determinadas pela correlação de Pearson (dados paramétricos) ou de Spearman (dados não paramétricos) com o pacote de softwares de Estatística Paleontológica para educação e análise de dados (PAST, v4.06b) (HAMMER *et al*., 2001).

Por fim, o software Statistical Analysis of Metagenomic Profile (STAMP) (PARKS *et al*., 2014) foi usado para explorar e comparar o potencial metabólico das comunidades microbianas previstas entre os grupos. O perfil funcional foi construído com base no MetaCyc Metabolic Pathway Database (CASPI *et al*., 2020). O teste t de Welch (bilateral) foi adotado como teste de hipótese estatística. Para ambas as análises, um valor de p inferior a 0,05 foi considerado uma diferença significativa.

2.8 Aspectos éticos e registro

Os projetos integrantes deste capítulo foram submetidos à Comissão de Ética no Uso de Animais da Universidade Federal de Viçosa (CEUA/UFV), Protocolo Nº 33/2018 (Anexo 1) e Protocolo Nº 09/2017 (Anexo 2), da qual obteve aprovação. Nesse procedimento, todas as Normas do Conselho Nacional de Controle de Experimentação Animal (CONCEA, 2008) foram seguidas.

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3 RESULTADOS

3.1 Artigo original

Título: **"**Modulation of the Gut Bacteriome and Intestinal Health by *Lactobacillus gasseri* LG-G12, Ceftriaxone, and a *High-Fat Diet*".

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ABSTRACT

Gut microbiota imbalance is associated with the occurrence of numerous diseases and its modulation rises as a promising strategy to improve intestinal health parameters. In this context, probiotics, antimicrobials, and diet stand out. Here, we aimed to evaluate how these factors, combined or not, can modulate the gut microbial composition and improve intestinal health. For this C57BL/6J mice were first induced to obesity and then redistributed and fed with an obesogenic diet (intervention A) or with the standard AIN-93 diet (intervention B). Concomitantly, all the groups underwent a treatment phase with the administration of *Lactobacillus gasseri* LG-G12, ceftriaxone, or ceftriaxone followed by *L. gasseri* LG-G12. At the end of the experimental period, metataxonomic analysis, functional profiles of gut microbiota, as well as intestinal permeability and caecal concentration of short-chain fatty acids were carried out. High-fat diet intake impaired bacterial diversity/richness, which was counteracted by the association *L. gasseri* LG-G12 and low-calorie diet. Additionally, SCFA-producing bacteria were negatively correlated with high intestinal permeability parameters, which was further confirmed by the prediction of functional profiles of gut microbiota. This study brings novel perspectives into the use of probiotics as an adjuvant treatment of obese or overweight subjects undergoing or not antimicrobial therapy.

Keywords: *Lactobacillus gasseri* G12; Intestinal health; Ceftriaxone; Gut microbiota; Short-chain fatty acids.

1. INTRODUCTION

Approximately 70% of the microbiota identified in humans are found in the gut and are composed of different microorganisms such as bacteria, fungi, viruses, and protozoa (Ley *et al.*, 2006; Sokol, 2019). Recently more attention has been directed to the composition and imbalance of the intestinal microbiota, due to its effect on metabolic disturbance, gut dysbiosis as well as other metabolic diseases (Thaiss *et al.*, 2016; Sokol, 2019). In this context, understanding the factors affecting intestinal microbiota modulation represents a great opportunity to develop strategies to prevent and treat dysbiosis-related diseases.

Probiotics, antimicrobials, and diet are among the main modulators of the intestinal microbiota (Hunt *et al.*, 2017; Levy *et al.*, 2017; Cani *et al.*, 2019). Probiotics are live microorganisms that, when consumed in adequate amounts, generate benefits for the health of the host. They act immunologically, through the modulation of cytokines, and also by inducing oral tolerance to food antigens. Probiotic cultures also affect physiological mechanisms stimulating mucosal integrity and control of intestinal pH, and can protect against microbial pathogens by producing bacteriocins (Hunt *et al.*, 2017).

Antimicrobials, on the other hand, can alter the intestinal microbiota in the long term, contributing to the loss of microbial diversity in the gut and causing shifts in metabolic capacity, potentially reducing the colonization resistance against invading pathogens, which can lead to the development of dysbiosis (Lange *et al.*, 2016). However, in some cases, the use of antimicrobials may be necessary, as in the treatment for *Clostridium difficile*. In addition, the ability of antimicrobials to alter the intestinal microbiota varies according to the drug's spectrum of action and its absorption capacity, highlighting that antimicrobials with a broader spectrum of action and systemic activity have a greater capacity to generate intestinal dysbiosis (Kim, Covington & Pamer, 2017).

Similarly, balanced and diversified diets, such as the Mediterranean standard diet, rich in fruits, vegetables, whole grains, and seafood, contribute to higher gut microbiota diversity, stimulating the intestinal barrier and immune tolerance. In contrast, Western-standard diets rich in red meat, processed food, refined sugar, and saturated fat can promote intestinal dysbiosis, which contributes to the loss of intestinal barrier function and immune intolerance to food antigens (Khalili *et al.*, 2018).

In addition to the intestinal microbiota, other parameters such as the production of short-chain fatty acid (SCFA) and intestinal permeability are also indicative of intestinal health (Ríos-Covián *et al.*, 2016; Khoshbin & Camilleri, 2020). These observations, highlight that

shifts in the intestinal microbiota composition can be related to changes in intestinal permeability (van Hul *et al.*, 2018). Furthermore, it is observed that in the presence of metabolic alterations, such as obesity, there is an impairment of intestinal health (da Silva, dos Santos & Bressan, 2013).

Given the above, and the need for more understanding about which mechanisms are used, by probiotics and antimicrobials, to control dysbiosis, this study aimed to evaluate how the potential probiotic *Lactobacillus gasseri* LG-G12 (LG-G12), the antimicrobial ceftriaxone, and the high-fat diet (de Moura e Dias *et al.*, 2021a, 2021b) act modulating the intestinal microbiota and affect parameters related to intestinal health.

2. MATERIALS AND METHODS

2.1 Animals

The experiment was approved by the Animal Use Ethics Committee of the Universidade Federal de Viçosa, according to the protocols' numbers 09/2017 and 33/2018, and followed the principles established by the National Animal Experimentation Control Council (Brasil, 2008).

Seventy-two male C57BL/6J mice (della Vedova *et al.*, 2016; de Moura e Dias *et al.*, 2021b) from the Central Vivarium of the Center for Biological and Health Sciences at the Universidade Federal de Viçosa (UFV) were used in the experiment. The animals were kept in collective cages, with 2 animals per cage, with a 12 h light/dark cycle and an average temperature of 22±2 ºC. Throughout the experimental period, the animals had free access to the fructose solution and the diet, which were administered following the pair-feeding scheme. The entire animal experiment was carried out at the Experimental Nutrition Laboratory of the Department of Nutrition and Health at UFV.

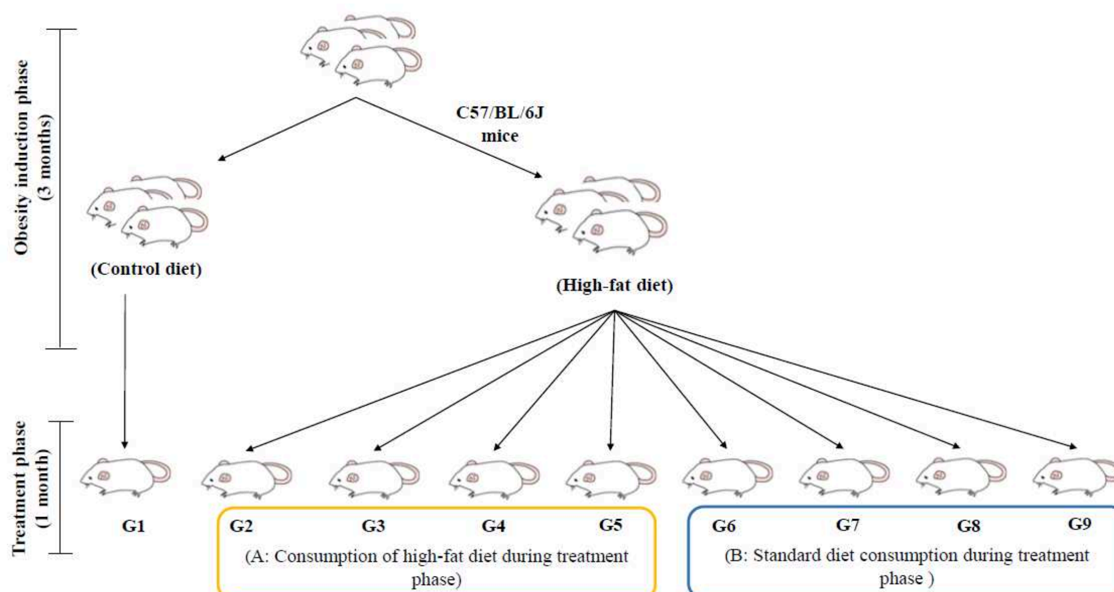
2.2 Experimental design and diets

Initially, mice, with 5 weeks, underwent a protocol for inducing overweight that lasted 12 weeks (induction phase). During this period, the animals were fed with a high-fat diet with 60% of the calories based on lipids (nutritional composition based on the diet D12492 of Research Diets, Inc) (Membrez *et al.*, 2008) and a 10% fructose solution (Synth®, Diadema, Brazil) instead of drinking water (della Vedova *et al.*, 2016).

After this period, the treatment phase began and the mice were randomly divided into nine experimental groups: negative control (n = 8), positive control A (n = 7), positive control B (n = 8), antimicrobial A (n = 7), antimicrobial B (n = 8), potential probiotic A (n = 7), potential probiotic B (n = 7), antimicrobial combined with potential probiotic A (n = 7), and antimicrobial combined with potential probiotic B (n = 8). Intervention A groups were those who continued to receive an obesogenic diet during the treatment phase. In contrast, intervention B groups were the ones that started to receive the AIN-93 standard diet during the treatment phase. All treatments were daily administered through gavage, always at the same time, in the evening.

Animals in the control group were treated with distilled water. The antimicrobial group was treated with 500 mg of ceftriaxone (Triaxton, Blau Farmacêutica S/A®, Brazil) kg of body mass (Rajpal *et al.*, 2015). The potential probiotic group received 10^9 colony forming units (CFU) of LG-G12 (Lemma Supply Solutions®, Brazil). The antimicrobial combined with the potential probiotic group received 500 mg of ceftriaxone kg of body weight in the first two weeks of the treatment phase and, in the following two weeks, 10^9 CFU of *L. gasseri* LG-G12 **(Fig. 1)**.

Figure 1 – Experimental design.



Where: G1: negative control group, G2: positive control group with consumption of high-fat diet, G3: group treated with probiotic potential with consumption of high-fat diet, G4: group treated with antimicrobial with consumption of high-fat diet, G5: group treated with antimicrobial followed by probiotic potential with consumption of high-fat diet, G6: positive control group with consumption of standard diet, G7: group treated with probiotic potential and consumption of standard diet, G8: group treated with antimicrobial and consumption of diet standard and G9: group treated with antimicrobial followed by probiotic potential with consumption of standard diet.

At the end of the treatment phase, the animals were euthanized after being anesthetized with 3% isoflurane (Cristália®, Belo Horizonte, Brazil) and, subsequently, subjected to total exsanguination. The use of this anesthetic followed by a physical method is a form of euthanasia recommended for rodents by CONSEA (Brasil, 2008). Tissue samples were collected and properly stored for further analysis. For additional information regarding the experimental design, the authors invite the reader to consult the study conducted by de Moura e Dias et al. (2021).

2.3 Intestinal permeability

As previously described in de Moura e Dias et al. (2021), after the treatment phase, the animals received a solution of lactulose (Daiichi Sankyo®, Barueri, Brazil) and mannitol (Synth®, Diadema, Brazil) via gavage, and all the urine from the subsequent 24 hours were collected. Quantification of sugars was performed using high-performance liquid chromatography (detector model RID 10A, Shimadzu®, Tokyo, Japan).

2.4 Determination of short-chain fatty acids

As previously described in de Moura e Dias et al. (2021), the quantification of short-chain fatty acids (acetic, propionic, and butyric) from the cecal contents was performed following the methods of Siegfried et al. (1984), and the analytes were analyzed by high-performance liquid chromatography (Ultimate 3000, Dionex, Thermo Fisher Scientific®, Waltham, Massachusetts, USA).

2.5 Composition and functional prediction of the intestinal microbiota

As previously described in de Moura e Dias, et al. (2021), after the treatment phase, fecal samples were collected from all experimental groups and pooled within each group. The pooling of fecal samples was adopted in this study because the animals are isogenic and live in a controlled environment, which means that they are considered biological replicates.

The amount of 200 mg of collected feces was weighed and metagenomic DNA was extracted using the methodology adapted from Zhang et al. (2006). Afterward, the quantity of the extracted DNA was evaluated utilizing Qubit (Invitrogen, Thermo Fisher, USA), whereas its integrity and quality were verified through electrophoresis in 1.8% agarose gel. The V3 and V4 regions of 16S rRNA genes were PCR amplified utilizing specific primers (Bakt 341F

and Bakt 805R) and sequenced using an Illumina MiSeq desktop sequencer (Illumina, San Diego, CA, USA) at the Macrogen Company (Macrogen Inc®, Seoul, South Korea).

Microbiota data were processed and analyzed with QIIME2 (version 2020.2) (Bolyen *et al.*, 2019). In brief, raw sequence data obtained across the C57BL/6J mice stool samples from the group G1 to the group G9 were imported via the Casava1.8 paired-end pipeline followed by denoising with DADA2 (Callahan *et al.*, 2016) (via q2-dada2). Subsequently, an amplicon sequence variants (ASV) table was constructed to generate a phylogenetic tree by using the align-to-tree-mafft-fasttree pipeline from the q2-phylogeny plugin (Katoh, 2002; Price, Dehal & Arkin, 2010). When appropriate, samples were rarefied to a sampling depth of 120,326 sequences. Taxonomy was assigned to the 16S data using a Naïve Bayes pre-trained Greengenes 13_8 99% OTUs classifier (DeSantis *et al.*, 2006).

For the functional prediction of the gut microbiota, ASVs (read sequences and read counts) were used as inputs for the PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) pipeline (Douglas *et al.*, 2020). In brief, ASVs were inserted and aligned into a reference tree composed of 20,000 full 16S rRNA genes from bacterial and archaeal genomes using, respectively, the tools HMMER (http://www.hmmer.org) and EPA-ng/ GAPPA (Barbera *et al.*, 2019; Czech & Stamatakis, 2019). Subsequently, the castor R package (Louca & Doebeli, 2018) was used to predict the missing gene families (Enzyme Commission numbers) for each ASV, as well as their respective copy number of 16S rRNA gene sequences, by using the output tree generated in the previous step. Finally, MinPath (Ye & Doak, 2009) was adopted to infer MetaCyc pathways based on EC number abundances.

The raw fastq data were submitted to Sequence Read Archive (SRA) from NCBI under accession numbers PRJNA705760 and PRJNA745938.

2.6. Statistical analysis

The principal component analysis (PCA) was performed using the relative abundance of the most abundant genera (greater than 0.1% in at least one sample). OTU abundance was scaled and then the PCA analysis was performed using the prcomp function of the R program (R Core Team 3.6.2, 2019). Normalization was performed to assures that the PCA results are mathematically independent of the overlap measure. The factorial analysis for OTU information was used aiming to obtain the variables which contribute to the highest

differentiation across the treatment groups using the FactoMineR R package (Lê, Josse & Husson, 2008).

The normality of variables (i.e., Shannon, Chao1, acetate, propionate, butyrate, total SCFA, and lactulose/mannitol ratio) was determined by the Shapiro-Wilk test. For parametric data, one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison *post hoc* test was adopted, whereas for non-parametric data, the Kruskal-Wallis test was applied and complemented by Dunn's multiple-comparison test. Results were expressed as mean ± standard error of the mean (SEM). Differently abundant taxa after the treatment phase that most likely explain the differences among the groups (i.e. fecal biomarkers) were assessed using the linear discriminant analysis (LDA) effect size (LEfSe) (Segata *et al.*, 2011) tool setting up an alpha-value of 0.05 and Log LDA threshold of 2.0.

Beta-diversity was assessed using the Unweighted and Weighted UniFrac metrics to evaluate bacterial community dissimilarities between the groups. Permutational multivariate analysis of variance (PERMANOVA) with 999 permutations was used to test whether distances between samples within a certain group are more similar to each other or not. Correlations between continuous variables were determined by Pearson's (parametric data) or Spearman's (non-parametric data) correlation with the Paleontological statistics software package for education and data analysis (PAST, v4.06b) (Hammer, Harper & Ryan, 2001).

Lastly, the software Statistical Analysis of Metagenomic Profile (STAMP) (Parks *et al.*, 2014) was used to explore and compare the metabolic potential of the predicted microbial communities across the groups. The functional profiling was built based on the MetaCyc Metabolic Pathway Database (Caspi *et al.*, 2020). The Welch's t-test (two-sided) was adopted as a statistical hypothesis test. For both analyses, a p-value less than 0.05 was considered as a significant difference.

