UNIVERSIDADE FEDERAL DE MINAS GERAIS

INSTITUTO DE CIÊNCIAS BIOLÓGICAS

DEPARTAMENTO DE GENÉTICA, ECOLOGIA E EVOLUÇÃO

PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA

TESE DE DOUTORADO

# O CONSUMO PROBLEMÁTICO DE ETANOL E SUAS INTER-CONEXÕES COM O SISTEMA IMUNE, DIETA, MICROBIOTA INTESTINAL E O GENE *Lrrk2*

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BELO HORIZONTE

Fevereiro de 2022

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**Renato Elias Moreira Júnior**

Tese apresentada ao Programa de Pós-Graduação em Genética do Departamento de Genética, Ecologia e Evolução da Universidade Federal de Minas Gerais como requisito parcial para a obtenção do título de Doutor em Genética

Área de concentração: Genética Molecular, de Microrganismos e Biotecnologia.

Orientadora: Ana Lúcia Brunialti Godard

BELO HORIZONTE

Fevereiro de 2022

043 Moreira Júnior, Renato Elias.
O consume problemático de etanol e suas interconexões com o sistema immune, dieta, microbiota intestinal e o gene Lrrk2 [manuscrito] / Renato Elias Moreira Júnior. – 2022.

88 f. : il. ; 29,5 cm.

Orientadora: Ana Lúcia Brunialti Godard.
Tese (doutorado) – Universidade Federal de Minas Gerais, Instituto de Ciências Biológicas. Programa de Pós-Graduação em Genética.

1. Alcool. 2. Microbiota intestinal. 3. Comportamento. 4. Sistema nervoso central. 5. Dietas. I. Godard, Ana Lúcia Brunialti. II. Universidade Federal de Minas Gerais. Instituto de Ciências Biológicas. III. Título.

CDU: 575

Ficha catalográfica elaborada pela bibliotecária Sônia M S Moraes – CRB 6 – 1357

UNIVERSIDADE FEDERAL DE MINAS GERAIS
Instituto de Ciências Biológicas
Programa de Pós-Graduação em Genética

**ATA DE DEFESA DE DISSERTAÇÃO / TESE**

| | |
|---|---|
| **ATA DA DEFESA DE TESE** | **152/2022**<br>**entrada** |
| **Renato Elias Moreira Júnior** | **2º/2017**<br>**CPF: 096.681.236-03** |

Às nove horas e trinta minutos do dia **22 de fevereiro de 2022**, reuniu-se remotamente (virtualmente) a Comissão Examinadora de Tese, indicada pelo Colegiado do Programa, para julgar, em exame final, o trabalho intitulado: **"O consumo problemático de etanol e suas interconexões com o sistema imune, dieta, microbiota intestinal e o gene Lrrk2"**, requisito para obtenção do grau de Doutor em **Genética.** Abrindo a sessão, a Presidente da Comissão, **Ana Lúcia Brunialti Godard**, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra ao candidato, para apresentação de seu trabalho. Seguiu-se a arguição pelos Examinadores, com a respectiva defesa do candidato. Logo após, a Comissão se reuniu, sem a presença do candidato e do público, para julgamento e expedição de resultado final. Foram atribuídas as seguintes indicações:

| Prof./Pesq. | Instituição | CPF | Indicação |
|---|---|---|---|
| Ana Lúcia Brunialti Godard | UFMG | 107.961.538-50 | Aprovado |
| Yves LE LOIR | INRA | 13AL62823 | Aprovado |
| Mauro Andrade de Freitas Guimarães | Fundação Ezequiel Dias | 089.822.456-00 | Aprovado |
| Elisabeth Neumann | UFMG | 562.737.116-15 | Aprovado |
| Álvaro Cantini Nunes | UFMG | 678.159.606-34 | Aprovado |

Pelas indicações, o candidato foi considerado: **APROVADO**.

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

**Belo Horizonte, 22 de fevereiro de 2022.**

Ana Lúcia Brunalti Godard - UFMG

Yves LE LOIR - INRA

Mauro Andrade de Freitas Guimarães - Fundação Ezequiel Dias

Elisabeth Neumann - UFMG

Álvaro Cantini Nunes - UFMG

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## AGRADECIMENTOS

Primeiramente, agradeço aos meus pais, irmãs e à minha família, por terem me dado o privilégio e todas as oportunidades e estímulos, que me permitiram fazer o mais amo e me possibilita contribuir para um mundo melhor**.**

Agradeço, também, à minha orientadora, Dra. Ana Lúcia Brunialti Godard, por ter acreditado em mim e nas minhas capacidades com diversos ensinamentos, incentivando sempre a qualidade no trabalho desenvolvido, contribuindo, assim, para o meu amadurecimento e crescimento profissional e pessoal.

A todos os integrantes que passaram pelo Laboratório de Genética Animal e Humana (LGAH) ao longo da minha formação, em especial: Agatha, Luana, Samara, Isadora, Lorena, Raquel, Barbara e Jeanne, pelos momentos de alegria, companheirismo e trabalho.

A todos os amigos da graduação em Ciências Biológicas, que me fizeram acreditar ser possível lutar para que todos tenham direito aos benefícios que a educação e ciência podem trazer para humanidade.

Manifesto meus agradecimentos aos professores, funcionários e colegas do Programa de Pós-Graduação em Genética do Departamento de Biologia Geral do ICB/UFMG.

**Por fim, agradeço sincera e profundamente a todos que, de algum modo, contribuíram para que eu pudesse realizar este trabalho.**

# LISTA DE ABREVIATURAS

**AIN93G** *American Institute of Nutrition 93-Growth*
**ATV** Área Tegmental Ventral
**AUD** Desordem do Uso do Álcool
**BHT** Hidroxitolueno butilado
**CD45** *Cluster of differentiation 45*
**CEUA** Comissão de ética no uso de animais
**COX-2** Ciclo-oxigenase-2
**ct** *Cycle Threshold*
**DLS** Estriado dorsolateral
**DMS** Estriado dorsomedial
***Drd1*** Receptor de dopamina D1
***Drd2*** Receptor de dopamina D1
**EtOH** Etanol
**g** Gramas
**GABA** Ácido γ-aminobutírico
**Gabbr1** Receptor do ácido gama-aminobutírico 1
**Gabbr2** Receptor do ácido gama-aminobutírico 2
**GAPDH** Gliceraldeído-3-fosfato desidrogenase
**$H_2O$** Água
**HPT** Hipotálamo
**HSB** *High Sugar and Butter*
**IgA** Imunoglobulina A
**IgM** Imunoglobulina M
**IL10** Interleucina 10
**IL1β** Interleucina 1β
**IL6** Interleucina 10
**iNOS** óxido nítrico-sintase induzida
**KO** Knockout
**l** litros
**LPS** Lipopolissacarídeo
**LRRK2** *Leucine-rich repeat kinase 2*
**MAMPs** Moléculas padrão associadas aos microrganismos
**MAPK** Mitogen-activated protein kinases
**MyD88** *Myeloid differentiation primary response 88*
**Nac** Núcleo Accumbens
**NFAT** Fator nucelar de células t ativadas
**NRON** RNA não codificador repressor de NFAT
**OMS** Organização mundial de saúde
**pb** Pares de base
**PCR** Reação em cadeia da polimerase
**pH** Potencial hidrogeniônico
**PKA** Proteína quinase A
**PPIA** peptidilprolil isomerase A
**PYY** Peptídeo tirosina tirosina
**rRNA** Ácido ribonucleico ribossómico
**SNC** Sistema Nervoso Central
**SPF** *Specific Pathogen Free*
**TLR4** Receptor *toll-like* 4
**TLR5** *Receptor toll-like 5*
**TNFα** Fator de necrose tumoral alfa
**UFC** Unidade formadora de colônia
**UFMG** Universidade Federal de Minas Gerais

## RESUMO

Os mecanismos que determinam a preferência pelo etanol e a adição por essa droga não estão limitados apenas ao sistema nervoso central (SNC). Um crescente corpo de evidências sugere que o consumo abusivo de etanol afeta diretamente a microbiota intestinal e o sistema imune, que, por sua vez, interagem com o SNC, desencadeando respostas e alterações neuronais que resultam na adição pela droga. De fato, a microbiota intestinal se comunica com o SNC via eixo intestino-cérebro, e há um aumento da neuroinflamação frente à ingestão de etanol. Entretanto, são poucos os estudos que relacionam esses achados ao perfil transcricional de genes associados ao consumo problemático de etanol. Nesse sentido, acreditamos que, no estriado, região integradora do sistema de recompensa cerebral, a interação da resposta imune e microbiota intestinal com o perfil transcricional do gene *Lrrk2 (*associado à perda de controle e à adição por etanol) possa influenciar no consumo excessivo e na preferência pelo etanol. Com base nessas informações, o presente trabalho teve como objetivo avaliar as interconexões entre a microbiota intestinal, o sistema imune, o perfil transcricional do gene *Lrrk2* e o consumo abusivo de etanol. Para atingir nosso objetivo, usamos um modelo animal, desenvolvido pelo nosso grupo de pesquisa, no qual camundongos machos C57BL/6 e knockouts para os genes *Il6* e *Nfat* foram submetidos às seguintes etapas: Etapa 1 (T1) – Tratamento dietético, por oito semanas, em que os animais receberam a dieta hipercalórica, *High Sugar and Butter* (grupo HSB), ou a dieta padrão, *American Institute of Nutrition 93-Growth* (grupo AIN93G); e Etapa 2 (T2) – Consumo de etanol, em que os animais foram submetidos, por quatro semanas, ao etanol no paradigma da livre escolha, sendo eles divididos em dez grupos: [1] AIN93G + $H_2O$; [2] AIN93G + EtOH; [3] HSB + $H_2O$; [4] HSB + EtOH; [5] HSB-AIN93G + $H_2O$; e [6] HSB-AIN93G + EtOH; [7] HSB-AIN93G *Il6* KO + $H_2O$; [8] HSB-AIN93G *Il6* KO + EtOH; [9] HSB-AIN93G *Nfat* + $H_2O$ e [10] HSB-AIN93G *Nfat* + EtOH. Os cinco grupos (+ $H_2O$) tiveram acesso apenas a água, enquanto os cinco restantes (+ EtOH) tiveram livre escolha entre água e uma solução de etanol 10%. Nos grupos HSB-AIN93G, a dieta HSB é substituída pela dieta AIN93G. Ao final das 12 semanas de experimento, os animais do grupo HSB-AIN93G + EtOH, HSB-AIN93G *Il6* + EtOH e HSB-AIN93G *Nfat* + EtOH tiveram alta preferência e consumiram excessivamente etanol. Com isso, observamos que o consumo de etanol resultou em (1) alterações na composição e abundância da microbiota intestinal; (2) aumento da permeabilidade intestinal; (3) elevação de células inflamatórias no estriado; (4) hiperregulação de genes associados a citocinas pró-inflamatórias; e (5) hiporregulação do gene *Lrrk2.* Nossos achados sugerem que as interações entre microbiota intestinal, sistema imune e o perfil transcricional do gene *Lrrk2* influenciam o consumo preferencial e abusivo de etanol. Contudo, mais estudos para entender a interação desses fatores são necessários.



Palavras-chave: Álcool. Microbiota Intestinal. Comportamento. Sistema Imune. Lrrk2.

## *ABSTRACT*

Mechanisms that dictate the preference for ethanol and its addiction are not only restricted to the central nervous system (CNS). An increasing body of evidence has suggested that abusive consumption of ethanol directly affects the gut microbiota and the immune system, which in turn interact with the CNS, triggering neuronal responses and changes, resulting in addition by the drug. It is a fact that the gut microbiota communicates with the CNS through the gut-brain axis, and there is an increase in neuroinflammation as a result of the ethanol consumption. Nevertheless, there are few studies linking these findings to the transcriptional profile of genes associated with heavy ethanol consumption. In this regard, there is a belief that, in the groove, an integrating region of the brain reward system, the interaction of the immune response and gut microbiota with the transcriptional profile of the *Lrrk2* gene (associated with loss of control and addiction to ethanol) may influence the heavy consumption and preference of ethanol. Given this information, this study aimed at evaluating the interconnections between the gut microbiota, immune system, transcriptional profile of the *Lrrk2* gene, and heavy ethanol consumption. For this purpose, an animal model, developed by our research group, is adopted, in which male C57BL/6 mice and knockouts for the *Il6* and *Nfat* genes were subjected to each of the following stages: Stage 1 (T1) – Dietary treatment, for eight weeks, in which the animals receive high-calorie diet, High Sugar and Butter (HSB group), or standard diet, American Institute of Nutrition 93-Growth (AIN93G group); and Stage 2 (T2) – Ethanol consumption, in which the animals are submitted, for four weeks, to ethanol within the free choice paradigm, being each of them divided into six groups: [1] AIN93G + $H_2O$; [2] AIN93G + EtOH; [3] HSB + $H_2O$; [4] HSB + EtOH; [5] HSB-AIN93G + $H_2O$; [6] HSB-AIN93G + EtOH; [7] HSB-AIN93G *Il6* KO + $H_2O$; [8] HSB-AIN93G *Il6* KO + EtOH; [9] HSB-AIN93G *Nfat* + $H_2O$ and [10] HSB-AIN93G *Nfat* + EtOH. The five groups (+$H_2O$) had access to only water, while the five others (+ EtOH) had free choice between water and a 10% ethanol solution. The HSB diet is substituted by the AIN93G diet in the HSB-AIN93G groups. At the end of the 12-week experiment, animals in the HSB-AIN93G + EtOH, HSB-AIN93G *Il6* + EtOH e HSB-AIN93G *Nfat* + EtOH groups had a high preference and consume excessive amounts of ethanol. Thus, we observed that ethanol consumption leads to (1) alterations in the composition and abundance of gut microbiota; (2) increased gut permeability; (3) elevation of inflammatory cells in the groove; (4) overregulation of genes associated with proinflammatory cytokines; and (5) under regulation of the *Lrrk2* gene. The findings suggest interactions among the gut microbiota, immune system, and transcriptional profile of the *Lrrk2* gene influence the preferential and heavy ethanol consumption. Nevertheless, further studies will be necessary to understand the interaction of these factors.



Keywords: Alcohol. Gut microbiota. Behavior. Imune system. Lrrk2.

## SUMÁRIO

# 1 INTRODUÇÃO GERAL

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## 1.1 Neurobiologia do consumo problemático de etanol: Uma breve visão

O etanol é a droga de abuso mais consumida mundialmente, com cerca de 2,3 bilhões de usuários segundo dados da Organização Mundial de Saúde (OMS) [1]. Seu alto consumo é diretamente responsável por aproximadamente 3 milhões de mortes ao ano e está relacionado com o desenvolvimento de diversas condições patológicas [1-3]. O uso problemático do etanol, conhecido clinicamente, como Desordem do Uso do Álcool (AUD) é uma condição multifatorial, na qual, o componente genético é responsável pela herdabilidade de aproximadamente 55% [3, 4]. Nesse panorama, sabe-se que o consumo - tanto agudo quanto crônico - dessa droga resulta em mudanças na regulação transcricional de vários genes, em diferentes regiões cerebrais (tais como hipotálamo, estriado e córtex pré-frontal); e consequentemente impacta na homeostase de todo o organismo [3, 5].

O comportamento de busca e consumo de etanol e suas resultantes fisiológicas estão particularmente associados ao Sistema Mesocorticolímbico Dopaminérgico (figura 1), no qual, o neurotransmissor dopamina é responsável pelos processos de motivação e aprendizagem [6, 7]. Esse sistema é divido em duas vias: (I) a Mesolímbica Dopaminérgica (sistema de recompensa) e (II) a Mesocortical (Figura 1). Nesse Contexto, a busca e o consumo do etanol são dirigidos pela via Mesolímbica Dopaminérgica, que compreende projeções da área tegmental ventral para o Núcleo *Accumbens* (parte do estriado) [8]. Já os processos cognitivos e emocionais que regulam a tomada de decisão pela ingestão dessa substância são exercidos pela via Mesocortical, que compreende projeções da área tegmental ventral para o córtex pré-frontal [9, 10]. No uso do etanol, há o disparo de neurônios dopaminérgicos na área tegmental ventral que são ativados devido a sua interação com receptores GABA do tipo A. Tem-se como resultado a inibição de terminais Gabaérgicos e a desinibição dos neurônios dopaminérgicos, gerando, por sua vez, a liberação de dopamina no Núcleo *Accumbens* e córtex pré-frontal, onde, a alta concentração sináptica deste neurotransmissor é associada a uma gratificação na forma da sensação subjetiva de prazer e bem-estar [7, 11-14]. Tais estímulos geram memória que impulsiona o comportamento de manter a busca pelo álcool ocasionando um reforço positivo nesse sistema, ou seja, toda vez que há sua ingestão a gratificação (recompensa) se repete, motivando o organismo a buscá-la novamente [15].

Com o consumo repetitivo de bebidas alcoólicas ao longo do tempo, há o desenvolvimento de uma tolerância aos estímulos gratificantes da dopamina sendo assim necessário concentrações cada vez maiores da droga para ultrapassar o limiar desta tolerância [16]. Ademais, o uso da substância deixa de ser exclusivamente direcionado pelo reforço positivo e passa a ser dirigido pelo reforço negativo. Pois, devido às mudanças

homeostáticas quanto à necessidade de altas concentrações da droga surgem sintomas negativos associados à abstinência da mesma, como, por exemplo, ansiedade, tremores, náuseas e arritmia [17, 18]. Tal desequilíbrio constitui um dos principais fatores que instigam o processo de transição do uso recreativo para a dependência e pelas mudanças neuroadaptativas vistas no córtex pré-frontal e estriado no consumo crônico de etanol [17, 19].

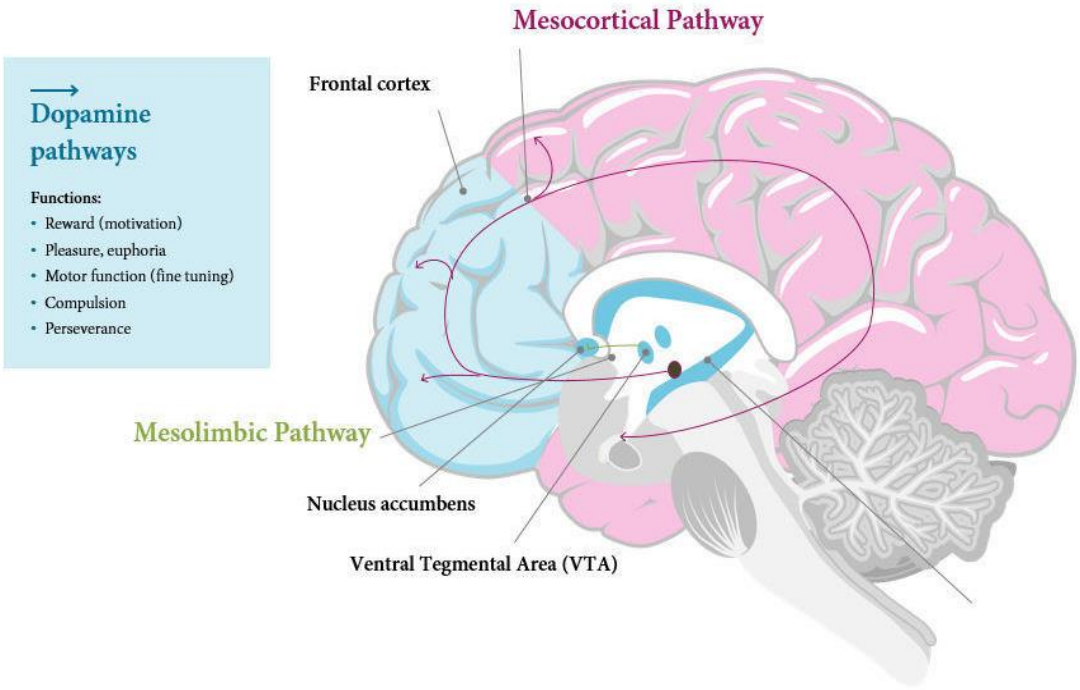


**Figura 1: Representação da Via Mesocorticolímbica Dopaminérgica em cérebro humano.** A Via Mesocorticolímbica é composta pela via Mesolímbica que compreende projeções dopaminérgicas da Área Tegmental Ventral (ATV) para o Núcleo *Accumbens* (NAc) e pela via Mesocortical, que compreende projeções dopaminérgicas da ATV para o Córtex Pré-frontal. Adaptado de [6].

O córtex pré-frontal assume importante papel no consumo de etanol já que está envolvido na tomada de decisões e na manutenção de comportamentos, como a preferência por essa droga e o desenvolvimento de sua dependência [20]. Assim, o consumo crônico da substância desencadeia mudanças adaptativas nessa região, causando déficits comportamentais, estruturais, fisiológicos e cognitivos, e levando à busca pela bebida (mesmo após períodos de abstinência) e ao aumento da necessidade das ações recompensadoras geradas pelo reforço positivo (mesmo diante de consequências negativas) [20, 21]. Neste cenário, o estriado, também se configura como estrutura chave no consumo problemático de etanol pois, está associado aos comportamentos direcionados e objetivos, bem como, à cognição e à emoção [22-24]. Didaticamente, o estriado pode ser dividido em três partes distintas: estriado dorsolateral (DLS), estriado dorsomedial (DMS) e estriado ventral (também conhecido como núcleo *Accumbens*, NAc) (Figura 2). O etanol altera a função desses circuitos estriatais de várias maneiras, o que contribui para a intoxicação

aguda, busca pela droga, dependência e efeitos da abstinência [22, 25]. Dessa forma, evidências crescentes demonstram a ação de cada estrutura do estriado no uso problemático de etanol: o DLS exerce função vital na formação de hábitos e pode participar no desenvolvimento do uso habitual da droga [22]. o DMS participa do controle de ações direcionadas e objetivas e, portanto, pode influenciar a busca pela substância direcionada como um objetivo; [22, 26]. o NAc tem participação no condicionamento ambiental das ações e desempenha importante papel no controle ambiental do consumo da droga e nas crises de abstinência [22, 27].

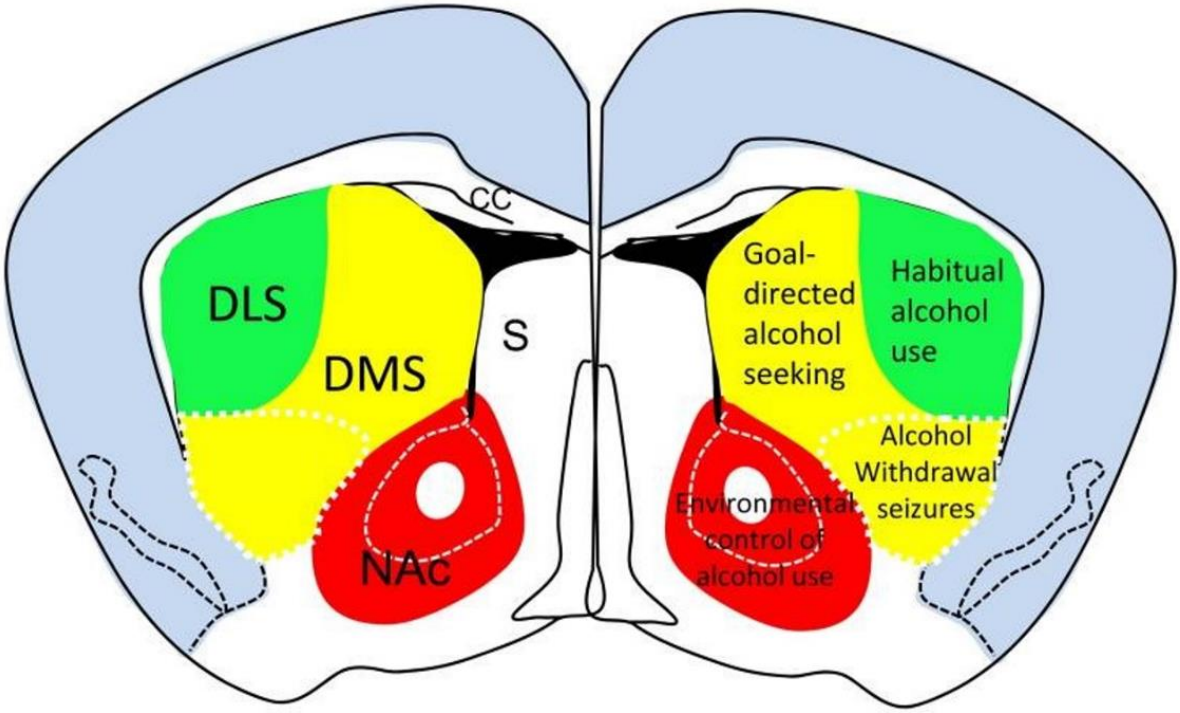


**Figura 2**: **Representação das áreas estriatais e sua relação com comportamentos associados ao uso abusivo de etanol.** As localizações do estriado dorsolateral (DLS), estriado dorsomedial (DMS) e estriado ventral (NAc) são ilustradas no lado esquerdo do cérebro. Os comportamentos associados ao etanol nas três áreas são descritos no lado direito. Adaptado de [22].

Embora o etanol exerça grandes efeitos sobre o organismo, a maioria dos estudos realizados até o momento se concentram na base fisiológica da dependência, principalmente na influência de seu consumo nas funções neuronais do cérebro. Entretanto, diversos trabalhos emergem em outra vertente, em modelos animais e em humanos, demonstrando que essa ingestão provoca aumento da permeabilidade intestinal, elevação dos níveis plasmáticos de metabólitos bacterianos derivados do intestino que ativam vias inflamatórias específicas, bem como desordens funcionais em diversos sistemas fisiológicos [28-31]. Desta forma, evidencia-se que as bases neurobiológicas que culminam no uso problemático de etanol podem ser influenciadas por elementos que interagem com o sistema nervoso central, entre eles, o sistema imune e a microbiota intestinal.

### 1.2 Sistema imune e o consumo problemático de etanol: possível influência do gene *Lrrk2*

Além da relação com o sistema de recompensa e suas implicações na adição, o uso problemático de etanol também tem íntima relação com o sistema imune, o qual, por sua vez, pode influenciar na regulação de aspectos que desencadeiam a adição [32-34]. Sabe-se que sistemicamente esse consumo suprime o sistema imune inato, dificultando a defesa frente a microrganismos patogênicos e até mesmo suprimindo sistemas de vigilância contra o desenvolvimento de tumores [35, 36]. Neste sentido, exemplificando, há uma diminuição na capacidade de recrutar leucócitos na eliminação de agentes patogênicos, e a incidência de diversos tipos de cânceres é maior em pessoas que consomem álcool [37-39].

Apesar da imunossupressão sistêmica, tal ingestão pode levar à ativação de vias imunes específicas e desencadear neuroinflamação (regulação positiva de citocinas e mediadores inflamatórios no cérebro) [39]. Quando consumido de forma crônica, o etanol pode prolongar o estado ativado da micróglia (células imunes residentes no sistema nervoso central), levando a um novo estado homeostático, no qual há produção contínua dessas citocinas inflamatórias [40]. Nessa conjuntura, no sistema nervoso central, os receptores do tipo *toll-like* 4 (TLR4), geralmente relatados por reconhecerem endotoxinas de bactérias gram-negativas (principalmente lipopolissacarídeos - LPS), também podem ativar vias inflamatórias e, por conseguinte, a produção de citocinas quando estimulados pelo etanol [41, 42]. De fato, já foi demonstrado um efeito direto dessa substância sobre os TLR4 na micróglia e astrócitos, na ausência de qualquer toxina bacteriana, com ativação da resposta imune e liberação de citocinas e de diversos mediadores inflamatórios [39, 43-45]. Curiosamente, com a inativação de TLR4 em células gliais por siRNA ou utilizando-se camundongos TLR4-KO, não são observadas nem a neuroinflamação e nem alterações patológicas relacionadas ao uso crônico da droga [44, 46].

É importante citar que mesmo que o etanol, por si só, já seja capaz de estimular TLR4 e causar neuroinflamação, o uso abusivo gera uma exacerbação dessa neuroinflamação por ação de LPSs bacterianos (Figura 3). Dado o aumento da permeabilidade intestinal provocado pela ingestão crônica dessa substância, há a possibilidade de translocação dessas endotoxinas para a corrente sanguínea e barreira hematoencefálica, o que permite que essa endotoxina chegue ao cérebro [29, 47]. Desse modo, ao estimular TLR4 no cérebro, o LPS e o próprio etanol podem induzir a geração e a liberação de citocinas pró-inflamatórias e, assim, dar origem a processos neuroinflamatórios, já relatados por contribuírem indiretamente na adição pela droga [47, 48]. Então, sabe-se que a presença de moléculas pro-inflamatórias de forma crônica no cérebro podem afetar o funcionamento de vias neurais e da plasticidade neuronal o que resulta em danos ao indivíduo e contribui

para o desenvolvimento de distúrbios psiquiátricos [49-51]. Nesse processo, a liberação dessas citocinas inflamatórias neurais se dá por meio da indução de diversas vias de sinalização e fatores de transcrição que são regulados por genes neuromoduladores como, por exemplo, o *Lrrk2* [52, 53].