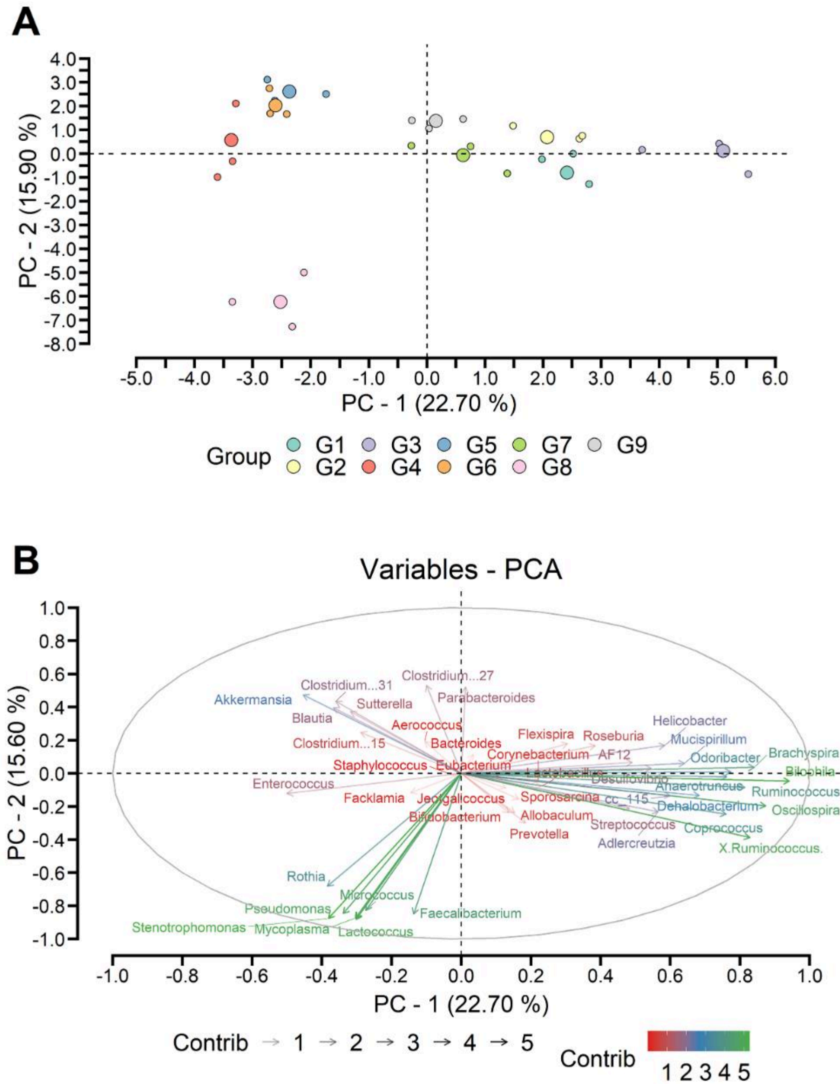
3. RESULTS AND DISCUSSION

3.1 Multidimensional scaling analysis (MDS)

Overall, MDS analysis based on the relative abundance of the intestinal microbiota explains around 38.9% of the variability among the groups (G1 to G9) **(Fig. 2)**. With regards to the intervention "B" (G1, G6, G7, G8, and G9 groups) there is a microbial homogeneity between G7 and the G9, which indicates that LG-G12 acted as a positive modulator of the intestinal microbiota **(Fig. 2A)**. Additionally, as observed in **Fig. 2B**, distinct bacterial genera

contributed differently to the total intestinal microbial load across the different experimental groups, which suggests different biological and/or metabolic capabilities **(Fig. 2B)**.

Figure 2 – Principal Component Analysis (PCA) (A) and factorial analysis (B) are based on the most abundant OTUs (relative abundance > 0.1%) at the genus level across the groups.



Where: G1: negative control group, G2: positive control group with consumption of high-fat diet, G3: group treated with probiotic potential with consumption of high-fat diet, G4: group treated with antimicrobial with consumption of high-fat diet, G5: group treated with antimicrobial followed by probiotic potential with consumption of high-fat diet, G6: positive control group with consumption of standard diet, G7: group treated with probiotic potential and consumption of standard diet, G8: group treated with antimicrobial and consumption of diet standard and G9: group treated with antimicrobial followed by probiotic potential with consumption of standard diet. The red to green gradient indicates a low to high magnitude of the contribution of a given genus for a specific factor.

3.2 Alpha and beta diversity

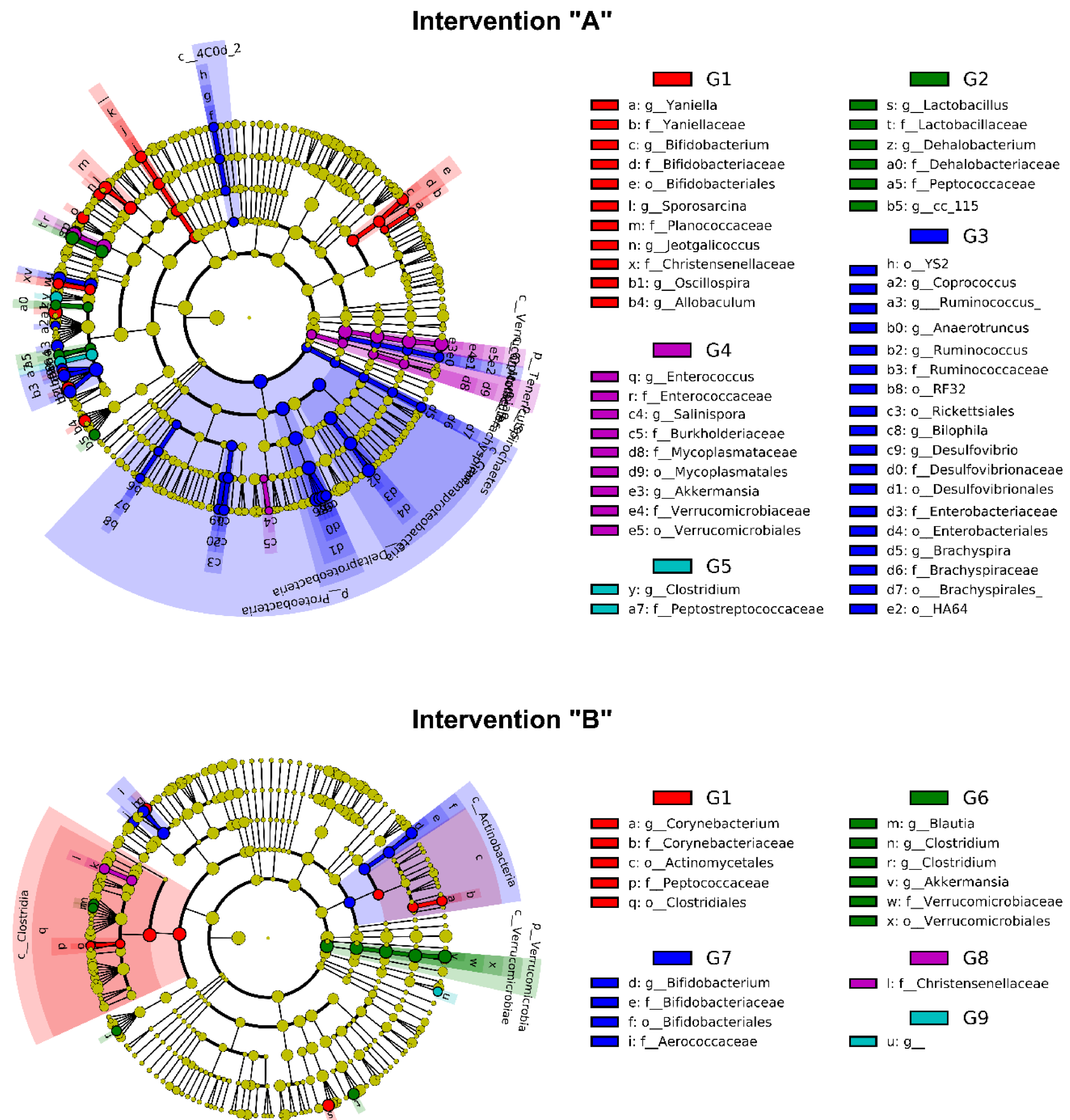
There were no significant differences regarding alpha diversity indices between the interventions "A" and "B" **(Table 1)**. However, a trend towards a reduction in Shannon and Chao1 indices was observed in the groups who received antimicrobial and high-fat diets (G4 and G5) when compared to the groups where ceftriaxone was administered combined with a standard diet (G8 and G9). This result shows that a synergy between antibiotic and high-fat diet can impair bacterial diversity/richness. As described elsewhere (Ianiro, Tilg & Gasbarrini, 2016; Cheng *et al.*, 2020), antibiotic usage can cause damages to the intestinal epithelium, as well as selectively favor the growth of specific microbial taxa. When associated with calorie diets, both factors can negatively contribute to the development of the intestinal microbiota (Ianiro *et al.*, 2016; Kong *et al.*, 2019), which justifies our findings for the group G4 (intervention "A"). Lastly, the change to a standard diet (intervention "B") was effective in increasing intestinal diversity in all groups, which justifies the absence of statistical significance.

3.3 Linear discriminant analysis Effect Size (LEfSe)

3.3.1 Phylum level

Overall, LEfSE analysis identified 127 significantly discriminative features ($LDA > 2$, $p < 0.05$). The phyla *Proteobacteria* ($LDA > 5$) and *Spirochaetes* ($LDA > 2.0$) were enriched in group G3, whereas the phylum *Tenericutes* ($LDA >2.0$) appeared as a biomarker for the group G4 **(Fig. 3)**. The expansion of *Proteobacteria* is associated with the consumption of a processed diet, commonly rich in emulsifiers and artificial sweeteners, which can reduce local mucus production and increase intestinal permeability (Shin, Whon & Bae, 2015; Crovesy, Masterson & Rosado, 2020). With regards to the phylum *Spirochaetes*, although its identification in fecal samples has not been associated with obesity, Jabbar et al. (2021) recently reported the association between *Brachyspira* and irritable bowel syndrome. Our results indicate a limited impact of the probiotic candidate LG-G12 in controlling the two aforementioned taxa. Since a single strain was used in this study, its inclusion in a multiple strain formulation, as suggested by the World Gastroenterology Organisation (Hunt *et al.*, 2017), might represent a more effective strategy in limiting the growth of such undesired phyla, which must be evaluated in further studies.

Figure 3 – Linear discriminant analysis effect size (LEfSe) analysis.



Where: G1: negative control group, G2: positive control group with consumption of high-fat diet, G3: group treated with probiotic potential with consumption of high-fat diet, G4: group treated with antimicrobial with consumption of high-fat diet, G5: group treated with antimicrobial followed by probiotic potential with consumption of high-fat diet, G6: positive control group with consumption of standard diet, G7: group treated with probiotic potential and consumption of standard diet, G8: group treated with antimicrobial and consumption of diet standard and G9: group treated with antimicrobial followed by probiotic potential with consumption of standard diet. Only taxonomic groups showing linear discriminant analysis (LDA) scores > 2.0 with false discovery rate (FDR) $p < 0.05$ are shown. Letters: p, phylum; c, class; o, order; f, family; g, genus. This analysis was carried out to identify significant differences in abundant taxa (fecal biomarkers) after the interventions “A” and “B”.

Notwithstanding *Firmicutes* and *Bacteroidetes* have not been identified as biomarkers in any of the interventions, the G4 group showed a much higher F/B ratio (**Table 2)**, which is typically found in obese subjects (Crovesy *et al.*, 2020). This finding reinforces that the concomitant administration of ceftriaxone and a high-fat diet impaired intestinal bacterial balance, stimulating the growth of few phyla at the expense of others, which is characteristic of intestinal dysbiosis (Stojanov, Berlec & Štrukelj, 2020). Interestingly, following LG-G12 administration, the F/B ratio increased in the G5 group achieving values similar to the lean group (G1 group). This outcome suggests a mechanism of counteraction to the damage caused by ceftriaxone administration contributing to restoring the intestinal homeostasis (Stojanov *et al.*, 2020). No statistically significant difference was observed in terms of F/B ratio among the groups in the intervention "B" (**Table 2**).

We also evaluated the ratio between Gram-positive and Gram-negative genera in each group (**Table 2**). As expected, the G4 group showed the least proportion of Gram-negative among all groups (23.50%) and the greatest G+/G- ratio (3.24), indicating the selectivity of the antimicrobial ceftriaxone against this specific group of bacteria. Ceftriaxone is a third-generation cephalosporin that targets most Gram-negative bacteria inducing changes in gut microbiota (Zhao *et al.*, 2020). Our results also reveal that the intervention with LG-G12 alleviated the effects of the synergism between the antimicrobial and the diet offered, restoring intestinal Gram-negative taxa at levels similar to the control groups. Lastly, the intake of a low-calorie diet (AIN93) supplemented with the administration of LG-G12 was able to re-establish the ratio between Gram-positive and Gram-negative near to the lean group (G1). In a systematic review of randomized controlled clinical trials, Crovesy et al. (2017) suggested that the modulatory effect of *Lactobacillus* in weight loss is strain-dependent and can require its association with calorie restriction, phenolic compounds, or other bacterial strains.

3.3.2 Genus level

Amongst the biomarkers identified by LEfSE analysis, the genera *Oscillospira* (LDA > 4), *Sporosarcina* (LDA > 4), *Allobaculum* (LDA > 3), *Jeotgalicoccus* (LDA > 3), *Bifidobacterium* (LDA > 3), and *Yaniella* (LDA > 3) were assigned as biomarkers for the G1 group. The enrichment of the genera *Bifidobacterium* is in agreement with the literature, where and an inverse relationship was shown between this genus and obesity. Bifidobacteria deconjugate bile acids, decreasing fat absorption (Gomes, Hoffmann & Mota, 2018). The higher

abundance of *Oscillospira* in the gut of lean subjects has been reported by several studies and is positively associated with lower body mass index (BMI) in both children and adults (Konikoff & Gophna, 2016). It is also well reported that a high-fat diet can significantly reduce the intestinal abundance of *Allobaculum*, a relevant SCFA producing bacteria, which may display an anti-obesogenic role by reducing intestinal inflammation and improving insulin resistance (Zhou *et al.*, 2019; Guo *et al.*, 2020; Wang *et al.*, 2020). The increase in the relative abundance of the genera *Jeotgalicoccus* and *Sporosarcina*, although less described in the literature, are associated with benefits outcomes in animal models fed high-fat diets (Machate *et al.*, 2020; Song *et al.*, 2021). To the best of our knowledge, there is no available information regarding the role of the *Yaniella*, a high salt-tolerant microorganism, in healthy or obese subjects. Together, our results reinforce the beneficial effects of the ingestion of low-calorie diets in the maintenance of taxa negatively associated with obesity development.

The genera *Lactobacillus* (LDA > 4), *Dehalobacterium* (LDA >2), and *Erysipelotrichaceae cc_115* (LDA > 3) were identified as biomarkers of the obese control group (G2 group, intervention "A"). Although most lactobacilli strains can have beneficial and auxiliary effects on weight loss in overweight adults (Crovesy *et al.*, 2017), some species, such as *Limosilactobacillus reuteri*, have been associated with weight gain in humans and animals (Armougom *et al.*, 2009; Million *et al.*, 2012). Regarding the genera, *Dehalobacterium* and *Erysipelotrichaceae cc-115* little information are available regarding the abundance and role of these taxa within the intestinal community of obese or overweight subjects. It is reported that the genus *Dehalobacterium* comprehends microorganisms strictly anaerobic capable of degrading dichloromethane and was found enriched in both obese and non-obese asthmatic patients (Michalovich *et al.*, 2019), whereas *Erysipelotrichaceae cc-115* was found depleted in the gut microbiota of community-dwelling older men physically active (Langsetmo *et al.*, 2019).

Six different genera were enriched in the G3 group (LG-G12 administration, intervention "A") after the end of the experimental period, namely: *Ruminococcus* (LDA > 4), *Anaerotruncus* (LDA > 4), *Bilophila* (LDA > 3), *Desulfovibrio* (LDA > 3), *Brachyspira* (LDA > 2), and *Coprococcus* (LDA > 2). We observed that when the probiotic candidate LG-G12 was offered to animals that were fed a high-calorie diet, there was a remarkable enrichment of SCFA producing bacteria such as *Ruminococcus*, *Anaerotruncus*, and *Coprococcus* which are commonly found in the microbiota of overweight or obese patients (Castaner *et al.*, 2018; Bailén *et al.*, 2020; Palmas *et al.*, 2021). Intriguingly, we also detected the enrichment of taxa involved in mucus degradation and hydrogen sulfide production such as

Brachyspira, *Bilophila*, and *Desulfovibrio*, which may indicate a limited action of LG-G12 against these taxa.

The genera *Enterococcus* (LDA > 5), *Salinispora* (LDA > 3), and *Akkermansia* (LDA > 4) were identified as biomarkers of the group G4 (ceftriaxone group, intervention “A”), whereas only *Clostridium* (LDA > 4) was significantly enriched in the G5 group (ceftriaxone followed by LG-G12, intervention “A”). Interestingly, *Akkermansia*, which is a Gram-negative, obligate anaerobe, non-motile, nonspore-forming bacterium, seems to be resilient to the adverse effects of the antimicrobial ceftriaxone. This genus has attracted great interest due to its capability to enhance mucus formation, activate the innate immune system, and promote intestinal homeostasis (Naito, Uchiyama & Takagi, 2018). As reported by (Vesić & Kristich, 2012), the genus *Enterococcus* is intrinsically resistant to cephalosporins, antibiotics that act on cell wall biosynthesis, which may explain its identification as a biomarker of this group. A protective effect of *Enterococcus* against weight gain is speculated in infants undergoing excessive weight gain during breastfeeding, which shows a reduced abundance of gut *Enterococcus* when compared with the control groups. Additionally, (Mishra & Ghosh, 2020) reports that the strain *E. faecalis* AG5 mitigates HFD induced obesity through several mechanisms such as activation of adipocyte apoptosis and the improvement of glucose, insulin, and leptin sensitivity.