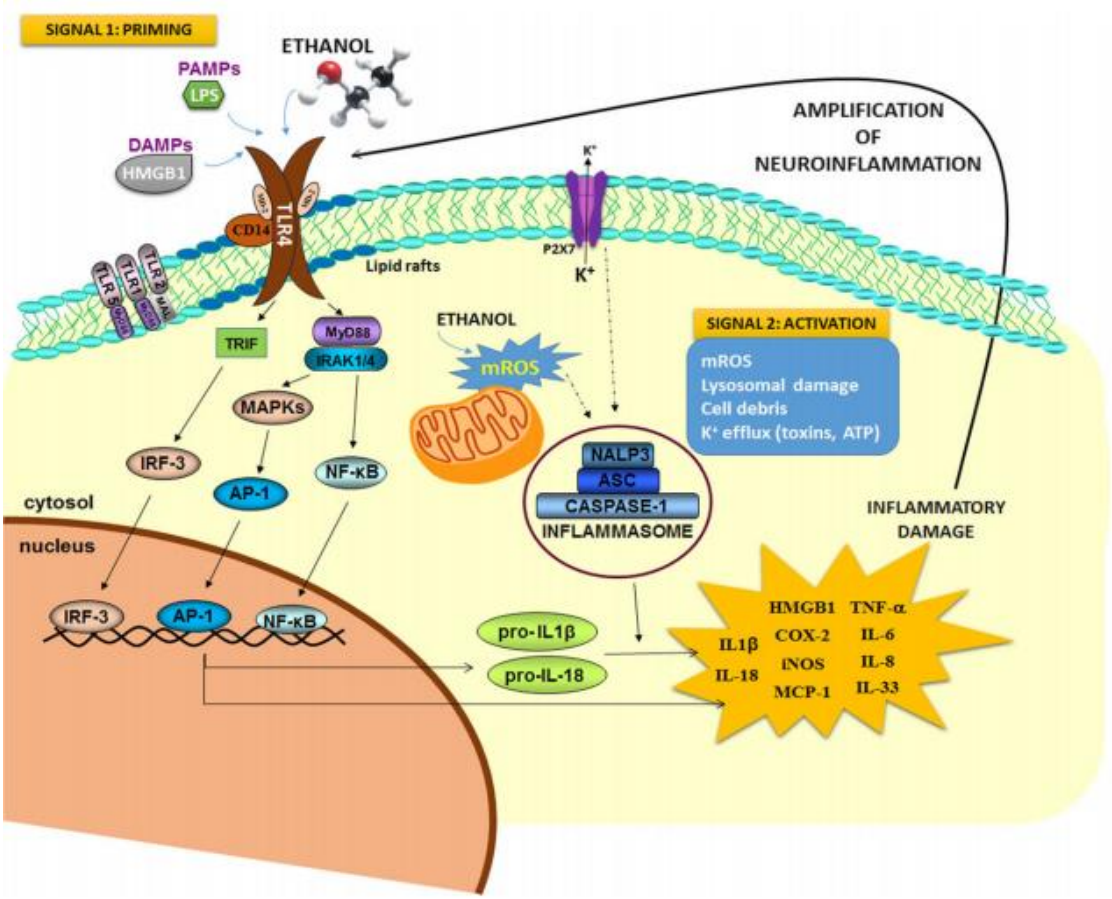


**Figura 3**:**Representação da ativação dos receptores TLR4 e da neuroinflamação induzida pelo etanol.** A ativação de TLR4 por LPS e/ou etanol recruta várias proteínas e quinases (como MyD88) e desencadeia vias de sinalização e fatores de transcrição que culminam na geração de citocinas (IL1β, IL6 e TNFα), quimiocinas (ou seja, MCP-1) e mediadores inflamatórios (iNOS e COX-2), levando à neuroinflamação. Adaptado de [39].

Tendo isso em vista, no estriado, Silva & Silva e colaboradores (2016), ao estudarem um modelo animal de consumo abusivo de etanol observaram que o gene *Lrrk2* está super expresso no cérebro de camundongos com fenótipo inflexível para o consumo de etanol [54]. Continuando com as evidências da participação do gene *Lrrk2* no fenótipo de uso abusivo de álcool, em um recente estudo publicado por nosso grupo, usando como modelo o Zebrafish (*Danio rerio*), foi observada uma super expressão do transcrito de *lrrk2* em animais que tinham preferência pelo etanol [55, 56]. Ainda neste estudo, foi demonstrado que o uso do inibidor da atividade quinase de LRRK2, GNE-082, reduz a preferência pelo etanol, depois do tratamento, no grupo de animais com comportamento tipo inflexível [56]. Outro estudo recentemente publicado por Carvalho e colaboradores (2020), foi identificado baixa transcrição de LRRK2 no córtex pré-frontal e núcleo *accumbens* em cérebros

*post-mortem* de indivíduos com desordens do uso do álcool quando comparados com controles [57]. Todos esses estudos evidenciam a participação do gene LRRK2 em comportamentos relacionados ao uso abusivo de álcool em diversos modelos de pesquisa, incluindo em humanos, indicando, portanto, que este gene participa do estabelecimento de fenótipos associados ao consumo excessivo de álcool.

O gene *LRRK2* (*Leucine-rich repeat kinase 2*) foi primariamente associado a uma forma familiar ou esporádica da doença de Parkinson [58]. Este gene codifica a proteína LRRK2 ou dardarina, possuindo múltiplos domínios de interação proteína-proteína e enzimáticos [59]. A atividade quinase é a principal função desempenhada por esta proteína, sendo capaz de orquestrar diversas atividades neuronais, como neuroplasticidade, tráfego de vesículas sinápticas, e crescimento de dendritos e axônios[60-62]. Sendo um modulador da atividade da proteína quinase A (PKA), a qual está associada à regulação dos receptores de dopamina pode-se inferir que este gene tenha participação no sistema de recompensa [63]. Ademais, este gene está envolvido em diversas vias de sinalização, pois desempenha funções GTPase e quinase que influenciam processos como a proliferação e diferenciação celulares, apoptose, inflamação, resposta imune e produção de citocinas [61, 64, 65]. Curiosamente, já foi demonstrado que os LPSs, ao estimularem TLR4 no cérebro, aumentam a atividade e a expressão de LRRK2 [61, 66, 67]. De fato, diversos estudos associam *Lrrk2* à resposta imune mediada por TLR4 [52, 61, 68].

Quando TLR4 é ativado, o mesmo sinaliza, via MyD88, o aumento da atividade quinase de p38, MAPK, IKKα e IKKβ, os quais ativam fatores de transcrição inflamatórios produtores de citocinas [69]. Nesse processo, a via MyD88 também leva a um aumento da fosforilação de LRRK2 em células imunes [70, 71]. Entretanto, o significado biológico dessa interação ainda não é totalmente compreendido, mas acredita-se que ele seja importante na determinação da localização subcelular de LRRK2 na inflamação [70, 71]. De fato, a inibição de *Lrrk2* ou o uso de camundongos LRRK2-KO mostram que a resposta inflamatória à ativação de TLR4 por LPS está prejudicada, e, como a fosforilação de LRRK2 requer MyD88, sempre que TLR4 for estimulado haverá fosforilação de LRRK2 [72]. Tais resultados apontam que *Lrrk2* pode estar envolvido na resposta neuroinflamatória vista no consumo abusivo de etanol.

Além da ligação bioquímica com TLR4, *Lrrk2* também está envolvido na regulação do fator nuclear da família de fatores de transcrição de células t ativadas (NFAT), reconhecido como importante mediador da resposta imune, estimulando a produção de citocinas inflamatórias [61, 68, 73]. NFAT também é observado como aumentado na ingestão de álcool [73, 74]. LRRK2 atua inibindo a translocação de NFAT para o núcleo e, assim, inibe sua função [75, 76]. Nesse sentido, camundongos LRRK2-KO têm maior acúmulo de NFAT no núcleo e produção aumentada de NFAT [77]. Sabe-se, então, que *Lrrk2* pode participar

da modulação de pelo menos duas vias relacionadas à produção de citocinas inflamatórias no uso abusivo de etanol [68]. Porém, mais estudos são necessários para entender sua real contribuição.

### 1.3 Microbiota intestinal, eixo intestino-cérebro e o consumo de etanol

A microbiota intestinal corresponde ao conjunto de todos os microrganismos com maior abundância de bactérias que colonizam o lúmen do trato gastrointestinal. Elas estabelecem com este uma relação simbiótica na qual desempenham funções vitais para o hospedeiro enquanto se beneficiam de um ambiente protegido, onde existe um suprimento rico e contínuo de nutrientes, que garantem também sua sobrevivência [78-80]. Neste cenário, o microbioma (genoma coletivo de todos os microrganismos presentes no trato gastrointestinal) contém 150 vezes mais genes do que no genoma humano, com pelo menos 6000 destes sendo funcionais [81-84]. Por exemplo, algumas dessas importantes funções seriam a digestão de polissacarídeos, o metabolismo de fármacos, a produção de vitaminas, a regulação do armazenamento de gorduras, a barreira contra a colonização por patógenos, a estimulação da angiogênese, o desenvolvimento e a modulação do sistema imunológico, e até mesmo o condicionamento comportamental [78, 84-88].

A microbiota intestinal interage e modula o sistema nervoso central por meio de diferentes mecanismos, ela pode influenciar na modulação neuronal que ocorre na preferência pelo etanol [89]. Essa interação ocorre através do eixo intestino-cérebro, o qual consiste num sistema de comunicação bidirecional que envolve o intestino e o sistema nervoso central [90]. Tal comunicação compreende múltiplas vias altamente complexas, que englobam a bioquímica do cérebro, nervo vago, citocinas pró-inflamatórias, hormônios e metabolismo do triptofano e tirosina [90, 91]. Os microrganismos realizam fermentação de fibras alimentares ocasionando a liberação de ácidos graxos de cadeia curta como butirato, acetato e propionato, os quais são metabólitos com potenciais propriedades neuroativas que atuam sobre o nervo vago. De fato, um crescente corpo de evidências sugere que a microbiota tem capacidade de alterar o comportamento do hospedeiro, principalmente quanto à ansiedade, ao sinalizar para o nervo vago [92-94]. Além disso, ácidos graxos de cadeia curta circulantes são capazes de atravessar a barreira hemato encefálica exercendo efeitos em neurônios, células gliais e na neurotransmissão, e influenciando áreas cruciais para os comportamentos associados ao sistema de recompensa no estriado e hipocampo [95-98]. Ainda, as bactérias têm capacidade de reconhecer e sintetizar homólogos de hormônios e neurotransmissores, incluindo acetilcolina, ácido γ-aminobutírico (GABA), dopamina e serotonina, podendo também modificar os níveis de aminoácidos relevantes para a síntese de neurotransmissores do hospedeiro [91, 99, 100]. Exemplificando, as concentrações

totais de triptofano (o precursor da serotonina) e tirosina (precursor da dopamina e da noradrenalina) em camundongos *germfree* são significantemente menores do que as de camundongos colonizados, o que mostra a importância da microbiota nas concentrações desses aminoácidos [99, 100].

Metabolitos produzidos pela microbiota intestinal, conhecidos em conjunto como Moléculas Padrão Associadas a Microrganismos (MPAMs), podem interagir com o sistema imune, endócrino e nervoso do intestino, agindo indiretamente na função cerebral via eixo intestino-cérebro, ou podem atingir diretamente o cérebro através da corrente sanguínea [91, 101]. Entre essas MPAMs que migram para a corrente sanguínea, podemos destacar os peptidoglicanos e os LPSs [91]. Os LPSs provenientes da microbiota intestinal, a partir do aumento da permeabilidade intestinal, chegam à corrente sanguínea e podem alterar a função cerebral através de três caminhos diferentes: (I) estimulando o sistema imunológico a produzir citocinas; (II) sensibilizando/estimulando neurônios aferentes vagais; e (III) modulando diretamente a função cerebral [91]. Neste contexto, já é bem caracterizado que os LPSs podem causar doenças neurológicas em humanos e induzir diferentes comportamentos em modelos animais [102-104]. A possibilidade dos LPSs modularem diretamente a função cerebral deve ser considerada porque, além do sistema imunológico inato, existe uma expressão generalizada de receptores do tipo *Toll-like* 4 (TLR4), aos quais os LPSs se ligam, em vários níveis do eixo intestino-cérebro, incluindo-se células epiteliais do sistema gastrointestinais, neurônios do sistema nervoso entérico, neurônios aferentes primários, células da micróglia e astrócitos no cérebro [105]. Ao estimular TLR4 no cérebro, os LPSs e outros metabólitos bacterianos podem induzir a geração e a liberação de citocinas pró-inflamatórias e, então, dar origem a processos neuroinflamatórios que, por sua vez, contribuem para neurodegeneração e neuroadaptação [105-107]. Processos neuroadaptativos são observados no córtex pré-frontal e no estriado a partir do consumo crônico de etanol e em distúrbios psiquiátricos, como a depressão [107, 108]. Nesses distúrbios, níveis aumentados de IgA e IgM contra os LPSs de bactérias intestinais são encontrados na circulação sanguínea indicando a ação do sistema imune em uma tentativa de minimizar os mecanismos que levam a essas disfunções e a comportamentos patológicos [108]. A figura 4 esquematiza as vias de comunicação entre o eixo intestino-cérebro e a microbiota intestinal.

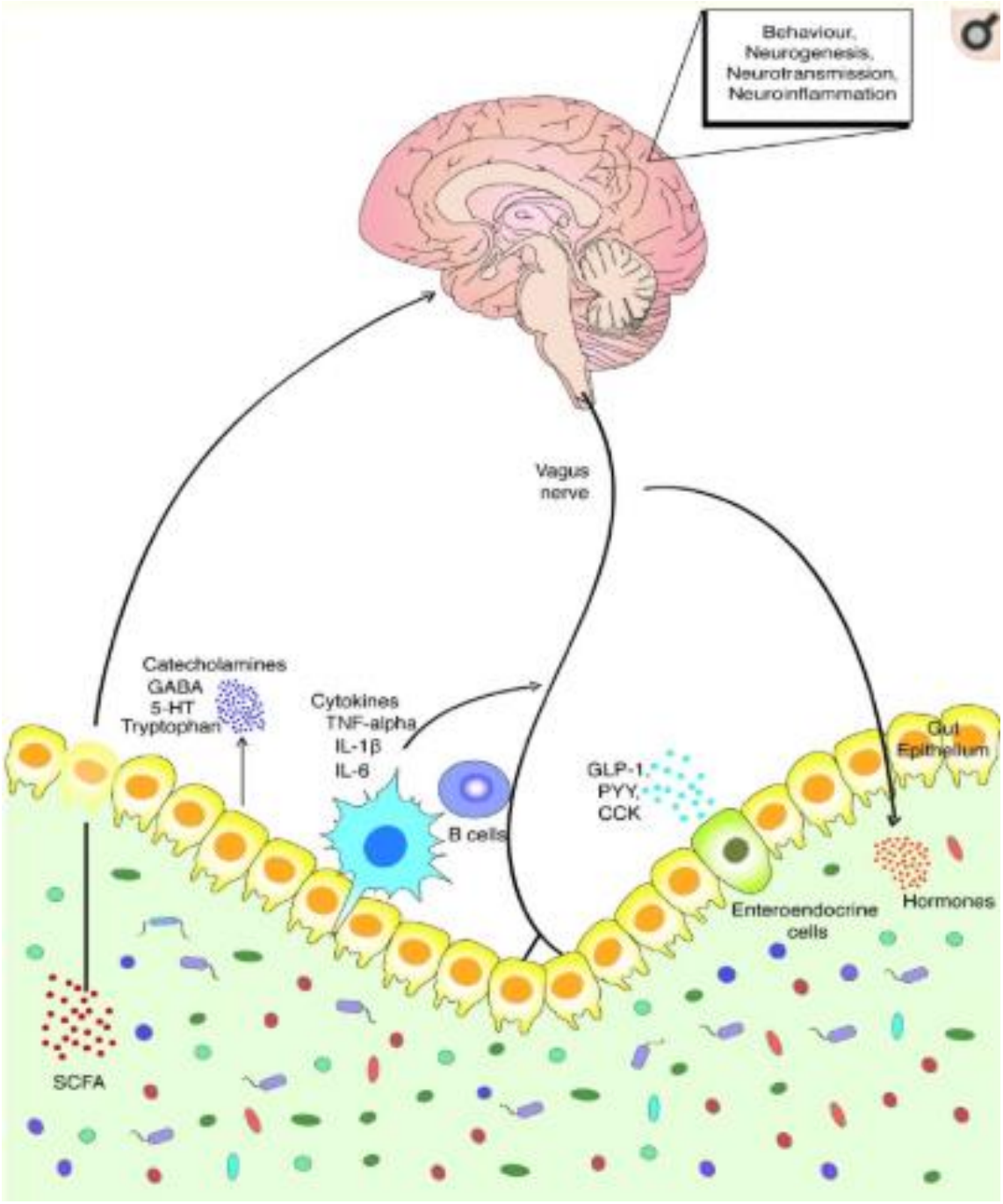


**Figura 4: Vias de comunicação entre o eixo intestino-cérebro e microbiota.** As bactérias intestinais podem sinalizar para o cérebro por diferentes mecanismos, incluindo-se: ativação do nervo vago, produção de mediadores imunológicos, metabolitos bacterianos (como os ácidos graxos de cadeia curta) e células endócrinas. Por meio dessas vias de comunicação, o eixo da microbiota-intestino-cérebro controla os processos fisiológicos centrais, como neurotransmissão, neurogênese, neuroinflamação e sinalização neuroendócrina. Adaptado de [109].

Considerando a existência de várias formas de comunicação e controle associados ao eixo intestino-cérebro, pesquisas recentes mostram que as perturbações induzidas por drogas na microbiota intestinal estão diretamente implicadas com o funcionamento do sistema de recompensa mostrando o papel chave do microbioma na condução do comportamento de busca por drogas [89, 110, 111]. A figura 5 esquematiza alguns dos alvos mais comuns da microbiota após perturbação por drogas.

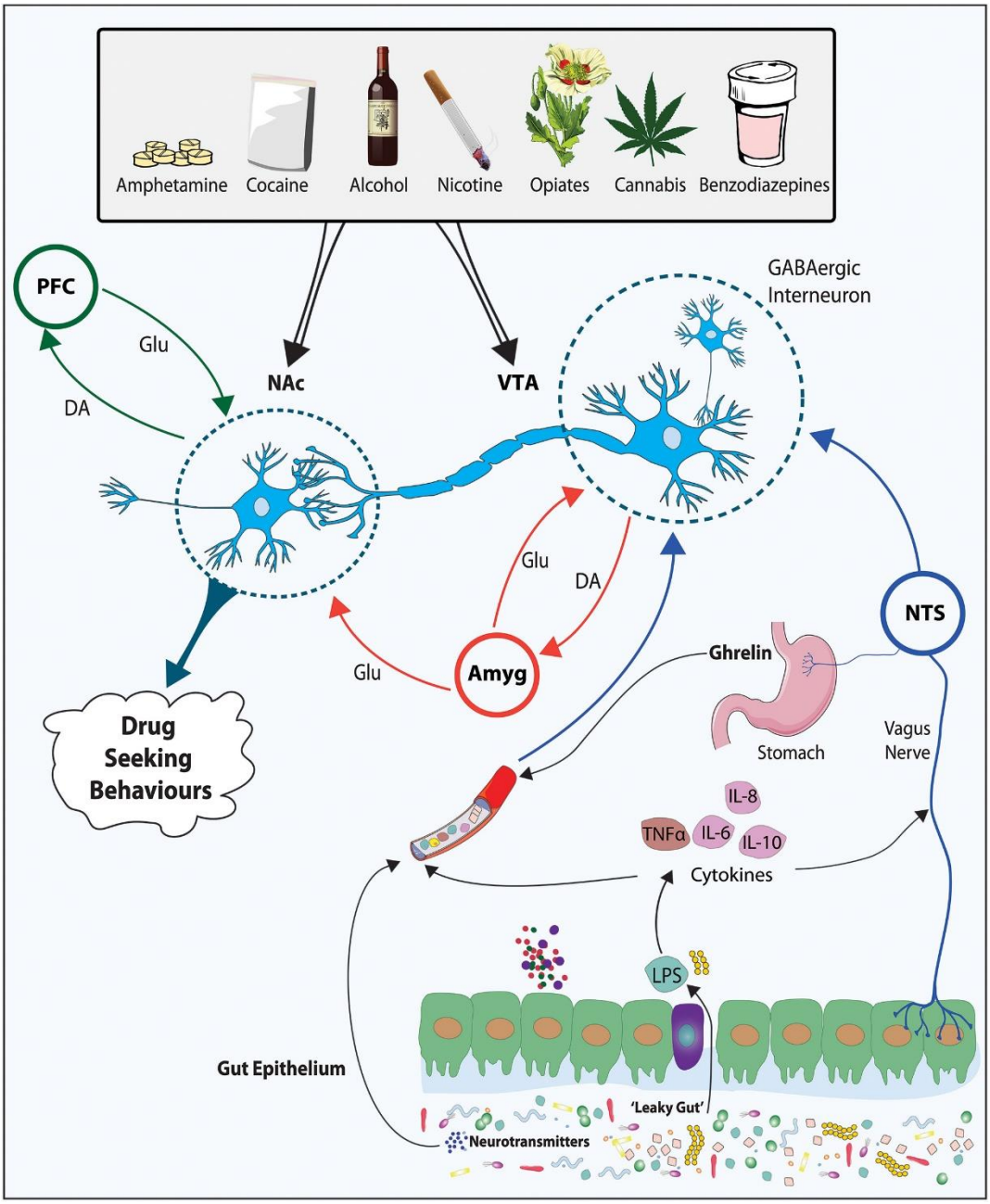


**Figura 5:Sistema de recompensa cerebral e principais alvos para a ação de drogas de abuso.** A microbiota intestinal e seus componentes podem regular o sistema de recompensa por meio de vias hormonais, imunes ou vagais. Os neurotransmissores por ela produzidos são liberados na circulação e interagem com o sistema de recompensa. Da mesma maneira, o aumento da permeabilidade intestinal faz com que produtos bacterianos, como LPS, induzam alterações sistêmicas que levam à produção de citocinas que causam danos neural ou induzam o nervo vago a regular a resposta de recompensa. Adaptado de [89].

Um número crescente de estudos mostra a relação entre a microbiota intestinal e os transtornos por uso de álcool [112-115]. Tal consumo pode alterar profundamente a composição e diversidade de microrganismos presentes na microbiota, que por sua vez, pode contribuir para a manutenção dessa ingestão ao estabelecer uma comunidade associada a ela [116-120]. Nesse sentido, alterações na microbiota intestinal induzidas pelo etanol foram associadas ao aumento da impulsividade e desejo pela droga com alterações estriatais dos receptores de dopamina, bem como, mudanças na depressão, sociabilidade,

desregulação emocional e aceleração do ciclo de dependência em pacientes com AUD [29, 121-123]. Levando em conta que essas alterações na microbiota intestinal com o consumo de etanol podem impactar diretamente no funcionamento do sistema de recompensa, estas assumem importante papel como mais um fator que contribui para as alterações funcionais vistas em estruturas como o córtex pré-frontal e o estriado no consumo crônico de etanol contribuindo para a preferência, adição e manutenção do uso da substância.

Em vista dos argumentos apresentados, fica claro que a preferência pelo etanol pode, em parte, ser motivada por componentes da microbiota intestinal que além de participar de processos associados ao sistema imunológico podem se comunicar com o cérebro através dos mecanismos citados anteriormente. Considerando que diferentes alimentos podem beneficiar distintos grupos bacterianos, as bactérias favorecidas pela ingestão do álcool podem modular regiões cerebrais relacionadas ao processamento de informações, tomada de decisão, cognição e sensações de prazer, como o córtex pré-frontal e o estriado, podendo, dessa maneira, estimular o estabelecimento do fenótipo de preferência ao etanol [28, 124-126]. Isso somado à predisposição genética, condições ambientais e neuroadaptação, pode contribuir para o abuso no uso dessa substância e o desenvolvimento de dependência. Diante dessa realidade, prova-se a imprescindibilidade de pesquisas que investiguem mais aprofundamente essas relações, para o que podem ser utilizados modelos animais.

### 1.4 O modelo animal de consumo de dieta rica em açúcar e gordura e livre escolha por etanol

Até aqui esclarecemos que o uso abusivo de etanol, além de estar associado à neurobiologia do sistema de recompensa cerebral, pode ser influenciado pela microbiota intestinal, em conjunto com o sistema imune, no qual o gene *Lrrk2* parece ter papel regulador. Contudo, mais investigações devem ser feitas para entender seus fatores e sua relação com a preferência e consumo abusivo dessa droga. Para isso, diferentes modelos animais podem ser aplicados.

Apesar de não existir um modelo animal que reproduza fielmente uma condição humana e da subjetividade da avaliação comportamental dos animais e interpretação de dados, esses modelos possibilitam a análise de elementos específicos que auxiliam o entendimento da neurobiologia do processo da adição [127]. Diversos modelos animais já foram desenvolvidos para estudar diferentes aspectos do uso e dependência de etanol, o que se inclui o comportamento de busca, danos a órgãos específicos, tolerância e até mesmo abstinência da droga [128]. Nesses estudos, os fatores-chave para sua eficiência reside no método de administração do etanol e a validade preditiva do modelo. Assim, ao observar as características associadas à preferência e busca pelo álcool, torna-se vital o

uso de um modelo em que o animal tenha a oportunidade de escolha entre consumir ou não a bebida, buscando, assim, simular o comportamento existente em humanos.

Para essa finalidade, nosso grupo de pesquisa desenvolveu um modelo animal de livre escolha e consumo abusivo de etanol [129]. Nesse modelo, inicialmente, os animais foram alimentados de forma crônica com uma dieta com alto teor de gorduras, e, após o período de 8 semanas, essa dieta foi retirada e os animais expostos por 4 semanas ao paradigma da livre escolha por etanol [129]. Durante essa exposição, os animais que consumiram a dieta hipercalórica, quando ela foi retirada, passaram a ingerir o álcool de forma excessiva e o escolheram em relação à água [120, 129]. Por fim, ao final do experimento, observou-se que esses animais apresentaram um fenótipo semelhante à adição pelo etanol, apresentando alterações comportamentais e na via mesocorticolímbica dopaminérgica determinada na avaliação da regulação transcricional dos receptores de dopamina (*Drd1*/*Drd2*) e da subunidade dos receptores GABAB (Gabbr1/Gabbr2) [129]. Desse modo, desenvolvemos um modelo com alto consumo de etanol e com características neurobiológicas que aparentam estar direcionando a ingestão e preferência pela da droga.

Esse modelo é incitante pois, além de permitir estudar aspectos relacionados à preferência pelo etanol, parece simular seu alto consumo também observado em humanos após cirurgia bariátrica, seguindo a premissa bem conhecida de existir uma sobreposição complexa entre a recompensa associada ao consumo de dietas palatáveis com alto teor de gordura e açúcar e outras compulsões e a dependência por drogas [130, 131]. Nesse contexto, estudos demonstram que, no período pós-operatório da cirurgia bariátrica, cerca de 6% dos pacientes desenvolvem transtornos relacionados ao uso problemático de álcool [132, 133]. De modo geral, acredita-se que há uma transferência da alimentação compulsiva para o alto consumo de etanol, o que potencializa a adição pela substância [134]. Além disso, há evidências substanciais de que a cirurgia bariátrica esteja associada a alterações na farmacocinética e sensibilidade aos efeitos dessa droga nesses pacientes, o que também reforça a adição [135, 136]

No modelo animal descrito, a dieta com alto teor de gorduras utilizada é a denominada HSB (do inglês *high sugar and butter*), desenvolvida por nosso grupo com o objetivo de ser uma dieta comparável à AIN93G (*American Institute of Nutrition Rodent Diets – 93 Growth*)(Tabela 1), mas que resulte em condições vistas na obesidade, já que a dieta AIN93G apresenta ingredientes refinados e restritos que levam ao crescimento dos animais de forma saudável [137, 138]. Assim, a dieta HSB desencadeia nos animais condições semelhantes às observadas em pessoas obesas antes e depois da cirurgia bariátrica, visto que sua retirada induz alto consumo de etanol, observado no período pós-cirúrgico. Desse modo, o modelo foi escolhido por simular condições vistas em humanos e por permitir

estudar a relação do sistema imune, do gene *Lrrk2* e da microbiota intestinal no uso abusivo de etanol.

**Tabela 1 - Composição nutricional das dietas AIN93G e HSB.**

| Ingredientes | Quantidades (g) | |
|---|---|---|
| | **AIN93G** | **HSB** |
| **Amido de milho** | 397,486 | 208,586 |
| **Caseína** | 200 | 200 |
| **Amido dextrinizado** | 132 | 0 |
| **Sacarose** | 100 | 232 |
| **Óleo de soja** | 70 | 70 |
| **Celulose** | 50 | 50 |
| **Mix mineral** | 35 | 35 |
| **Mix de vitaminas** | 10 | 10 |
| **Cistina (metionina)** | 3 | 3 |
| **Bitartarato de colina** | 2,5 | 2,5 |
| **BHT** | 0,014 | 0,014 |
| **Manteiga comercial** | 0 | 188,9 |
| **TOTAL (g)** | 1000 | 1000 |
| **Composição Nutricional** | **AIN93G (%)** | **HSB (%)** |
| **Carboidrato** | 64,00 | 36 |
| **Proteína** | 20,00 | 16 |
| **Lipídeo** | 16 | 48,00 |

Adaptado de [138]

### 1.5 Hipótese

Com base no exposto, percebe-se que os fatores envolvidos no desencadeamento do uso problemático de etanol não estão limitados somente ao sistema nervoso central. Nesse contexto, hipotetizamos que alterações na microbiota intestinal e na resposta imune têm implicações do uso problemático, que, por sua vez, se associa a alterações na regulação transcricional do gene *Lrrk2*.

## 1.6 Objetivos

### 1.6.1 Objetivo Geral

Estabelecer possíveis interconexões entre o consumo problemático de etanol com parâmetros associados à dieta, sistema imune, microbiota intestinal e o gene *Lrrk2.*

### 1.6.2 Objetivos Específicos

- **Capítulo 1**: Avaliar parâmetros relacionados à obesidade induzida pela dieta HSB no que diz respeito a mudanças comportamentais, na regulação de genes associados ao comportamento alimentar no hipotálamo, na composição da microbiota intestinal e como elas estão inter-relacionadas. Para em seguida, melhor compreender a influência da retirada dessa dieta no alto consumo de etanol por meio do modelo animal de consumo de dieta rica em açúcar e gordura e livre escolha por etanol utilizados nos capítulos 2 e 3;
- **Capítulo 2:** Avaliar os efeitos da ingestão de dieta rica em açúcar e gordura, de sua retirada e do consumo de etanol na composição e função da microbiota intestinal, bem como estabelecer possíveis ligações desses aspectos com os fenótipos apresentados pelos animais;
- **Capítulo 3:** Avaliar o perfil transcricional do gene *Lrrk2* e estabelecer conexões deste com o sistema imune, em especial a neuroinflamação, para então estudar como isso se associa com o comportamento e com o consumo e preferência de etanol em um modelo de consumo de dieta rica em gordura (HSB) e livre escolha de etanol.

# 2 CAPÍTULO 1 DIET-INDUCED OBESITY LEADS TO ALTERATIONS IN BEHAVIOR AND GUT MICROBIOTA IN MICE [139]

(Artigo publicado no *Journal of Nutricional Biochemistry*)

Available online at www.sciencedirect.com

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Journal of Nutritional Biochemistry 92 (2021) 108622

Journal of Nutritional Biochemistry

# Diet-induced obesity leads to alterations in behavior and gut microbiota composition in mice

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Received 25 June 2020; received in revised form 30 December 2020; accepted 4 February 2021

**Abstract**

The high prevalence of obesity and associated metabolic disorders are one of the major public health problems worldwide. Among the main causal factors of obesity, excessive consumption of food rich in sugar and fat stands out due to its high energy density. The regulation of food intake relies on hypothalamic control by the action of several neuropeptides. Excessive consumption of hypercaloric diets has impact in the behavior and in the gut microbiota. In the present study, we used a high-sugar and fat (HSB) diet for 12 weeks to induce obesity in C57BL/6 mice and to investigate its effects on the gut microbiota, hypothalamic peptides, and behavior. We hypothesize that chronic consumption of HSB diet can change the behavior. Additionally, we also hypothesize that changes in gut microbiota can be associated with changes in the transcriptional regulation of hypothalamic peptides and behavior. To evaluate the gut microbiota, we performed the sequencing of 16S rRNA gene, which demonstrate that HSB diet modulates the gut microbiota with an increase in the *Firmicutes* and *Actinobacteria* phylum and a decrease of *Bacteroidetes* phylum. The real time qPCR revealed that HSB-fed mice presented changes in the transcriptional regulation of hypothalamic neuropeptides genes such as *Npy*, *Gal* and *Galr1*. The Marble-burying and Light/dark box tests also showed an alteration in anxiety and impulsive behaviors for the HSB-fed mice. Our data provides evidence that obesity induced by HSB diet consumption is associated with alterations in gut microbiota and behavior, highlighting the multifactorial characteristics of this disease.


*Keywords:* High-sugar and fat diet; Obesity; Gut microbiota; Behavior.

## 1. Introduction

Obesity is a multifactorial disease defined as abnormal or excessive fat accumulation that may be accompanied by increased plasma glucose, dyslipidemia, and elevated blood pressure [1–3]. Obesity also increases the likelihood of developing several pathological conditions including diabetes mellitus, hepatic steatosis, anxiety, depression, and gastrointestinal disorders [4–7]. By 2030, it is estimated that 38% of the adult population will be overweight and another 20% will have obesity [8,9].

The excessive consumption of high energy density food (rich in sugar and fat), combined with low energy expenditure, is one of the main factors responsible for obesity development [2,10–12]. Studies with rodents have shown the role of hypothalamic peptides in the control of feeding behavior and energy homeostasis [13–15]. The neuropeptide Y (NPY), it is known for its orexigenic effects mediated by hypothalamic NPY neurons. These neurons are controlled by a variety of peripheral factors which signalize energy status to the brain. Among these factors' leptin is a hormone secreted by adipocytes that acts reducing the NPY expression, inducing satiety and promoting energy expenditure [16,17]. Interestingly, the leptin resistance, commonly observed in obesity, leads to a higher transcription levels of NPY associated with a higher motivation for feeding behavior that commonly turns on obsessive-compulsive phenotype observed in obese patients [18–20]. The orexigenic galanin-like peptide and orexin are also involved in the regulation of feeding behavior and energy homeostasis throughout its signaling in the hypothalamus. Different from NPY that specifically increasing consumption of foods rich in carbohydrates, hypothalamic injection of galanin and orexin agonists preferentially stimulate the ingestion of foods rich in fat [21–24].

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https://doi.org/10.1016/j.jnutbio.2021.108622

The chronic consumption of high-sugar and fat diets can trigger alterations in the composition and function of the gut microbiota [25,26]. These changes can implicate an important function of gut microbiota in the development of obesity and metabolic diseases, once gut microbiota play an important role in the metabolism of nutrients, extraction of energy and fat storage [27–30]. For example, in animal models it is widely accepted that the proportions of the Firmicutes and Bacteroidetes phyla differs between lean and obese individuals [31–34]. These proportions seem to be still conflicting in human studies since they depend on lifestyle-associated factors and population, although several studies have demonstrated specific microbial populations in obese individuals [31,32,35,36]. Thus, it is believed that obese individuals present an increase in the *Firmicutes*/*Bacteroidetes* ratio, while a reduction in this proportion is observed during the weight loss, guided by diet changes [28,31,35,37–39]. Recently, some rodents studies have reported that gut microbiota influences feeding behavior throughout the gut-brain axis [40,41]. In this regard, the potential influence of intestinal bacteria on regulation of hypothalamic neuropeptides that are key factors for food intake should be considered since food influences the composition of the gut microbiota and in a competition environment the bacteria that control your host tend to benefit [42–45]. For example, the mRNA abundance of the hypothalamic NPY and agouti-related peptide (AGRP) are altered by Lipopolysaccharide (LPS) from the membrane of gram-negative bacteria [46]. Similarly, the expression of NPY is also altered by antibiotic-induced dysbiosis [47]. Interestingly, NPY and galanin also have been linked with addiction and anxiety behaviors, commonly observed in obese individuals [48–52]. Together these results highlights the association between gut microbiota, hypothalamic neuropeptides and behavior, and their importance to the development of obesity.

Considering the high prevalence of obesity, its risk to health, and its relationship with feed behavior, the use of animal models is vital to investigate different processes related to the pathophysiology of this disease. We previous developed a high-sugar and fat diet (HSB diet) that induces an obesity-like phenotype in mice. The chronic consumption of HSB diet trigger alterations common observed in humans with metabolic syndrome, and reduce regulatory immune responses that control inflammation [53]. The aim of this study was to explore how diet-induced obesity correlates with behavioral changes, changes in the regulation of genes associated with feeding behavior in the hypothalamus and changes in the gut microbiota composition and how these changes are interrelated. Specifically, we hypothesize that chronic consumption of HSB diet can induce changes in anxiety-like and impulsive-like behavior and modulate the gut microbiota composition. Additionally, we also hypothesize that changes in gut microbiota can be associated with changes in the transcriptional regulation of hypothalamic peptides.

## 2. Methodology

### 2.1. Animals

Twenty-four male mice specific pathogen free (SPF) C57BL/6, at the age of six weeks, were provided by the Animal Facility of the Universidade Federal de Minas Gerais (UFMG). Mice were housed individually housed in mini-isolators in a ventilated rack (ALESCO, São Paulo, Brazil) under a 12h/12h light/dark cycle and unrestricted access to water and food. This study was approved by the ethics committee for animal use (CEUA), of UFMG (protocol # 119/2012) and all efforts were made to minimize animal suffering.

### 2.2. Experimental design

The experiment design was carried out in two replicates. For each round, 12 animals were used. Initially, mice were placed individually in their respective cages and fed on the American Institute of Nutrition 93-Growth (AIN93G) (Carbohydrate: 64%, Proteins: 20%; Lipids: 16%; 4 kcal/g) [54]. At the end of the acclimatization period animals were randomly divided into two groups and for the following 12 weeks, one group received a pelleted high-fat diet called high-sugar and butter (HSB) (Carbohydrate: 36%, Proteins: 16%; Lipids: 48%; 5 kcal/g) [53] (n = 6), while the others kept receiving the standard pelleted AIN93G diet (n = 6). The diet was introduced during the light cycle and was continuously available 24 hour per day. Mice were weighted during the light cycle once a week throughout the experiment. The animals were euthanized one day after the end of 12$^{th}$ week, also during the light cycle. Animals from the first experiment round were subjected to the marble burying test during the light cycle, and samples were collected to conduct biochemistry, histological, molecular, and fecal microbiome analysis. The animals from the second experiment round were subjected to the light/dark box test.

### 2.3. Adiposity index, Lee index, and biochemical parameters

The perigonadal adipose tissue was collected and weighed at the end of experimental procedure. The adiposity index was determined by the ratio of the weight of perigonadal adipose tissue (g) and the body weight of the animals (g) in the last week of experiment. The Lee Index was used to determine the animals' body mass as described in [55] by the formula [body weight (g) × 1000/body length (cm)]$^{1/3}$.

Blood samples were also collected at the end of experimental procedure, and the total cholesterol, triglyceride, and glucose levels were measured using enzymatic kits (Bioclin, Belo Horizonte, Brazil). Blood samples were also used to measure leptin levels using the Mouse/Rat Leptin Immunoassay Quantikine ELISA kit (R&D Systems, Minneapolis, USA). The kits were used according to the manufacturer's instructions.

### 2.4. Histological evaluation

Fragments of liver were collected for histopathological analysis. Immediately after collection, tissue was washed with saline solution, placed in histological cassettes, and fixed in a 10% formaldehyde buffered solution. After 24 hours, tissue was transferred to a 70% ethanol solution, dehydrated, embedded in paraffin, and 4-μm-thick histological sections were stained with hematoxylin and eosin (H&E). Photographs were obtained from the SPOT Basic capture system version 3.4.5, adapted in an Olympus microscope (BX-40).

Two independent investigators who were blind to the samples evaluated the liver histological slides. The tissue sections were graded according to published criteria for magnitude of steatosis [56]. Briefly, the degree of steatosis was ranked from 0 to 4 based on the percentage of fat-containing hepatocytes per field: 0 = <5%, 1 = 5 ˜ 25%, 2 = 26 ˜ 50%, 3 = 51 ˜ 75% and 4 = > 75%.

### 2.5. Marble-burying test

This test was used to evaluate the effect of HSB diet consumption on the mice's natural and repetitive behavior of burying marbles. The marble-burying test procedure was performed and analyzed as described previously [57]. Briefly, on the last day of the experiment, each mouse (n = 12) was individually placed in a cage of standard size lined with 5-cm thick sawdust bedding, and 18 glass marbles arranged in three rows of six units evenly distributed. The animals were monitored for their burying behavior and after 10 minutes, the number of marbles, with at least two thirds (2/3) of its size covered by sawdust, were counted. The percentage of marbles buried was used to measure the behavior for each animal [58–60].

### 2.6. Light/dark box test

Anxiety-like behavior induced by HSB diet consumption was measured using the Light/dark box test, which allows the evaluation of the mouse's aversive reaction in highly illuminated environment. Test procedure was performed and analyzed as described previously [61,62]. The apparatus consists of two PVC boxes [46 × 27 × 30 cm (l x w x h)], one in dark and the other is highly illuminated (60W light bulb), connected by a tunnel which allows unrestricted movement between the light and dark compartments [62]. Mice are placed individually in the dark side (n = 12). The time spent in the light side (sec), the latency to leave the dark side (sec), numbers of light-dark sides transitions, and distance traveled are measured for 5 min as an index of anxiety-like behavior. The distance traveled in the light side was used to show the exploratory activity of the animals. The experiment was filmed and analyzed using the Ethovision XT software version 12 (Noldus Information Technology, Utrecht, Netherlands) [63].

### 2.7. Total RNA isolation and reversal transcription

Brains were dissected on ice and the hypothalamus collected and stored at -80°C. Perigonadal adipose tissue was also collected, weighted, and stored at -80°C. The total RNA was extracted using ReliaPrep RNA Tissue Miniprep System (Promega, São Paulo, Brazil), according to the manufacturer's recommendations. After being extracted and diluted in 20μL of ultrapure water, the total RNA was quantified using the DeNovix DS-11 equipment (DeNovix, Delaware, USA). Samples were stored

at -80°C until complementary DNA (cDNA) synthesis. For each sample, 500 ng of total RNA were used to make cDNA with oligo (dT20) primers (Prodimol Biotechnology, Belo Horizonte, Brazil), dNTP mix (10 mM), Reaction Buffer 5X (Thermo Fisher Scientific, São Paulo, Brazil), Ribolock RNase Inhibitor (Thermo Fisher Scientific, São Paulo, Brazil), and Revertaid transcriptase (Thermo Fisher Scientific, São Paulo, Brazil), according to the manufacturer's recommendations.

### 2.8. Primer design and real-time PCR (qPCR)

Primers were designed and the qPCR was conducted as described in [64]. The sequences (5'→3') of the target genes used in this study are available in Supplementary table 1. Transcript expression levels of target genes were measured using the CFX 96TM Real-Time system thermocycler (BioRad, California, USA). The qPCR reactions for each gene were performed using 10μL KAPA SYBR FAST Universal qPCR Kit (Kapa Biosystems, São Paulo, Brazil), 1μL cDNA (10ng/μL), 0.4μL primer solution (10pM) and 8.2μL ultra-pure water (Thermo Fisher Scientific, São Paulo, Brazil). In all reactions, a negative control without cDNA template (NTC) was used, and the final reaction volume was kept at 20μL. PCR amplification was performed without the extension step (95°C for 3 min, followed by 40 cycles of 95°C for 3 secs and 60°C for 20 sec). Fluorescence levels were measured during the last step of each cycle (60°C). The relative quantities of the transcripts were calculated by the delta–delta Ct method [65]. The reference genes used were *Gapdh* and *Ppia* based on their stability and continuous levels of transcription [66].

### 2.9. Feces collection and DNA extraction

Feces for the study of the microbiome were collected on the last day of the experiment as previously established by [67]. The extraction of bacterial DNA in feces samples was performed using the *QIAamp DNA Stool Mini Kit* (QIAGEN, São Paulo, Brazil) following the manufacturer's instructions. The DNA concentration and purity were analyzed by spectrophotometry, using DeNovix DS-11 (DeNovix, Delaware, USA). In this evaluation, the ratios 260/280 and 260/230 were considered and no impurities were detected in the DNA samples, which were then immediately frozen in a freezer at -20°C until subsequent molecular analysis [68].

### 2.10. DNA library preparation, sequencing the 16S rRNA gene, and bioinformatics analysis

DNA library preparation, sequencing and bioinformatics analysis were performed as previously described in [50]. Briefly, the library was prepared through the polymerase chain reaction (PCR) using 5′CCTACGGGRSGCAGCAG3' [69] and 5'GGACTACHVGGGTWTCTAAT3' [70] primers, specific for the V3-V4 region of the 16S rRNA. Then the amplicons were purified using the AMPureXP beads kit (Beckman Coulter, Brea, CA), normalized and, grouped into libraries using the KAPA Library Quantification Kit for Illumina platforms (KAPA Biosystems, Woburn, MA). The sequencing was performed on the MiSeq system on the Illumina platform [71]. The sequencing data were processed and analyzed with QUIIME v. 1.9.1 and the taxonomy was assigned to each OTU using the SILVA reference database [70,72,73].

Principal Coordinates Analysis (PCoA) based on the Bray-Curtis index and examined by similarity analysis (ANOSIM) was performed on Microbiome Analyst (www.microbiomeanalyst.ca) [74]. The LDA Effect Size (LEfSe) analysis using standard parameters in the Galaxy online interface (huttenhower.sph.harvard.edu/galaxy/root) was used to identify abundant different bacterial groups according to the diet consumed by animals [75].

### 2.11. Statistical analysis

The data were analyzed for Gaussian distribution using the Shapiro-Wilk normality test. Two-way ANOVA followed by *Sidak post hoc* test was used to analyze body weight during the 12 weeks of the experiment. ANOVA data are represented as F (between group df, within group df) = F statistic, p-value). To evaluate adiposity index, Lee index, biochemical parameters, behavioral tests, mRNA relative quantification by real-time qPCR, relative abundance of bacterial groups in the fecal microbiome and Firmicutes/Bacteroidetes ratio, it was used the unpaired Student t test with data represented by the values of t and p. Linear regression analysis was carried out and the Pearson correlation coefficient was calculated to estimate the association between gut microbiota groups and mRNA levels or behavior. Correlation tests were performed between all the representatives of gut microbiota and animal behaviors, but we only graph the results with significant differences. The data were expressed as mean ± SEM, and each bar represents an experimental group. All analyses were performed using the GraphPad Prism statistical package version 7.01 (GraphPad Software Inc, San Diego, USA). Differences were considered significant at $p \leq 0.05$ and were identified by an asterisk (*).

## 3. Results

### 3.1. HSB diet intake affected body weight, adiposity index, biochemical parameters, and liver morphology

HSB diet consumption resulted in an increase ($P < .05$) in body weight gain in HSB group in comparison with AIN93G animals (Fig. 1A and B). Higher indexes of adiposity ($t = 9.624$, $P < .0001$) and Lee ($t = 9.187$, $P < .0001$) were also observed in animals from the HSB group in comparison with the AIN93G group (Fig. 1C and D). The indexes of adiposity and Lee are metrics used to indicate the obesity-like phenotype in studies using animal models with diet-induced weight gain [76,77]. As such the association of these indices with weight gain are indicative of the development of an obesity-like phenotype in HSB animals.

As expected, there was a hyperregulation of the *leptin* gene in the adipose tissue ($t = 3.173$, $P = .0156$) (Fig. 1E) as well as a higher plasmatic leptin levels ($t = 4.582$, $P = .0018$) in animals from the HSB group compared to AIN93G (Fig. 1F). Additionally, HSB animals presented higher levels of total cholesterol ($t = 6.493$, $P < .0001$), triglycerides ($t = 20.41$, $P < .0001$), and glucose ($t = 6.682$, $P < .0001$) when compared to animals in the AIN93G group (Fig. 1G–I, respectively). The histological evaluation showed disseminated fat vacuoles in the HSB animals' liver compared to AIN93G (Fig 1K and L). The HSB animals' liver also presented hepatomegaly due of the greater weight of this organ ($t = 4.655$, $P < .0001$) (Fig. 1J) and was diagnosed with grade 4 (>75%) (Fig. 1M) of fatty degeneration, suggesting the development of a hepatic steatosis in comparison to AIN93G animals. Macroscopic characteristics can be observed in Supplementary Figure S1.

### 3.2. The HSB diet affected the behaviors of animals

HSB animals presented a higher percentage of buried marbles ($t = 4.709$, $P = .0006$) than animals in AIN93G group, indicating intensification in the obsessive-compulsive-like behavior (Fig. 2A). In the Light/Dark box test, animals show their preference for dark, enclosed places over bright, exposed places [78]. Thus, the time spent in the light side is a reliable parameter for assessing anxiolytic effects [78]. HSB animals spent more time exploring the light side ($t = 3.606$, $P = .0048$) compared to AIN93G group, and presented a shorter initial latency to leave the dark side ($t = 3.165$, $P = .0101$) (Fig. 2B and C). Regarding the number of light-dark sides transitions, no significant difference was found between groups ($t = 0.3419$, $P = 7395$) (Fig. 2D). Interestingly, HSB animals present a longer distance traveled and high exploratory activity in the light side in comparison with AIN93G group ($t = 2.624$, $P = .0254$) (Fig. 2E and F). In sum, the Light/Dark box test results suggest that 12 weeks of HSB diet consumption induces an anxiolytic-like effect in the HSB animals compared to AIN93G.

### 3.3. The HSB diet modifies the transcriptional regulation of orexigenic genes

The transcription of hypothalamic neuropeptides genes directly associated with feeding behavior was evaluated in HSB and AIN93G mice after 12 weeks of diet consumption. A hyperregulation in the transcripts for neuropeptide y (*Npy*) ($t = 2.66$, $P = .0288$) (Fig. 3A), galanin (*Gal*) ($t = 3.286$, $P = .0111$) (Fig. 3B), and galanin receptor (*Galr1*) ($t = 3.79$, $P = .0053$) (Fig. 3C) was observed in the animals of the HSB group in relation to the animals of the AIN93G group. No significant differences were found in the orexin (*Hcrt*) transcripts ($t = 0.3269$, $P = .7512$) (Fig. 3D) and in the orexin receptor (*Hcrtr1*) ($t = 0.5846$, $P = .5718$) (Fig. 3E).

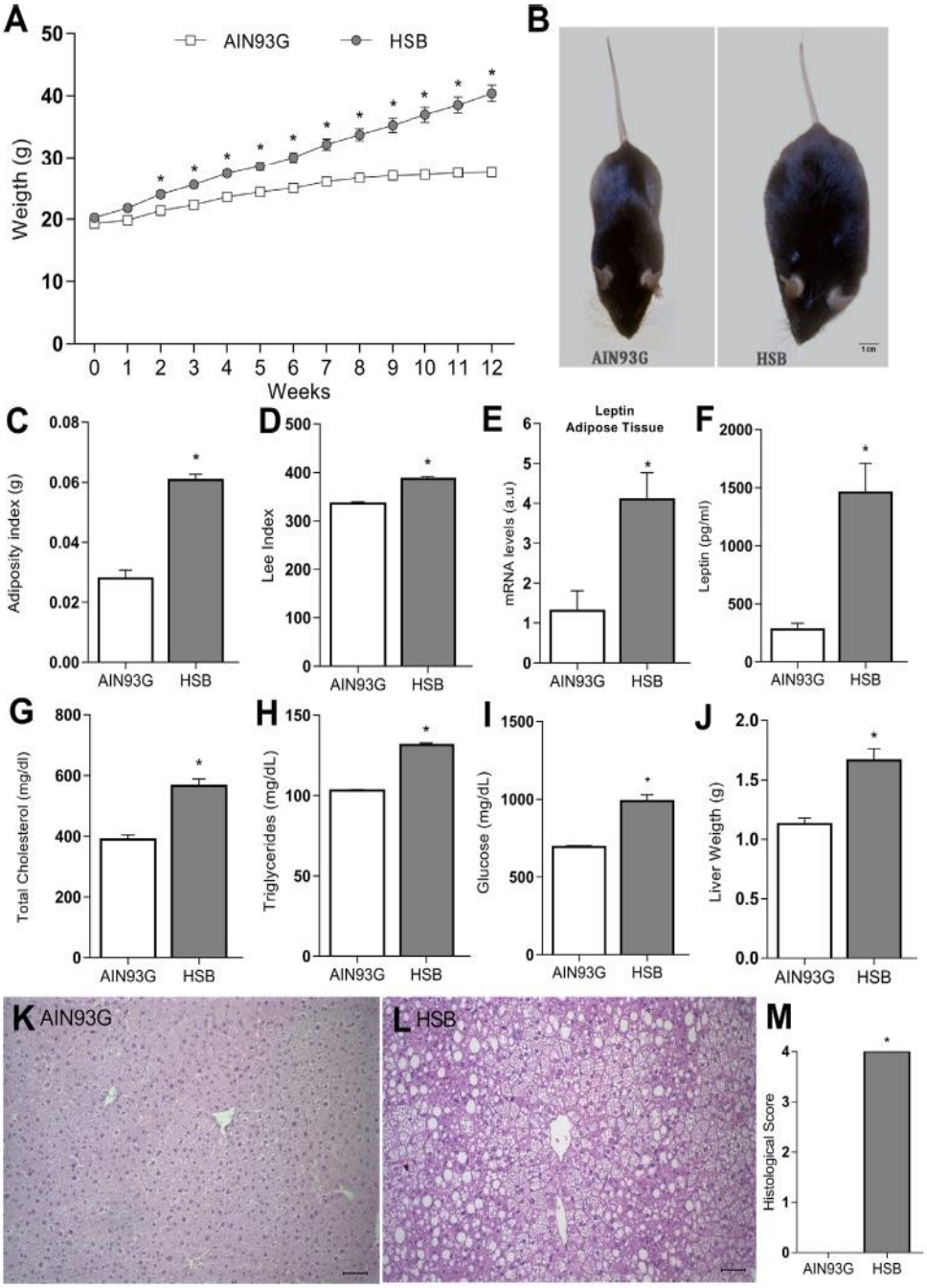


**Fig. 1. HSB diet consumption induces obesity and liver alterations**. (A) Body weight (g). (B) Animal phenotype at the end of the experiment. (C) Adiposity index (g). (D) Lee index. (E) mRNA relative levels (a.u.) of Leptin in adipose tissue. (F) Leptin in plasma (pg/ml). (G) Total cholesterol (mg/dl). (H) Triglycerides (mg/dl). (I) glucose (mg/dl). (J) Liver Weight (g). Histological representation of the liver (20X) by the groups: (K) AIN93G and (L) HSB. Bars represent 100 μm. (M) Liver histological score. In (A) Two-way ANOVA followed by Sidak *post hoc* test were used to evaluate body weight (g) over the weeks. In (C-J, and M) student's t-test was used to determine statistically significant differences between the groups. Results are expressed as mean ± SEM. *Asterisks represent the statistical differences (*$P < .05$).

### *3.4. HSB diet intake affected the fecal microbiome composition*

The dietary treatment is likely to be the major factor responsible for differences in similarity (R = 0.852, $P < .004$) and structure of the fecal microbiome between HSB and AIN93G groups, clustered according to the beta diversity measured by the Bray-Curtis index (Fig. 4A). This result can also be observed in the dendrogram of this index (Supplementary Fig. S2). The alpha diversity indexes (Shannon, Fisher, Simpson and Chao1) are shown in Supplementary Figure S3. The linear discriminant analysis (LDA) of the effect size of LEfSE analysis ($\alpha$=0.05, LDA score>2.0) showed different abundance of bacterial groups between HSB and AIN93G groups (Fig. 4B). Analysis of the fecal microbiome also showed different compositions and abundances in terms of phyla and families between the experimental groups (Fig. 4C and D).

Regarding to the relative abundance, the *Firmicutes* phylum showed a higher number of representatives in the feces of animals that had eaten the HSB diet (t = 2.323, $P = .0487$) in relation to AIN93G group (Fig. 4E). We also observed a decreasing (t = 7.171, $P < .0001$) of *Bacteroidetes* phylum representatives in HSB animals' feces (Fig. 4F), and an increasing of *Firmicutes*/*Bacteroidetes* ratio (t = 4.277, $P = .0027$) in HSB group compared to AIN93G. (Fig. 4G). The *Actinobacteria* phylum (t = 4.83, $P = .0013$) was also increased in HSB animals compared to AIN93G (Fig. 4H). We also

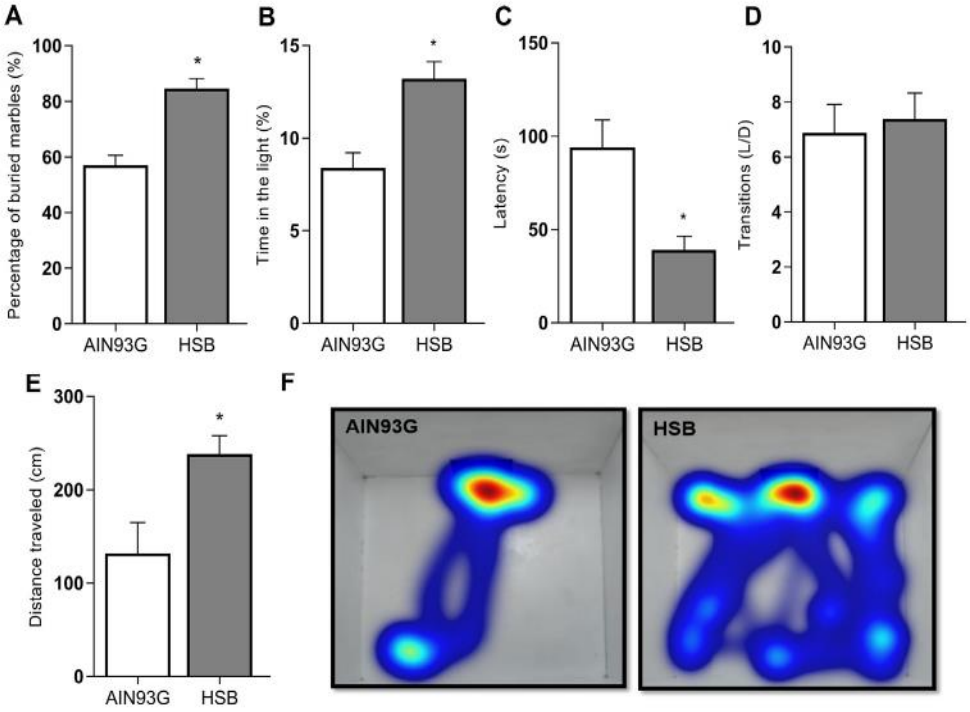


**Fig. 2. HSB diet induced behavioral changes**. Obsessive-compulsive behavior was assessed using the Marble burying test to measure the percentage of buried marbles (A). Anxiety was assessed using the light/dark box test to measure: (B) time on the light compartment (%), (C) latency (s), (D) number of transitions, and (E) distance traveled (cm). (F) Representation of the exploratory activity of mice in the light side of box. In (A-E) Student's t-test was used to determine statistically significant differences between groups. Results are expressed as mean ± SEM. *Asterisks represent the statistical differences (*$P < .05$).