When the biomarkers associated with the intervention “B” are analyzed, fewer genera are observed when compared to the intervention “A”, which reinforces the strong effect of the diet on the reestablishment of the dysbiotic gut microbiota in the groups that underwent a high-fat diet. The genus *Corynebacterium* (LDA > 3) was identified as a biomarker of group G1, while the genera *Blautia* (LDA > 3), *Clostridium* (LDA > 4), and *Akkermansia* (LDA > 5) appear enriched in the control group G6 (AIN-93 intake during the treatment phase). The enrichment of the SCFA producing bacteria *Akkermansia*, *Blautia,* and *Clostridium* may contribute to restoring intestinal integrity and the development of intestinal homeostasis. For instance, when overweight or obese subjects undergo calorie-restricted diet therapy, a pronounced improvement in insulin resistance has been reported and correlated with a higher abundance of *Akkermansia* in the human gut (Naito *et al.*, 2018).

Finally, an enrichment of the *Bifidobacterium* genus (LDA > 4) was observed following LG-G12 administration and return to the standard diet in the treatment phase (group G7). This result indicates a synergism between LG-G12 and endogenous bifidobacteria and might be strain-specific. Following probiotic intervention with *Latilactobacillus curvatus* and *Lactiplantibacillus plantarum*, (Park et al., 2013) observed enrichment of the species

Bifidobacterium pseudolongum in HFD-probiotic mice when compared to the HFD-placebo group. Differently abundant genera were not identified in the groups G8 (ceftriaxone) and G9 (ceftriaxone followed by LG-G12).

3.4 Correlation analyses

3.4.1 Groups treated with LG-G12

With regards LG-G12 treated groups (G1, G2, G3, G6 and G7) **(Fig. 4A),** the genera *Enterococcus* (r = -0.59, p = 6.27E-03) and *Bifidobacterium* (r = -0.54, p = 1.32E-02) were negatively correlated with high Lactulose/Mannitol (L/M) ratio (**Table 3**). Species of the genus *Enterococcus* can interact with mucosal immune cells, providing activation of the intestinal immune response (Fine *et al.*, 2020). (Wu, Zhen, Geng, Wang, & Guo, 2019) report that the probiotic *E. faecium* NCIMB 11181 can ameliorate necrotic enteritis by improving intestinal mucosal barrier function and modulate gut microbiota. *Bifidobacterium*, which is indicative of microbial diversity (Liu *et al.*, 2016), can protect against obesity and diabetes, as well as improve the intestinal integrity and control metabolic endotoxemia, important parameters for the assessment of intestinal balance and health (Cani *et al.*, 2007; Kong *et al.*, 2019).

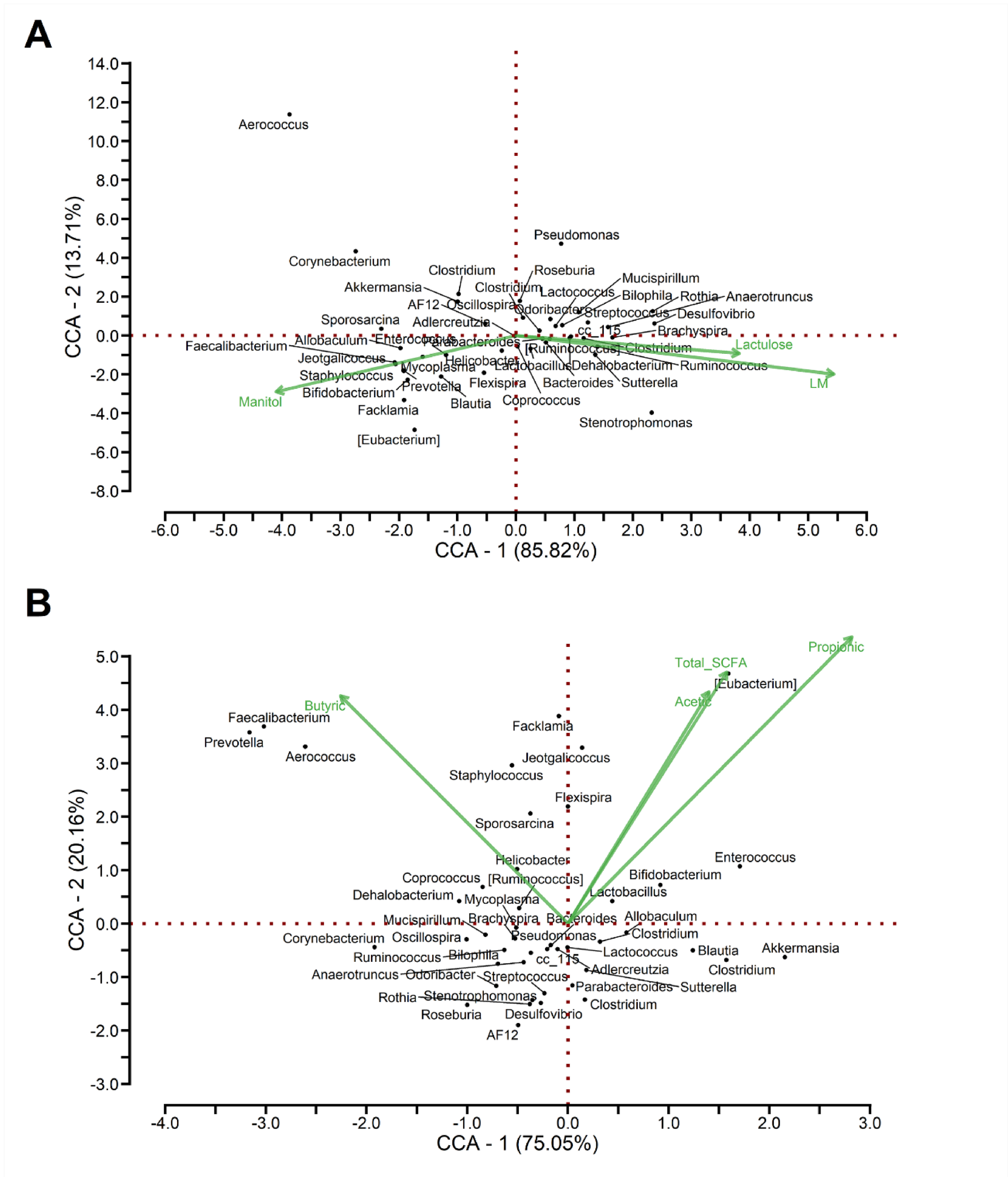
In terms of SCFA production **(Fig. 4B, Table 3)**, the genera *Enterococcus* (r = 0.48, p = 3.12E-02), *Allobaculum* (r = 0.76, p = 8.96E-05), *Sporosarcina* (r = 0.83, p = 4.73E-06), *Jeotgalicoccus* (r = 0.87, p = 7.67E-07), *Staphylococcus* (r = 0.91, p = 2.50E-08), *Bifidobacterium* (r = 0.60, p = 4.79E-03), *Blautia* (r = 0.59, p = 6.11E-03) were positively correlated with total amount of SCFA, whereas *Prevotella* (r = 0.50, p = 2.33E-02) was only positively correlated with the production of butyrate.

As reported by (Kong et al., 2019), the consumption of high-fat and high-sucrose diets reduces the abundance of *Prevotella* and, consequently, butyrate levels. In the same study, the authors observed that probiotic administration (*Lactobacillus acidophilus*, *Bifidobacterium longum*, and *Enterococcus faecalis*) was able to restore the intestinal microbiota, increasing microorganisms such as *Lactobacillus*, *Bifidobacterium,* and *Akkermansia*. Our results suggest that the LG-G12 positively modulated SCFA-producing bacteria such as *Allobaculum*, *Bifidobacterium,* and *Prevotella*.

Overall, bacterial genera that negatively correlated with the L/M ratio were positively correlated with the production of SCFA, indicating a relevant role of such organic acids with

the integrity of the intestinal barrier, which is in line with previous studies (De Vuyst *et al.*, 2014; Kong *et al.*, 2019; Huang *et al.*, 2020).

Figure 4 – Canonical correspondence analysis (CCA) among groups treated with LG-G12.



CCA was performed on the most abundant OTUs (relative abundance > 0.1%) at the genus level for the groups G1, G2, G3, G6, and G7, with **A)** the intestinal permeability parameters (lactulose, mannitol, and lactulose/mannitol ratio) and **B)** the short-chain fatty acids acetate, propionate, butyrate, and total SCFA. Green lines indicate the direction and magnitude of measurable variables associated with community structures.

3.4.2 Groups treated with ceftriaxone

The correlation analysis performed considering the groups G1, G2, G4, G6, and G8 **(Supplemental Fig. 1A, Table 3)** revealed that. *Staphylococcus* (r = -0.81, p = 7.49E-04) was negatively correlated with lactulose, whereas *Clostridium* (r = 0.48, p = 3.79E-02) was positively correlated with the L/M ratio. In a study conducted by (Zeng et al., 2018), the authors demonstrate that lactulose inhibited the effect of *Staphylococcus aureus* due to the production of sialyllactulose, an antimicrobial enzyme capable of provoking damage to the *S. aureus* cell membrane, which may be beneficial to intestinal health. The positive correlation between *Clostridium* and the high L/M ratio is not surprising since some species such as *Clostridium difficile* can secrete toxins with cytotoxic effects on the intestinal epithelium (Buccigrossi *et al.*, 2019).

In terms of SCFA **(Supplemental Fig. 1B, Table 3)**, the genera *Allobaculum* (r = 0.67, p = 1.59E-03) and *Bifidobacterium* (r = 0.54, p = 1.72E-02), were positively correlated with total SCFA production, whereas only the genus *Stenotrophomonas* (r = -0.48, p = 3.76E-02) showed a negative correlation. Even following antimicrobial treatment, the genera *Allobaculum* and *Bifidobacterium* were negatively correlated with the L/M ratio and positively correlated with the production of SCFA, which indicates that these genera may act in the maintenance of intestinal integrity and homeostasis as previously described (Kong et al., 2019). With regards to the genus *Stenotrophomonas*, some species of belonging to this genus, such as *Stenotrophomonas maltophilia*, are considered pathogenic bacterium (Looney, Narita & Mühlemann, 2009) and highly resistant to antibiotics (Kalidasan *et al.*, 2018), which may justify its presence among ceftriaxone-treated groups.

3.4.3 Groups treated with ceftriaxone followed by LG-G12

Correlation analyses encompassing the groups G1, G2, G5, G6, and G9 **(Supplemental Fig. 2A)** revealed that the genus *Desulfovibrio* (*r* = 0.48, *p* = 3.29E-02) was positively correlated with the L/M ratio and, by consequence, loss of intestinal integrity. Members of the genus *Desulfovibrio* are frequently increased in cases of intestinal dysbiosis, contributing to intestinal permeability and inflammation (Chen *et al.*, 2019), which is in agreement with our results.

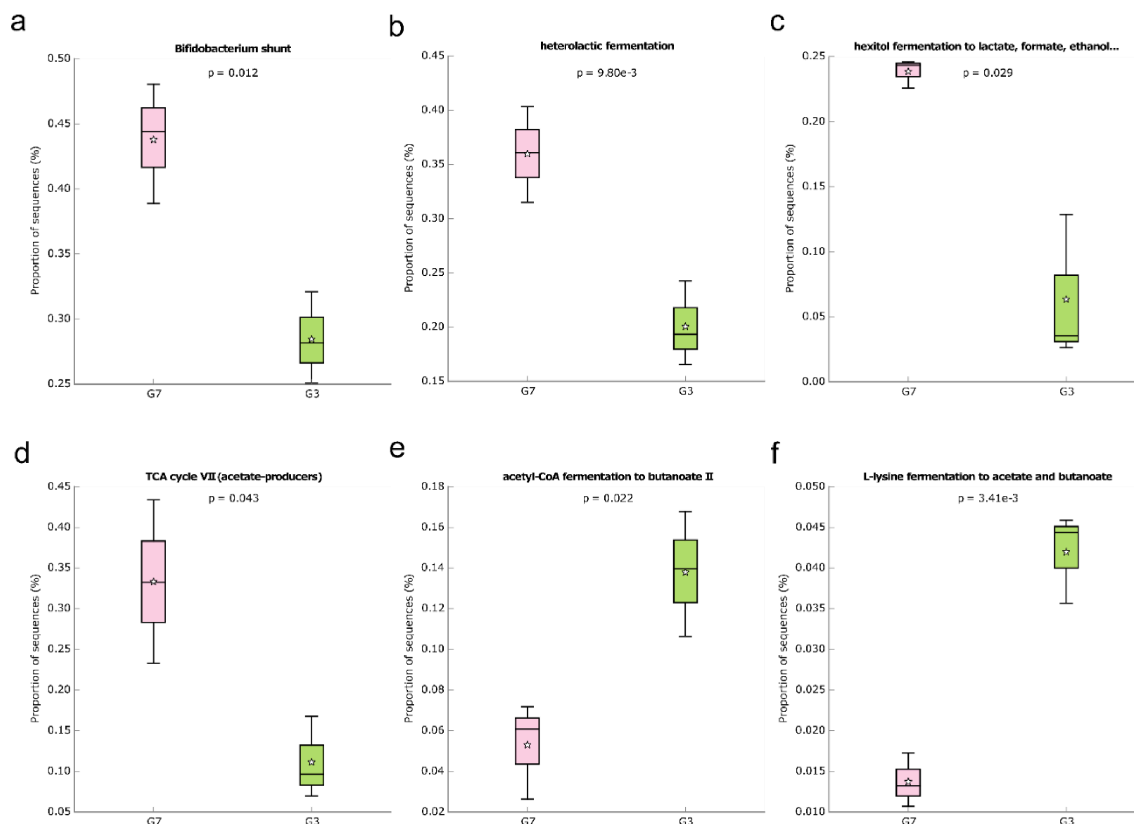
Concerning SCFAs **(Supplemental Fig. 2B)**, the genera *Prevotella* (*r* = 0.49, *p* = 2.98E-02) and *Faecalibacterium* (*r* = 0.50, *p* = 2.44E-02) were positively correlated with its

total amount (here represented by the sum of acetate, propionate and butyrate), whereas some genera were positively correlated with only certain compounds, but not with their total production, as follows: *Allobaculum* (Acetic: r = 0.88, p = 2.95E-03; Propionic, r = 0.73, p = 2.70E-04; Butyric, r = 0.64, p = 2.62E-03) and *Bifidobacterium* (Acetic, r = 0.81, p = 1.71E-02; Propionic, r = 0.67, p = 1.11E-03; Butyric, r = 0.74, p = 1.87E-04). As discussed previously, *Bifidobacterium* is considered an SCFA-producing bacteria able to produce especially acetate and lactate (Manome *et al.*, 2019), whereas the production of butyrate is more related to the presence of prebiotics (De Vuyst *et al.*, 2014). The identification of *Faecalibacterium* and *Allobaculum*, both genera described as producers of SCFA (Kong *et al.*, 2019; Huang *et al.*, 2020), indicate that the association ceftriaxone and LG-G12 can also positively modulate the gut microbiota by favoring SCFA-producing genera and, consequently, improving intestinal health.

3.5 Functional predictions of the gut microbiota

The functional prediction of the gut microbiota aimed to identify the enrichment of microbial metabolic pathways that could be associated with the effects of maintaining or not a high-fat diet during the different interventions evaluated in the current study. In addition, we focused on identifying metabolic pathways related to SCFA production that can directly impact intestinal health. Taking into account the comparison between groups G7 and G3, 58 functional pathways differed significantly between groups **(Supplemental Table 1)**, and the great majority (approximately 69.0%) were enriched in group G7. Regarding the MetaCyc pathways associated with SCFA production, six metabolic pathways were identified **(Fig. 5)**, and four of them were present in group G7 (Bifidobacterium shunt, heterolactic fermentation, hexitol fermentation to lactate, formate, ethanol, and acetate). The enrichment of Bifidobacterium shunt, which is a classic pathway of carbohydrate metabolisms such as fructose and glucose, can generate compounds serving as an energy source to intestinal epithelial cells (FUSHINOBU, 2010; De Vuyst *et al.*, 2014; Manome *et al.*, 2019), which explains the greater intestinal integrity noticed in this group. Only the acetyl-Coa fermentation to butanoate II and L-lysine fermentation to acetate and butanoate pathways were enriched in the G3 group.

Figure 5 – Box plot showing the distribution in the proportion of the predicted MetaCyc pathways related to SCFA (A to F) production between the groups G3 and G7.



Boxes indicate the IQR (75th to 25th of the data). The median value is shown as a line within the box and the mean value as a star. Whiskers extend to the most extreme value within 1.5*IQR. Where: a: Bifidobacterium shunt; b: Heterolactic fermentation; c: Hexitol fermentation to lactate, formate, ethanol; d: TCA cycle VII; e: Acetyl-CoA fermentation to butanoate II; f: L-lysine fermentation to acetate and butanoate.