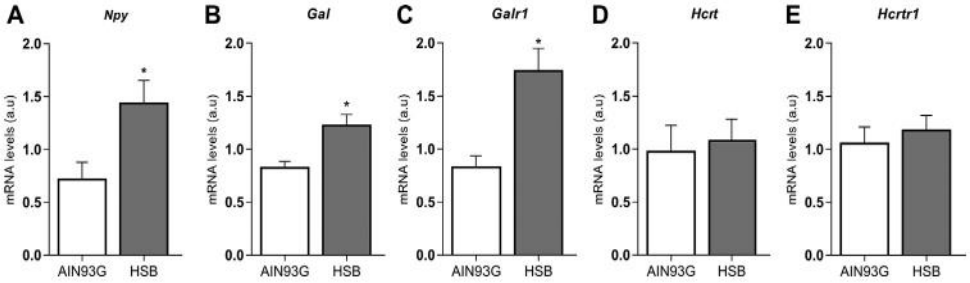


**Fig. 3. Hypothalamus' relative mRNA quantification of genes involved in feeding behavior**. mRNA relative levels (a.u) by: (A) *Npy*, (B) *Gal*, (C) *Galr1* (D) *Hcrt*, and (E) *Hcrtr1* in the hypothalamus of HSB and AIN93G groups. Student's t-test was used to determine statistically significant differences between the groups. Results are expressed as mean ± SEM. *Asterisks represent the statistical differences (*$P < .05$).

observed differences between the experimental groups in terms of gender. HSB animals presented increase in relative abundance of *Lachnoclostridium* (t = 3.597, $P = .0070$), *Bifidobacterium* (t = 27.49, $P < .0001$), *Parvibacter* (t = 5.793, $P = .0004$), *Ruminiclostridium* (t = 0.9078, $P < .0001$), and *Blautia* (t = 2.399, $P = .0433$) (Fig. 4I–M, respectively), genera compared to AIN93G group. On the other hand, an increasing of *Lactobacillus* genera representatives (t = 3,468, $P = .0085$) in the HSB animals feces was observed in comparison with AIN93G group (Fig. 4N).

### *3.5. The relative abundance of fecal microbiome representatives correlates with transcriptional regulation of orexigenic genes and behavior*

To investigate the association between the relative abundance of fecal microbiome representatives, transcriptional regulation of orexigenic genes, and burial behavior, we performed correlations between the Marble-burying behavior result test, the mRNA levels of hypothalamic neuropeptides genes and the relative abundance of phylum and genera in gut microbiome. The heat map (Fig. 5A) shows significant correlations found ($P < .05$ in bold) between *Npy*, *Galn* and *Galr1* mRNA levels and gut microbiota representatives. The percentage of marbles buried by the HSB animals was positively correlated with representatives of the Actinobacteria phylum (r = -0.8279, $P = .0031$) (Fig. 5B). However, the percentage of marbles buried by the HSB animals was negatively correlated with the relative abundance of *Bacteroidetes* phylum representatives (r = -0.7862, $P = .0070$) as well as with the *Lactobacillus* genera (r = 0.8079, $P = .0047$) (Fig. 5C and D, respectively). Additionally, we demonstrated a positive correlation between *Firmicutes*/*Bacteroidetes* ratio and adiposity index (r = 0.752, $P = .0062$) (Fig. 5E).

## 4. Discussion

The results presented here shows that chronic consumption of HSB diet induced in mice a phenotype with obesity-like characteristics [3,25,53,79]. We also showed that 12 weeks of HSB diet intake leads to changes in the structure and composition of fecal microbiome, changes in the anxiety-like and impulsive-like behavior, as well as changes in the transcriptional regulation of *Gal*, *Galr1*, and *Npy* genes in the hypothalamus. Additionally, we

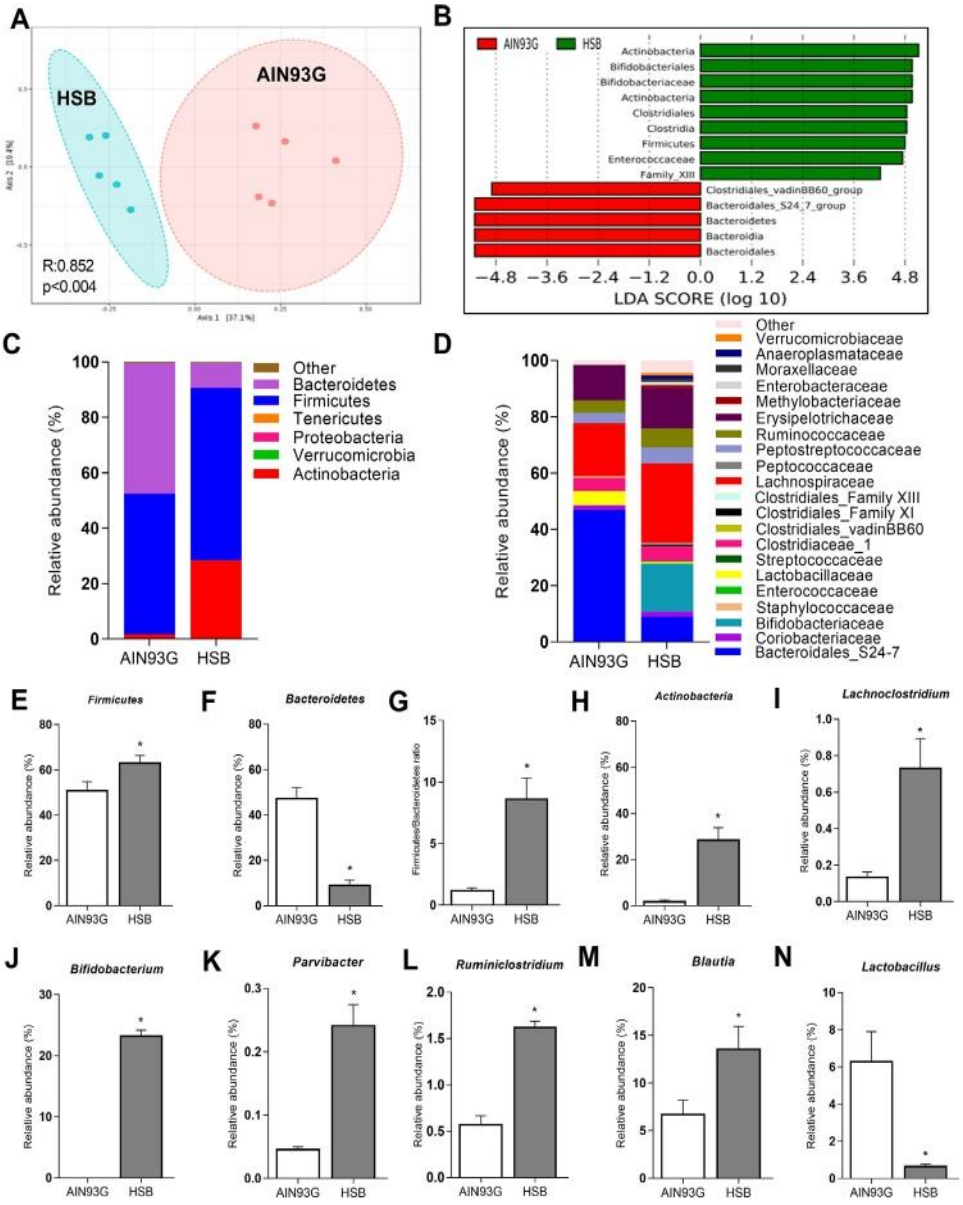


**Fig. 4. Fecal microbiome composition is altered by HSB diet**. (A) Principal Coordinates Analysis (PCoA) plot of beta diversity measured by the *Bray-Curtis* index. (B) Linear discriminant analysis (LDA) Effect Size (LEfSe) analysis of bacterial taxa (C) Composition of fecal microbiome showing different communities at the phylum level, each bar represents an experimental group and each color represents a bacterial phylum (D) Composition of fecal microbiome showing different communities at the family level. Relative abundance of Phyla: (E) *Firmicutes*, (F) *Bacteroides* and, (H) *Actinobacteria*. (G) *Firmicutes/Bacteroidetes* ratio. Relative abundance of genres: (I) *Lachnoclostridium*, (J) *Bifidobacterium*, (K) *Parvibacter*, (L) *Ruminiclostridium*. (M) *Blautia* and, (N) *Lactobacillus*. In (C and D) each bar represents an experimental group and each color represents a bacterial Family. In (E-N) Student's t-test was used to determine statistically significant differences between the groups. Results are expressed as mean ± SEM. *Asterisks represent the statistical differences (*$P < .05$).

also demonstrate an association between these genes and behavior with changes observed in the gut microbiota.

Behavioral disorders are commonly observed in obese individuals and may be associated to the onset and/or maintenance of the disease [80–82]. Studies have suggested that, in periods of anxiety and stress, individuals are more likely to engage in the consumption of high sugar and fat foods, because of its ability to activate pathways of the reward system in the brain, bringing positive effects and the relief of these symptoms [82,83]. In this way, the continued consumption of these foods becomes mandatory for the relief of negative emotional states, triggering compulsive behavior that ultimately can lead to obesity [82,83].

The light/dark box test showed that HSB animals spent more time in the light compartment and had a higher exploratory activity than AIN93G animals, indicating an anxiolytic effect associated with the consumption of HSB diet. Our results corroborate with studies in the literature, which describes the association between high-fat diet consumption and anxiety-like behavior. For example, Ortoloni and collaborators (2011) reported that the ingestion of comfort food (high-sugar and fat) potentiate the anxiolytic-like effect in animals submitted to footstock stress [84]. Similar results were also reported by Maniam *et al.* (2010), who demonstrated the efficiency of a high-sugar and fat diet in reducing the effects of postpartum anxiety in rats [85]. The marble-burying test showed an increased burial behavior in the animals of the HSB group. Studies prove that this behavior is similar to the obsessive-compulsive disorder in humans in which impulsive behavior is observed [58,86–88]. These findings are consistent with studies in which high-calorie diets were associated with an increase in the compulsive-like behavior [89,90] and suggests that chronic consumption of HSB diet can trigger impulsivity in mice.

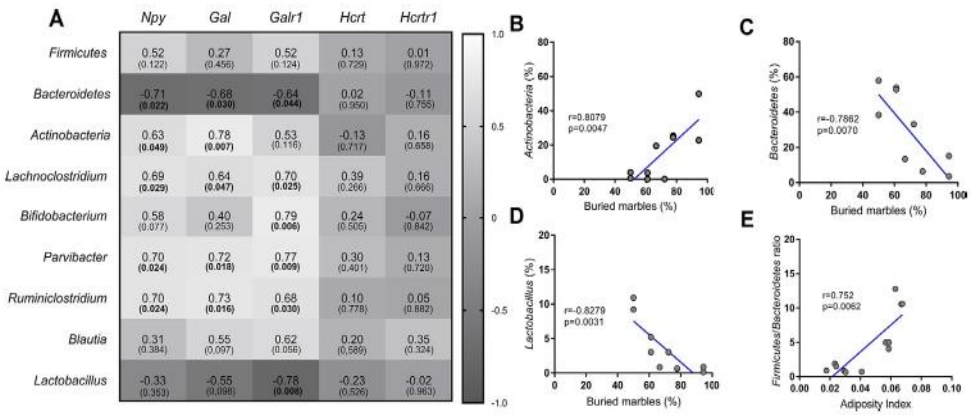


**Fig. 5. Correlations between relative abundance of fecal microbiome representatives, transcriptional regulation of orexigenic genes, and burial behavior.** (A) Heat map of person correlation coefficient between transcription of hypothalamic neuropeptides genes and representatives of the fecal microbiota (in each cell: r above, and p-value below in bold). Person Correlation coefficient between buried marbles (%) and (B) *Actinobacteria*, (C) *Bacteroidetes*, and (D) *Lactobacillus*. (E) Person Correlation coefficient between *Firmicutes/Bacteroidetes ratio* and adiposity index.

The regulation of feeding behavior is primarily done in the hypothalamus by the activation of neurons that express a wide range of neuropeptides such as NPY [17,91]. Under physiological conditions, leptin inhibits the NPY action leading to a reduction in food intake and an increase in the energy expenditure [17,48]. Contrary, in obesity there is an interruption of this homeostatic mechanism and the development of leptin resistance, which impairs the proper inhibition of NPY production [92]. Although our data cannot directly demonstrate the leptin resistance in our HSB animals, our results agrees with the literature data since we found an elevated leptin levels in plasma, as well as a hyperregulation of *Npy* in the hypothalamus of HSB animals. Another neuropeptide that is also hyperregulated by the consumption of high-fat diets is the *Gal* [22,93]. Studies report that an increase in Galanin transcription is connected to reduced levels of corticosterone, a stress-related hormone [93,94]. Additionally, the Galanin administration in animals is known to trigger an anxiolytic effect, and its upregulation in response to the consumption of fatty foods can be a mode to cope with anxiety in order to relief this symptom that is associated with chronic intake of high-fat diet [22,50,95]. The *Galr1* was also hyperregulated in HSB animals. Studies have revealed that this receptor is also implicated in the consumption of a high-fat diet. Zorrila *et al.* (2007) showed that *Galr1* deficient mice presented a reduced consumption of this type of diet [96].

A growing body of evidence from humans and animal models have been shown the impact of changes in the gut microbiota composition on obesity pathogenesis [97,98]. In the present study, we showed a clustering of the fecal microbiome structure according to the consumed diet. We noticed, especially in the HSB-fed group, an increase in *Firmicutes* representatives' phylum and a depletion in the *Bacteroidetes* phylum. This increase in the *Firmicutes/Bacteroidetes* ratio is related to obesity in several studies [25,31,35]. We also found a positive correlation between this ratio and the adiposity index. It is suggested that representatives of the phylum *Firmicutes* has more enzymes that contribute to the metabolization of carbohydrates allowing greater energy absorption and consequently increase of adiposity, processes that facilitate weight gain observed in obesity [32,33,99,100]. Additionally, metabolic products of *Firmicutes* bacteria are indirectly connected to the regulation of hunger and satiety since they produce short chain acids that downregulate the synthesis of the hunger-suppressing hormones such as peptide YY and glucagon-like peptide 1 [31,101]. Others bacterial phyla may also be altered in the gut microbiome during weight gain and obesity. As an example, the *Actinobacteria* phylum, herein observed in greater abundance in HSB-fed mice corroborate with human results in which obese individuals presented higher levels of this phylum when compared with their lean twin siblings [102].

Specific groups at the genus level such as *Blautia, Lachnoclostridium, Parvibacter, Bifidobacterium*, and *Ruminiclostridium* had its abundance increased in HSB-fed group. These results corroborate with the literature since these genus have been associated with high fat-diet consumption, obesity and their comorbidities in several studies [77,103–108]. On the other hand, bacteria from the genus *Lactobacillus* are reduced in HSB-fed mice. Species included in this group were already been described for their anti-obesity effects and for protection against fat accumulation and deposition of triglycerides in adipocytes [109,110]. Therefore, it is plausible that differences found in our study in fecal microbiome between experimental groups may play a major role in obesity development in HSB-fed mice. Additionally, it is likely that intestinal bacteria had also influenced the animal's feeding behavior of animals. The gut microbiota is known to interact and modulate the central nervous system through the gut-brain axis, which consists of a bidirectional communication system that involves the intestine and the central nervous system [111,112]. This communication is facilitated in the high-sugar and fat diets intake since there is an increase in intestinal permeability [113–115].

Diet is the key factor for selective pressure in relation to the nutrients available for gut microbiota. Thus, selected bacteria can control the host's feeding behavior to increase their fitness [43,116,117]. Representatives of gut microbiota can induce craving for foods that they are specialize or foods that suppress their competitors and may secrete metabolites that influence their host appetite and eating behavior [43,116,117]. In a review, Fetissov (2017) mentions several bacterial products detected in the systemic circulation, which can act directly on hypothalamic neurons controlling host appetite and satiety [43]. Studies show that specific components of intestinal bacteria can affect gene transcription [118–120]. Interestingly, in the present study the *Npy, Gal*, and *Galr1* mRNA levels correlate with representatives of the gut microbiota. In this context, it is known that indole (generated by reductive deamination from tryptophan) secreted during the growth of several bacteria binds to an aryl hydrocarbon receptor pathway in the hypothalamic NPY/AgRP neurons showing its possible association in central feeding behavior [43,121,122]. The study of Fröhlich and collaborators (2016) shows that *Npy* mRNA levels are increased in the hypothalamus of mice with intestinal dysbiosis [47]. Indeed, Schele and collaborators (2013) when analyzing germ-free and conventional mice noticed that only control conventional mice developed

hypothalamic signs of leptin resistance [123]. Considering the role of Npy, Gal and Galr1 in feeding behavior and their correlations with gut microbiota, we cannot disregard their possible connection with microbiota-gut-brain axis in the present study. In addition, the correlations between representatives of the microbiota and the burial behavior are also can be important, since several studies also show the capacity of high-fat diets to alter the gut microbiota and, consequently, the host behavior [124–126]. The transplantation of fecal material from HSB mice to control mice with the purpose of gut microbiota transference between these animals may confirm if the results observed in the present study.

In summary, we demonstrated that chronic consumption of HSB diet induces obesity-like phenotype in mice with typical features such as weight gain and adiposity, and metabolic disorders. Furthermore, we demonstrated that obesity induced in HSB-fed mice was associated with inter-correlated changes in animal anxiety-like and compulsion-like behaviors; in the regulation of genes related to food intake, and in the fecal microbiome composition. Our findings also pointed out the important role of diet in the processes that determine the structure and composition of intestinal microbiota, suggesting that it may influence weight gain and feeding behavior in animals. These data contribute to our understanding of the gut microbiota rule on the pathogenesis of obesity and to the identification of possible microbiome targets for future therapeutic intervention.

## Author contributions

REMJ conducted all the experiments, performed the statistical analysis, carried out bioinformatics analysis and wrote the paper. LMC contributed to the experiments and statistical analysis. DCR and GDC performed histopathological analysis. AMCF and TUM developed the HSB diet and helped to discuss the results. ALBG contributed to the project development and supervised all the study. All authors contributed to manuscript revisions. All authors read and approved the final manuscript.

## Funding

This works was supported by grants from Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG: APQ-02285-14 and APQ-00704-14) and PNPD/CAPES (23038.005051/2012-48).

## Declaration of competing interests

CAPES and FAPEMIG had no role in the design, analysis or writing of this article. This manuscript was reviewed by a professional science editor and by a native English-speaking copy editor to improve readability.

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2021.108622.

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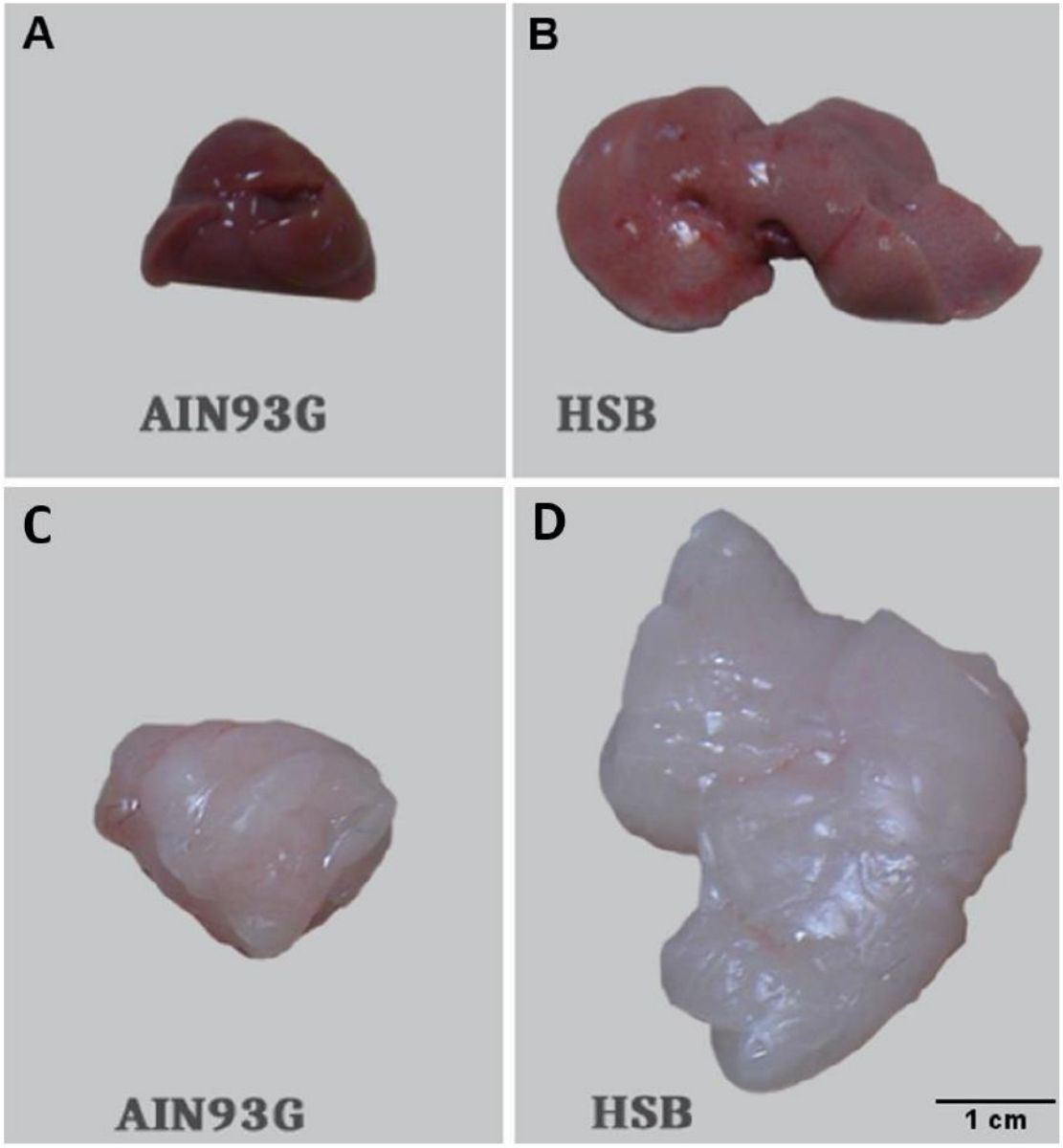
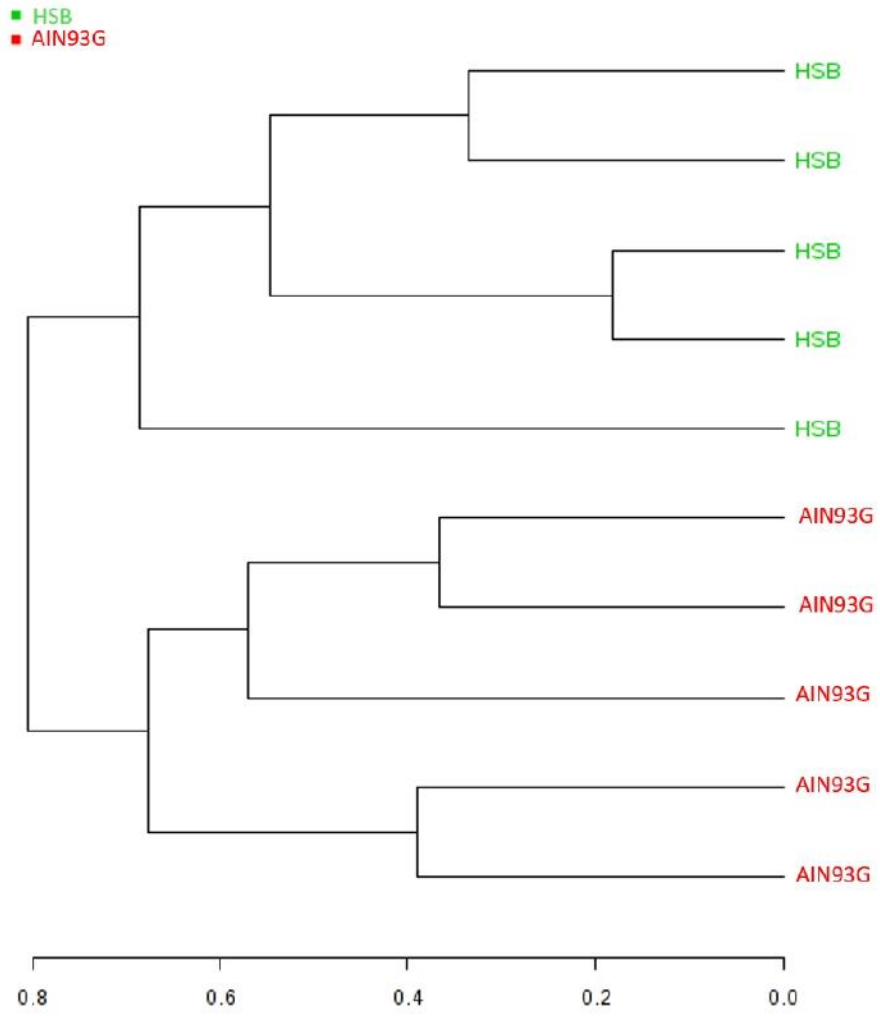
## SUPPLEMENTARY INFORMATION




**Supplementary Figure S1**: **Macroscopic view of the liver and perigonadal adipose tissue at the end of the experiment.** (A) Representative liver of AIN93G group showing reddish-brown color, characteristic of this organ. (B) Representative liver of HSB group showing yellowish-pink color indicating fat accumulation. (C) Representative perigonadal adipose tissue of AIN93G group. (D) Representative perigonadal adipose tissue of HSB group.




**Supplementary Figure S2: Fecal microbiome dendrogram**. Analysis performed using the *Bray-curtis* index. The distance between the vertical lines indicates differences between the various samples.

# 3 CAPÍTULO 2 INTERACTION BETWEEN HIGH-FAT DIET AND ETHANOL INTAKE LEADS TO CHANGES ON THE FECAL MICROBIOME [120]

(Artigo publicado no *Journal of Nutricional Biochemistry*)

Available online at www.sciencedirect.com

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Journal of Nutritional Biochemistry 72 (2019) 108215

Journal of Nutritional Biochemistry

# Interaction between high-fat diet and ethanol intake leads to changes on the fecal microbiome

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Received 28 February 2019; received in revised form 24 June 2019; accepted 19 July 2019

**Abstract**

It is known that high-fat diet and alcohol intake can modulate the gut microbiota and consequently affect physiological processes such as fat storage and conditional behavior. However, the effects of the interaction between high-fat diet, its withdrawal and ethanol intake in gut microbiota remain unclear. To address this question, we used an animal model in which C57BL/6 mice were fed on standard (AIN93G) or high-sugar and -butter (HSB) diet for 8 weeks. Then, a protocol of free choice between water and a 10% alcohol solution was introduced, and the HSB diet was replaced with AIN93G in two experimental groups. This model allowed us to distinguish the individual effects of HSB diet and ethanol, and the effects of its interaction on the microbiome. The interaction of those factors was the main driver in the structure changes of the fecal microbial community. HSB diet and ethanol consumption directly affected the abundance of Firmicutes and Actinobacteria phylum, and Clostridiaceae and Coriobacteriaceae family. On the other hand, we also showed that abundance of Bacteroidales_S24-7 family and the Firmicutes/Bacteroidetes ratio were affected only by HSB diet consumption and that ethanol consumption was uniquely responsible for the bacterial translocation to the liver, indicating a breaking of the gut barrier. Finally, we also pointed out that the withdrawal of the HSB diet affects the preference for alcohol and shows a structural resilience in the fecal microbiome. These results highlight the importance of the gut microbiome modulation and its possible role on the phenotype developed by animals.


*Keywords:* High-fat diet; Alcohol intake; Gut microbiome; Microbiota resilience; Interaction; Bacteroidetes phylum

## 1. Introduction

The gut microbiota establishes a symbiotic relationship with its host, taking on important roles in the maintenance of the host's homeostasis [1]. Among the different physiological processes influenced by the gut microbiota are food digestion, fat storage and conditional behavior [2–6].

It is known that different food components, probiotics, antibiotics and drugs use can modify the intestinal ecosystem [7–10]. These alterations can benefit some bacteria groups, unleashing different phenotypes in the host [11,12]. For example, it has been recognized that, both in human and in animal models, the increase of Firmicutes and Bacteroidetes ratio is associated with higher efficiency in fat storage, which contributes to increased body adiposity and weight [13–16]. The relation between gut microbiota, body fat and obesity is highlighted by the Walker et al. (2013) findings, in which germ-free mice inoculated with the gut microbiota of either obese or lean twins developed the same phenotype as the donor [17].

Ethanol consumption also leads to microbiota dysbiosis throughout the intestinal barrier dysfunction, and intestinal permeability increases [18]. These changes can result in a boost of lipopolysaccharides (LPSs) and other endotoxins in circulation as well as an increase in the bacteria translocation to specific liver sites [18–20]. These alterations can influence the development of hepatic diseases and also lead to alterations in the transcription of specific genes in brain regions related with decision making over the ethanol consumption [18,21–23]. Additionally, intestinal bacteria seem to participate in the anxiety triggered by ethanol abstinence [24]. Indeed, Leclercq and collaborators (2014) reported an association between the increase of the gut permeability with the high anxiety rate and craving for ethanol in patients with alcohol use disorders (AUDs), suggesting that the phenotype achieved was directly related to the interaction between microbiota, intestine and brain [19,25,26]. Interestingly, Leclercq and collaborators also reported maintenance in the anxiety state and craving for ethanol in these patients even after the detoxification period, pointing out the role of the microbiota resilience in AUDs [25].

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https://doi.org/10.1016/j.jnutbio.2019.07.006
0955-2863/

Resilience is related to the microbiota capacity to return to a stable state after a perturbation, and it is a key point to be considered during the evaluation of microbiota modulation as therapeutic treatment [27,28].

Considering the microbiota modulation by different types of diet and the use of drugs of abuse, as well as the health problems involved in this scenario, the current study aims to evaluate the effects of a hypercaloric diet and ethanol consumption in the composition and structure of the intestinal microbiota. Several studies demonstrated the impact of fat- and sugar-rich diet or ethanol consumption in the composition and function of gut microbiota [15,20,29,30]. However, few have evaluated how the interaction between these two factors impacts the gut microbiota and which are the implications of the withdrawal of one of them in the microbiota ecosystem. To address these issues, the current work used an animal model previously developed by our group [31], in which, for 8 weeks, animals were chronically fed with a diet rich in sugar and fat, called high-sugar and -butter diet (HSB) [32]. After this period, HSB was withdrawn, and animals had free choice between water and 10% ethanol solution. In this model, the HSB consumption and its withdrawal affected the structure of the gut microbiota, modifying the abundance of the Bacteroidetes phylum, for example. Moreover, the interaction between diet and ethanol consumption seems to have a key role in the composition of the gut microbiota and in the behavior observed in animals.

## 2. Methodology

### 2.1. Animals

Forty-four male C57BL/6JUnib (CEMIB, UNICAMP) mice, SPF (Specific Pathogen Free), at 6 weeks of age, were provided by the Animal Facility of the *Universidade Federal de Minas Gerais* (UFMG). The animals were housed individually in mini-isolators in a ventilated rack (ALESCO) under a 12-h/12-h light/dark cycle. Only male mice were used to avoid interference of hormonal fluctuation. All animals had unrestricted access to water and food throughout the experimental procedure, and to a 10% ethanol (EtOH) solution in accordance with the experimental design. This study was approved by the ethics committee of the university (CEUA-UFMG; protocol number: 119/2012), and all efforts were made to minimize animal suffering.

### 2.2. Experimental design

The experiment was conducted in two stages using the protocol fully described in [31] (Suplementary Fig. S1). Before the experiment, mice were individually housed in the experimental room for 1 week (week 0) as acclimatization. In this period, animals were fed with the American Institute of Nutrition 93-Growth (AIN93G) standard diet [33]. After this initial period, the first stage of the experiment (T1) started, in which mice were randomly divided into two groups: one group was fed with the standard diet AIN93G ($n$=14) and the other was fed with HSB diet ($n$=30) [32]. After 8 weeks, the second stage (T2) started, and animals were subdivided into six groups, named in accordance with their particular treatments: AIN93G + $H_2O$ ($n$=7), AIN93G + EtOH ($n$=7), HSB + $H_2O$ ($n$=7), HSB + EtOH ($n$=7), HSB-AIN93G + $H_2O$ ($n$=8) and HSB-AIN93G + EtOH ($n$=8). Over the 4-week period of T2, three groups (+$H_2O$) had only access to water, while the remaining three (+EtOH) had a free choice between water and a 10% ethanol solution. During T2, in the HSB-AIN93G groups, the HSB diet was replaced by the AIN93G diet. In all stages, body weight was measured weekly. Animals were euthanized 1 day after the end of T2, during the light cycle.

### 2.3. Alcohol consumption and preference

During the T2 stage, alcohol and water consumption was measured daily by subtracting the initial weight by the final weight of the bottles (in grams), which had their liquids renewed after each measurement to avoid alcohol loss by evaporation. Alcohol consumption was normalized by animals' weekly weight (g/kg of body weight/24 h). Ethanol preference was determined by the percentage of ethanol consumed in relation to the total liquid consumed.

### 2.4. Adiposity index

The perigonadal adipose tissue was collected and weighed at the end of T2. The adiposity index was determined by the ratio of the weight of perigonadal adipose tissue (in grams) and the body weight of the animals (in grams) at the end of T2.

### 2.5. Feces collection

To study the gut microbiota (here represented by the fecal microbiome), feces were collected on the last day of T2, following the protocol previously described previously [34]. After collection, the fecal pellets were stored in 2-ml microtubes and then immediately frozen at −20°C until DNA extraction.

### 2.6. DNA extraction and preparation of the DNA library for sequencing the 16S rRNA gene

The extraction of bacterial DNA in feces samples was performed using the QIAamp DNA Stool Mini Kit (QIAGEN, São Paulo, Brazil) following the manufacturer's instructions to extract DNA from minimal amounts of feces, optimized for extraction of bacterial DNA. The DNA sample concentration and their purity were assessed by spectrophotometry using the DeNovix DS-11 Spectrophotometer (DeNovix, Delaware, USA). The samples were then immediately frozen in a freezer at −20°C until subsequent molecular analysis.

The library was prepared through the polymerase chain reaction (PCR) using the specific primers for the V3–V4 region of rRNA 16S: 5′ CCTACGGGRSGCAGCAG3′ (314F) [35] and 5′GGACTACHVGGGTWTCTAAT3′ (806R) [36]. The PCR contained 1 μl of DNA (20 ng/μl) from the gut microbiota, 0.3 μl of primer reverse (10 pmol/μl), 0.3 μl of primer forward (10 pmol/μl), 20.25 μl of ultrapure water, 0.5 μl of dNTPs (10 nM/μl), 2.5 μl of buffer solution (10× Dream taq, Thermo Scientific) and 0.15 μl of DNA Polymerase (Dream taq, Thermo Scientific), totaling a final volume of 25 μl. The tubes with the reaction mixture were transferred for amplification to a thermocycler with the subsequent programming: initial cycle of denaturation at 94°C for 3 min; 35 cycles of 94°C for 40 s, 60°C for 40 s and 72°C for 30 s for denaturation, annealing and extension, respectively; and final extension at 72°C for 3 min. Negative control was included as 1 μl of ultrapure water in replacement of the DNA, for monitoring possible contamination. The amplicons were purified with AMPureXP beads (Beckman Coulter, Brea, CA, USA), normalized, grouped into libraries for quantification by using KAPA Library Quantification Kit for Illumina platforms (KAPA Biosystems, Woburn, MA, USA) and sequenced by next-generation sequencing on the Illumina MiSeq platform.

### 2.7. 16S sequence analysis

Sequence data were processed and analyzed with QIIME v. 1.9.1 [37]. Initially, the raw data from the sequencing were demultiplexed, and the low-quality readings were discarded. The chimeric sequences were identified by VSEARCH v. 4.2 [38] and removed with the UCHIME algorithm [39]. The operational taxonomic units (OTUs) were grouped by cluster readings with 97% similarity using open reference in

comparison to the SILVA and SortMeRNA reference databases combined with the SUMACLUST algorithm [40] [41,42]. The taxonomy was assigned to each OTU using the SILVA reference database [41,43].

The alpha diversity was achieved by applying the Chao1 and Shannon metrics, and the main coordinate analysis [principal coordinate analysis (PCoA)] using the UniFrac weighted distance of the beta diversity associated with the Permutational multivariate analysis of variance (PERMANOVA) was performed in MicrobiomeAnalyst (www.microbiomeanalyst.ca) [44–46].

### 2.8. Bacterial translocation

Bacterial translocation was determined by the presence of bacterial DNA in animals' liver tissue at the end of T2. The total liver DNA, collected at the end of T2, was extracted utilizing the DNeasy Blood & Tissue Kit (QIAGEN, São Paulo, Brazil) in accordance with the manufacturer recommendations. Primers with the 16S rRNA gene consensus sequence, 5′AAACTCAAAKGAATTGACGG3′ (926F) [47] and 5′CTCACRRCACGAGCTGAC3′ (1062R) [47], were used to quantify bacterial DNA in these samples by real-time PCR. All reactions were conducted in the CFX 96TM Real Time System (BioRad) using the intercalating agent SYBR Green (Kapa Biosystems, São Paulo, Brazil). Serially diluted bacterial genomic DNA with factor 10 was used to generate the standard curve. To define the number of bacterial cells, the methodology proposed by [48] was applied. The bacteria 16S rRNA gene copy number of 4.9 was obtained from the rrnDB v.5.5 database (https://rrndb.umms.med.umich.edu/) [49,50]. The counts were expressed as number of bacteria present in each μg of total liver DNA.

### 2.9. Statistical analysis

Data were analyzed for Gaussian distribution using the Shapiro–Wilk normality test. Two-way ANOVA followed by *post hoc* Sidak test was used to analyze body weight on T1 and T2; adiposity index; ethanol and water consumption; alpha diversity metrics (Chao1 and Shannon index); Firmicutes/Bacteroidetes ratio; bacterial translocation to liver; and relative abundance among Firmicutes, Bacteroidetes and Actinobacteria phyla, and among Bacteroidales_S24-7, Clostridiaceae and Coriobacteriaceae families. ANOVA data are represented as *F* (between-group df, within-group df) = *F* statistic, *P* value). Linear regression analysis was carried out, and the Spearman correlation coefficient was calculated to estimate the association between bacterial groups and ethanol consumption, as well as between the adiposity index and the Firmicutes/Bacteroidetes ratio. The preference

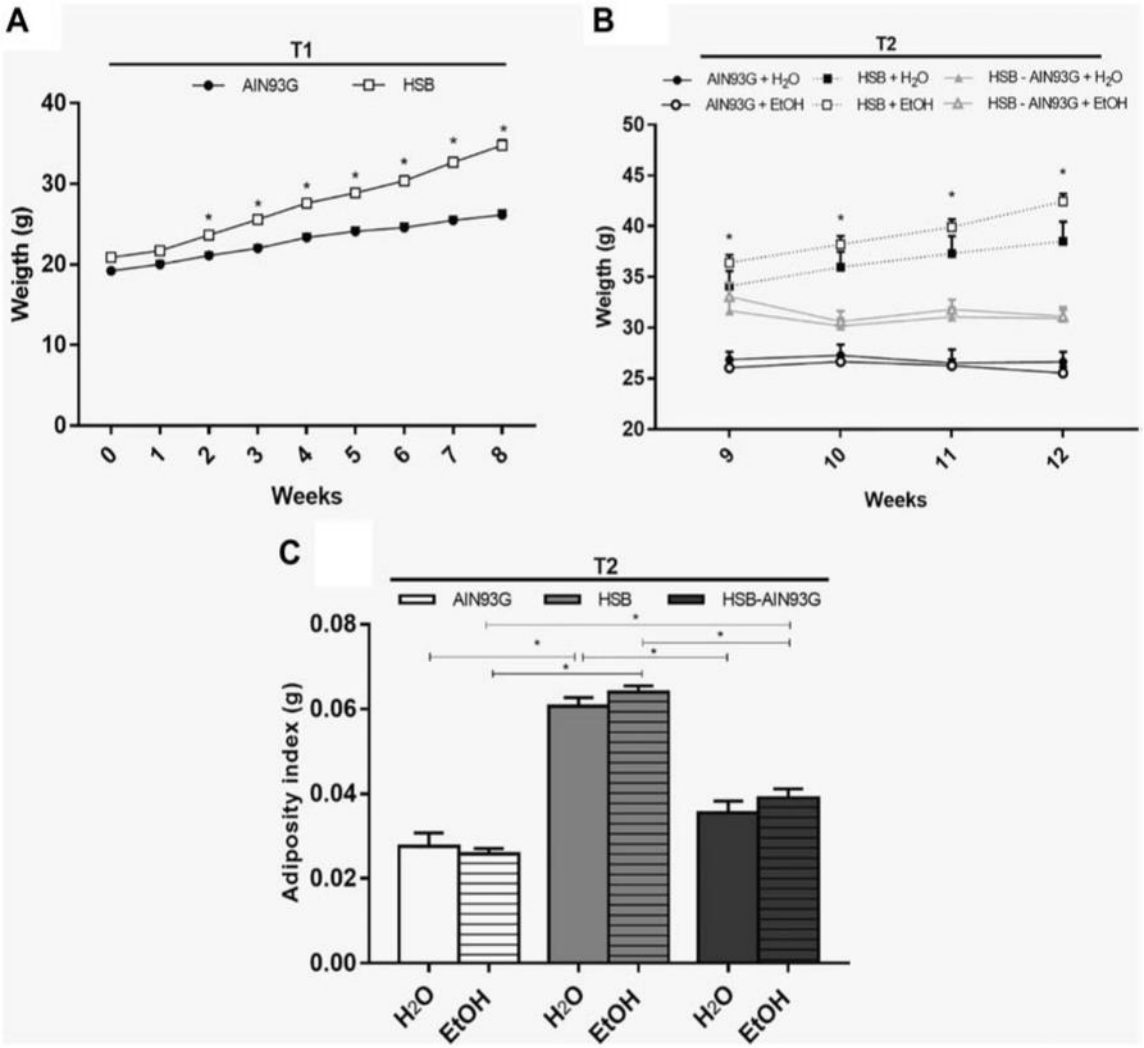


Fig. 1. Body weight (g) over the weeks and adiposity index (g). (A) Body weight during T1, *$P<.05$ for AIN93G vs. HSB. (B) Body weight during T2, week 9: *$P<.05$ for AIN93G + $H_2O$ vs. HSB + $H_2O$ and HSB-AIN93G + $H_2O$, for AIN93G + EtOH vs. HSB + EtOH and HSB-AIN93G + EtOH, for AIN93G + EtOH vs. HSB + EtOH and HSB-AIN93G + EtOH; weeks 10, 11 and 12: *$P<.05$ for AIN93G + $H_2O$ vs. HSB + $H_2O$ and HSB-AIN93G + $H_2O$, for AIN93G + EtOH vs. HSB + EtOH and HSB-AIN93G + EtOH, for HSB + EtOH vs. HSB-AIN93G + EtOH. (C) Adiposity index during T2, *$P<.05$ for AIN93G + $H_2O$ vs. HSB+ $H_2O$ and HSB-AIN93G + $H_2O$, for HSB + $H_2O$ vs. HSB-AIN93G + $H_2O$, for AIN93G + EtOH vs. HSB+ EtOH and HSB-AIN93G + EtOH, for HSB + EtOH vs. HSB-AIN93G + EtOH. Two-way ANOVA followed by Sidak *post hoc* test was used to determine statistically significant differences between the groups. Results are expressed as mean ± S.E.M. *Asterisks represent only the *post hoc* difference between groups.

for alcohol was compared with a hypothetical value of 50.1% using the Student's *t* test. Data were expressed as mean and standard error of the mean (± S.E.M.), and all analyses were performed using the GraphPad Prism version 7.01 statistical package. Differences were considered significant at $P \leq .05$ and were identified by an asterisk (*).

## 3. Results

### *3.1. The HSB diet consumption affected body weight and adiposity index, and its withdrawal affects consumption and preference for alcohol*

During T1, two-way ANOVA demonstrated that body weight was significantly affected by the type of diet [$F(1, 41) = 45.56$, $P<.0001$], by the ingestion period [$F(8,328) = 453.7$, $P<.0001$] and by the interaction of these factors [$F(8,328) = 50.62$, $P<.0001$]. The *post hoc* test evidenced that, at the end of T1, the animals of the HSB group had significantly higher ($P<.05$) body weight than the animals of the AIN93G group (Fig. 1A). At the end of T2, the two-way ANOVA showed a significant effect of the diet [$F(2,37) = 86.78$, $P<.0001$] but not of ethanol [$F(1,37) = 1.332$, $P=.2558$] on the animals' body weight. The *post hoc* test revealed that the HSB animals (HSB + $H_2O$ and HSB + EtOH) presented a significantly higher ($P<.05$) body weight in comparison with AIN93G (AIN93G + $H_2O$, AIN93G + EtOH) and HSB-AIN93G groups (HSB-AIN93G + $H_2O$ and HSB-AIN93G + EtOH). Nevertheless, the animals of the withdrawal group (HSB-AIN93G + $H_2O$ and HSB-AIN93G + EtOH) presented higher body weight when compared to the control group (AIN93G + $H_2O$ and AIN93G + EtOH) at the end of T2 (Fig. 1B).

The adiposity index, at the end of T2, corroborates the results of body weight gain in animals. The two-way ANOVA indicated that diet [$F(2,37) = 125.3$, $P<.0001$] affected the accumulation of epididymal fat in animals, but ethanol [$F(1,37) = 0.8409$, $P=.3651$] did not. The *post hoc* test showed that animals of the HSB group (HSB + $H_2O$ and HSB + EtOH) had a higher adiposity index ($P<.05$) when compared to animals of the AIN93G and HSB-AIN93G groups (AIN93G + $H_2O$, AIN93G + EtOH, HSB-AIN93G + $H_2O$ and HSB-AIN93G-EtOH). Additionally, the animals of the HSB-AIN93G + EtOH group presented a higher adiposity index ($P<.05$) in comparison with the animals of the AIN93G + EtOH group (Fig. 1C).

The two-way ANOVA showed that the type of diet [$F(2,19) = 141.7$, $P<.0001$], period of consumption [$F(27, 513) = 5.152$, $P<.0001$] and the interaction between these factors [$F(54, 513) = 5.028$, $P<.0001$] significantly affected the consumption of ethanol by the animals. The *post hoc* test showed that animals in the HSB-AIN93G + EtOH group consumed considerably higher amounts of ethanol ($P<.05$) than animals in the AIN93G + EtOH and HSB + EtOH groups. There were no differences in ethanol consumption between the AIN93G + EtOH and HSB + EtOH groups (Fig. 2A). Additionally, the animals of the HSB-AIN93G + EtOH group had a greater preference for ethanol ($t=9.781$, $P<.05$) by comparison with the

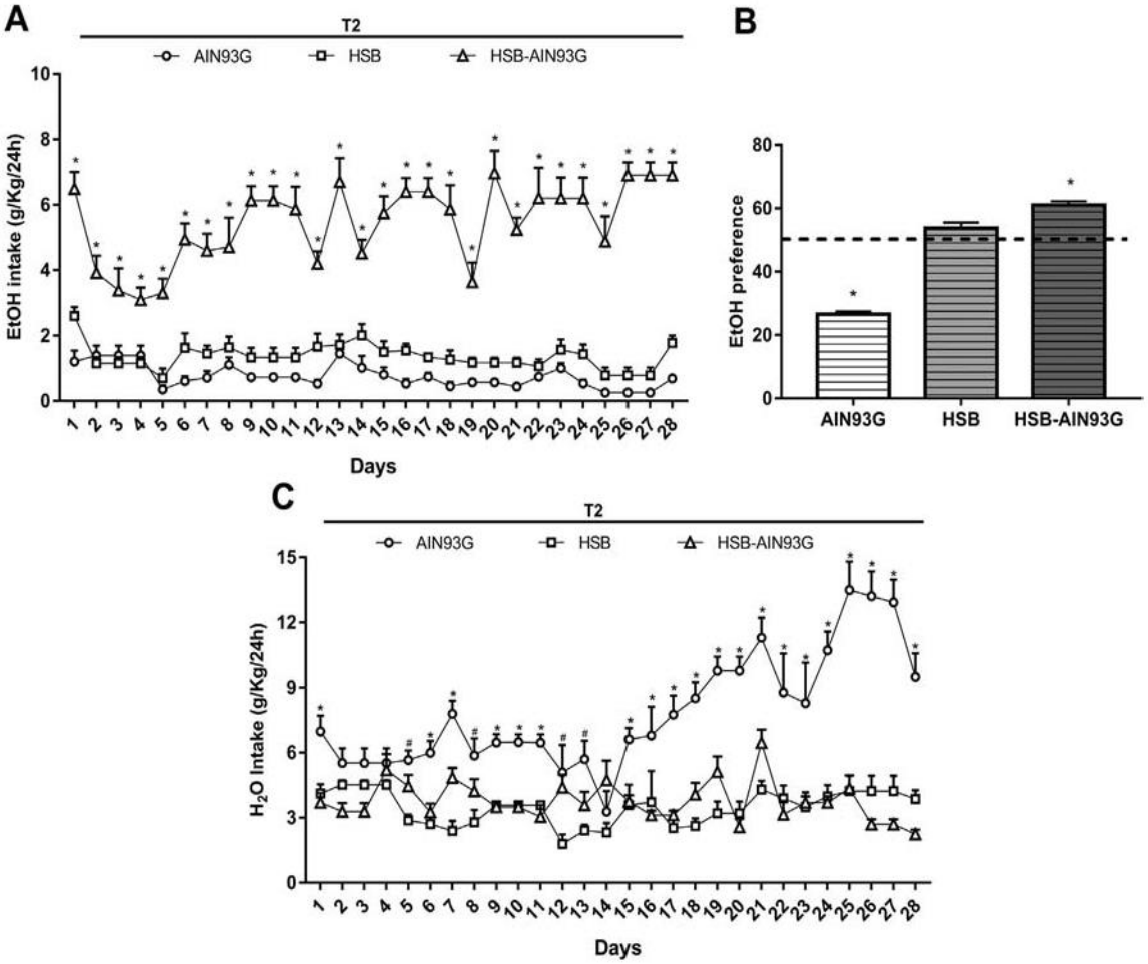


Fig. 2. Alcohol consumption and preference. (A) Alcohol consumption expressed as the ratio between alcohol intake/body weight (g/kg/24 h), *$P<.05$ for HSB-AIN93G + EtOH vs. AIN93G + EtOH and HSB + EtOH. (B) Alcohol preference (%) measured as the percentage of EtOH intake in relation to the total amount of liquid consumed (EtOH + $H_2O$), *$P<.05$ for hypothetical value of 50.1%. (C) Water consumption expressed as the ratio between water intake/body weight (g/kg/24 h), *$P<.05$ for AIN93G + EtOH vs. HSB + EtOH and HSB-AIN93G + EtOH, #$P<.05$ for AIN93G + EtOH vs. HSB + EtOH. In (A) and (C), two-way ANOVA followed by Sidak *post hoc* test was used to analyze alcohol and water consumption. In (B), Student's *t* test was used to compare the alcohol preferences with a hypothetical value of 50.1%. In (C), results are expressed as mean ± S.E.M. *Asterisks represent the statistical differences.

hypothetical value of 50.1% used to determine the preference. The animals of the AIN93G + EtOH group had no preference ($t$=30.28, $P$<.05) regarding ethanol. For the animals of the HSB + EtOH group, the choice of alcohol was not considerably different from the hypothetical value of 50.1% ($t$=2.142, $P$>.05) (Fig. 2B).

Regarding water intake, two-way ANOVA showed that time [$F$(27, 513) = 5.48, $P$<.0001], diet [$F$(2, 19) = 83.87, $P$<.0001] and its interaction with time [$F$(54, 513) = 4.964, $P$<.0001] significantly affected water consumption. *Post hoc* analysis showed that animals fed with standard diet (AIN93G + EtOH) consumed higher amounts of water ($P$<.05) in comparison with HSB + EtOH and HSB-AIN93G + EtOH groups during almost the entire T2 stage: on days 1, 6, 7, 9, 10, 11 and 15 through 28. On days 5, 8, 12 and 13, the animals of AIN93G + EtOH group consumed higher amounts of water ($P$<.05) when compared to HSB + EtOH group. No differences were observed between the HSB + EtOH and HSB-AIN93G + EtOH groups (Fig. 2C). These results show that the higher ethanol preference in the HSB-AIN93G + EtOH group in relation to HSB + EtOH group was due to ethanol intake since the groups consumed similar amount of water.

### *3.2. Consumption of the HSB diet and ethanol affected the structure of the fecal microbial community*

In the alpha diversity assessment, at the end of step T2, the Shannon index (species diversity) (Fig. 3A) and Chao1 (species richness) (Fig. 3B) of OTUs were used as metrics. The two-way ANOVA indicated a significant effect of diet and ethanol for both the Shannon index [diet: $F$(2,24) = 2.645, $P$=.0916; ethanol: $F$(1,24) = 7.034, $P$=.0139] and for Chao1 [diet: $F$(2, 24) = 4893, $P$=.0165; ethanol: $F$(1, 24) = 5204, $P$=.0317]. Nevertheless, although the *post hoc* test pointed to a significantly higher Shannon index ($P$<.05) for the HSB-AIN93G + EtOH group in relation to the HSB-AIN93G + $H_2O$ group, no difference was observed between the groups for the Chao1 metric.

The beta diversity between the OTUs at the end of T2 was calculated from the weighted UniFrac distance metric and visualized by using the PCoA (Fig. 3C). Bacterial communities were distributed into six treatment groups, and the analysis demonstrated a statistical significance of the clusters ($P$<.001 by PERMANOVA), being possible to notice an overlap between the groups AIN93 + $H_2O$, AIN93G + EtOH,

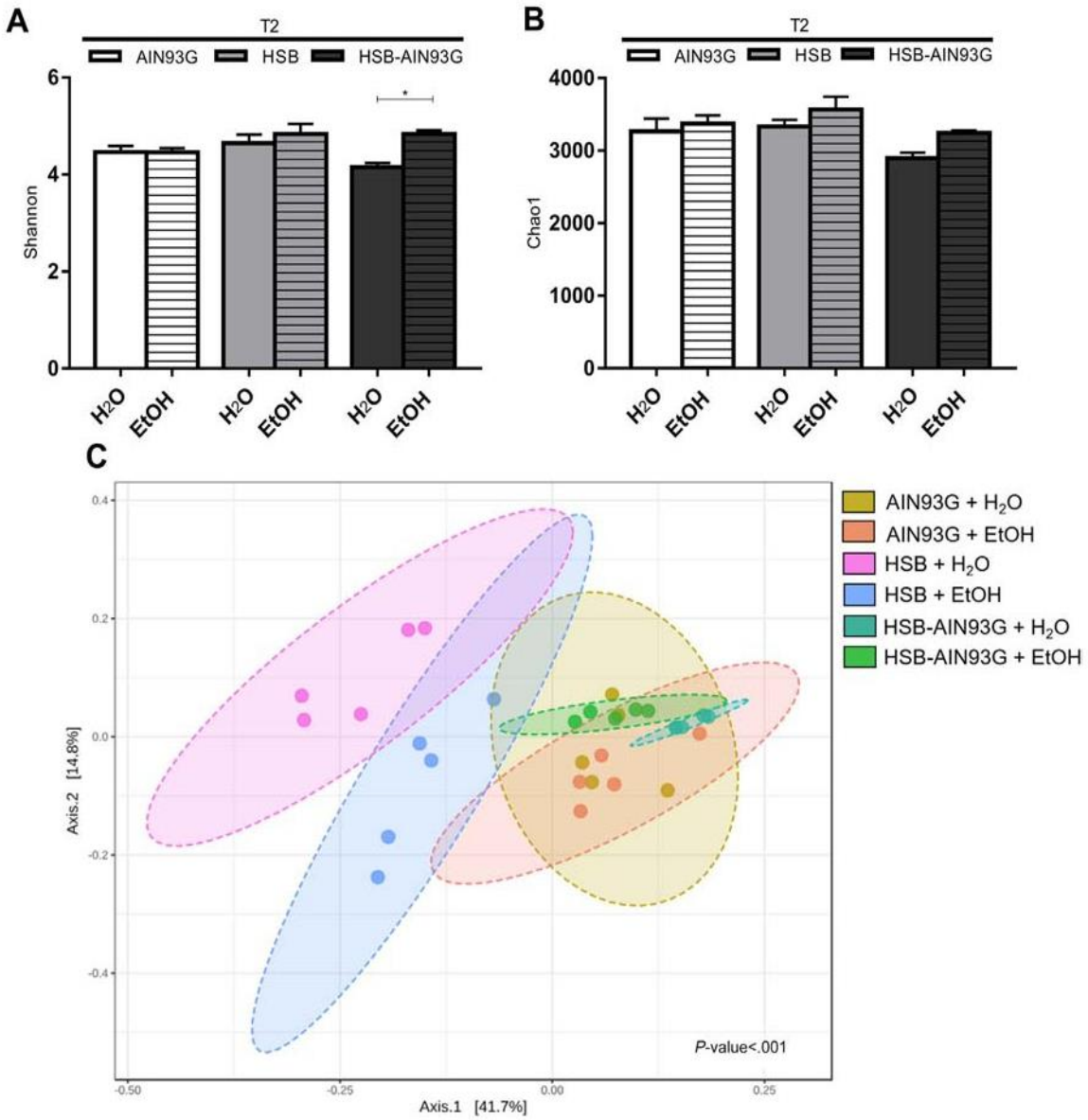


Fig. 3. Alpha and beta diversity at the end T2. (A) Chao1. (B) Shannon index, *$P$<.05 for HSB-AIN93G + $H_2O$ vs. HSB-AIN93G + EtOH. (C) PCoA plot of weighted UniFrac distance used to plot beta diversity; each dot represents one sample. In (A) and (B), two-way ANOVA followed by Sidak *post hoc* test was used to determine statistically significant differences between the groups. *Asterisks represent only the *post hoc* difference between groups.

HSB-AIN93G + $H_2O$ and HSB-AIN93G + EtOH, and a separation of the groups HSB + $H_2O$ and HSB + EtOH.

### 3.3. Consumption of the HSB diet and ethanol affected the relative abundance at the phylum and family level in the fecal microbiome

The analysis of relative abundance of the fecal microbiome indicated an important pattern of phylum composition among the experimental groups (Fig. 4A). At the end of T2, a greater number of representatives of the Actinobacteria phylum were seen in animal feces of the HSB + $H_2O$ group, while the Firmicutes phylum was more prevalent in HSB animals that had access to ethanol (HSB + EtOH). It was interesting to note that the control (AIN93G + $H_2O$ and AIN93G + EtOH) and the diet withdrawal groups (HSB-AIN93G + $H_2O$ and HSB-AIN93G + EtOH) had a similarity in the composition of their fecal microbiota.

The analysis of relative abundance indicated a predominance of the Firmicutes, Bacteroidetes and Actinobacteria phyla in the experimental groups (Fig. 4A). Fig. 4B–D shows the comparison of the abundance of phyla between the groups at the end of T2. Two-way ANOVA revealed that diet, ethanol and the interaction between them affected the relative abundance of the Firmicutes [diet: $F(2, 24) = 38.66$, $P<.0001$; ethanol: $F(1, 24) = 14.85$, $P=.0008$; interaction: $F(2, 24) = 3.813$, $P=.0365$] and Actinobacteria [diet: $F(2,24) = 21.97$, $P<.0001$; ethanol: $F(1,24) = 13.07$, $P=.0014$; interaction: $F(2,24) = 23.39$, $P<.0001$] phyla. *Post hoc* test indicated a higher abundance ($P<.05$) of Firmicutes phylum in HSB + EtOH group than in the HSB + $H_2O$, AIN93G + EtOH and HSB-AIN93G + EtOH groups. Additionally, the animals of the HSB + $H_2O$ group presented higher abundance ($P<.05$) than the HSB-AIN93G + $H_2O$ group (Fig. 4B). For the phylum Actinobacteria, the *post hoc* test showed a higher abundance in the HSB + $H_2O$ group ($P<.05$) than in the AIN93G + $H_2O$, HSB-AIN93G + EtOH and HSB + EtOH groups, respectively.