In the groups that underwent ceftriaxone followed by LG-G12 (groups G5 and G9), it was observed that only 14 functional pathways differed significantly between both groups **(Supplemental Table 2)** with approximately 64.0% of the features enriched in the G9 group. The enrichment of the super pathway of D-glucarate and D-galactarate degradation in group G5 also demonstrates the use of alternative carbon sources for growth. The use of dicarboxylic acid sugars as growth substrate although observed in many different bacteria is especially found in Gram-negative bacteria such as members of the families *Enterobacteriaceae*, *Moraxellaceae*, and *Pseudomonadaceae* (Aghaie *et al.*, 2008). In group G9, metabolic pathways associated with SCFA production were enriched only in this group and refer to the Bifidobacterium shunt and heterolactic fermentation pathways **(Supplemental Figure 3)**. Similarly, in the G9 group, we also observed the enrichment of the Bifidobacterium shunt and heterolactic fermentation pathways, both associated with carbohydrate metabolism (Verce, De Vuyst & Weckx, 2020), which evidence an important role

of low-calorie diets in the enrichment of these functions. Interestingly, the super pathway of D-glucarate and D-galactarate degradation and the pathway of purine nucleotides degradation II (aerobic) were enriched in the G5 group, which is indicative of the use of alternative carbon sources by the microbial community in this group.

Finally, 12 functional pathways differed significantly between the G4 and G8 groups (ceftriaxone administration only) **(Supplemental Table 3)**, with the vast majority of pathways (approximately 66.0%) being enriched in the G4 group. The main metabolic pathways enriched in the G4 group were associated with menaquinol biosynthesis and de novo nucleotide biosynthesis. The enrichment of menaquinol biosynthesis might be related to bacterial energy processes since menaquinones are relevant growth factors for the gut microbiota (Aussel *et al.*, 2014; Fenn *et al.*, 2017). Traditionally, the intestinal microbiota is an important source of purines, which are used in different functions of the intestinal barrier and innate immunity, being necessary for intestinal protection and health (Lee *et al.*, 2020). Since dysbiosis was a finding of the G4 group, it is believed that the enrichment of *de novo* nucleotide biosynthesis pathways confirms an expansion of specific microbial taxa in this group.

4. CONCLUSION

High-fat diet intake remarkably impaired bacterial diversity indices, which was accentuated when associated with ceftriaxone. LG-G12 was effective in re-store gut homeostasis, and the most effective outcomes were observed when this strain was associated with a low-calorie diet. The higher caecal amount of SCFA contributed to increasing intestinal integrity, and the genera that negatively correlated with a high L/M ratio were similar to those that positively correlated with total SCFA production, which was further confirmed by the prediction of metagenomes function of the gut microbiota. This study brings novel insights into the use of LG-G12 as an adjuvant treatment of obese or overweight subjects undergoing or not antimicrobial therapy.

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Ethics Approval

The study was approved by the Ethics Committee on Animals Use of Universidade Federal de Viçosa, under protocol 33/2018. The principles recommended by the Nacional Council for the Control of Animal Experimentation were followed (Brasil, 2008).

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Table 1 – Filtering and denoising Amplicon Sequence Variants (ASVs) statistics following DADA2 pipeline and alpha-diversity indexes of fecal samples obtained across the different groups after their respective intervention on phase 2 (treatment phase).

Group	Denoising stats from DADA2				Alpha diversity	
	Input	**Filtered**	**Denoised**	**Non-chimeric**	**Shannon**	**Chao1**
G1	227,880 ± 7,222	164,610 ± 4,423	160,280 ± 4,281	125,329 ± 5214	6.4 ± 0.1 ^{B}	394 ± 7.0 ^{B}
G2	196,882 ± 12,396	144,675 ± 8,857	140,087 ± 8,573	103,217 ± 5604	6.1 ± 0.3 ^{B}	363.3 ± 11.2 ^{B}
G3	190,352 ± 18,375	137,423 ± 11,006	133,888 ± 11,325	109,444 ± 13665	6.2 ± 0.1 ^{B}	352.7 ± 16.9 ^{B}
G4	185,306 ± 4,739	140,334 ± 2,383	139,779 ± 2,367	134,685 ± 3727	2.1 ± 0.8 ^{A}	124.7 ± 27.6^{A}
G5	182,069 ± 9,462	130,719 ± 8,297	128,434 ± 7,935	95,498 ± 11375	4.7 ± 0.4 ^{AB}	192 ± 50.1^{AB}
G6	169,774 ± 20,766	125,635 ± 15,813	123,564 ± 15,890	103,703 ± 19,606	3.7 ± 0.5 ^{AB}	217 ± 20.2 ^{AB}
G7	194,984 ± 15,583	143,570 ± 11,056	139,281 ± 10,908	103,892 ± 12,059	5.9 ± 0.2 ^{B}	338.3 ± 15.9 ^{B}
G8	213,138 ± 2,010	155,436 ± 4,102	153,189 ± 6,196	137,891 ± 20,783	3.5 ± 1.5 ^{AB}	250.5 ± 79.9 ^{AB}
G9	188,450 ± 16,499	137,769 ± 10,912	134,893 ± 9,980	103,992 ± 6,541	5.2 ± 0.6 ^{AB}	270 ± 69.64 ^{AB}

Where: G1: negative control group, G2: positive control group with consumption of high-fat diet, G3: group treated with probiotic potential with consumption of high-fat diet, G4: group treated with antimicrobial with consumption of high-fat diet, G5: group treated with antimicrobial followed by probiotic potential with consumption of high-fat diet, G6: positive control group with consumption of standard diet, G7: group treated with probiotic potential and consumption of standard diet, G8: group treated with antimicrobial and consumption of diet standard and G9: group treated with antimicrobial followed by probiotic potential with consumption of standard diet. Different superscript letters (A and B) indicate significant differences among the groups ($p < 0.05$).

Table 2 – *Firmicutes* to *Bacteroidete* ratio (F/B), and relative abundance of Gram-negative/positive genera (> 0.1%), Mycoplasma and low abundant genera (LAG) across the different groups.

	G1	G2	G3	G4	G5	G6	G7	G8	G9
F/B ratio	3.40	1.72	2.41	16.81	0.96	1.46	2.06	1.18	2.07
Gram-negative	62.03%	74.51%	77.21%	23.50%	76.56%	81.64%	49.92%	42.74%	65.15%
Gram-positive	37.80%	25.37%	22.35%	76.23%	23.25%	18.29%	49.96%	39.36%	34.63%
G+/G- ratio	0.61	0.34	0.29	3.24	0.30	0.22	1.00	0.92	0.53
Mycoplasma	0.01%	0.00%	0.00%	0.07%	0.00%	0.01%	0.02%	14.95%	0.06%
LAG	0.16%	0.12%	0.44%	0.21%	0.19%	0.07%	0.11%	2.94%	0.16%

Where: G1: negative control group, G2: positive control group with consumption of high-fat diet, G3: group treated with probiotic potential with consumption of high-fat diet, G4: group treated with antimicrobial with consumption of high-fat diet, G5: group treated with antimicrobial followed by probiotic potential with consumption of high-fat diet, G6: positive control group with consumption of standard diet, G7: group treated with probiotic potential and consumption of standard diet, G8: group treated with antimicrobial and consumption of diet standard and G9: group treated with antimicrobial followed by probiotic potential with consumption of standard diet.

Table 3 – Short-chain fatty acid (SCFA) concentration (µmol/g feces) in caecal samples and lactulose/mannitol ratio (L/M) obtained across the different groups after their respective intervention on phase 2 (treatment phase).

Group	Acetate	Propionate	Butyrate	Total SCFA	L/M
G1	571.8 ± 37.4^{D}	150.7 ± 17.4^{B}	31.35 ± 8.9^{B}	753.8 ± 61.0^{C}	1.41 ± 0.22^{A}
G2	0.19 ± 0.02^{A}	0.04 ± 0.004^{A}	0.058 ± 0.045^{A}	0.288 ± 0.044^{A}	55.1 ± 2.25^{C}
G3	0.16 ± 0.03^{A}	0.04 ± 0.005^{A}	0.015 ± 0.003^{A}	0.1983 ± 0.035^{A}	49.3 ± 3.2^{C}
G4	0.06 ± 0.02^{A}	0.03 ± 0.01^{A}	-	0.1 ± 0.03^{A}	59.6 ± 3.02^{C}
G5	0.20 ± 0.08^{A}	0.07 ± 0.03^{A}	0.02 ± 0.003^{A}	0.27 ± 0.07^{A}	27.5 ± 6.7^{B}
G6	382.8 ± 44.3^{C}	142.2 ± 28.2^{B}	7.2 ± 3.6^{A}	530.7 ± 68.24^{BC}	2.1 ± 0.8^{A}
G7	374.7 ± 61.9^{C}	140.7 ± 17.7^{B}	40.1 ± 4.0^{B}	535.4 ± 80.13^{BC}	0.98 ± 0.17^{A}
G8	165.3 ± 23.4^{B}	185.7 ± 17.3^{B}	-	351.0 ± 31.0^{B}	0.58 ± 0.21^{A}
G9	396.0 ± 45.2^{C}	211.2 ± 27.9^{B}	36.7 ± 6.9^{B}	644.0 ± 68.7^{C}	2.0 ± 0.23^{A}

Where: G1: negative control group, G2: positive control group with consumption of high-fat diet, G3: group treated with probiotic potential with consumption of high-fat diet, G4: group treated with antimicrobial with consumption of high-fat diet, G5: group treated with antimicrobial followed by probiotic potential with consumption of high-fat diet, G6: positive control group with consumption of standard diet, G7: group treated with probiotic potential and consumption of standard diet, G8: group treated with antimicrobial and consumption of diet standard and G9: group treated with antimicrobial followed by probiotic potential with consumption of standard diet. Total SCFA refers to the sum of acetate, propionate, and butyrate. Different superscript letters (A and B) indicate significant differences among the groups ($p < 0.05$).

Supplemental Table 1. Pairwise comparison (G7 vs G3) of PICRUSt2 predicted pathway abundances.

G7 vs G3				
pathway	**description**	**G7: mean rel. freq. (%)**	**G3: mean rel. freq. (%)**	**p-values (corrected)**
ARGORNPROST-PWY	arginine, ornithine and proline interconversion	0.19	0.08	0.03
FASYN-ELONG-PWY	fatty acid elongation -- saturated	0.79	0.63	0.01
FOLSYN-PWY	superpathway of tetrahydrofolate biosynthesis and salvage	0.59	0.49	0.05
HEXITOLDEGSUPER-PWY	superpathway of hexitol degradation (bacteria)	0.31	0.11	0.05
LACTOSECAT-PWY	lactose and galactose degradation I	0.14	0.03	0.02
P122-PWY	heterolactic fermentation	0.36	0.20	0.01
P124-PWY	Bifidobacterium shunt	0.44	0.28	0.01
P125-PWY	superpathway of (R,R)-butanediol biosynthesis	0.06	0.00	0.04
P461-PWY	hexitol fermentation to lactate, formate, ethanol and acetate	0.24	0.06	0.03
PENTOSE-P-PWY	pentose phosphate pathway	0.51	0.27	0.03
POLYAMINSYN3-PWY	superpathway of polyamine biosynthesis II	0.04	0.01	0.00
PWY-2941	L-lysine biosynthesis II	0.50	0.16	0.00
PWY-3781	aerobic respiration I (cytochrome c)	0.66	0.11	0.01
PWY-5265	peptidoglycan biosynthesis II (staphylococci)	0.28	0.02	0.02
PWY-5837	1,4-dihydroxy-2-naphthoate biosynthesis I	0.18	0.03	0.02
PWY-5838	superpathway of menaquinol-8 biosynthesis I	0.29	0.07	0.03
PWY-5840	superpathway of menaquinol-7 biosynthesis	0.30	0.07	0.03
PWY-5861	superpathway of demethylmenaquinol-8 biosynthesis	0.26	0.06	0.02
PWY-5863	superpathway of phylloquinol biosynthesis	0.20	0.04	0.02
PWY-5897	superpathway of menaquinol-11 biosynthesis	0.29	0.07	0.03
PWY-5898	superpathway of menaquinol-12 biosynthesis	0.29	0.07	0.03
PWY-5899	superpathway of menaquinol-13 biosynthesis	0.29	0.07	0.03
PWY-5910	superpathway of geranylgeranyldiphosphate biosynthesis I (via mevalonate)	0.21	0.04	0.00
PWY-6126	superpathway of adenosine nucleotides de novo biosynthesis II	0.78	0.70	0.00
PWY-6147	6-hydroxymethyl-dihydropterin diphosphate biosynthesis I	0.50	0.42	0.03
PWY-6151	S-adenosyl-L-methionine cycle I	0.62	0.45	0.00

PWY-6470	peptidoglycan biosynthesis V (β-lactam resistance)	0.33	0.06	0.01
PWY-6471	peptidoglycan biosynthesis IV (Enterococcus faecium)	0.46	0.35	0.01
PWY-7200	superpathway of pyrimidine deoxyribonucleoside salvage	0.43	0.32	0.03
PWY-7220	adenosine deoxyribonucleotides de novo biosynthesis II	0.72	0.56	0.01
PWY-7222	guanosine deoxyribonucleotides de novo biosynthesis II	0.72	0.56	0.01
PWY-7234	inosine-5'-phosphate biosynthesis III	0.49	0.16	0.00
PWY-7254	TCA cycle VII (acetate-producers)	0.33	0.11	0.04
PWY-7527	L-methionine salvage cycle III	0.02	0.00	0.01
PWY-7539	6-hydroxymethyl-dihydropterin diphosphate biosynthesis III (Chlamydia)	0.52	0.43	0.02
PWY-922	mevalonate pathway I	0.16	0.03	0.00
PWY0-166	superpathway of pyrimidine deoxyribonucleotides de novo biosynthesis (E. coli)	0.50	0.43	0.03
PWY0-781	aspartate superpathway	0.43	0.37	0.02
REDCITCYC	TCA cycle VIII (helicobacter)	0.39	0.13	0.05
TEICHOICACID-PWY	teichoic acid (poly-glycerol) biosynthesis	0.16	0.05	0.04
COBALSYN-PWY	adenosylcobalamin salvage from cobinamide I	0.31	0.46	0.02
GLUCUROCAT-PWY	superpathway of β-D-glucuronide and D-glucuronate degradation	0.17	0.23	0.04
GLUTORN-PWY	L-ornithine biosynthesis	0.54	0.73	0.05
GLYCOCAT-PWY	glycogen degradation I (bacterial)	0.62	0.82	0.05
GLYCOGENSYNTH-PWY	glycogen biosynthesis I (from ADP-D-Glucose)	0.54	0.74	0.02
P163-PWY	L-lysine fermentation to acetate and butanoate	0.01	0.04	0.00
PWY-181	photorespiration	0.14	0.37	0.04
PWY-5005	biotin biosynthesis II	0.06	0.15	0.01
PWY-5505	L-glutamate and L-glutamine biosynthesis	0.33	0.56	0.04
PWY-5509	adenosylcobalamin biosynthesis from cobyrinate a,c-diamide I	0.30	0.44	0.03
PWY-5676	acetyl-CoA fermentation to butanoate II	0.05	0.14	0.02
PWY-6269	adenosylcobalamin salvage from cobinamide II	0.30	0.45	0.03
PWY-6478	GDP-D-glycero-α-D-manno-heptose biosynthesis	0.03	0.09	0.03
PWY-6891	thiazole biosynthesis II (Bacillus)	0.13	0.33	0.02
PWY-6895	superpathway of thiamin diphosphate biosynthesis II	0.28	0.40	0.00
PWY-7013	L-1,2-propanediol degradation	0.01	0.05	0.02
PWY-7315	dTDP-N-acetylthomosamine biosynthesis	0.05	0.17	0.01
PWY-7328	superpathway of UDP-glucose-derived O-antigen building blocks biosynthesis	0.18	0.35	0.03

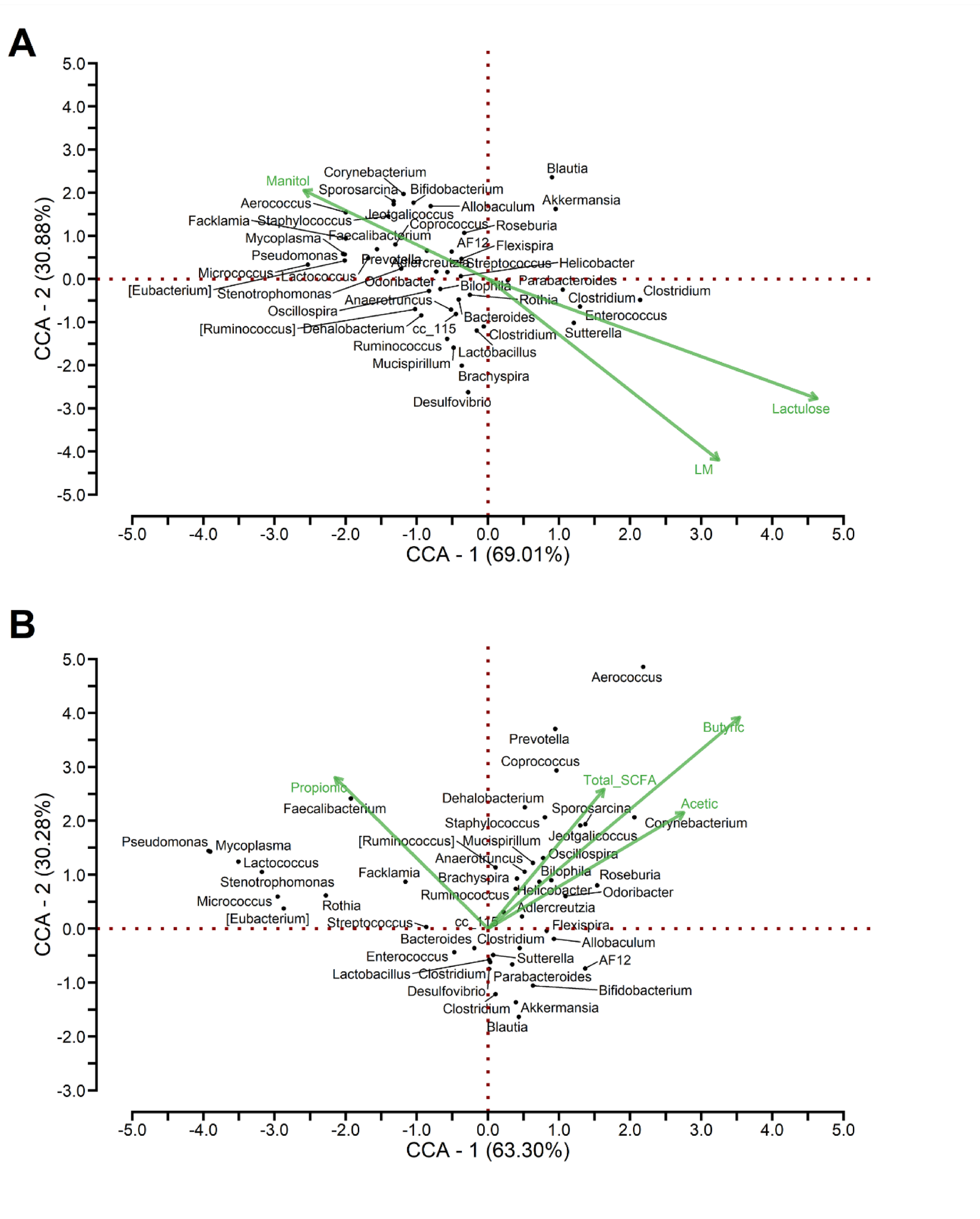
Supplemental Table 2. Pairwise comparison (G9 vs G5) of PICRUSt2 predicted pathway abundances.