The alterations in the relative abundance of the Bacteroidetes phylum representatives were due only to the different types of diet [$F(2, 23) = 148$, $P<.0001$] and not to the consumption of ethanol [$F(1, 23) = 4.097$, $P=.0547$]. In T2, the *post hoc* test evidenced that the animals of the groups that consumed the AIN93G diet (AIN93G + $H_2O$, AIN93G + EtOH, HSB-AIN93G + $H_2O$ and HSB-AIN93G + EtOH) presented a greater abundance of this phylum in comparison to the animals that ingested the HSB diet (HSB + $H_2O$ and HSB + EtOH) (Fig. 4D).

At the end of T2, taxa distribution at a family level also presented differences in relation to experimental treatments. At this level, the predominant groups in the fecal microbiome were Bacteroidales_S24-7, Lachnospiraceae, Peptostreptococcaceae, Erysipelotrichaceae, Ruminococcaceae and Clostridiaceae (Fig. 5A). Two-way ANOVA showed that diet [$F(2, 24) = 119.5$, $P<.0001$] but not ethanol [$F(1,24) = 1.832$, $P=.1885$] affected the abundance of the Bacteroidales_S24-7 family in the experimental groups. The *post hoc* test evidenced a greater abundance ($P<.05$) of this family in the HSB-AIN93G + $H_2O$ and AIN93G + $H_2O$ groups in comparison with HSB-AIN93G + $H_2O$ group, and in the HSB-AIN93G + EtOH group ($P<.05$) in relation to the HSB + EtOH group. Furthermore, the AIN93G + EtOH group had a higher abundance of Bacteroidales_S24-7 ($P<.05$) than HSB + EtOH group (Fig. 5B).

The two-way ANOVA indicated that diet [$F(2,24) = 16.81$, $P<.0001$], ethanol [$F(1,24) = 13.76$, $P=.0011$] and the interaction

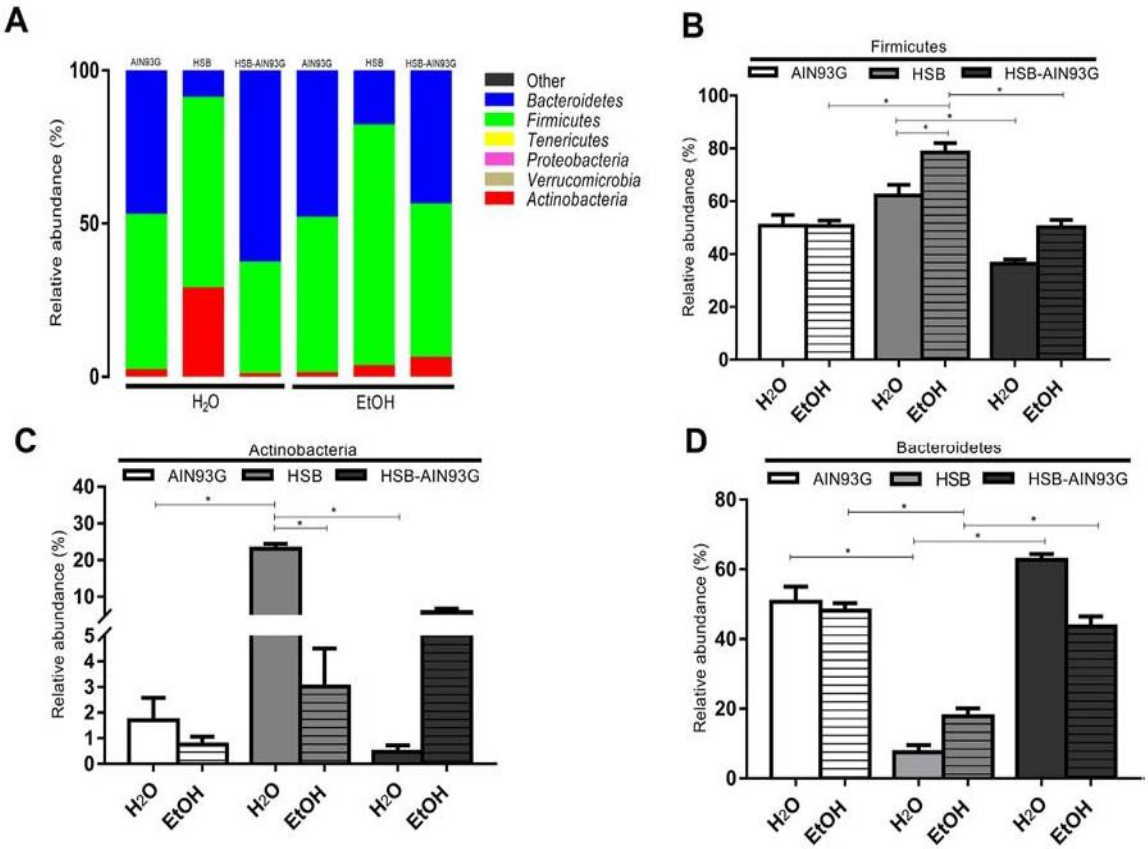


Fig. 4. Composition and relative abundance of the fecal microbiome at the phylum level at the end of T2. (A) Composition of fecal microbiome showing different communities at the phylum level; each bar represents an individual, and each color represents a bacterial phylum. (B) Relative abundance at the Firmicutes phylum, *$P<.05$ for AIN93G + EtOH vs. HSB + EtOH, HSB + $H_2O$ vs. HSB + EtOH and HSB-AIN93G + $H_2O$ and HSB + EtOH vs. HSB-AIN93G + EtOH. (C) Relative abundance at the Actinobacteria phylum, *$P<.05$ for AIN93G + $H_2O$ vs. HSB + $H_2O$, HSB + $H_2O$ vs. HSB + EtOH and HSB-AIN93G + $H_2O$. (D) Relative abundance at the Bacteroidetes phylum, *$P<.05$ for AIN93G + $H_2O$ vs. HSB + $H_2O$, AIN93G + EtOH vs. HSB + EtOH, HSB + $H_2O$ vs. HSB-AIN93G + $H_2O$, HSB + EtOH vs. HSB-AIN92G + EtOH. In (B), (C) and (D), two-way ANOVA followed by Sidak *post hoc* test was used to determine statistically significant differences between the groups. Results are expressed as mean ± S.E.M. *Asterisks represent only the *post hoc* difference between groups.

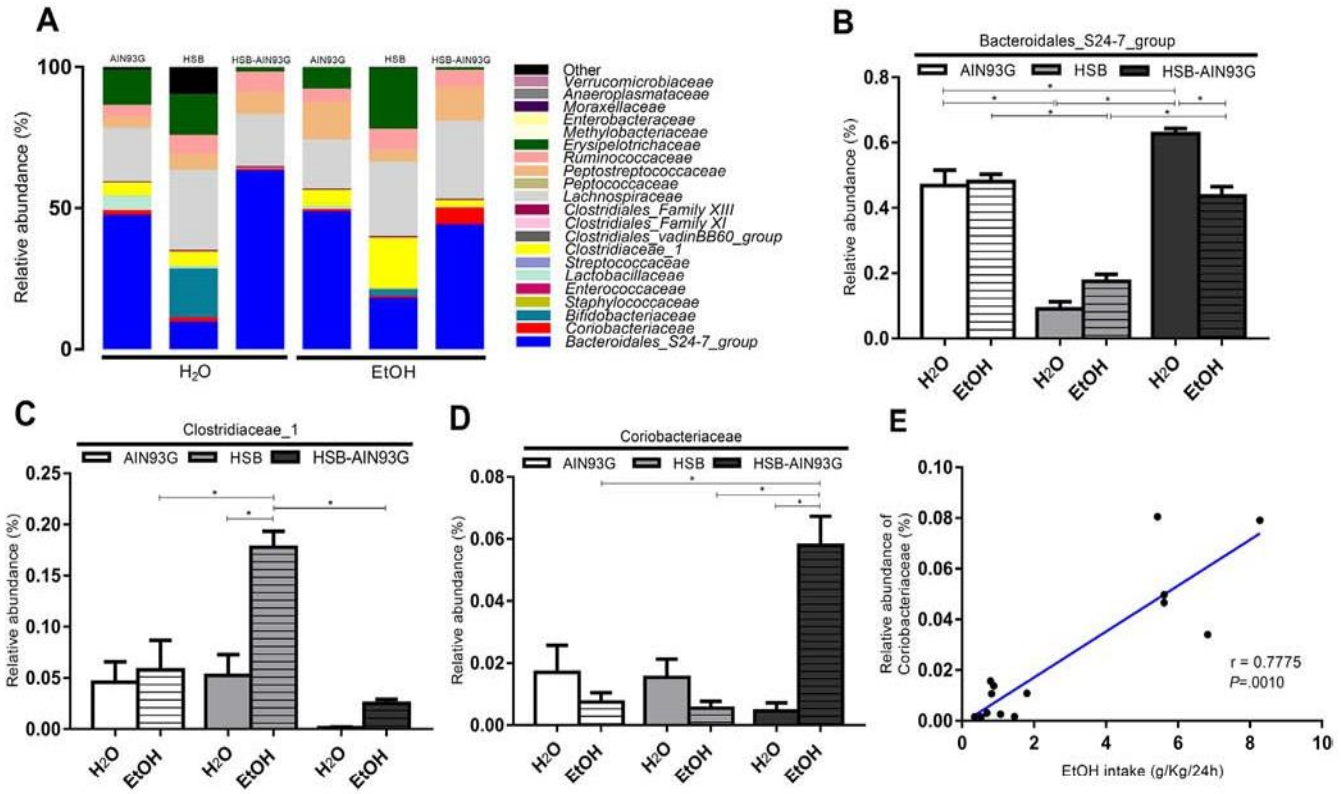


Fig. 5. Composition and relative abundance of the fecal microbiome at the family level at the end of T2. (A) Composition of fecal microbiome showing different communities at the phylum level; each bar represents an individual, and each color represents a bacterial Family. (B) Relative abundance at the Bacteroidales_S24-7 family, *$P<.05$ for AIN93G + $H_2O$ vs. HSB + $H_2O$, AIN93G + EtOH vs. HSB + EtOH, HSB-AIN93G + $H_2O$ vs. HSB + EtOH and HSB-AIN93G + EtOH. (C) Relative abundance at the Clostridiaceae family, $P<.05$ for HSB + EtOH vs. HSB + $H_2O$, AIN93G + EtOH and HSB-AIN93G + EtOH. (D) Relative abundance at the Coriobacteriaceae family, *$P<.05$ for HSB-AIN93G + EtOH vs. HSB-AIN93G + $H_2O$, HSB + EtOH and AIN93G + EtOH. (E) Spearman correlation between the relative abundance of the Coriobacteriaceae family and the consumption of ethanol at the end of T2. In (B), (C) and (D), two-way ANOVA followed by Sidak *post hoc* test was used to determine statistically significant differences between the groups. Results are expressed as mean ± S.E.M. *Asterisks represent only the *post hoc* difference between groups.

between these two factors [$F(2,24) = 6.142$, $P=.0070$] affected the relative abundance of Clostridiaceae in the experimental groups. The *post hoc* test pointed out a higher abundance ($P<.05$) of this family in the HSB + EtOH group than in the HSB + $H_2O$, AIN93 + EtOH, and HSB-AIN93G + EtOH groups (Fig. 5C). Similarly, the abundance of Coriobacteriaceae family was also affected by diet [$F(2,23) = 7.347$, $P=.0034$], ethanol [$F(1,23) = 5.207$, $P=.0321$] and the interaction between these two factors [$F(2,23) = 18.38$, $P<.0001$]. The *post hoc* test indicated that the HSB-AIN93G + EtOH group presented higher abundance ($P<.05$) of this family than the HSB-AIN93G + $H_2O$, HSB + EtOH and AIN93G + EtOH groups (Fig. 5D). Curiously, the Coriobacteriaceae family correlated positively ($r=0.7775$, $P=.0010$) with the consumption of ethanol at the end of T2 (Fig. 5E).

### 3.4. The Firmicutes/Bacteroidetes ratio was affected by the consumption of the HSB diet

The two-way ANOVA showed that the type of diet [$F(2, 23) = 38.36$, $P<.0001$] but not the consumption of ethanol [$F(1, 23) = 2.131$, $P=.1578$] affected the Firmicutes/Bacteroidetes ratio at the end of T2. The *post hoc* test found that the groups that consumed the HSB diet (HSB + $H_2O$ and HSB + EtOH) had a higher Firmicutes/Bacteroidetes ratio than the animals in the groups AIN93G + $H_2O$, AIN93G + EtOH, HSB-AIN93G + $H_2O$ and HSB-AIN93G + EtOH at the end of T2 (Fig. 6A). Additionally, the Firmicutes/Bacteroidetes ratio correlated positively with the adiposity index ($r=0.7009$, $P<.0001$) established at the end of T2 (Fig. 6B).

### 3.5. Ethanol intake resulted in bacterial translocation to the liver

At the end of T2, the presence of bacterial DNA in the liver of the animals was evaluated to investigate the possible breakage of the intestinal barrier and the consequent bacterial translocation out of this area. The two-way ANOVA indicated that ethanol [$F(1,29) = 17.44$, $P=.0002$] and not the diet [$F(2,29) = 3.264$, $P=.0527$] affected the bacterial translocation to the liver of the animals. The *post hoc test* showed that the HSB-AIN93G + EtOH group presented greater ($P<.05$) bacterial translocation to the liver than the animals in the AIN93G + $H_2O$, HSB + $H_2O$, HSB + EtOH and HSB-AIN93G + $H_2O$ groups (Fig. 7).

## 4. Discussion

Using a model of chronic consumption of a high-fat diet and ethanol intake [31], we assessed the impact of the HSB diet and its withdrawal, as well as the ethanol effects, in the fecal microbiome. After 12 weeks of treatment, we noticed that the alpha and beta diversities were affected by the diet and the consumption of ethanol. Even though the fecal microbiome had no differences regarding the species richness, the species diversity was greater in the HSB-AIN93G + EtOH group in comparison to the HSB-AIN93G + $H_2O$ group. Most interestingly, the HSB-AIN93G + EtOH group showed a higher consumption and preference for ethanol in comparison with the other groups. Considering that this result was not due to differences in diet intake that is similar between HSB-AIN93G + $H_2O$ and HSB-AIN93G + EtOH groups (Suplementary Fig.

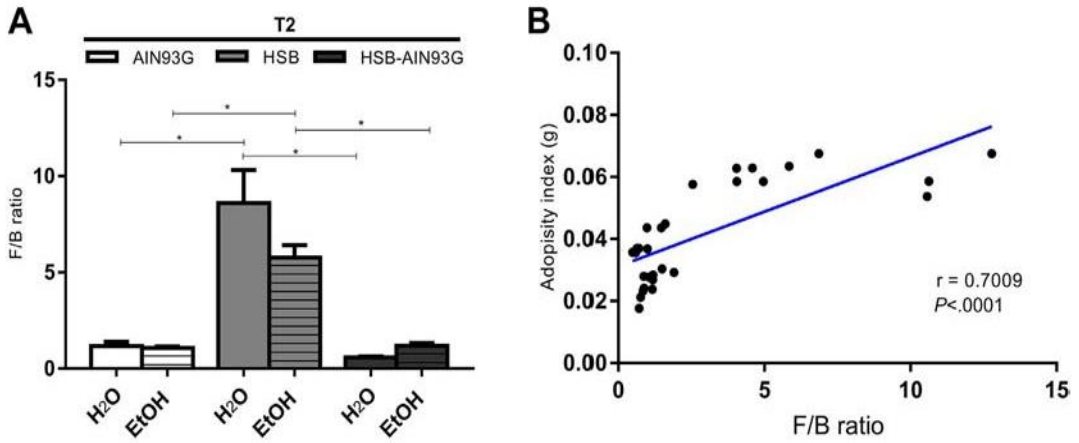


Fig. 6. Firmicutes/Bacteroidetes ratio. (A) Firmicutes/Bacteroidetes ratio at the end of T2, *$P<.05$ for AIN93G + $H_2O$ vs. HSB + $H_2O$, AIN93G + EtOH vs. AIN93G + EtOH, HSB + $H_2O$ vs. HSB-AIN93G + $H_2O$, HSB + EtOH vs. HSB-AIN93G + EtOH. (B) Spearman correlation between the Firmicutes/Bacteroidetes ratio and the adiposity index at the end of T2. In (A), two-way ANOVA followed by Sidak *post hoc* test was used to determine statistically significant differences between the groups. Results are expressed as mean ± S.E.M. *Asterisks represent only the *post hoc* difference between groups.

S2), this shows that the high ethanol consumption is a factor that can modify the alpha diversity of gut microbiota. Indeed, animal and human studies confirm that ethanol consumption can alter the microbiome diversity, mainly by leading to intestinal dysbiosis [19,22,51].

In general, the increase in microbial diversity is associated with a higher functional potential and, consequently, a greater influence of this diversity on the host's organism [52,53]. According to De Carvalho and collaborators (2018), it was assumed that the higher consumption of ethanol, verified in HSB-AIN93G + EtOH animals, may have been driven by the removal of the hypercaloric diet and the different transcriptional patterns observed for example in class II dopamine receptors [31]. Given the body of evidence that indicates the capability of the gut microbiome to influence brain function and behavior [54–57], the possibility of the microbiota to be another factor that could be driving the behavior of choice for alcohol in these animals should not be ignored.

Studies indicate that microbiota has an influence on the compulsive ethanol consumption and on decision making over drug use [18,23,24,58]. As such, Jadhav and collaborators (2018) identified multiple correlations between the behavior and dopamine receptors with the gut microbiome of dependent mice, which presented compulsive consumption of ethanol even when exposed to punishments, such as electric shocks [23]. In this regard, Leclercq and collaborators assumed that ethanol consumption can lead to dysbiosis in bacterial communities, which ultimately contributes to the process of ethanol dependence [18,25]. Particularly, it is proposed that the consumption of ethanol results in an increase of gut permeability, facilitating the passage of bacterial endotoxins into the bloodstream and their consequent interaction with the gut–brain axis [19,25]. This interaction triggers changes in the central nervous system, for instance, neuroinflammation and the metabolites production, contributing to the development of AUD [18,25,26,59]. In the current study, the HSB-AIN93G + EtOH group also showed higher bacterial translocation to the liver when compared with HSB-AIN93G + $H_2O$ group as a result of the connection between the gut and the liver. This increased bacterial translocation indicates a breaking of the gut barrier and a possible increase in intestinal permeability in this group [25,60]. Given this evidence, we can suppose that the change in bacterial diversity in the fecal microbiome and the bacterial translocation to the liver in HSB-AIN93G + EtOH animals may be associated with the increase in preference and consumption of ethanol.

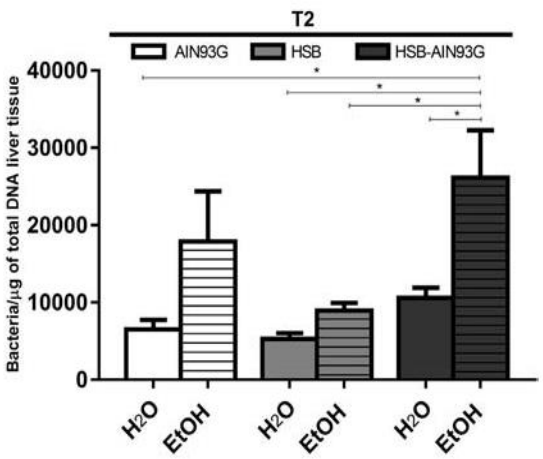


Fig. 7. Bacterial translocation to the liver at the end of T2, represented by the quantification of bacterial cells present in hepatic tissue. *$P<.05$ for AIN93G + $H_2O$ vs. HSB-AIN93G + EtOH, HSB + $H_2O$ vs. HSB-AIN93G + EtOH, HSB + EtOH vs. HSB-AIN93G + EtOH, HSB-AIN93G + $H_2O$ vs. HSB-AIN93G + EtOH. Two-way ANOVA followed by Sidak *post hoc* test was used to determine statistically significant differences between the groups. Results are expressed as mean ± S.E.M. *Asterisks represent only the *post hoc* difference between groups.

In terms of beta diversity, the PCoA demonstrated that the clusters correspond to the experimental treatments, implying that the processes that determine the composition of these bacterial communities are reproducible. The overlap noted among the HSB-AIN93G + $H_2O$, HSB-AIN93G + EtOH, AIN93G + $H_2O$ and AIN93G + EtOH groups indicates the structural similarity in their bacterial communities. Moreover, it also emphasizes how dynamic the change in microbiome is when responding to changes in diet, given that the beta diversity of the fecal microbiome in HSB-AIN93G animals is similar to that observed in AIN93G animals after the withdrawal of HSB diet and its replacement by AIN93G diet in T2. As a result, we could access the resilience in the structure of the HSB-AIN93G animals' fecal microbiome, considering that the HSB intake in T1 was a disturbance that did not permanently affect the microbiota of the group. Zhang and collaborators (2012) noticed similar results, reporting reversibility in the composition of microbiota in mice after 4 weeks of high-fat diet removal, previously administered over a period of 12 weeks [61]. In this way, our results confirm the findings on the resilient capacity of the gut microbiota in the face of specific disturbances, supporting studies that aim to understand the dynamics of the gut ecosystem.

Additionally, the reversibility of the microbiota noted in HSB-AIN93G + $H_2O$ and HSB-AIN93G + EtOH animals may be connected to the stagnation of the weight gain observed in these animals during T2. Shang and collaborators (2017) reported a stagnation in weight gain in mice submitted to a withdrawal period of 2 weeks after 5 weeks of high-fat diet consumption [62]. Such result was associated with alterations in the diversity and functional properties of the gut microbiota, mainly with the reduction of the Firmicutes/Bacteroidetes ratio, linked to weight gain and obesity [62]. Similar, in the HSB-AIN93G groups, a relationship between the reduction in the Firmicutes/Bacteroidetes ratio and the stagnation of weight in these animals was also observed.

The highest body weight and adiposity index verified in HSB groups (HSB + $H_2O$ and HSB + EtOH) may be related to Firmicutes phylum found with greater predominance in this model. It is known that the enrichment of this phylum depends on the nutrients coming from the diet and plays a role in the absorption of lipids and their accumulation, leading to an increase in body weight [63–65]. We found a higher abundance of this phylum in HSB + EtOH animals, indicating that ethanol in association with diet can increase the abundance of this phylum, which could partly explain the weight gain in this group (Fig. 4B). We also noticed a greater abundance of the Clostridiaceae family from the Firmicutes phylum in HSB + EtOH animals. Corroborating our results, representatives of this family has been found enriched in studies that employed high-fat diets and/or ethanol intake and that observed alterations in animals' weight [66–71]. In this manner, we hypothesized that, in the current study, representatives of this family may be carrying out these functions to enable the increase of weight gain and adiposity in animals in this group.

As a result of the microbial community dynamics, the high proliferation of representatives of the Firmicutes phylum in animals that consumed the HSB diet may have been responsible for the depletion of Bacteroidetes phylum representatives in these groups (HSB + $H_2O$ and HSB + EtOH). Representatives of this phylum perform key functions in the digestion of food and are associated with the health and homeostasis of the gut microbiota [68,72–75]. Within the representatives of this phylum, the Bacteroidales_S24-7 family is distinguished for its relationship with the gut microbiota eubiosis and its inverse relationship with obesity [70,76,77]. This group is also less abundant in animals that consumed the HSB diet. Indeed, the increase in the Firmicutes/Bacteroidetes ratio is associated with body weight gain and adiposity related to the diet consumption in both humans and animal models [7,52,61,78,79]. We found in our study that animals that ingested the HSB diet had a higher Firmicutes/Bacteroidetes ratio, which, in turn, presented a positive correlation with the adiposity index in these animals, confirming the studies that link this ratio to body mass gain [80,81].

The Actinobacteria phylum is associated with the maintenance of the gut barrier homeostasis and the production of acetate [82]. This phylum showed to be increased in the HSB + $H_2O$ group, confirming the data found in literature, which associate its abundance increase with high-fat diet intake [61,63,68]. Considering the association between acetate production excess with obesity, it would be plausible to consider that this phylum is also involved in weight gain with the consumption of the HSB diet.

We also observed the enrichment of the Coriobacteriaceae family (Actinobacteria phylum) in HSB-AIN93G + EtOH animals. Representatives of this family are found increased in inflammatory diseases [83–87]. Ferrere and collaborators (2017) described the overgrowth of this family in mice that developed hepatic inflammation after ethanol consumption and associated this family with a specific profile of microbiota established during ethanol consumption [71]. On the other hand, Bangsgaard Bendtsen and collaborators (2012) found representatives of the Coriobacteriaceae family increased in mice that experienced a situation of intense stress [88]. Curiously, the microbiome has been associated as a regulator in physiology and behavior relating to stress [89,90]. Stressful situations are considered as a conditioning factor in the decision making over ethanol use, and it is associated with hepatic inflammatory diseases [21,88,91]. In this way, it is possible to consider a potential relationship between this family and the preference for alcohol. Supporting this hypothesis, we also observed a positive correlation between the Coriobacteriaceae family and ethanol consumption; however, additional studies are still needed to deepen the understanding of the relationship between this group and the pathological conditions related to the high ethanol consumption.

In summary, this study demonstrates that the chronic consumption of a high-fat diet and the ethanol intake led to specific clusters in animals' fecal microbiome. This model allowed us to demonstrate that the consumption of the HSB diet and ethanol affected (a) the alpha and beta diversity of the fecal microbiome, (b) the taxon abundance at the phylum and family level in the fecal microbiome and (c) the Firmicutes/Bacteroidetes ratio. Furthermore, we pointed out that the withdrawal of the HSB diet affected the preference for alcohol and evoked a structural resilience in the fecal microbiome. Data obtained in our study contribute to the scope of evidence on the influence of diet and ethanol on the microbiome composition and its relationship with the phenotype developed. Our findings also support the concept of the association between the increase in the Firmicutes/Bacteroides ratio and the increase in body weight and adiposity index. Further studies with fecal transplant and germ-free mice may help elucidate the exact mechanism by which microbiome influences the phenotypes observed in the present study.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jnutbio.2019.07.006.

## Declaration of interest

This works was supported by grants from Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG: APQ-02285-14 and APQ-00704-14 and APQ-02942-16) and PNPD/CAPES (23038.005051/2012–48). CAPES and FAPEMIG had no role in the design, analysis or writing of this article. This manuscript was reviewed by a professional science editor and by a native English-speaking copy editor to improve readability.

## Acknowledgments

The authors thanks Jean Brunialti Godard for English support.

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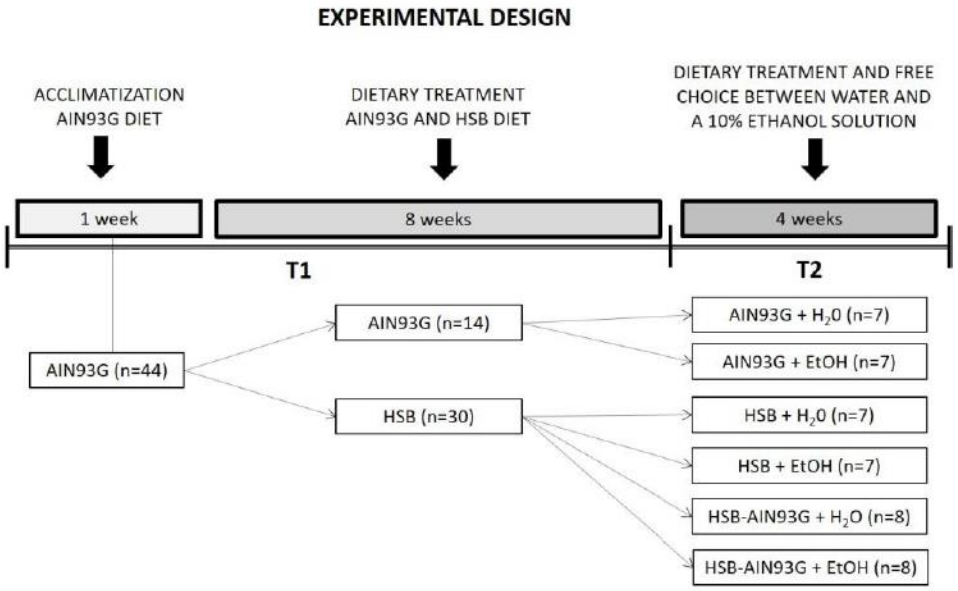
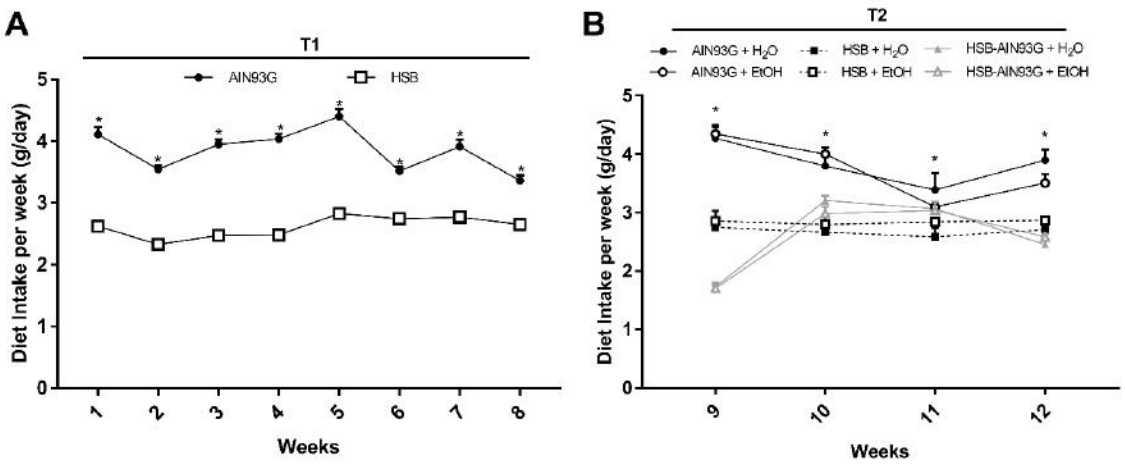
# SUPPLEMENTARY INFORMATION




**Suplementary figure S1: Experimental design.** Firstly, the animals underwent a 1-week acclimatization period and were fed with the American Institute of Nutrition 93-Growth (AIN93G) standard diet. In the following 8 weeks (stage T1) the mices were divided into two groups: one group was fed with the standard diet AIN93G (n=14) and the other was fed with High Sugar and Butter (HSB) diet (n=30). In the stage T2, with total duration of 4 weeks, the animals were subdivided into six groups, named in accordance with their particular treatments: AIN93G + $H_2O$ (n=7), AIN93G + EtOH (n=7), HSB + $H_2O$ (n=7), HSB + EtOH (n=7), HSB-AIN93G + $H_2O$ (n=8), and HSB-AIN93G + EtOH (n=8). Over the four-week period of T2, three groups (+$H_2O$) had only access to water, while the remaining three (+EtOH) had a free choice between water and a 10% ethanol solution. During T2, in the HSB-AIN93G groups, the HSB diet was replaced by the AIN93G diet. The experimental design was adapted of Carvalho et al. (2019) High-fat diet withdrawal modifies alcohol preference and transcription of dopaminergic and GABAergic receptors, Journal of Neurogenetics, 33:1, 10-20, DOI: 10.1080/01677063.2018.1526934




**Suplementary figure S2: Diet intake.** (A) Average diet intake per week (g/day) during T1, *p < 0,05 for AIN93G vs HSB. (B) Average diet intake per week (g/day) during T2, Weeks 9, 11 and 12 *p < 0,05 for AIN93G + $H_2O$ vs HSB + $H_2O$ and HSB-AIN93G + $H_2O$, for AIN93G + $H_2O$ vs HSB + EtOH and HSB-AIN93G + EtOH, for AIN93G + EtOH vs HSB + EtOH and HSB-AIN93G + EtOH, for AIN93G + EtOH vs HSB + $H_2O$ and HSB-AIN93G + $H_2O$; Week 11 *p < 0,05 for AIN93G + $H_20$ vs HSB + $H_2O$. Two-way ANOVA followed by Sidak *post hoc* test were used to determine statistically significant differences between the groups. Results are expressed as mean ± SEM. *Asterisks represent only the *post hoc* difference between groups.

# 4 CAPÍTULO 3 AVALIAÇÃO COMPORTAMENTAL E REGULAÇÃO TRANSCRICIONAL ESTRIATAL DOS GENES *Lrrk2*, *Nfat* E DE CITOCINAS EM UM MODELO ANIMAL DE CONSUMO DE DIETA RICA EM GORDURA E AÇÚCAR E LIVRE ESCOLHA POR ETANOL

## INTRODUÇÃO

A desordem do uso de álcool (AUD) é uma condição multifatorial caracterizada pelo consumo compulsivo, na qual o componente genético se configura como um importante fator de risco, sendo a herdabilidade estimada em torno de 55% [1-5]. O consumo exagerado desta droga é responsável por aproximadamente 3,3 milhões de mortes ao ano e está associado ao desenvolvimento de doenças como gastrite, hepatite, cirrose, infarto, anorexia, infecções, câncer, Parkinson, transtornos de ansiedade e demência [6-11]. Dessa forma, estudos sobre a neurobiologia e fatores desencadeadores da AUD têm um papel-chave na busca de tratamentos para essa patologia.

Uma característica normalmente observada no consumo de etanol são as alterações comportamentais [11-14]. Diversos estudos demonstram que o etanol possui propriedades de reforço positivo e negativo. O reforço positivo está associado aos efeitos estimulantes da droga [15, 16]. Enquanto, os reforços negativos estão relacionados ao consumo contínuo e excessivo do etanol, eles são provocados para remover um evento negativo, como a ansiedade e o estresse, ou para prevenir sintomas de abstinência [12, 17, 18]. De fato, Pascual e colaboradores (2015) constataram que em um modelo animal que passou pela autoadministração de etanol por 5 meses, apenas 1 dia de retirada da droga foi o bastante para induzir ansiedade nos animais. Além disso, neste estudo, foi demonstrado que a ativação do sistema imune inato pode influenciar no comportamento de busca pelo etanol [17].

São crescentes as evidências que sugerem a ativação da resposta imune inata em transtornos comportamentais, bem como no uso abusivo de etanol e nos seus danos cerebrais associados [19-22]. Neste cenário, a ativação de receptores imunes inatos do tipo *toll-like* 4 (TLR4) no cérebro durante o abuso crônico de etanol desencadeia a produção de citocinas e diversos mediadores inflamatórios [20, 23]. De fato, em camundongos TLR4-KO não é observada a indução de citocinas e quimiocinas pelo etanol, nem diferenças comportamentais durante a abstinência [17, 24]. Notavelmente, esse processo inflamatório, é visto no estriado, alterando sua funcionalidade e estimulando a busca compulsiva pela droga, mesmo diante de consequências negativas [17]. Mudanças no estriado estão associadas à compulsão, pois essa região cerebral possui um papel central nos comportamentos direcionados e objetivos; além disso, tal região integra o Sistema Dopaminérgico Mesolímbico, popularmente conhecido como sistema de recompensa [25-27].

Neste âmbito, nosso grupo de pesquisa investigou a relação da transcrição gênica no estriado com o consumo inflexível de etanol (definido pela alta preferência, mesmo após a adulteração da substância) [28]. Em nossos resultados, observamos diversos genes diferencialmente transcritos na via LRRK2 [28]. O gene principal desta via, o *Lrrk2*, produz uma AKAP (proteína de anexação de quinase) que modula a atividade da proteína quinase

A (PKA) que está envolvida na regulação dos receptores de dopamina, transcritos em neurônios que projetam para o estriado no sistema de recompensa, o qual está associado com a preferência pelo etanol [29-31].

Adicionalmente, o gene *Lrrk2* tem especial relação com o TLR4, que, quando ativado por lipopolissacarídeos (LPS), sinaliza através da proteína adaptadora MyD88 (proteína de diferenciação de resposta mieloide primária 88) que pode influenciar de forma ainda desconhecida na sua localização subcelular, na sua superexpressão e produção de citocinas inflamatórias [32, 33]. Por outro lado, o *Lrrk2* também está associado com o fator nuclear de células T ativadas (NFAT), responsável tradicionalmente pela produção de citocinas inflamatórias via sinalização de cálcio, sendo assim um importante mediador da resposta imune [34-36]. A sinalização de NFAT é inibida pelo RNA não codificador repressor de NFAT (NRON), um complexo composto de 11 proteínas, das quais 5 são associadas à LRRK2 [37]. De fato, LRRK2 é relatado como um regulador negativo de NFAT que pode inativar sua função e bloquear sua resposta [32, 33, 36]. Dessa forma, podemos afirmar que o gene *Lrrk2* está envolvido na resposta imune, porém essa relação ainda não está clara e mais estudos são necessários para sua compreensão.

Embora o etanol exerça grandes efeitos sobre o organismo, a maioria dos estudos realizados até o momento se concentram na neurobiologia do alcoolismo, evidenciando a influência do consumo de etanol em circuitos cerebrais relacionados a tomada de decisão e processamento de recompensa [1, 3]. Pouco se investiga sobre a regulação de genes associados ao consumo de etanol em associação a genes relacionados ao sistema imune e importantes na inflamação como das citocinas IL-6, IL1β e IL-10, e de sinalizadores como iNOS e o próprio NFAT.

Com base no exposto, é evidente que há uma interação entre consumo/preferência de etanol, o *Lrrk2* e a resposta imune inata no estriado. Em um modelo de consumo de dieta rica em gordura e açúcar (HSB) e etanol desenvolvido por nosso grupo de pesquisa, observamos que, ao se retirar a dieta HSB e expor camundongos ao paradigma da livre escolha, há um aumento no consumo e preferência de etanol nesses animais [38]. Neste trabalho, suscitamos a possibilidade de esse aumento estar sendo direcionado via regulação transcricional dos receptores de dopamina. Considerando a relação desses receptores com o gene *Lrrk2* e deste com a resposta imune, é plausível considerar a possibilidade de que esta interação esteja associada com o alto consumo e preferência por etanol nesse modelo. Assim, o presente estudo objetiva avaliar a relação entre *Lrrk2* e o sistema imune e como isso se associa com o consumo e preferência de etanol em um modelo de consumo de dieta rica em gordura (HSB) e livre escolha de etanol. Neste contexto, para melhor compreensão do papel do sistema imune no consumo de etanol, aplicaremos o modelo descrito

anteriormente, também em animais *knockout* (KO) para a citocina IL6 e para o fator de transcrição NFAT.

## METODOLOGIA

### Animais

Oitenta camundongos C57BL/6, machos, SPF, foram fornecidos pelo biotério central da Universidade Federal de Minas Gerais (UFMG) com seis semanas de idade. Outros quarenta, vinte C57BL/6 *Il6* KO e vinte C57CLBL/6 *Nfat* KO, machos, foram disponibilizados pelo Laboratório de Imunologia de Doenças Infecciosas da UFMG. Somente espécimes machos foram usados a fim de evitar a interferência da flutuação hormonal presente em fêmeas. Durante as 12 semanas de experimento, os animais foram individualizados em mini-isoladores acomodados em rack ventilada (ALESCO, São Paulo, Brasil) com ciclo claro/escuro de 12 horas. Eles tiveram livre acesso à dieta, água e/ou a uma solução de etanol (EtOH) 10%, conforme desenho experimental a seguir. O projeto foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Federal de Minas Gerais (CEUA-UFMG), protocolo 119/2012. Todos os esforços foram feitos para garantir o bem-estar animal.

### Desenho experimental

O experimento foi performado em duas etapas, conforme protocolo descrito detalhadamente em [1, 2]. A primeira etapa (T1) durou 8 semanas, onde os camundongos foram divididos aleatoriamente em dois grupos: os que foram alimentados com a dieta controle *American Institute of Nutrition 93-Growth* (AIN93G) (n=20) e os que receberam a dieta *High Sugar and Butter* (HSB) (n=70) [3, 4]. Os grupos foram nomeados conforme dieta consumida: AIN93G e HSB.

A segunda etapa (T2) durou 4 semanas, nas quais os animais foram subdivididos em dez grupos, nomeados segundo seu tratamento específico e *status* genético: AIN93G + $H_2O$ (n=10), AIN93G + EtOH (n=10), HSB + $H_2O$ (n=10), HSB + EtOH (n=10), HSB-AIN93G + $H_2O$ (n=10), HSB-AIN93G +EtOH (n=10), HSB-AIN93G *Il6* KO + $H_2O$ (n=10), HSB-AIN93G *Il6* KO + EtOH (n=10), HSB-AIN93G *Nfat* KO + $H_2O$ (n=5*) e HSB-AIN93G *Nfat* KO + EtOH (n=5*). Nesse período, cinco grupos (+$H_2O$) tiveram acesso somente à água, enquanto os outros cinco (+EtOH) tiveram livre escolha entre a água e uma solução de etanol a 10%. Nos grupos HSB-AIN93G a dieta HSB foi trocada pela dieta AIN93G ao se iniciar T2. A Figura 1 ilustra o desenho experimental.

***Observação**: Ainda não completamos o n experimental dos grupos *Nfat* KO. Por isso, não temos resultados referentes a *qPCR* para os grupos HSB-AIN93G *Nfat* + $H_2O$ e HSB-AIN93G *Nfat* + ETOH.

Durante todo o protocolo experimental, o peso corporal dos camundongos foi mensurado semanalmente. Durante T2, foi observada a ingestão de etanol e água diariamente. Os espécimes foram eutanasiados um dia após o final de T2, durante o ciclo claro. Na eutanásia, foram coletados o tecido adiposo perigonadal e o baço de todos os camundongos. Ademais, foram coletados os cérebros de 40 animais (4 de cada grupo) para a realização da Citometria de fluxo e dos outros 60 foi extraído o estriado, utilizado nas análises moleculares.

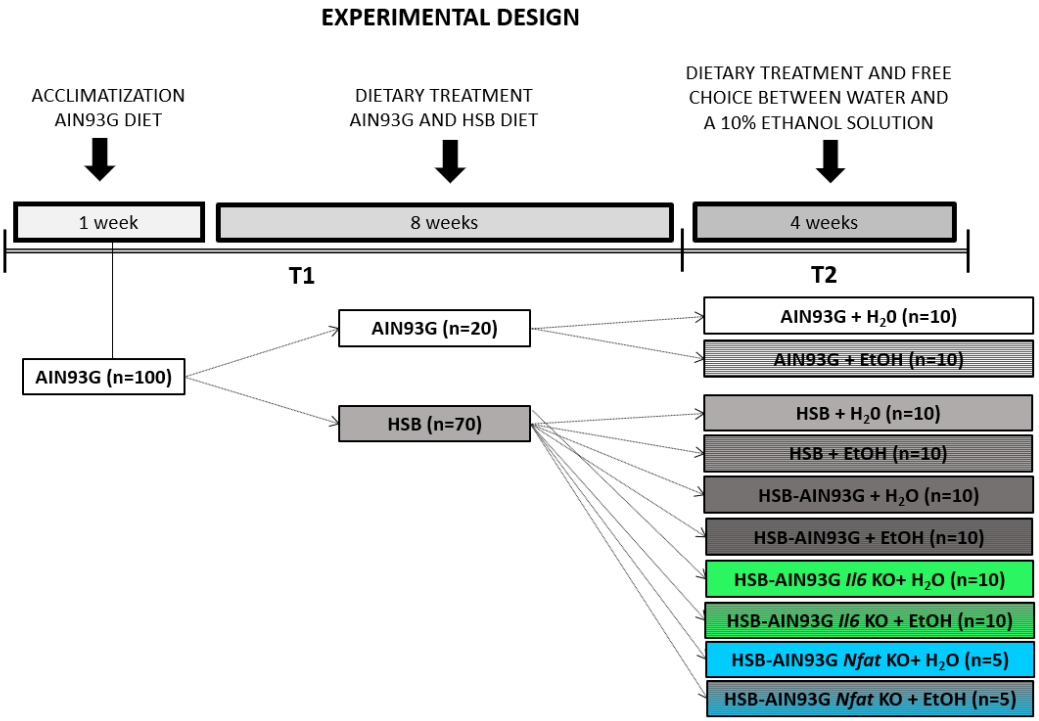



**Figura 1**: **Desenho experimental**. Primeiramente, os animais passaram por um período de aclimatação de 1 semana e foram alimentados com a dieta padrão *American Institute of Nutrition 93-Growth* (AIN93G). Nas 8 semanas seguintes (estágio T1) os camundongos foram divididos em dois grupos: um grupo foi alimentado com a dieta padrão AIN93G (n=20) e o outro foi alimentado com dieta rica em açúcar e gordura (HSB) (n=70). No estágioT2, com duração total de 4 semanas, os animais foram subdivididos em dez grupos, nomeados de acordo com seus tratamentos particulares: AIN93G + $H_2O$ (n=10), AIN93G + EtOH (n=10), HSB + $H_2O$ (n=10), HSB + EtOH (n=10), HSB-AIN93G + EtOH (n=10), HSB-AIN93G + $H_2O$ (n =10), HSB-AIN93G *Il6* KO + $H_2O$ (n =10), HSB-AIN93G *Il6* KO + EtOH (n =10), HSB-AIN93G *Nfat* KO + $H_2O$ (n =5) e HSB-AIN93G *Nfat* KO + EtOH (n = 5).. Durante o período de quatro semanas de T2, cinco grupos (+$H_2O$) tiveram acesso apenas à água, enquanto os cinco restantes (+EtOH) tiveram livre escolha entre água e solução de etanol a 10%. Durante o T2, nos grupos HSB-AIN93G, a dieta HSB foi substituída pela dieta AIN93G.

## Índice de adiposidade

Após coletados, os tecidos adiposos perigonadais foram lavados com solução salina e pesados para determinação do índice de adiposidade, calculado pela razão entre o peso do tecido adiposo perigonadal (em gramas) e o peso corporal dos animais (em gramas).

**Consumo e preferência pelo etanol**

As medidas foram feitas conforme [38, 39]. O consumo diário de água e álcool foi estabelecido pela subtração do peso inicial pelo peso final das garrafas (em gramas) e então dividido pelo peso do animal na semana. As garrafas com etanol a 10% tiveram seus líquidos trocados após cada medição para contornar a perda da substância por evaporação. A preferência foi determinada quando a porcentagem de álcool ingerido em relação ao total de líquido consumido constituiu valor estatisticamente maior que o hipotético de 50,1%.

**Testes comportamentais**

O comportamento dos animais foi observado a partir dos testes *Marble burying* e o da caixa claro/escuro. No penúltimo dia de T2, os espécimes foram submetidos ao teste *Marble burying*, que é classicamente utilizado com o objetivo de investigar o comportamento do tipo impulsivo e obsessivo-compulsivo [42-44]. O teste e sua análise foram realizados conforme [38, 45]. Nesse processo, cada camundongo foi colocado individualmente em uma gaiola padrão forrada com 5 cm de serragem. Foram dispostas 18 bolas de vidro em três filas de seis unidades, distribuídas uniformemente. Os animais foram avaliados quanto ao seu comportamento de enterrar após 10 minutos, quando foram contadas o número de bolas com pelo menos 2/3 de seu tamanho encobertos por serragem. Essa contagem foi conduzida por dois pesquisadores independentes, estabelecendo-se, então, uma média de bolas enterradas que foi utilizada para mensurar o comportamento obsessivo-compulsivo de cada animal [42].

O teste da caixa/claro escuro foi realizado conforme descrito por [46]. Esse teste, baseado na aversão natural do camundongo a locais abertos e iluminados e no comportamento exploratório espontâneo de roedores em resposta a estressores leves, tem como função estudar o comportamento semelhante à ansiedade [47]. Inicialmente, no último dia de T2, cada espécime foi alocado no compartimento escuro da caixa, que conta com passagens para possibilitar o trânsito livre entre esta área e a clara. Os camundongos foram filmados durante os 5 minutos em que exploraram essas seções e os vídeos foram observados no *software* EthoVision® XT versão 12 (Noldus Information Technology, Utrecht, The Netherlands) [48]. Foram registrados o tempo gasto no compartimento claro, latência, número de transições e a distância percorrida na parte clara. Nesse cenário, menor tempo no compartimento claro e maior latência associa-se ao comportamento semelhante à ansiedade nos animais [47, 49].

**Extração de leucócitos cerebrais e citometria de fluxo**

A extração de leucócitos do celebro foi adaptada de [50]. Uma vez coletados, os cérebros foram macerados e mantidos em tubos Falcon (50ml) com 4ml de meio DMEM suplementado com colagenase D a 250µg/ml em estufa de $CO_2$ a 37ºC por 45 minutos. Então, foi adicionado 10ml de DMEM com EDTA (2Mm) e as amostras foram centrifugadas a 450g a 4ºC por 5 minutos. O sobrenadante foi descartado e as células foram ressuspensas em Percoll 37%. Essa suspensão foi adicionada em outro tudo Falcon (15ml) contendo 3ml de Percoll 60% e centrifugada a 950g a 24ºC por 20 minutos. Após centrifugação, o anel de células mononucleares foi coletado, transferido para tubos Falcon (15ml) com 10ml de DMEM completo e novamente centrifugado a 450g por 5 minutos. Finalmente, as amostras foram ressuspensas em 200µl de PBS com 0,2% de soro fetal bovino. As células foram contadas em microscópio óptico, com câmara de Neubauer.

As células isoladas do cérebro foram plaqueadas em uma quantidade aproximada de $1x10^6$. Em seguida, foi adicionado 10µl por poço do coquetel de anticorpos monoclonais anti-marcadores fenotípicos conjugados com os fluorocromos FITC, PercP-cy5.5, PE-Cy7, APC, AmCyan e biotina (sendo, essa última, ligada à estreptavidina Pacific Blue posteriormente). As células foram incubadas a 4ºC por 30 minutos, no escuro, foram lavadas com 100µl por poço de PBS-BSA-NaN3 e centrifugadas a 1200rpm por 10 minutos. O sobrenadante foi descartado e, então, o procedimento de lavagem foi repetido. Posteriormente, as células marcadas com anticorpo biotina foram submetidas a uma nova incubação (30 minutos) com estreptavidina-Pacific Blue e lavadas mais 2 vezes, conforme descrito. Por fim, elas foram ressuspendidas em 200µl de solução fixadora (0,5% de formaldeído em PBS1X) e mantidas a 4ºC, no escuro, até o dia seguinte.

Os anticorpos utilizados foram: antiCD11b (FITC); anti-F4/80 (PE-Cy7), antiCD45.2 (biotina + estreptavidina Pacific Blue) e marcador de viabilidade celular (AmCyan). A leitura foi realizada usando o FACS Fortessa (Beckton Dickinson, Mountain View, Califórnia), e as análises conduzidas através do programa FlowJo (Tree Star Inc).

**Desenho de primers e quantificação relativa por PCR em tempo real (qPCR)**

O desenho dos *primers* quando necessário foi realizado conforme descrito por [38]. A tabela 1 descreve as sequências (5'→ 3') dos genes alvos utilizados nesse estudo. Para as análises de *qPCR* o RNA total do estriado foi extraído conforme descrito por [38]. A concentração e pureza do RNA foram investigadas com um espectrofotômetro DeNovix DS-11 (Delaware, EUA). A integridade do RNA foi visualizada em gel de agarose a 1%, corado com GelRed (Biotium, Califórnia, EUA). A transcrição reversa foi performada com *primers* oligo (Dt20) (Prodimol Biotecnologia, Belo Horizonte, Brasil), dNTP mix (10mM), Reaction Buffer 5X (Thermo Fisher Scientific, São Paulo, Brasil) e M-MLV Reverse

Transcriptase (Promega Biotecnologia, São Paula, Brasil), conforme orientações do fabricante. A quantificação relativa dos transcritos dos genes alvo foram realizadas conforme [2]. As quantificações relativas foram calculadas pelo método delta-delta Ct [14]. Os genes normalizadores utilizados foram *Gapdh* (Glyceraldehyde-3-Phosphate Dehydrogenase) e *Ppia* (Peptidylprolyl isomerase A) [52, 53].

**Tabela 1**: Sequencias (5'→ 3') do primers usados para real-time PCR (qPCR)

| ***Gene*** | **Forward** | **Reverse** | **Referência** |
|---|---|---|---|
| *Lrrk2* | TTCCCCACCAATGAAAACAT | AAGGCTGCGTTCTCAGGATA | *This Study* |
| *Nfat* | CAGTGTGACCGAAGATACCTGG | TCGAGACTTGATAGGGACCCC | [54] |
| *Il6* | CTCTGGGAAATCGTGGAAATG | AAGTGCATCATCGTTGTTCATACA | [55] |
| *Il1β* | CACTCATTGTGGCTGTGGAGAA | CCACGGGAAAGACACAGGTAG | [55] |
| *Il10* | GCTCTACTGACTGGCATGAG | CGCAGCTCTTAGGAGCATGTG | [56] |
| *iNOS* | AGCACTTTGGGTGACCACCAGGA | AGCTAAGTATTAGAGCGGCGGCA | [56] |
| *Gapdh* | AGGAGCGAGACCCCACTAAC | GTGGTTCACACCCATCACAA | [52] |
| *Ppia* | AATGCTGGACCAAACACAAA | CCTTCTTTCACCTTCCCAAA | [52] |

**Análises estatísticas**

Os dados foram avaliados quanto a sua distribuição com o teste de normalidade Shapiro-Wilk. Eles foram expressos por média ± SEM. ANOVA de duas vias, seguida pelo teste *post hoc* Tukey, foi empregada na análise do peso corporal em T1 e T2, índice de adiposidade, consumo de etanol, testes comportamentais, porcentagem de células marcadas na citometria de fluxo e quantificação relativa de transcritos de genes selecionados no estriado. Os dados da ANOVA são representados como F (entre o grupo df, dentro do grupo df) = estatística F, valor p). O Teste *Mann-Whitney* foi aplicado para comparar a preferência pelo etanol com o valor hipotético de 50,1%. Correlação de *Spearman* com regressão linear simples foi executada para estudar a relação entre os níveis transcricionais de *Nfat* e *Lrrk2*. Todas as análises foram conduzidas no pacote estatístico *GraphPad Prism*, versão 9.0.2 (GraphPad Software, Inc. San Diego, EUA). O nível de significância foi de $p < 0,05$, sendo indicado por um asterisco (*).

## RESULTADOS

### O consumo da dieta HSB afetou o peso corporal e o índice de adiposidade e sua retirada afetou o consumo e a preferência por etanol

O desenho experimental performado no presente estudo consiste numa reprodução com 4 grupos extras de camundongos *knockout* (*Il6* e *Nfat* KO) dos trabalhos de Martins de Carvalho e Colaboradores (2018) e Moreira Júnior (2019), resultando, com os mesmos tratamentos e tempos, em um modelo animal com as mesmas características apresentadas nesses estudos [38, 39]. Desse modo, a ANOVA de duas vias demonstrou que o peso corporal em T1 foi afetado significativamente pela dieta ($F (1, 89) = 9.087$, $p = 0.0034$), pelo

período de ingestão (F (2.338,208.1) = 228.9, p < 0.0001) e pela interação desses fatores (F (8, 712) = 9.110, p < 0.0001). Já ao final de T2, o tratamento empregado (F (9, 81) = 20.73, p < 0.0001), o tempo de experimento (F (1.901, 154.0) = 13.44, p < 0.0001) e a interação entre esses aspectos (F (24, 243) = 11.03, p < 0.0001) tiveram influência sobre o ganho de peso dos camundongos. O teste *post hoc* revelou que os animais que ingeriram a dieta HSB durante todo o experimento tiveram peso corporal significativamente maior (p < 0.05) em relação aos outros grupos, que ingeriram somente a dieta AIN93G ou que em T2 a dieta HSB foi trocada para a formulação AIN93G (Figuras 2A e 2B). Ainda, em T2, os animais do grupo HSB-AIN93G + $H_2O$ apresentaram maior peso corporal (p < 0.05) em comparação aos do HSB-AIN93G *Nfat* KO+ $H_2O$ (Figura 2B).

Além disso, a ANOVA de duas vias apontou que o índice de adiposidade, o qual mostra o acúmulo de gordura perigonadal dos animais, foi influenciado pela dieta (F (4, 81) = 30.40, p < 0.0001) e não pelo etanol (F (1, 81) = 0.02760, p = 0.8685), sendo que o teste *post hoc* indicou que espécimes do grupo HSB (HSB + $H_2O$ e HSB + EtOH) tiveram maior índice de adiposidade (p < 0.05) quando comparados aos animais dos grupos AIN93G e HSB-AIN93G (AIN93G + $H_2O$, AIN93G + EtOH, HSB-AIN93G + $H_2O$, HSB-AIN93G + EtOH, HSB-AIN93G *Il6* KO + $H_2O$, HSB-AIN93G *Il6* KO + EtOH, HSB-AIN93G *Nfat* KO+ $H_2O$ e HSB-AIN93G *Nfat* KO + EtOH) (Figura 2C).

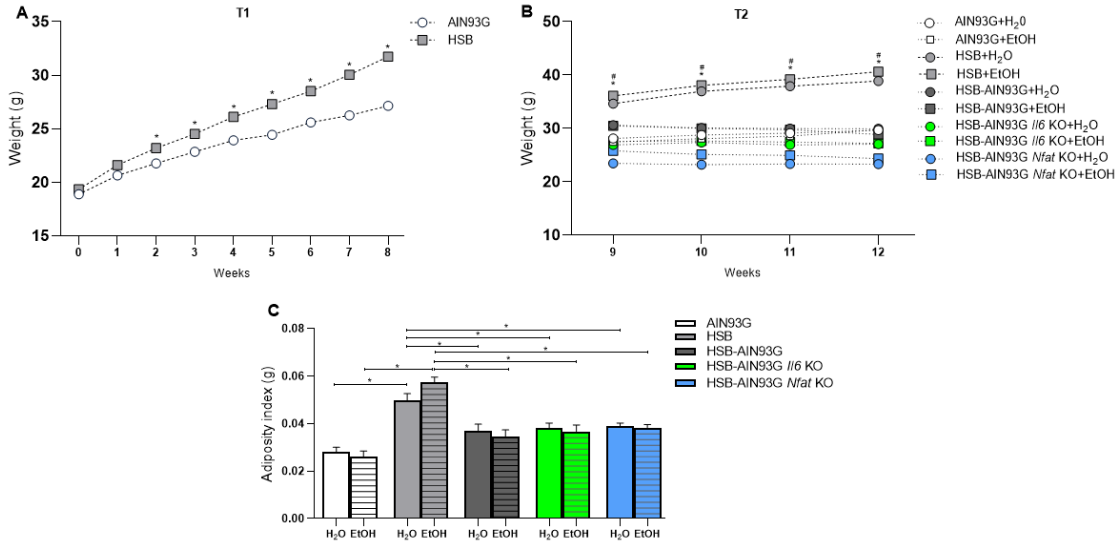



**Figura 2: Peso corporal e índice de adiposidade.** (A) Peso corporal (g) em T1. (B) Peso Corporal (g) em T2. (C) Índice de adiposidade (g). Resultados são expressos como média ± SEM. Análises performadas com ANOVA de duas vias seguida por pós *post hoc Tukey*. Em (A) *p < 0,05 para AIN93G *vs* HSB. Em (B), *p < 0,05 para HSB + $H_2O$ e HSB + EtOH *vs* AIN93G + $H_2O$, AIN93G + EtOH, HSB-AIN93G + $H_2O$, HSB-AIN93G + EtOH, HSB-AIN93G *Il6* KO + $H_2O$, HSB-AIN93G *Il6* KO + EtOH, HSB-AIN93G *Nfat* KO+ $H_2O$ e HSB-AIN93G *Nfat* KO e $^{\#}$p < 0,05 para HSB-AIN93G + $H_2O$ *vs* HSB-AIN93G *Nfat* KO+ $H_2O$. Asteriscos (*) e (#) representam as diferenças do teste *post hoc* entre os grupos.