G9 vs G5				
pathway	**description**	**G9: mean rel. freq. (%)**	**G5: mean rel. freq. (%)**	**p-values (corrected)**
COMPLETE-ARO-PWY	superpathway of aromatic amino acid biosynthesis	0.76	0.63	0.03
ARO-PWY	chorismate biosynthesis I	0.74	0.61	0.01
ARGSYNBSUB-PWY	L-arginine biosynthesis II (acetyl cycle)	0.67	0.49	0.05
ARGSYN-PWY	L-arginine biosynthesis I (via L-ornithine)	0.65	0.52	0.05
DAPLYSINESYN-PWY	L-lysine biosynthesis I	0.64	0.47	0.01
OANTIGEN-PWY	O-antigen building blocks biosynthesis (E. coli)	0.58	0.45	0.03
PWY-5505	L-glutamate and L-glutamine biosynthesis	0.50	0.34	0.02
P124-PWY	Bifidobacterium shunt	0.29	0.13	0.02
P122-PWY	heterolactic fermentation	0.23	0.12	0.04
PWY-5913	TCA cycle VI (obligate autotrophs)	0.34	0.48	0.03
PWY-6353	purine nucleotides degradation II (aerobic)	0.15	0.29	0.03
GLUCARDEG-PWY	D-glucarate degradation I	0.01	0.04	0.01
GALACTARDEG-PWY	D-galactarate degradation I	0.01	0.03	0.05
GLUCARGALACTSUPER-PWY	superpathway of D-glucarate and D-galactarate degradation	0.01	0.03	0.05

Supplemental Table 3. Pairwise comparison (G8 vs G4) of PICRUSt2 predicted pathway abundances.

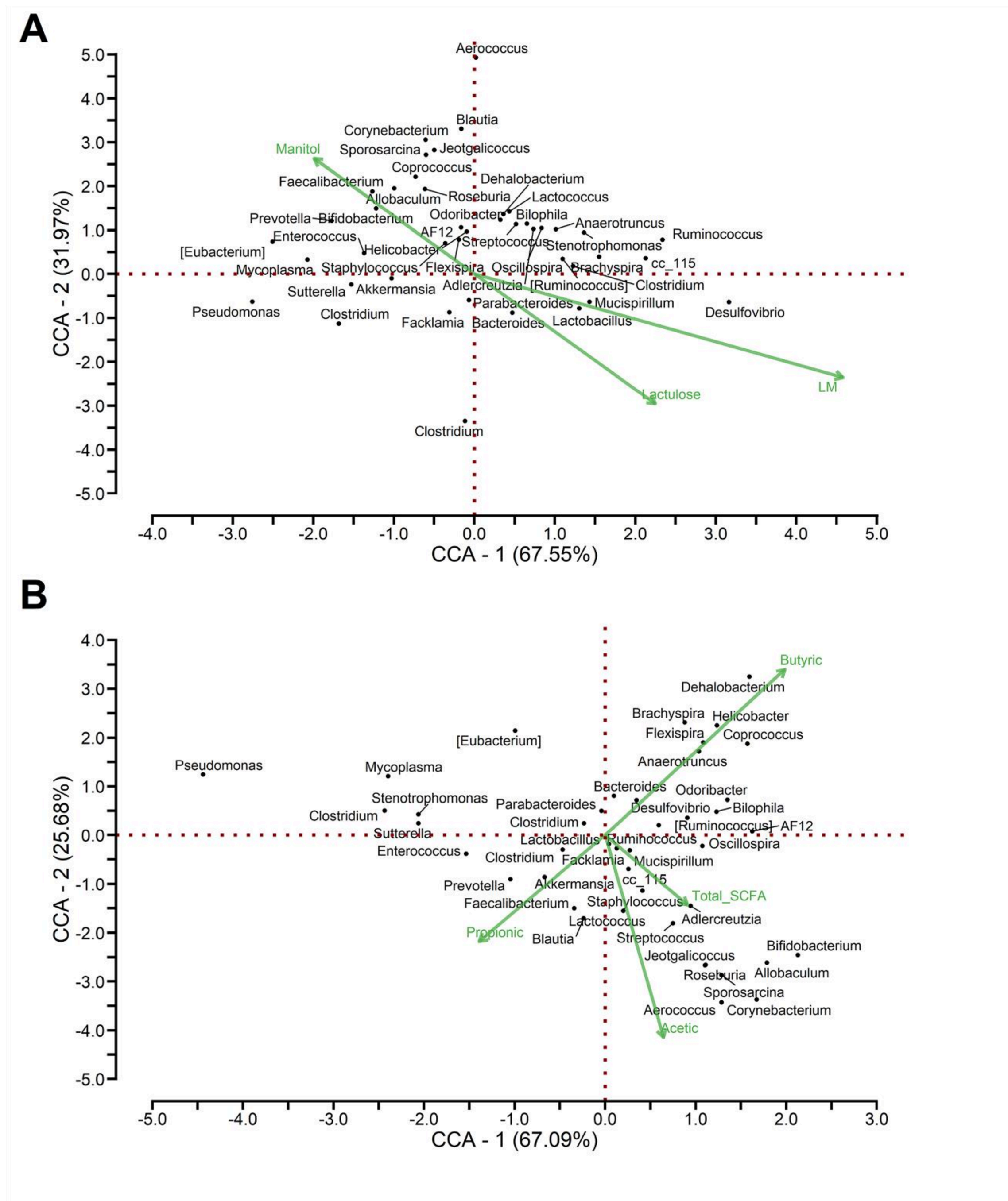
G8 vs G4				
pathway	**description**	**G8: mean rel. freq. (%)**	**G4: mean rel. freq. (%)**	**p-values (corrected)**
FAO-PWY	fatty acid β-oxidation I	0.07	0.02	0.013
PWY-5971	palmitate biosynthesis II (bacteria and plants)	0.21	0.10	0.048
PWY-5989	stearate biosynthesis II (bacteria and plants)	0.14	0.06	0.002
PWYG-321	mycolate biosynthesis	0.16	0.08	0.027
ALL-CHORISMATE-PWY	superpathway of chorismate metabolism	0.01	0.03	0.019
PWY-5845	superpathway of menaquinol-9 biosynthesis	0.01	0.04	0.025
PWY-5850	superpathway of menaquinol-6 biosynthesis I	0.01	0.04	0.025
PWY-5896	superpathway of menaquinol-10 biosynthesis	0.01	0.04	0.025
PWY-6125	superpathway of guanosine nucleotides de novo biosynthesis II	0.35	0.97	0.042
PWY-7197	pyrimidine deoxyribonucleotide phosphorylation	0.22	0.81	0.043
PWY-7228	superpathway of guanosine nucleotides de novo biosynthesis I	0.32	0.98	0.044
PWY0-162	superpathway of pyrimidine ribonucleotides de novo biosynthesis	0.43	1.01	0.040

Supplemental Figure 1. Canonical correspondence analysis (CCA) among groups treated with ceftriaxone.



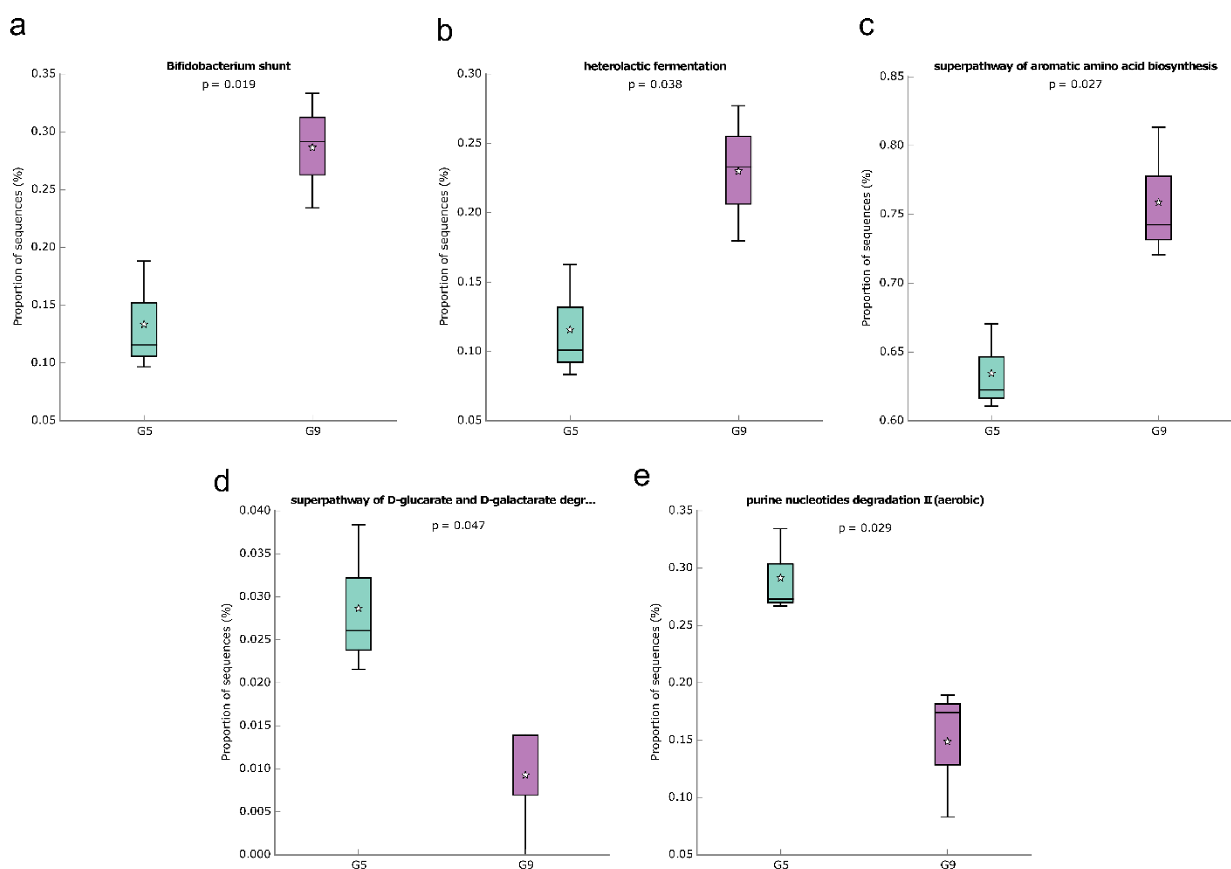
CCA was performed on the most abundant OTUs (relative abundance > 0.1%) at the genus level for the groups G1, G2, G4, G6, and G8, with **A)** intestinal permeability parameters (lactulose, mannitol, and lactulose/mannitol ratio) and **B)** the short-chain fatty acids acetate, propionate, butyrate, and total SCFA. Green lines indicate the direction and magnitude of measurable variables associated with community structures.

Supplemental Figure 2. Canonical correspondence analysis (CCA) among groups treated with LG-G12 and ceftriaxone.



CCA was performed on the most abundant OTUs (relative abundance > 0.1%) at the genus level for the groups G1, G2, G5, G6, and G9, with **A)** intestinal permeability parameters (lactulose, mannitol, and lactulose/mannitol ratio) and **B)** the short-chain fatty acids acetate, propionate, butyrate, and total SCFA. Green lines indicate the direction and magnitude of measurable variables associated with community structures.

Supplemental Figure 3. Box plot showing the distribution in the proportion of selected MetaCyc pathways between the groups G5 and G9.



Boxes indicate the IQR (75th to 25th of the data). The median value is shown as a line within the box and the mean value as a star. Whiskers extend to the most extreme value within 1.5*IQR. Where: a: Bifidobacterium shunt; b: Heterolactic fermentation; c: Superpathway of aromatic amino acids biosynthesis; d: Superpathway of D-glucarate and D-galactarate degradation; e: Purine nucleotides degradation II (aerobic).

5. CONCLUSÃO GERAL

O uso conjunto de *Lactobacillus gasseri* LG-G12 e ceftriaxona é promissor para o tratamento da obesidade, visto que, após a utilização dessas intervenções, houve modulação da microbiota intestinal e dos parâmetros relacionados à obesidade, como redução de parâmetros biométricos e do perfil lipídico e aumento do tecido adiposo marrom e da produção de butirato.

Essa modulação intestinal foi realizada pela produção de ácidos graxos de cadeira curta, o que contribuiu para o aumento da integridade intestinal, e pelo enriquecimento de vias metabólicas para controle da disbiose, sendo esses mecanismos de proteção advindos da eubiose intestinal após o tratamento com as intervenções testadas.

Em relação à indução da obesidade em modelo animal são muitos os fatores envolvidos, como espécie, tempo de intervenção, dieta e oferta de diferentes tipos e quantidades de carboidratos e gorduras. Contudo, a oferta de dieta *high-fat* com 60% das calorias advindas de gordura e solução de frutose 10% foi eficiente em causar disbiose intestinal.

Por fim, destaca-se que a modulação intestinal pode ser efetiva não apenas para o tratamento da obesidade, como também de outras doenças, sendo o uso de alimentos probióticos, como o kefir, uma estratégia interessante e acessível para a população em geral.

Logo, os resultados encontrados indicam que a modulação da microbiota intestinal é promissora para o tratamento da disbiose intestinal e de todas as doenças a ela relacionadas.

APÊNDICES

A. Cálculo experimental

Fórmula proposta por Mera e colaboradores (1998):

$$N = \frac{2 \times (t\alpha/2 \times DP)^2}{E^2}$$

em que:

$t\alpha/2$ = valor da tabela de distribuição t (two-tailed);

DP = desvio-padrão; e

E = diferença que se deseja detectar no estudo;

$t\alpha/2$ = 2,998, considerando um poder estatístico de 98%; e

$$N = \frac{2 \times (2{,}998 \times 2{,}22)^2}{3{,}67^2} = 6{,}57$$

Tabela 1 – Variáveis a serem utilizadas para o cálculo amostral segundo o estudo de Miyoshi *et al*. (2014)

	Grupo-controle	Grupo tratado	Média ± DP
Peso (g)*	39,15 ± 2,18	35,48 ± 2,26	37,315 ± 2,22
n	9	10	-

* Valores expressos em média ± DP.

Considerando a possibilidade de perdas durante o experimento, acrescentaram-se 20% ao n calculado, sendo necessários, portanto, oito animais por grupo experimental.

B. Artigo científico

Título: “Ceftriaxone Causes Dysbiosis and Changes Intestinal Structure in Adjuvant Obesity Treatment”.

Autores: Sandra Aparecida dos Reis Louzano, Mariana de Moura e Dias, Lisiane Lopes da Conceição, Tiago Antônio de Oliveira Mendes e Maria do Carmo Gouveia Peluzio.

Submetido à revista: Pharmacological Reports (FI: 3.024).

Abstract:

Background: Obesity is still a worldwide public health problem, requiring the development of adjuvant therapies to combat it. In this context, the modulation of the intestinal microbiota stands out, given that the composition of the intestinal microbiota contributes to the outcome of this disease. The aim of this work is to investigate the treatment with an antimicrobial and/or a potential probiotic against overweight. Methods: Male C57BL/6J mice were subjected to a 12-week overweight induction protocol. After that, 4 weeks treatment were started, with mice divided into 4 groups: control, treated with distilled water; potential probiotic, with Lactobacillus gasseri LG-G12; antimicrobial, with ceftriaxone; and antimicrobial + potential probiotic with ceftriaxone in the first 2 weeks and L. gasseri LG-G12 in the subsequent weeks. Results: The treatment with ceftriaxone in isolated form or in combination with the potential probiotic provided a reduction in body fat. However, such effect is supposed to be a consequence of the negative action of ceftriaxone on the intestinal microbiota composition, and this intestinal dysbiosis may have contributed to the destruction of the intestinal villi structure, which led to a reduction in the absorptive surface. Also, the effects of L. gasseri LG-G12 apparently have been masked by the consumption of the high-fat diet. Conclusions: The results indicate that the use of a ceftriaxone in the adjuvant treatment of overweight is not recommended due to the potential risk of developing inflammatory bowel disease.

Keywords: obesity, intestinal modulation, dysbiosis, intestinal microbiota, probiotics, high-fat diet.

Introduction

In the last two decades, the intestinal microbiota has been included in the group of environmental factors that determine an individual's state of health and disease [1]. In this sense, evidence suggests that intestinal dysbiosis can be considered a risk factor for the development of overweight [2]. In the presence of dysbiosis, the intestinal microbiota is supposed to increase the energy intake from the diet [3, 4], as well as the intestinal absorption of macronutrients [5, 6]; modulate the expression and functioning of proteins related to energy storage and metabolism processes [3, 7]; and induce a chronic low-grade inflammatory state [8]. In this way, the intestinal microbiota would be able to stimulate the gain of body fat, contributing to the occurrence of overweight [9]. Thus, the observed increase in the incidence of overweight around the world followed by the negative health consequences that this situation can trigger [10] have promoted the investigation of the effect of adjuvant treatment with modulators of the intestinal microbiota composition on overweight [11-14].

Based on the above, the potential use of antimicrobials in the adjuvant treatment of overweight has been investigated [13]. It is expected that the consumption of antimicrobials by overweight individuals promotes an “eubiotic effect”, favoring a possible positive modulation in the intestinal microbiota composition, which would contribute to the reduction in body mass gain [15]. However, the indiscriminate use of antimicrobials can contribute to the increase in the number of antibiotic resistant microorganisms [16, 17]. Other modulators that have been investigated aiming at their potential use in the adjuvant treatment of overweight are the probiotics, which have been found to reduce body mass, depending on the employed strain [18, 19]. Unlike antimicrobials, the continuous consumption of probiotics does not contribute to an increase in the number of drug-resistant microorganisms [20].

For the study of obesity, one of the most employed animal models are those that develop the disease through the ingestion of an obesogenic diet, rich in fat, since this model has similar development of obesity in humans. Among these, the C57BL/6J mouse stands out, since it is more susceptible to fat accumulation and weight gain. Also, young and male mice have a better response to the development of obesity through food consumption, and its use is recommended in studies that present obesity as a study topic [21].

Recently, our research group evaluated the effect of energy restriction combined with antimicrobials and/or probiotics in the treatment of overweight, obtaining positive results in relation to biometric measurements and modulation of the intestinal microbiota [22]. However, considering that overweight is related to excessive consumption of calories [23, 24] and that few studies comparing the effect of treatment with an antimicrobial and a probiotic,

in isolated or combined forms in the treatment of overweight are available, this study aimed to investigate the effects of the treatment with intestinal microbiota modulators (either an antimicrobial or a potential probiotic) and the combination of both treatments (antimicrobial and potential probiotic) on overweight and secondary metabolic changes in an animal model that consumed a high-fat diet with 60% of the calories based on lipids throughout the treatment phase.

Materials and methods

Animals

Twenty-eight male C57BL/6J mice [21, 25] from the Central Vivarium of the Center for Biological and Health Sciences at the Universidade Federal de Viçosa (UFV), Viçosa, Minas Gerais, Brazil, were used in the experiment. The animals were kept in collective cages, with 2 animals per cage, with a 12 h light/dark cycle and an average temperature of 22±2ºC. In the experimental period, the animals had free access to the fructose solution and the diet, which were administered following the pair-feeding scheme. The animal experiment was carried out at the Experimental Nutrition Laboratory of the Department of Nutrition and Health (DNS) at UFV. No adverse effects were observed in the animals.

The experiment was approved by the Animal Use Ethics Committee of the Universidade Federal de Viçosa, according to the protocol number 09/2017, and followed the principles established by the National Animal Experimentation Control Council [26].

Experimental design and diets

Initially, 5 weeks old mice (21.6g ± 1.18g), underwent a protocol for inducing overweight that lasted 12 weeks (induction phase). During this period, the animals were fed with a high-fat diet containing 60% of the calories based on lipids (nutritional composition based on the diet D12492 of Research Diets, Inc) [27] (Table 1) and a 10% fructose solution (Synth®, Diadema, Brazil) instead of drinking water [25].

Table 1 – Composition of the high fat diet with 60% fat

Ingredient	High-fat diet 60%
Maize starch	-
Soy oil	3.2
Lard	31.7
Casein	25.8
Maltodextrin	16.2
Sucrose	8.9
Cellulose	6.5
Mineral mix	1.3
Potassium citrate	2.1
L-cystine	0.39
Calcium phosphate	1.7
Vitamin mix	1.3
Calcium carbonate	0.7
Choline bitartrate	0.3
% of fat kcal	60.0
% of carbohydrate kcal	20.0
% of protein kcal	20.0
Caloric density (kcal)	406.7

Source: Research diets (http://www.researchdiets.com/open-source-diets/stock-diets/dio-series-diets); an casein has > 85% protein.

After this period, the treatment phase began and the mice were randomly divided into four experimental groups: control (n = 7), antimicrobial (n = 7), potential probiotic (n = 7) and antimicrobial combined with potential probiotic (antimicrobial + probiotic) (n = 7). During this phase, with a total period interval of four weeks, the animals continued to receive the same diets to prevent qualitative and quantitative changes in diet from interfering with the effects of treatments, and the fructose solution as in the induction phase. All treatments were daily administered through gavage, at the same time, in the evening.

Animals in the control group were treated with distilled water. The antimicrobial group was treated with 500 mg of ceftriaxone (Triaxton, Blau Farmacêutica S/A®, Brazil)/ kg of body mass diluted in phosphate buffered saline solution (PBS) [28]. The spectrum of action of ceftriaxone is the gram-negative bacteria, which, due to lipopolysaccharide, have been linked to an increased risk for the development of overweight [8].

The potential probiotic group, on the other hand, received 10^9 colony-forming units (CFU) of *Lactobacillus gasseri* LG-G12 (Lemma Supply Solutions®, Brazil) diluted in PBS.

The antimicrobial + potential probiotic group received 500 mg of ceftriaxone/ kg of body weight in the first two weeks of the treatment phase and, in the following two weeks, 10^9 CFU of *L. gasseri* LG-G12. By using such treatment scheme, it is expected that the

antimicrobial eliminates bacteria related to the increased risk for the development of overweight and the probiotic recolonizes the intestine, leading to a reduction in body mass gain [29].

At the end of the treatment phase, the animals were euthanized after being anesthetized with 3% isoflurane (Cristália®, Belo Horizonte, Brazil) and, subsequently, subjected to total exsanguination. The use of this anesthetic followed by a physical method is a form of euthanasia recommended for rodents by CONCEA [26]. Tissue samples were collected and properly stored for further analysis.

Body mass and dimensions

The animals body mass was checked weekly by weighing on a scale. Body dimensions (abdominal and thoracic perimeters, and snout-anus length) were measured at the end of the treatment phase, and the Lee index was calculated [29].

Histological analysis of abdominal adipose tissue

After fixation, tissue samples were dehydrated in an increasing gradient of ethanol and included in paraffin. Through a rotating microtome (model CUT 4055, Olympus®, Miami, EUA) 5 µm thick histological fragments were obtained. Subsequently, these fragments were stained with hematoxylin and eosin.

For histological analysis, photomicrographs were obtained directly from the light microscope (Zeiss 2012, Primo Star) and analyzed with the aid of Image Pro-Plus® software version 4.5 (Media Cybernetics, USA).

For counting and measuring the area of adipocytes, 10 fields/animal were captured with the 10X objective. From the photomicrographs, full-size cells that presented well-defined contours and that were complete in the photo field were counted [30], and the diameter of 20 cells/ field was measured, with the diameter of the same cell being measured twice in different positions. The average of these values was calculated and used in the statistical analyses.

Food, water and caloric consumption

Food consumption was assessed based on the weight difference between the amount of diet offered and the remaining amount not consumed. The fructose solution offered and consumed throughout the experiment was also quantified. The animals' caloric consumption

was estimated based on the amount of high-fat 60% diet (5.177 kcal/g diet) and fructose (Synth®, Diadema, Brazil) solution (4 kcal/g fructose) consumed.

Intestinal permeability

The intestinal permeability test was performed at the end of the treatment phase. Through gavage, the animals received 200 μL of a solution containing lactulose (Daiichi Sankyo®, Barueri, Brazil) and mannitol (Synth®, Diadema, Brazil). Following, all the urine excreted in the subsequent 24 h was collected [31].

A high-performance liquid chromatography (Shimadzu®, model detector: RID 10A, Japan) was used to quantify the sugars through a wavelength of 210 nm. A sample of 20 μL was injected into a mobile column (Aminex®, model HPX-87H, USA) 300 mm x 7.8 mm in diameter with a flow of 1 mL/ min and pressure of 54 kgf. Ultrapure water was used as a mobile phase.

Histological analysis of the small intestine

After fixation, the samples were dehydrated and included in resin. The histological fragments (5 μm thick) were obtained in a rotating microtome and stained with hematoxylin and eosin.

For histological analysis, photomicrographs were obtained with the aid of a light microscope (Zeiss 2012, Primo Star) and analyzed with the Image Pro-Plus® software version 4.5 (Media Cybernetics, USA).

For morphometric analysis (height and width of villi, and depth of crypts), 10 random fields/animal were captured, with photomicrographs obtained with the 10X objective.

Villus height was measured only in villi with well-defined epithelium and visible connective tissue. The same villus was measured at apical, medial and basal points, and the mean value was considered as the result. Depth was also measured in the images where it was possible to see the crypt base and apex (opening) [32].

Intestinal microbiota composition: Sequencing and analysis of bioinformatics

As previously described in Dias et al. [22], after the treatment phase, fecal samples were collected from all experimental groups, performing a pool of feces. Metagenomic DNA was extracted using the methodology adapted by Zhang et al. [33]. The V3 and V4 regions of 16S rRNA genes were PCR amplified utilizing specific primers (Bakt 341F and Bakt 805R)

and sequenced using an Illumina MiSeq desktop sequencer (Illumina, San Diego, CA, USA) at the Macrogen Company (Macrogen Inc®, Seoul, South Korea).

The raw sequencing data (files in the fastq format) were using the program Trimmomatic v0.36 [34]. Data were processed using the DADA2 package version 1.8 [35] on R platform, version 3.6.1 (https://cran.r-project.org/). The data processing followed all the steps recommended by the DADA2 developers, based on alignments performed with the Silva release 138 database [36], as previously described in Dias et al. [22].

To estimate the diversity of the microbial community, we calculated the within-sample alpha-diversity using the Shannon and Simpson index, and CHAO-1 index for richness assessment using the phyloseq package [37]. Beta-diversity was estimated by computing Jaccard distance and visualized by multidimensional scaling (MDS) plot using R platform. Bacterial communities' composition was analyzed at the phylum, family and gender level.

The raw fastq data were submitted to Sequence Read Archive (SRA) from NCBI under accession number PRJNA705760.

Extraction and analysis of short chain fatty acids

Acetic, propionic and butyric short chain fatty acids (SCFA) were extracted from samples of cecal content collected after the animals were euthanized, according to the methodology proposed by Siegfried et al. [38].

The samples were analyzed by high performance chromatography (HPLC), using the Ultimate 3000 chromatograph, Dionex®, and a column (RezexROA- Organic Acid H + (8%), Phenomenex®) with a length of 300 mm and a diameter of 7.8 mm. A volume of 20 µL was injected with a flow of 0.7 mL minute^{-1}, oven temperature at 45 ºC, and mobile phase composed of 5 µmolar sulfuric acid diluted in water. The detector used was Rid RH01 (Shodex®).

Statistical analysis

The normality of the variables was determined according to the Kolmogorov-Smirnov test. The comparisons between the experimental groups were performed according to two-way analysis of variance (two-way ANOVA), followed by Bonferroni post hoc test for parametric data. For the non-parametric data (abdominal perimeter (AP), thoracic perimeter (TP), ratio AP/TP, length, butyric acid, *Bacteroidetes, Firmicutes, Bacteroidaceae and Bacteroides*), the Friedman's Two-way Analysis of Variance by Ranks was applied. In both tests, two independents variables used were presence of antibiotic as column factor and

presence of potential probiotic as row factor. The control group was defined by the absence of both antibiotic and potential probiotic.

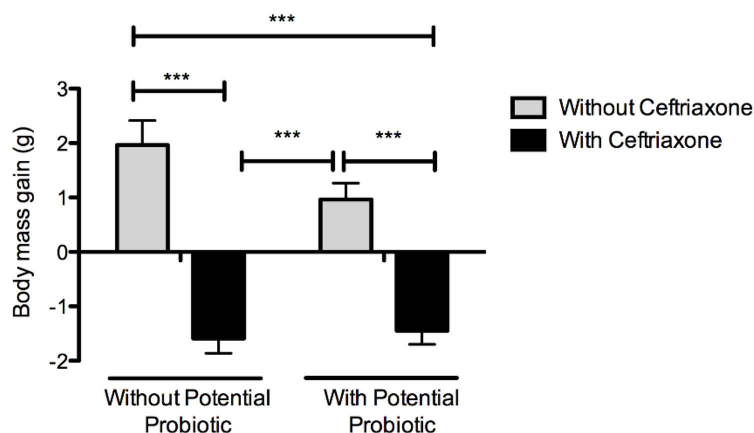
The results were expressed as mean ± standard error (SE) or median (Q1-Q2) for parametric and non-parametric data, respectively. The processing and statistical analyses of the data were performed using SPSS software, version 20 for Windows and GraphPad Prism version 6.0.

Results

Body mass and dimensions gain

At the beginning of the treatment phase, all groups presented similar mean body mass (control: 24.85 ± 0.53 g; antimicrobial: 24.85 ± 0.86 g; potential probiotic: 24.66 ± 0.30 g; antimicrobial + potential probiotic: 24.34 ± 0.37 g) with no significant statistical difference compared to control group by two-way ANOVA with Bonferroni post hoc test ($F_{1,24} = 0.032$ and $p = 0.86$ for comparison between control and antimicrobial group, $F_{1,24} = 0.287$ and $p = 0.59$ for control versus probiotic group and $F_{1,24} = 0.321$ and $p = 0.57$ for control versus antimicrobial + potential probiotic). After four weeks of treatment, the antimicrobial and antimicrobial + potential probiotic groups presented lower body mass gain compared to control ($F_{1,24} = 91.230$, $p \leq 0.001$ for control versus antimicrobial group, $F_{1,24} = 0.241$, $p = 0.62$ for control versus potential probiotic group and $F_{1,24} = 1.737$, $p = 0.20$ for control versus antimicrobial + potential probiotic) and the probiotic group ($F_{1,24} = 92.120$, $p \leq 0.001$ for antimicrobial versus potential probiotic group and $F_{1,24} = 50.865$, $p \leq 0.001$ for potential probiotic versus antimicrobial + probiotic group) (Figure 1). Such result represented 5 and 7% of reduction in body mass gain in the antimicrobial and antimicrobial + potential probiotic groups, respectively, in relation to the potential probiotic group.

Figure 1. Body mass gain variations. Values are expressed as mean ± SE (n = 7 for each group). Two way ANOVA test followed by Bonferroni post hoc. *** $p \leq 0.001$



The abdominal and thoracic perimeters, as well as the ratio between them, and the snot-anus length, were similar among the different groups ($p > 0.05$). The control and potential probiotic group presented higher Lee index values if compared with the antimicrobial and antimicrobial + potential probiotic groups (Table 2). These results suggest a reduction in the amount of body fat stored by animals in the antimicrobial and antimicrobial + probiotic groups, and that this reduction did not occur in a specific part of the body, such as the chest or abdomen.

Table 2. Body dimensions of the experimental groups

Body dimension	Control (n=7)	Antimicrobial (n=7)	Potential probiotic (n=7)	Antimicrobial + potential probiotic (n=7)	$F_{1,24}$ (p) Control *versus* antimicrobial	$F_{1,24}$ (p) Control *versus* probiotic	$F_{1,24}$ (p) Control *versus* antimicrobial + Probiotic	$F_{1,24}$ (p) Antimicrobial *versus* probiotic	$F_{1,24}$ (p) Antimicrobial *versus* antimicrobial + Probiotic	$F_{1,24}$ (p) Probiotic *versus* antimicrobial + Probiotic
AP (cm) †	7.0 (7.0-8.0)	7.0 (6.5-7.0)	7.7 (7.0-7.3)	7.2 (6.8-7.6)	0.937 (0.34)	1.172 (0.29)	2.876 (0.10)	2.352 (0.14)	2.524 (0.12)	2.299 (0.14)
TP (cm) †	6.5 (6.0-7.0)	6.5 (6.5-6.5)	7.0 (6.5-7.0)	6.5 (6.5-7.5)	0.621 (0.44)	2.386 (0.13)	0.987 (0.33)	2.986 (0.09)	0.614 (0.44)	2.092 (0.16)
Ratio AP/TP (cm)†	1.08 (1.07-1.14)	1.07 (1.00-1.08)	1.07 (1.07-1.08)	1.07 (1.00-1.08)	0.822 (0.37)	0.786 (0.38)	0.831 (0.37)	0.654 (0.43)	0.674 (0.42)	0.751 (0.39)
Length (cm) †	9.0 (9.0-9.1)	9.0 (9.0-9.5)	9.0 (9.0-9.0)	9.0 (9.0-9.5)	0.531 (0.47)	0.296 (0.59)	0.367 (0.55)	0.768 (0.39)	0.891 (0.35)	0.829 (0.37)
Lee index	0.331±0.004[a]	0.314±0.004[b]	0.327±0.001[a]	0.324±0.003[b]	6.128 (0.020)	2.671 (0.11)	5.763 (0.024)	5.679 (0.025)	3.122 (0.09)	5.989 (0.022)

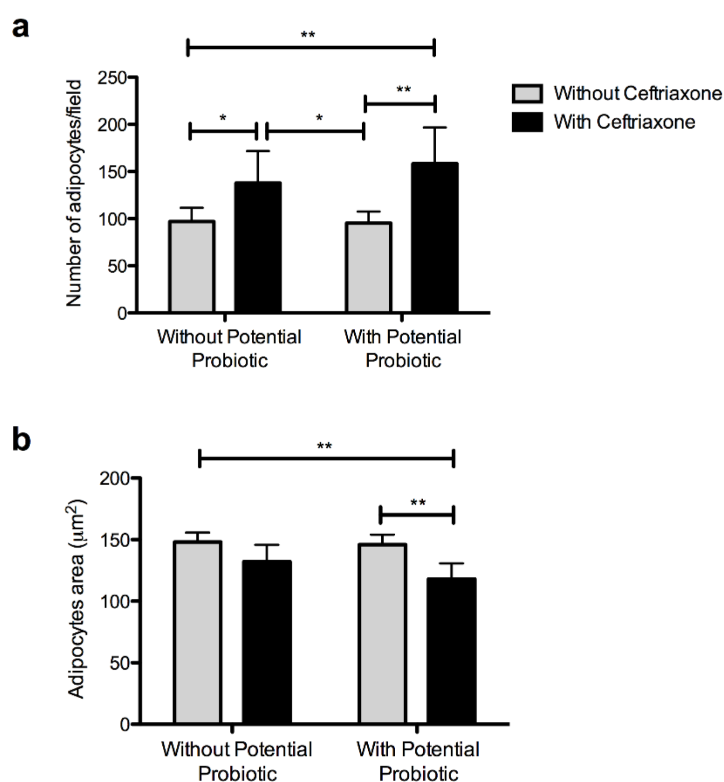
AP: abdominal perimeter; TP: thoracic perimeter. Values are expressed as mean ± SE or † median (Q1-Q3). Two-way ANOVA test followed by Bonferroni post hoc or † Friedman's Two-way Analysis of Variance by Ranks.

Adipose tissue

The amount of abdominal adipose tissue collected during the animals' euthanasia was higher in the potential probiotic group (0.19 ± 0.006 g) compared to the antimicrobial (0.11 ± 0.003 g) and antimicrobial + potential probiotic (0.12 ± 0.007 g) groups ($F_{1,22}$ = 99.160, $p < 0.0001$ for antimicrobial versus probiotic group, $F_{1,22}$ = 84.237, $p < 0.0001$ for probiotic versus antimicrobial + probiotic group and $F_{1,22}$ = 0.168, p = 0.68 for potential antimicrobial versus antimicrobial + probiotic factor).

The histological evaluation of the tissue revealed that the use of antimicrobial with and without probiotic induced a higher number of adipocytes per field compared to the potential probiotic treatment and control group ($F_{1,22}$ = 6.120 and p = 0.021 for control versus antimicrobial group, $F_{1,22}$ = 0.287 and p = 0.59 for control versus probiotic group, $F_{1,22}$ = 9.233 and p = 0.006 for control versus antimicrobial + probiotic group, $F_{1,22}$ = 6.842 and p = 0.015 for antimicrobial versus probiotic group, $F_{1,22}$ = 0.121 and p = 0.73 for antimicrobial versus antimicrobial + probiotic group and $F_{1,22}$ = 11.516 and p = 0.0026 for potential probiotic versus antimicrobial + probiotic; Figure 2a). In contrast, the antimicrobial + potential probiotic group displayed adipocytes with a smaller area compared to the control and potential probiotic group ($F_{1,22}$ = 0.982 and p = 0.33 for control versus antimicrobial group, $F_{1,22}$ = 0.171 and p = 0.68 for control versus probiotic group, $F_{1,22}$ = 8.912 and p = 0.0068 for control versus antimicrobial + probiotic group, $F_{1,22}$ = 1.222 and p = 0.28 for antimicrobial versus probiotic group, $F_{1,22}$ = 0.971 and p = 0.34 for antimicrobial versus antimicrobial + probiotic group and $F_{1,22}$ = 9.113 and p = 0.0063 for potential probiotic versus antimicrobial + probiotic; Figure 2b). This result suggests that the greater number of cells/fields observed in the antimicrobial + potential probiotic group would be a consequence of the reduction in the area occupied by adipocytes due to the reduction in the amount of fat stored by that cell.

Figure 2. Histological parameters of abdominal adipose tissue. a. Number of adipocytes per field. b. Adipocyte area. Values are expressed as mean ± SE (n = 7 for each group). Two way ANOVA test followed by Bonferroni post hoc. * p < 0.05 and ** p < 0.01



Food, water and caloric consumption

In the treatment phase, food ($F_{1,22}$ = 0.087 and p = 0.77 for control versus antimicrobial group, $F_{1,22}$ = 0.912 and p = 0.35 for control versus probiotic group, $F_{1,22}$ = 0.238 and p = 0.63 for control versus antimicrobial + probiotic group, $F_{1,22}$ = 1.021 and p = 0.32 for antimicrobial versus probiotic group, $F_{1,22}$ = 0.754 and p = 0.39 for antimicrobial versus antimicrobial + probiotic group and $F_{1,22}$ = 1.751 and p = 0.20 for potential probiotic versus antimicrobial + probiotic; Figure 2b), water ($F_{1,22}$ = 1.022 and p = 0.77 for control versus antimicrobial group, $F_{1,22}$ = 0.786 and p = 0.35 for control versus probiotic group, $F_{1,22}$ = 1.245 and p = 0.32 for control versus antimicrobial + probiotic group, $F_{1,22}$ = 1.512 and p = 0.23 for antimicrobial versus probiotic group, $F_{1,22}$ = 0.086 and p = 0.77 for antimicrobial versus antimicrobial + probiotic group and $F_{1,22}$ = 1.235 and p = 0.28 for potential probiotic versus antimicrobial + probiotic; Figure 2b), and caloric ($F_{1,22}$ = 1.118 and p = 0.30 for control versus antimicrobial group, $F_{1,22}$ = 2.435 and p = 0.13 for control versus probiotic group, $F_{1,22}$ = 1.865 and p = 0.18 for control versus antimicrobial + probiotic group, $F_{1,22}$ = 2.839 and p = 0.11 for antimicrobial versus probiotic group, $F_{1,22}$ = 0.023 and p = 0.88 for antimicrobial versus antimicrobial +

probiotic group and $F_{1,22} = 2.561$ and $p = 0.12$ for potential probiotic versus antimicrobial + probiotic; Figure 2b), consumption were similar among the experimental groups.

Intestinal health

The intestinal permeability test revealed that all groups excreted similar amounts of lactulose, and that the antimicrobial group excreted a lower percentage of mannitol compared to the other groups. The lactulose/mannitol excretion ratios were similar for the different investigated groups (Table 3). These results suggest that the treatments did not interfere with intestinal permeability. However, a reduction in the absorptive area in the antimicrobial group is apparently seen. Such a hypothesis was confirmed through the morphometric analysis of the small intestine (Figure 3), which revealed that the control and potential probiotic groups presented a greater villus height and width compared to the antimicrobial and antimicrobial + potential probiotic groups (Table 4). The depth of the crypts was similar between the experimental groups.

Figure 3. Photomicrographs of intestinal crypts and villi (LEICA DM750 light microscope, magnification 100x) (n = 7 for each group). Hematoxylin and eosin staining. Black arrows indicate the presence of changes in intestinal morphology

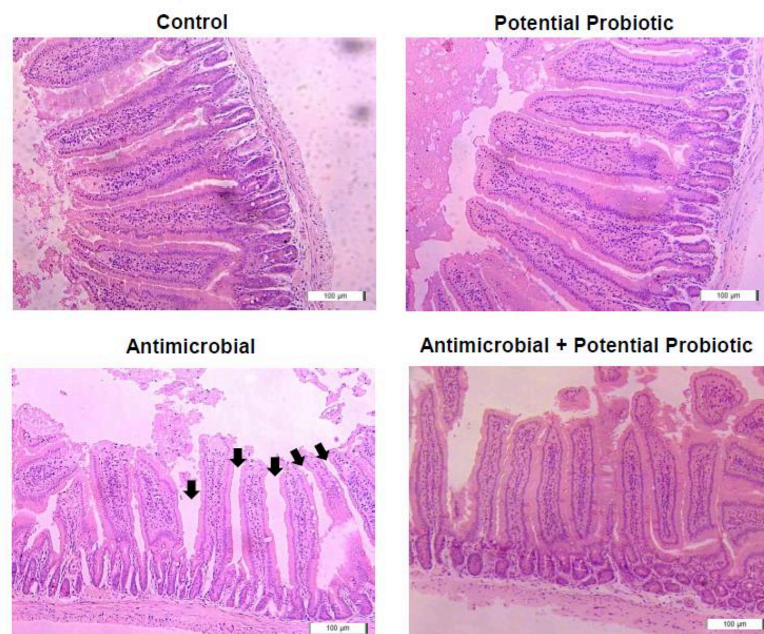


Table 3. Intestinal permeability test

Sugar (%)	Control (n=7)	Antimicrobial (n=7)	Potential probiotic (n=7)	Antimicrobial + potential probiotic (n=7)	$F_{1,24}$ (p) Control *versus* antimicrobial	$F_{1,24}$ (p) Control *versus* probiotic	$F_{1,24}$ (p) Control *versus* antimicrobial + Probiotic	$F_{1,24}$ (p) Antimicrobial *versus* probiotic	$F_{1,24}$ (p) Antimicrobial *versus* antimicrobial + Probiotic	$F_{1,24}$ (p) Probiotic *versus* antimicrobial + Probiotic
Lactulose	1.66±0.17	1.90±0.18	1.66±0.09	1.53±0.21	2.665 (0.11)	0.071 (0.79)	1.444 (0.24)	2.765 (0.11)	3.109 (0.09)	1.144 (0.29)
Mannitol	0.26±0.024[a]	0.16±0.038[b]	0.25±0.019[a]	0.25±0.009[a]	7.541 (0.011)	0.754 (0.39)	0.762 (0.39)	6.787 (0.015)	6.564 (0.017)	0.819 (0.37)
Ratio L/M	6.88±0.81	6.48±0.93	6.69±0.74	6.19±1.01	1.008 (0.32)	0.478 (0.49)	1.738 (0.20)	0.529 (0.47)	0.730 (0.40)	1.260 (0.27)

L: lactulose; M: mannitol. Values are expressed as mean ± SE. Two-way ANOVA test followed by Bonferroni post hoc.

Table 4. Morphometric analysis of small intestine

Morphometry (µm)	Control (n=7)	Antimicrobial (n=7)	Potential probiotic (n=7)	Antimicrobial + potential probiotic (n=7)	$F_{1,24}$ (p) Control *versus* antimicrobial	$F_{1,24}$ (p) Control *versus* probiotic	$F_{1,24}$ (p) Control *versus* antimicrobial + Probiotic	$F_{1,24}$ (p) Antimicrobial *versus* probiotic	$F_{1,24}$ (p) Antimicrobial *versus* antimicrobial + Probiotic	$F_{1,24}$ (p) Probiotic *versus* antimicrobial + Probiotic
Height	263.50±9.69	195.77±7.54	261.81±3.69	202.32±7.38	17.130 (0.0003)	0.427 (0.52)	15.470 (0.24)	16.700 (0.0006)	1.657 (0.21)	15.050 (0.0007)
Width	55.55±4.84	40.08±2.41	54.82±3.46[a]	40.07±0.98	8.914 (0.006)	0.420 (0.52)	8.920 (0.006)	0.005 (0.94)	8.499 (0.007)	0.819 (0.37)
Depth	55.39±3.43	57.28±3.62	57.38±3.53	55.57±2.93	1.044 (0.32)	1.099 (0.30)	0.099 (0.75)	0.055 (0.81)	0.944 (0.34)	0.999 (0.33)

Values are expressed as mean ± SE. Two-way ANOVA test followed by Bonferroni post hoc.

Intestinal microbiota composition

MDS results revealed that the structure of the intestinal microbiota of the antimicrobial group differs from the other experimental groups (Figure 4a). Additionally, the use of Shannon estimator revealed that the diversity of the microbiota in the antimicrobial group is lower if compared to the control and probiotic groups ($F_{1,22}$ = 7.916 and p = 0.009 for control versus antimicrobial group, $F_{1,22}$ = 1.186 and p = 0.28 for control versus probiotic group, $F_{1,22}$ = 3.500 and p = 0.073 for control versus antimicrobial + probiotic group, $F_{1,22}$ = 8.977 and p = 0.006 for antimicrobial versus probiotic group, $F_{1,22}$ = 4.031 and p = 0.056 for antimicrobial versus antimicrobial + probiotic group and $F_{1,22}$ = 4.161 and p = 0.052 for potential probiotic versus antimicrobial + probiotic; Figure 4b). The CHAO1 and Simpson indices did not differ between the experimental groups.

Through metagenomic sequencing, 5 phyla, 10 families and 15 genera were detected (Figures 4e, f and g, respectively), with variations in the composition of the intestinal microbiota at the phylum, family, and gender level in all groups.

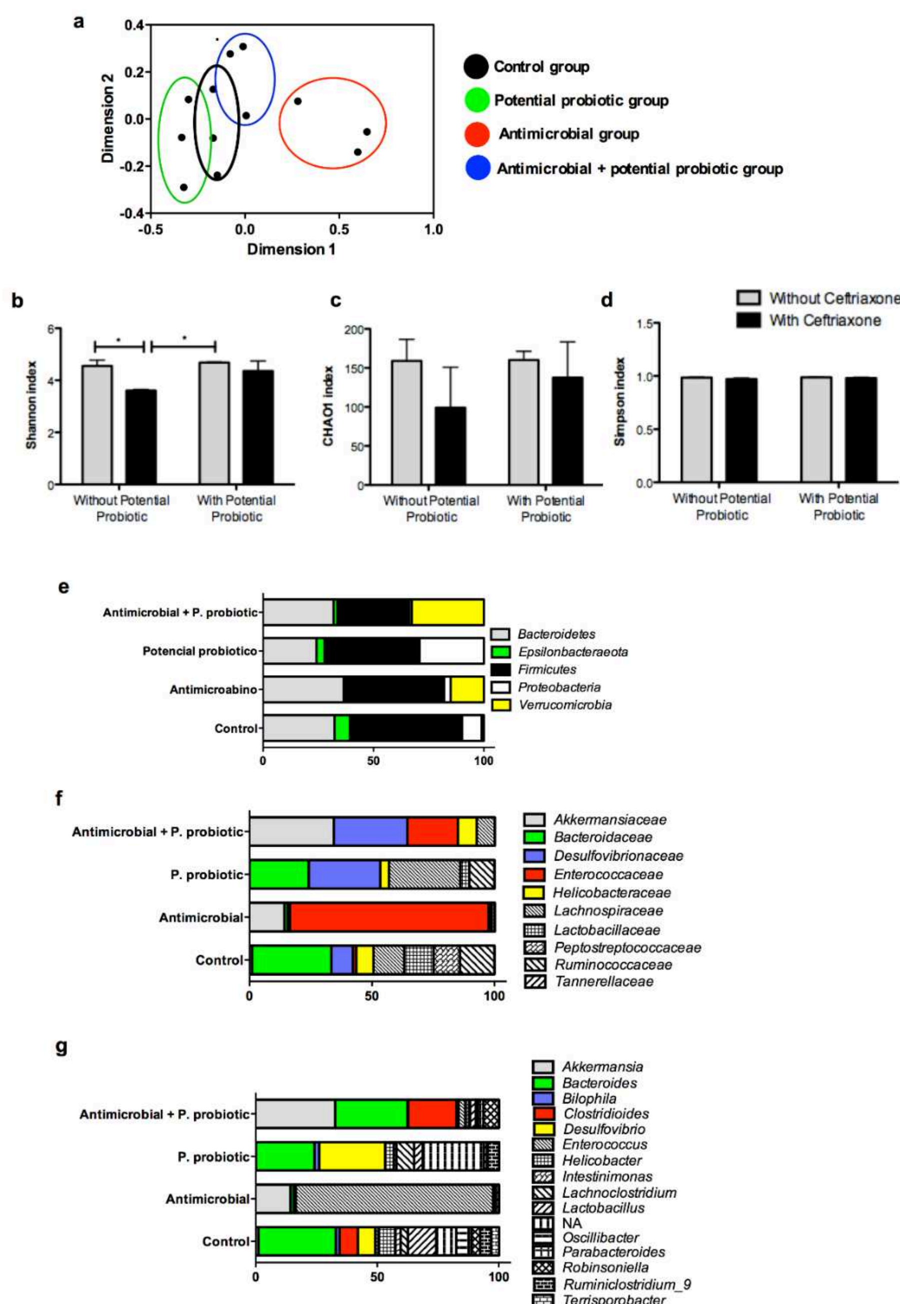
Within the same experimental group (intragroup comparison) the *Bacteroidetes* and *Firmicutes* phyla were the most abundant. In the intergroup comparison, the phylum *Epsilonbacteraeota* was found to be more abundant in the control group compared to the other experimental groups ($F_{1,22}$ = 4.299 and p = 0.049 for control versus antimicrobial group, $F_{1,22}$ = 1.389 and p = 0.25 for control versus probiotic group, $F_{1,22}$ = 2.779 and p = 0.11 for control versus antimicrobial + probiotic group, $F_{1,22}$ = 2.709 and p = 0.11 for antimicrobial versus probiotic group, $F_{1,22}$ = 1.320 and p = 0.26 for antimicrobial versus antimicrobial + probiotic group and $F_{1,22}$ = 1.389 and p = 0.25 for potential probiotic versus antimicrobial + probiotic).

Regarding the composition of bacterial families, *Bacteroidaceae* was found with lower abundance in the antimicrobial and antimicrobial + probiotic groups compared to the others experimental groups ($F_{1,22}$ = 10.210 and p = 0.0038 for control versus antimicrobial group, $F_{1,22}$ = 2.635 and p = 0.11 for control versus probiotic group, $F_{1,22}$ = 10.540 and p = 0.0034 for control versus antimicrobial + probiotic group, $F_{1,22}$ = 7.577 and p = 0.011 for antimicrobial versus probiotic group, $F_{1,22}$ = 0.329 and p = 0.57 for antimicrobial versus antimicrobial + probiotic group and $F_{1,22}$ = 7.906 and p = 0.009 for potential probiotic versus antimicrobial + probiotic). The *Lactobacillaceae* family was found in greater abundance in the control and potential probiotic groups compared to the antimicrobial and antimicrobial + probiotic groups ($F_{1,22}$ = 6.831 and p = 0.015 for control versus antimicrobial group, $F_{1,22}$ = 3.415 and p = 0.076 for control versus probiotic group, $F_{1,22}$ = 7.514 and p = 0.011 for control versus

antimicrobial + probiotic group, $F_{1,22} = 4.421$ and $p = 0.046$ for antimicrobial versus probiotic group, $F_{1,22} = 0.683$ and $p = 0.41$ for antimicrobial versus antimicrobial + probiotic group and $F_{1,22} = 4.298$ and $p = 0.049$ for potential probiotic versus antimicrobial + probiotic).

Regarding genus, a reduction in the relative abundance of *Bacteroides* was found in the antimicrobial group compared to the control (1.45 vs. 0.16%), potential probiotic (0.75 vs. 0.16%) and antimicrobial + potential probiotic (0.63 vs. 0.16%) groups ($F_{1,22} = 12.790$ and $p = 0.0015$ for control versus antimicrobial group, $F_{1,22} = 2.985$ and $p = 0.096$ for control versus probiotic group, $F_{1,22} = 5.117$ and $p = 0.033$ for control versus antimicrobial + probiotic group, $F_{1,22} = 9.807$ and $p = 0.0045$ for antimicrobial versus probiotic group, $F_{1,22} = 7.675$ and $p = 0.010$ for antimicrobial versus antimicrobial + probiotic group and $F_{1,22} = 2.132$ and $p = 0.157$ for potential probiotic versus antimicrobial + probiotic). A small abundance of *Bacteroides* genus was also observed in the antimicrobial + potential probiotic group compared to the control group. The *Helicobacter* genus was found in less abundance in the antimicrobial and antimicrobial + potential probiotic groups compared to the control group ($F_{1,22} = 5.344$ and $p = 0.029$ for control versus antimicrobial group, $F_{1,22} = 3.083$ and $p = 0.091$ for control versus probiotic group, $F_{1,22} = 5.138$ and $p = 0.032$ for control versus antimicrobial + probiotic group, $F_{1,22} = 2.261$ and $p = 0.14$ for antimicrobial versus probiotic group, $F_{1,22} = 0.205$ and $p = 0.65$ for antimicrobial versus antimicrobial + probiotic group and $F_{1,22} = 2.055$ and $p = 0.16$ for potential probiotic versus antimicrobial + probiotic). *Lactobacillus*, on the other hand, presented reduced abundance in the antimicrobial group compared to the control group ($F_{1,22} = 12.790$ and $p = 0.0015$ for control versus antimicrobial group, $F_{1,22} = 2.985$ and $p = 0.096$ for control versus probiotic group, $F_{1,22} = 5.117$ and $p = 0.033$ for control versus antimicrobial + probiotic group, $F_{1,22} = 9.807$ and $p = 0.0045$ for antimicrobial versus probiotic group, $F_{1,22} = 7.675$ and $p = 0.010$ for antimicrobial versus antimicrobial + probiotic group and $F_{1,22} = 2.132$ and $p = 0.157$ for potential probiotic versus antimicrobial + probiotic).

Figure 4. 16s RNA metagenomic analysis (n = 7 for each group). a. Multidimensional scaling analysis (MDS): The points represent the intestinal microbiota of the control (black color), probiotic (green color), antimicrobial (red color) and antimicrobial + probiotic (blue color) groups. b. CHAO1 index. c. Shannon index. d. Simpson index. e, f, g. Estimated distribution of bacterial abundance between treatments, at the phylum (e), family (f) and gender (g) levels. Values are expressed as % (a, e, f, g) or mean ± SE (b, c, d). Two way ANOVA test followed by Bonferroni post hoc. * $p < 0.004$ *vs* control group, potential probiotic group, and antimicrobial + potential probiotic group



Short chain fatty acids

The acetic and propionic acids were found in similar concentrations in the cecal content of the animals. Regarding butyric acid, the probiotic and antimicrobial + probiotic groups presented a higher concentration if compared with the antimicrobial group (Table 5).

Table 5. Short chain fat acids concentration in cecal content

SCFA (mmol g^{-1})	Control (n=7)	Antimicrobial (n=7)	Potential probiotic (n=7)	Antimicrobial + potential probiotic (n=7)	$F_{1,24}$ (p) Control *versus* antimicrobial	$F_{1,24}$ (p) Control *versus* probiotic	$F_{1,24}$ (p) Control *versus* antimicrobial + Probiotic	$F_{1,24}$ (p) Antimicrobial *versus* probiotic	$F_{1,24}$ (p) Antimicrobial *versus* antimicrobial + Probiotic	$F_{1,24}$ (p) Probiotic *versus* antimicrobial + Probiotic
Acetic	0.17 ± 0.03	0.17 ± 0.05	0.18 ± 0.02	0.19 ± 0.07	0.0001 (0.99)	0.401 (0.53)	0.802 (0.38)	0.401 (0.53)	0.8122 (0.37)	0.411 (0.52)
Propionic	0.036 ± 0.004	0.035 ± 0.008	0.038 ± 0.004	0.037 ± 0.006	0.325 (0.57)	0.651 (0.43)	0.312 (0.58)	0.977 (0.33)	0.643 (0.43)	0.327 (0.57)
Butyric †	0.004 (0.007 - 0.134)	0.000 (0.000 - 0.000)	0.006 (0.000 - 0.010)	0.009 (0.000 - 0.018)	3.347 (0.079)	1.673 (0.21)	4.183 (0.051)	5.020 (0.034)	7.530 (0.011)	2.510 (0.13)

Values are expressed as mean ± SE or † median (Q1-Q3). Two-way ANOVA test followed by Bonferroni post hoc or † Friedman's Two-way Analysis of Variance by Ranks.

Discussion

The results obtained in the current study indicate that, while the potential probiotic treatment contributed to body mass gain, the antimicrobial and antimicrobial + potential probiotic groups presented a reduction in body mass gain. Subsequent analysis confirmed that such a difference in body mass was due to a lower accumulation of fat in the adipose tissue of animals treated with the antimicrobial.

The reduction in body adiposity occurs essentially when an energy deficit takes place, which can be obtained by reducing energy intake or increasing energy expenditure [10]. In this sense, the present study indicated that the caloric consumption of the experimental groups was similar, which suggests that the treatments would have somehow influenced the absorption and/or the energy metabolism and, consequently, the gain or loss of body fat [9].

Regarding absorption, the analysis of intestinal permeability revealed that treatment composed of the antimicrobial, solely, led to a reduction in intestinal absorption of mannitol. This monosaccharide is used as a marker of transcellular intestinal permeability, since it is absorbed through small aqueous pores with high incidence present in the intestinal cell membrane [39]. Thus, this result suggests that animals in the antimicrobial group developed reduced intestinal surface area [40].

Corroborating to the intestinal permeability test results, the histological analysis revealed that the treatment with the antimicrobial, in both isolated and combined (with probiotic) forms, caused a reduction in the height and width of the intestinal villi. Thus, it is confirmed that the animals that received the two types of treatments presented a reduced absorptive area, which would have contributed to the lower body mass gain presented by these groups compared to the others.

In a study carried out with BALB c^{-1} mice treated with different doses of ceftriaxone (100, 200 and 400 mg mL^{-1} for 8, 30, 60, 90, 120 and 150 days) it was observed that, regardless of the dose or the duration of treatment, a shortening of the intestinal villi of these animals, compared to the control group, occurred. One of the consequences of such a smaller absorptive area was a reduction in body mass gain [41]. Thus, it is suggested that this effect is drug-mediated, since regardless of the dose and duration of treatment, changes in the villus structure were observed.

The effect of ceftriaxone on the intestinal structure would be a consequence of its negative action on the modulation of microbiota composition. The microbiota in turn would overstimulate the local immune system, which would be responsible for the effective destruction of the villi. In this sense, it was observed that animals treated with ceftriaxone,

solely, presented a microbiota with decreased diversity than the other groups, with this result being like that found in a previous study [42].

Intestinal dysbiosis commonly leads to changes in the distribution of two dominant bacterial phyla found in the intestinal microbiota, *Firmicutes* and *Bacteroidetes* [41]. In the present study, both phyla had their abundance similar between the analyzed groups.

As for genus, treatment with ceftriaxone caused reductions in the populations of *Bacteroidaceae* and *Helicobacter*. This result can be attributed to efficiency of ceftriaxone, which acts, mainly, against gram-negative bacteria (Medication leaflet, Triaxton®, Blau Farmacêutica S/A). Additionally, treatment with the antimicrobial was able to significantly increase the abundance of *Enterococcaceae* family bacteria and, consequently, *Enterococcus* genus. Guo et al. [42] found that the genus *Enterococcus* started to dominate the intestinal microbiota of BALB/c mice treated with different doses of ceftriaxone (100, 200 and 400 mg mL^{-1}) in the period of 8 to 30 days.

It is noteworthy that the genus *Enterococcus* contains the species *E. faecium* that has been associated with the development of nosocomial infection, since this species can rapidly become resistant to the action of multiple antimicrobials [43].

The reduction in the population of *Lactobacillus* caused by ceftriaxone may be a consequence of the changes in the composition of gut microbiota resulting from the antimicrobial treatment. It is known that the magnitude of the effect of treatment with an antimicrobial on the intestinal microbiota is strongly influenced by the interdependence between the different species [17]. In this sense, antimicrobials have a considerable impact on the composition and functionality of the intestinal microbiota, which can cause a strong reduction in microbial diversity or in a specific taxon, in addition to stimulating the development and proliferation of microorganisms resistant to the action of antimicrobials [44].

Intestinal dysbiosis can be characterized by the isolated or combined occurrence of an increased population of pathogenic microorganisms, a reduction in the population of commensal microorganisms and a reduction in the microbiota diversity. Thus, the results suggest that the treatment with ceftriaxone would have caused intestinal dysbiosis, since it reduced the diversity of the intestinal microbiota; increased the population of potentially pathogenic bacteria, like *Enterococcus*; and reduced the population of commensal bacteria, such as the genus *Lactobacillus*. Intestinal dysbiosis causes negative consequences for intestinal health, since it reduces the production of SCFA, especially butyrate, which are important for the maintenance of intestinal integrity, in addition to having local anti-inflammatory effects [45].

It is known that the composition of the diet directly influences the diversity and structure of the intestinal microbiota [46-48]. The study by Dias et al. [22] found that C57BL/6J mice that received the AIN diet -93M in replacement of high-fat 60% diet and were treated with ceftriaxone (500 mg/kg) for 4 weeks showed no change in intestinal permeability, as well as in the morphology of the small intestine weight compared to the control group (AIN-93M diet treated with water). Thus, it is likely that the high concentration of saturated fatty acids present in the high-fat diet received by the animals in this study has exacerbated the negative modulatory effect of the antimicrobial and contributed to changes in intestinal morphology.

The benefits of the treatment with *L. gasseri* LG-G12 could be masked by the effects of a high-fat diet with 60% of calories from lipids, what can be considered a limitation of this study. Such a diet, besides having a high caloric intake, can increase body mass and fat efficiently due to its high-saturated fatty acid profile [49-52]. Allied to this, the continuous consumption of such a high-fat diet can modify the composition of the intestinal microbiota, contributing to the overweight [50, 51, 53].

Additionally, it is noteworthy that despite the effects of the probiotic on the body mass, it had not negatively changed the intestinal health of the animals. Therefore, its continuous use combined with the consumption of a healthy diet can bring benefits, in accordance with previous studies [54-56].

Treatment with ceftriaxone alone or in combination with a probiotic is able to decrease the accumulation of body fat. However, based on the involved mechanism, such a benefit presents a high risk, which includes the destruction of intestinal villi and intestinal dysbiosis, thus increasing the risk for the development of inflammatory bowel diseases. Hence, according to the obtained results, the use of antimicrobial ceftriaxone is not recommended in the adjuvant treatment of overweight, mainly in individuals whose diets are rich in saturated fat and should only be used in the treatment of infectious diseases.

Author's contributions

SARL, MMD and LLC participated in all production stages of this article (handling the animals, execution of analysis, analysis of the results and writing of the article). TAOM and MCGP guided in the choice of the analyzes performed, participated in the analysis of the results and assisted in the writing of this article. All authors reviewed the manuscript.

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ANEXOS

1 Aprovação pelo Comitê de Ética – Projeto descrito no Capítulo 1

CERTIFICADO

A Comissão de Ética no Uso de Animais - CEUA/UFV certifica que o processo nº 33/2018, intitulado **"Comparação entre a atuação de probiótico e/ou antimicrobiano associado a restrição energética: um estudo experimental em modelo animal obeso"**, coordenado pela professora Maria do Carmo Gouveia Peluzio do Departamento de Nutrição e Saúde, está de acordo com a Legislação vigente (Lei Nº 11.794, de 08 de outubro de 2008), as Resoluções Normativas editadas pelo CONCEA/MCTI, a DBCA (Diretriz Brasileira de Prática para o Cuidado e a Utilização de Animais para Fins Científicos e Didáticos) e as Diretrizes da Prática de Eutanásia preconizadas pelo CONCEA/MCTI, portanto sendo aprovado por esta Comissão em 30/05/2018, com validade de 12 meses.

CERTIFICATE

The Ethic Committee in Animal Use/UFV certify that the process number 33/2018, named **"Comparison between probiotic and / or antimicrobial performance associated with energy restriction: an experimental study in an obese animal model"**, is in agreement with the an actual Brazilian legislation (Lei Nº 11.794, 2008), Normative Resolutions edited by CONCEA/MCTI, the DBCA (Brazilian Practice Guideline for the Care and Use of Animals for Scientific Purposes and Teaching) and the Guidelines of Practice the Euthanasia recommended by CONCEA/MCTI therefore being approved by the Committee on May 30, 2018 valid for 12 months.

Profª. Átima Clemente Alves Zuanon
Presidente
Comissão de Ética no Uso de Animais – CEUA/UFV

2 Aprovação no Comitê de Ética – Projeto descrito no Capítulo 3

CERTIFICADO

A Comissão de Ética no Uso de Animais - CEUA/UFV certifica que o processo nº 09/2017, intitulado **"Efeito do tratamento com antimicrobiano ou probiótico sobre parâmetros associados com a obesidade e a resistência insulínica em modelo animal"**, coordenado pela professora Maria do Carmo Gouveia Peluzio do Departamento de Nutrição e Saúde, está de acordo com a Legislação vigente (Lei Nº 11.794, de 08 de outubro de 2008), as Resoluções Normativas editadas pelo CONCEA/MCTI, a DBCA (Diretriz Brasileira de Prática para o Cuidado e a Utilização de Animais para Fins Científicos e Didáticos) e as Diretrizes da Prática de Eutanásia preconizadas pelo CONCEA/MCTI, portanto sendo aprovado por esta Comissão em 09/05/2017, com validade de 12 meses.

CERTIFICATE

The Ethic Committee in Animal Use/UFV certify that the process number 09/2017, named **"Effect of antimicrobial or probiotic treatment on parameters associated with obesity and insulin resistance in animal model"**, is in agreement with the a actual Brazilian legislation (Lei Nº 11.794, 2008), Normative Resolutions edited by CONCEA/MCTI, the DBCA (Brazilian Practice Guideline for the Care and Use of Animals for Scientific Purposes and Teaching) and the Guidelines of Practice the Euthanasia recommended by CONCEA/MCTI therefore being approved by the Committee on May 09, 2017 valid for 12 months.

Profª. Átima Clemente Alves Zuanon
Presidente
Comissão de Ética no Uso de Animais – CEUA/UFV