Em relação ao consumo diário de etanol, a ANOVA de duas vias exibiu que o tipo de dieta (F (4, 41) = 69.56, p < 0.0001) e o período de consumo da bebida (F (12.18, 499.5) = 3.559,

p < 0.0001) afetaram significamente os animais. Após a troca da dieta HSB pela AIN93G ao final de T1, o teste *post hoc* anunciou que os espécimes dos grupos HSB-AIN93G + EtOH, HSB-AIN93G *Il6* + EtOH e HB-AIN93G *Nfat* KO + EtOH consumiram álcool de forma semelhante (p > 0.05), exceto nos dias 11, 17, 20, 25, 27 e 28, nos quais, os camundongos do grupo HB-AIN93G *Nfat* KO + EtOH ingeriram maior quantidade da droga que os animais do grupo HSB-AIN93G *Il6* KO + EtOH. Todos os grupos com troca de dieta (HSB-AIN93G + EtOH, HSB-AIN93G *Il6* + EtOH e HB-AIN93G *Nfat* KO + EtOH) consumiram quantidades significativamente maiores da substância (p < 0.05) em relação aos animais dos grupos AIN93G + EtOH e HSB + EtOH, que não apresentaram diferenças entre si (Figura 3A). A preferência pelo etanol em relação à água também foi demonstrada como sendo maior (p < 0.0001) entre os espécimes dos grupos HSB-AIN93G + EtOH, HSB-AIN93G *Il6* + EtOH e HB-AIN93G *Nfat* KO + EtOH. Enquanto isso, os camundongos do grupo AIN93G + EtOH não demonstraram preferência pela bebida (p < 0.0001) e os do HSB + EtOH não indicaram resultado significativo (p = 0.1138) em comparação ao valor hipotético de 50,1%. (Figura 3B).

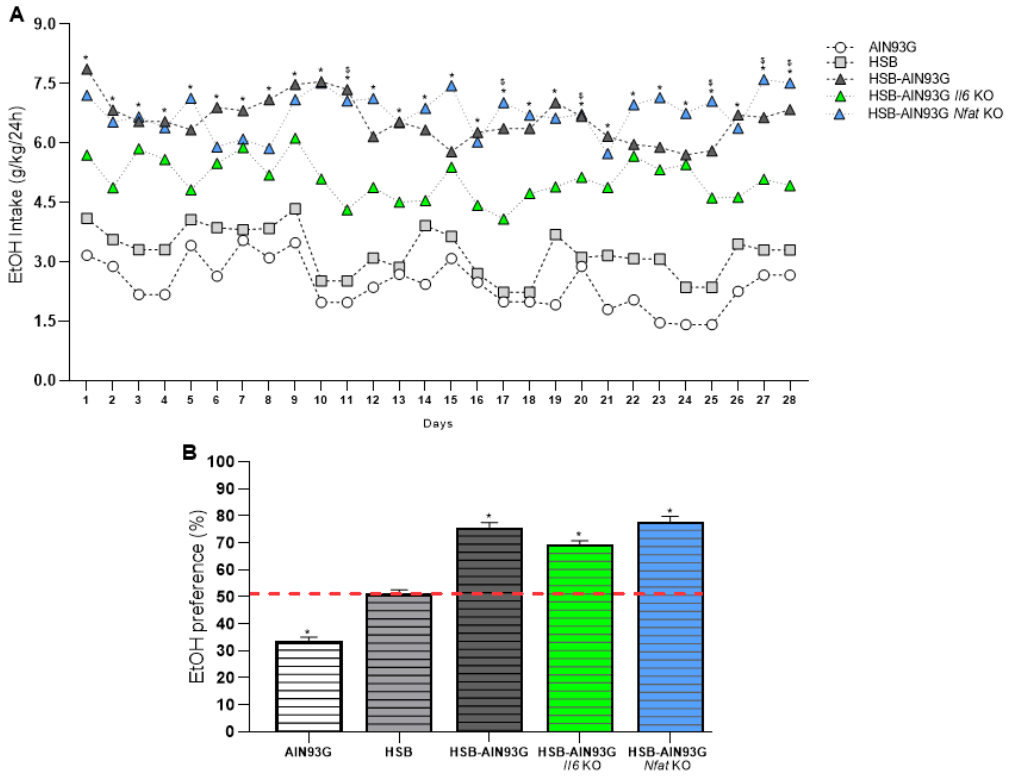



**Figura 3**: **Consumo diário e preferência por etanol.** (A) Consumo de etanol expresso pela razão entre o consumo diário e o peso corporal (g/Kg/24h). (B) Preferência pelo etanol determinada pela (%) consumida em relação ao total de líquido ingerido. Resultados são expressos como média ± SEM. Análises performadas com ANOVA de duas vias seguida por teste *post hoc Tukey* em (A) e por teste *Mann-Whitney* em relação ao valor hipotético de 50,1% em (B). Em (A) *p < 0,05 para HSB-AIN93G + EtOH, HSB-AIN93G *Il6* KO + EtOH e HB-AIN93G *Nfat* KO + EtOH vs HSB + EtOH e AIN93G vs EtOH e para HB-AIN93G *Nfat* KO + EtOH *vs* HSB-AIN93G *Il6* KO+ EtOH nos dias 11, 17, 20, 25, 27 e 28. Em (B) *p < 0,05 para o valor hipotético de 50,1% vs AIN93G + EtOH, HSB-AIN93G + EtOH, HSB-AIN93G *Il6* KO + EtOH e HB-AIN93G *Nfat* KO + EtOH. Asteriscos (*) representam as diferenças do teste *post hoc* entre os grupos.

**O consumo da dieta HSB e etanol afetaram o comportamento dos animais**

A ANOVA de duas vias ($F(3, 50) = 8.513$, $p = 0.0001$) mostrou que o teste *Marble Burying* foi influenciado principalmente pela interação entre tratamento dietético e o consumo de bebida. Esse teste é utilizado na avaliação da impulsividade, do comportamento dos tipos obsessivo-compulsivo e ansioso em camundongos [43, 44]. Nesse sentido, o teste *post hoc* apontou que os animais do grupo AIN93G (AIN93G + $H_2O$ e AIN93G + EtOH) expressaram baixa impulsividade em relação aos dos grupos HSB (HSB + $H_2O$ e HSB + EtOH), HSB-AIN93G (HSB-AIN93G + $H_2O$ e HSB-AIN93G + EtOH) e HSB-AIN93G *Il6* KO + EtOH e HSB-AIN93G *Nfat* KO (HSB-AIN93G *Nfat* KO + $H_2O$ e HSB-AIN93G *Nfat* KO + EtOH) (Figura 4A). Interessantemente, os camundongos do grupo HSB-AIN93G *Il6* KO+ $H_2O$ apresentaram baixa impulsividade em relação aos do HSB-AIN93G *Il6* KO+ EtOH, enquanto não foram observadas diferenças em relação aos espécimes do grupo HSB-AIN93G *Nfat* KO (Figura 4A).

O teste da caixa claro/escuro é tradicionalmente aplicado na avaliação do comportamento do tipo ansioso em modelos animais, usando métricas como a latência, o tempo gasto no compartimento claro, o número de transições e a distância percorrida dentro da caixa [47, 57]. Nesse contexto, a ANOVA de duas vias revelou que a dieta e o etanol afetaram a latência (dieta: $F(4, 67) = 20,68$, $p < 0.0001$; etanol: $F(1, 67) = 5.065$, $p = 0.0277$) e o tempo gasto no compartimento claro (dieta: $F(4, 73) = 9,551$, $p < 0.0001$; etanol: $F(1, 73) = 94.23$ , $p < 0.0001$), enquanto que somente o álcool ($F(1, 64) = 75.28$, $p < 0.0001$) influenciou os resultados vistos quanto à distância percorrida dentro da parte clara da caixa.

Para latência, o teste *post hoc* indicou que os animais dos grupos HSB (HSB + $H_2O$ e HSB + EtOH), HSB-AIN93G (HSB-AIN93G + $H_2O$ e HSB-AIN93G + EtOH) e HSB-AIN93G *Nfat* KO (HSB-AIN93G *Nfat* KO + $H_2O$ e HSB-AIN93G *Nfat* KO + EtOH) apresentaram menor tempo ($p < 0.05$) para começar a explorar o compartimento claro em comparação aos espécimes do grupo AIN93G (AIN93G + $H_2O$ e AIN93G + EtOH), demonstrando um efeito ansiolítico do tratamento para esses animais (Figura 4A). Enquanto isso, os do grupo HSB-AIN93G *Il6* KO + $H_2O$ exibiram maior tempo de latência em relação aos dos grupos HSB-AIN93G + $H_2O$ e HSB-AIN93 *Nfat* KO + $H_2O$ (Figura 4A). Não foram observadas diferenças em relação ao grupo HSB-AIN93G Il6 KO + EtOH (Figura 4A). Os camundongos dos grupos +EtOH (AIN93G + EtOH, HSB + EtOH, HSB-AIN93G + EtOH, HSB-AIN93G *Il6* KO + EtOH e HSB-AIN93G *Nfat* KO + EtOH) gastaram mais tempo ($p < 0,05$) no compartimento claro da caixa em relação aos dos +$H_2O$ (AIN93G + $H_2O$, AIN93G + $H_2O$, HSB-AIN93G + $H_2O$, HSB-AIN93G Il6 KO + $H_2O$ e HSB-AIN93G *Nfat* KO + $H_2O$) (Figura 4B). Esses dados novamente confirmam o efeito ansiolítico dos tratamentos experimentais. Não foram percebidas diferenças significativas em relação ao número de transições claro/escuro (Figura 4C).

Em relação à distância percorrida na parte clara da caixa, o teste *post hoc* mostrou que os animais dos grupos +EtOH (AIN93G + EtOH, HSB + EtOH, HSB-AIN93G + EtOH, HSB-AIN93G *Il6* KO + EtOH e HSB-AIN93G *Nfat* KO + EtOH) percorreram uma distância maior ($p < 0,05$) e, consequentemente, exploraram mais o ambiente em relação aos dos +$H_2O$ (AIN93G + $H_2O$, AIN93G + $H_2O$, HSB-AIN93G + $H_2O$, HSB-AIN93G Il6 KO + $H_2O$ e HSB-AIN93G *Nfat* KO + $H_2O$) (Figura 4D). Tal resultado pode ser visualizado pelos *Heat-maps* apresentados na figura 4F, nos quais é evidente a maior exploração da área pelos animais dos grupos +EtOH.

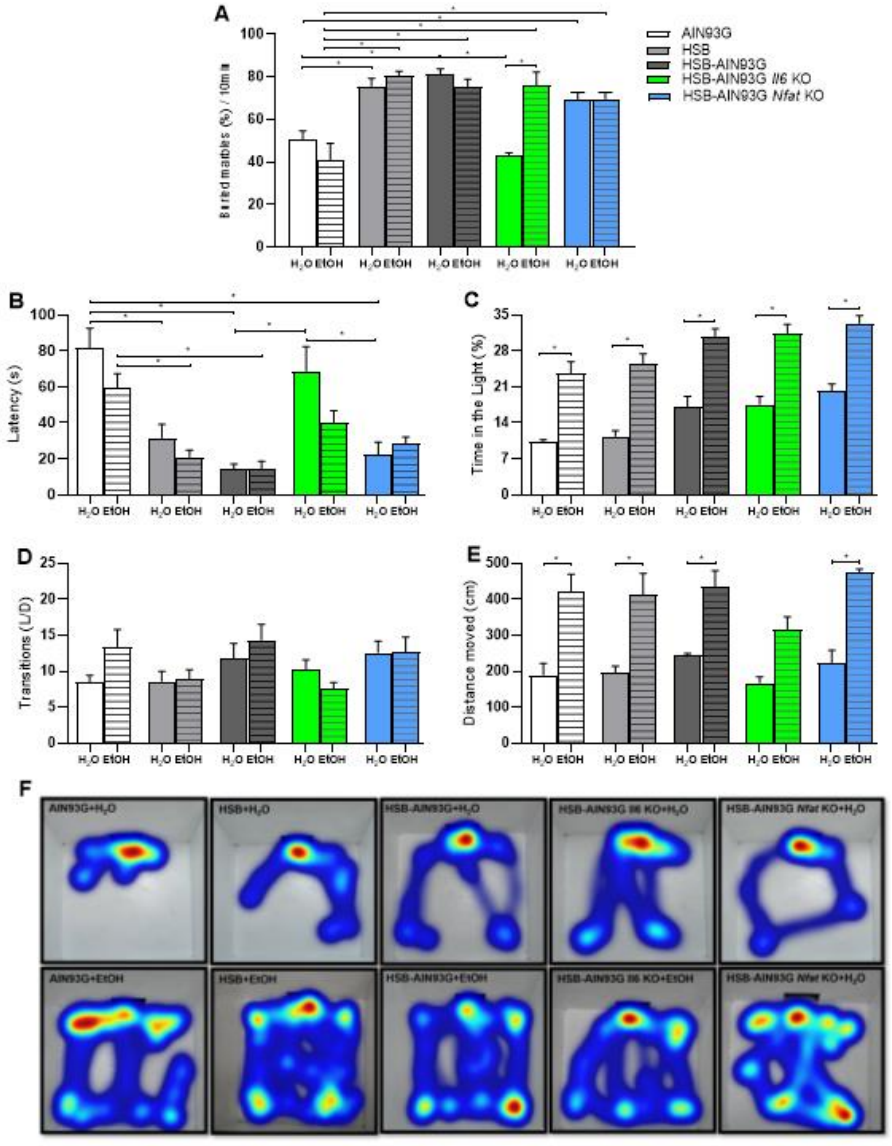



**Figura 4: Análises comportamentais**. Comportamento do tipo impulsivo avaliado no teste *Marble burying* pela: (A) Porcentagem de enterramentos (%). Comportamento do tipo ansioso avaliado no teste da caixa claro/escuro por: (B) Latência (s), (C) Tempo no lado claro (%), (D) Número de transições, (E) e (F) Distância percorrida (cm). Resultados são expressos como média ± SEM. Análises performadas com ANOVA de duas vias seguida por teste *post hoc Tukey*. Em (A) *$p < 0,05$ para AIN93G + $H_2O$ vs HSB + $H_2O$ e HSB-AIN93G + $H_2O$, para AIN93G + EtOH vs HSB + EtOH, HSB-AIN93G + EtOH e HSB-AIN93G *Il6* KO + EtOH e para HSB-AIN93G *Il6* KO + $H_2O$ vs HSB-AIN93G + EtOH e HSB-AIN93G *Il6* KO + EtOH. Em (B) *$p < 0,05$ para AIN93 +

$H_2O$ vs HSB + $H_2O$, HSB-AIN93G + $H_2O$ e HSB-AIN93G *Nfat* KO + $H_2O$, para AIN93G + EtOH vs HSB + EtOH e HSB-AIN93G + EtOH e para HSB-AIN93G *Il6* + $H_2O$ vs HSB-AIN93G + $H_2O$ e HSB-AIN93G *Nfat* + $H_2O$. Em (C) e (E) *p < 0,05 para AIN93 + $H_2O$ vs AIN93G + EtOH, para HSB + $H_2O$ vs HSB + EtOH, para HSB-AIN93G + $H_2O$ vs HSB-AIN93G + EtOH, para HSB-AIN93G *Il6* KO+ $H_2O$ vs HSB-AIN93G *Il6* KO + EtOH e para para HSB-AIN93G *Nfat* KO + $H_2O$ vs HSB-AIN93G *Nfat* KO+ EtOH. Asteriscos (*) representam as diferenças do teste *post hoc* entre os grupos.

**O tratamento dietético e consumo de etanol afetaram o percentual de células inflamatórias no cérebro e baço**

No cérebro, a ANOVA de duas vias revelou que a dieta é o principal fator para as variações nos percentuais de células CD45+F4/80+CD11b+ ($F(4, 27) = 36.43$, $p < 0.0001$). O teste *post hoc* mostrou que os animais do grupo HSB-AIN93G *Il6* KO + EtOH possui maior ($p < 0.05$) percentual de células CD45+F4/80+CD11b+ quando comparado com os animais dos grupos AIN93G + EtOH, HSB + EtOH e HSB-AIN93G + EtOH e da mesma forma, os animais do grupo HSB-AIN93G + EtOH possuem maior percentual desse marcador nas suas células que os dos grupos AIN93G + EtOH e HSB + EtOH (Figura 4A). Já o grupo HSB-AIN93G *Nfat* KO + EtOH possui maior percentual quando comparados com o AIN93G + EtOH (Figura 5A). Para o mesmo marcador, o grupo HSB-AIN93G *Nfat* KO + $H_2O$ possui maiores quantidades que os grupos AIN93G + $H_2O$, HSB + $H_2O$, HSB-AIN93G + $H_2O$ (Figura 5A). No baço, a dieta ($F(4, 27) = 108.0$, $p < 0.0001$) foi responsável pelas diferenças nas células marcadas com CD45+F4/80+CD11b. O teste *post hoc* revelou que os grupos AIN93G, HSB e HSB-AIN93G (AIN93G + $H_2O$, AIN93G + EtOH, HSB +$H_2O$, HSB + EtOH, HSB-AIN93G + $H_2O$, HSB-AIN93G + EtOH) possuem maiores percentuais ($p < 0.05$) das células CD45+F4/80+CD11b+ quando comparados com animais *Il6* e *Nfat* KO (HSB-AIN93G *Il6* + $H_2O$, HSB-AIN93G *Il6* + EtOH, HSB-AIN93G *Nfat* + $H_2O$ e HSB-AIN93G *Nfat* + EtOH) (Figura 5B).

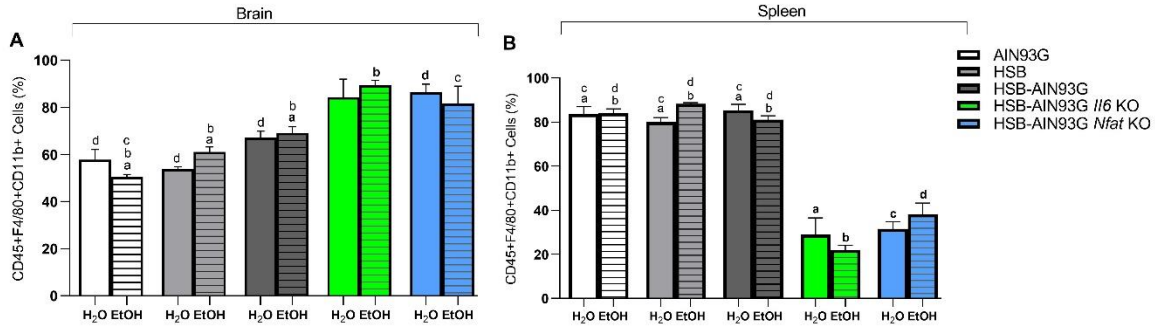



**Figura 5: Citometria de fluxo de células do cérebro e baço.** (A) CD45+F4/80+CD11b+ (%) no cérebro. (B) CD45+F4/80+CD11b+ (%) no baço. Resultados são expressos como média ± SEM. Análises performadas com ANOVA de duas vias seguida por teste *post hoc* Tukey. Em (A) *p < 0,05 para AIN93G + EtOH vs HSB + EtOH e HSB-AIN93G + EtOH, para HSB + EtOH vs HSB-AIN93G + EtOH e HSB-AIN93G *Il6* KO + EtOH, para AIN93G + EtOH vs HSB-AIN93G + EtOH e para HSB + $H_2O$ vs HSB-AIN93G + $H_2O$ e HSB-AIN93G *Nfat* KO + $H_2O$. Em (B) *p < 0,05 para HSB-AIN93G Il 6 + $H_2O$ vs AIN93G + $H_2O$, HSB + $H_2O$ e HSB-AIN93G + $H_2O$, para HSB-AIN93G Il 6 + EtOH vs AIN93G + EtOH, HSB + EtOH e HSB-AIN93G + EtOH, para HSB-AIN93G *Nfat* + $H_2O$

vs AIN93G + $H_2O$, HSB + $H_2O$ e HSB-AIN93G + $H_2O$ e para HSB-AIN93G *Nfat* + EtOH vs AIN93G + EtOH, HSB + EtOH e HSB-AIN93G + EtOH.

**O consumo de etanol afetou a transcrição de *Lrrk2, Nfat* e de citocinas no estriado**

A regulação transcricional de genes e citocinas chave em relação ao consumo e dependência pelo etanol foram afetados nos diferentes grupos experimentais do presente estudo [58]. A ANOVA de duas vias demonstrou que o álcool é o único fator responsável pelas diferenças na regulação transcricional dos genes *Lrrk2* ($F(1, 38) = 31.68$, $p < 0.0001$), *Nfat* ($F(1, 38) = 16.27$, $p = 0.0003$), *Il1β* ($F(1, 38) = 49.1$, $p < 0.0001$), Il10 ($F(1, 37) = 8.513$, $p = 0.0060$) e *iNOS* ($F(1,38) = 15.31$, $p = 0.004$). Já *Il6*, teve como responsáveis pela sua regulação a dieta ($F(2, 27) = 7.148$, $p = 0.0032$), o etanol ($F(1, 27) = 22.92$, $p< 0.0001$) e a interação entre esses fatores ($F(2, 27) = 3.788$, $p = 0.0355$).

Avaliamos no estriado, a transcrição de citocinas inflamatórias (*Il6* e *Il1β*) e anti-inflamatórias (*Il10*). A análise *post hoc* revelou que *Il-6* foi regulado positivamente ($p < 0,05$) no grupo HSB-AIN93G + EtOH em comparação aos animais dos grupos HSB + EtOH e AIN93G + EtOH (Figura 6A). O *Il-1β* estava hiporregulado nos animais HSB + $H_2O$, HSB-AIN93G + $H_2O$ e HSB-AIN93G *Il6* KO + $H_2O$ em relação aos animais HSB + EtOH, HSB-AIN93G + EtOH e HSB-AIN93G *Il6* KO + EtOH, respectivamente (Figura 6B). O *Il-10* estava regulado positivamente nos animais do grupo HSB-AIN93G + $H_2O$ e HSB-AIN93G *Il6* KO + $H_2O$ em comparação aos animais dos grupos HSB-AIN93G + EtOH e HSB-AIN93G *Il6* KO + EtOH (Figura 6C). O gene *iNOS* apresentou hiporregulação nos grupos HSB-AIN93G + EtOH e HSB-AIN93G *Il6* KO + $H_2O$ em comparação com o grupo HSB-AIN93H + $H_2O$ e HSB-AIN93G *Il6* KO + EtOH, respectivamente (Figura 6D). O gene *Lrrk2* que recentemente foi associado à perda de controle e preferência pelo etanol pelo nosso grupo de pesquisa, apresentou hiporregulação nos grupos que fizeram alto consumo de etanol (HSB-AIN93 + EtOH e HSB-ANI93G *Il6* KO + EtOH (Figura 6E), inversamente, o gene *Nfat* apresentou hiperregulação nesses grupos (Figura 6F). Curiosamente, a correlação de *Spearman* demonstrou uma relação negativa entre os genes *Lrrk2* e *Nfat* ($r = 0.7393$, equação da regressão $-1.748*X + 3.339$, $p < 0.0001$) (Figura 6J).

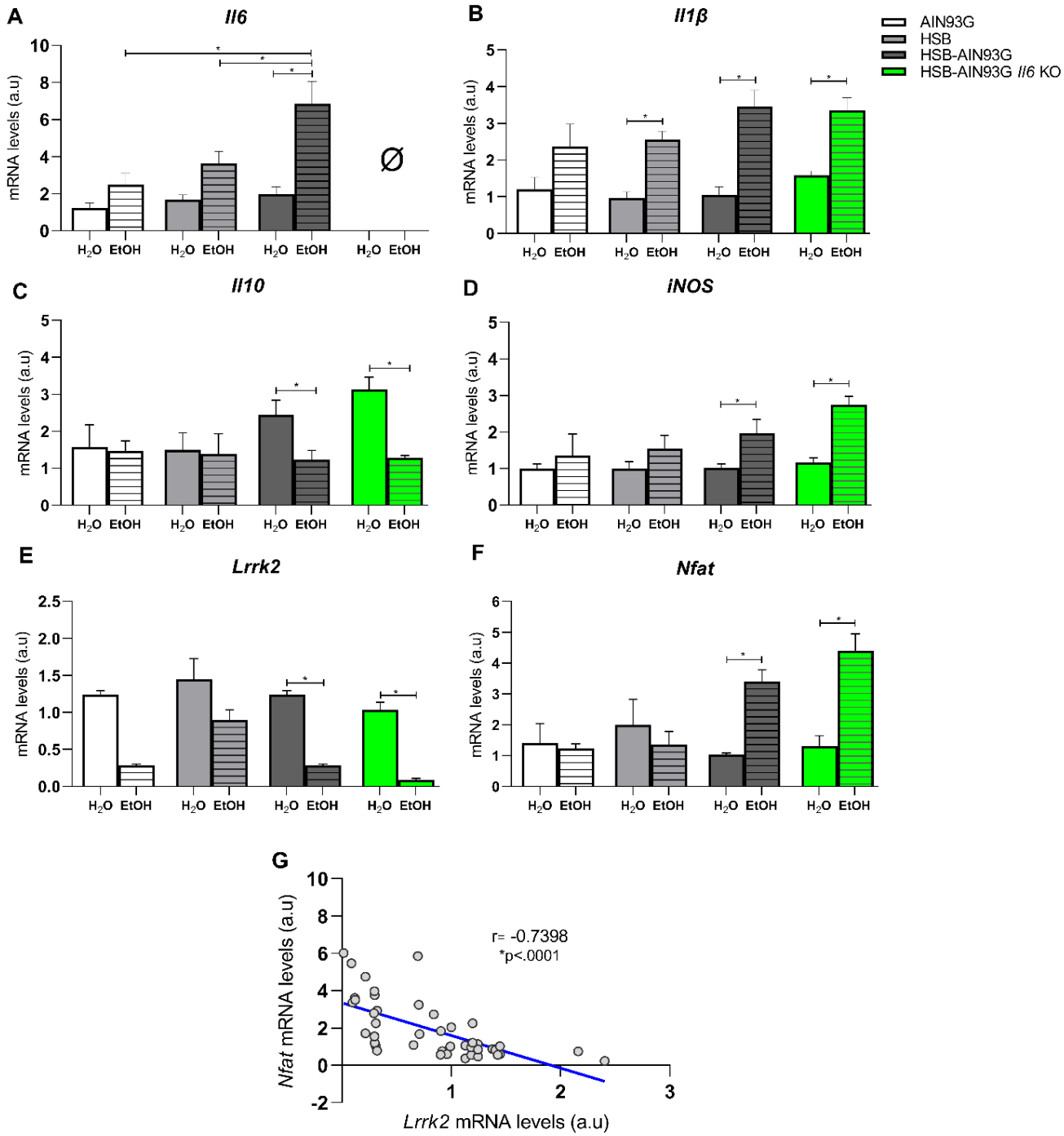



**Figura 6: Quantificação relativa do mRNA no Estriado**. Níveis relativos de mRNA em**:** (A) *Il6*, (B) *Il1β*, (C) *Il10,* (D) *iNOS, (*E) *Lrrk2* e (F) *Nfat*. Resultados são expressos como média ± SEM. Análises performadas com ANOVA de duas vias seguida por teste post hoc Tukey. Em (A) *p < 0,05 para AIN93G + EtOH vs HSB + EtOH e HSB-AIN93G + EtOH. Em (B), (C), (D), (E) e (F) *p < 0,05 para HSB-AIN93G + $H_2O$ vs HSB-AIN93G + EtOH e para HSB-AIN93G *Il6* KO + $H_2O$ vs HSB-AIN93G *Il6* KO + EtOH. Asteriscos (*) representam as diferenças do teste post hoc entre os grupos.

## DISCUSSÃO

Com o objetivo de avaliar a regulação transcricional do gene *Lrrk2* em associação com genes relacionados ao sistema imune e suas implicações no comportamento e na preferência por etanol em camundongos C57BL/6, reproduzimos um modelo de consumo crônico de dieta rica em açúcar e gordura (HSB) e livre escolha de etanol, conforme detalhado por [38, 39]; e acrescentamos animais *knockout Il6* e *Nfat.* Nesse modelo, foi observado que, após a alimentação dos camundongos por 8 semanas com a dieta HSB e sua subsequente troca pela dieta AIN93G, verificou-se nos animais alto consumo e preferência de etanol [38, 39]. Adicionalmente, animais que consumiram apenas a dieta HSB desenvolveram características semelhantes à obesidade. Reproduzimos aqui esses resultados como visto nas figuras 1 e 2 e observamos que a falta dos genes *Il6* e *Nfat* não altera de forma significativa a preferência e o consumo de etanol nos animais ao longo do tempo. Efetivamente, já havíamos demonstrado que os resultados observados no modelo estavam relacionados com um desequilíbrio na via dopaminérgica mesocorticolímbica associada à regulação transcricional dos receptores de dopamina (*Drd1*/*Drd2*) e de subunidades de receptores Gabab (*Gabbr1*/*Gabbr2*) e com alterações na estrutura e composição da microbiota intestinal [38, 39]. No presente trabalho, observamos que o alto consumo de etanol pelos animais dos grupos resultou em diferenças comportamentais e em maior neuroinflamação, em especial no estriado, onde houve elevação de citocinas inflamatórias e redução de anti-inflamatórias.

O consumo de etanol está relacionado a diversas alterações comportamentais [59]. De fato, no presente estudo, observamos um efeito ansiolítico associado ao consumo crônico de etanol evidenciado pela baixa latência, alto tempo de permanência, maior distância percorrida e exploração do compartimento claro no teste da caixa claro/escuro nos animais que estavam em grupos associados ao paradigma da livre escolha por etanol, sendo importante evidenciar que tal comportamento foi observado independentemente da quantidade ingerida da bebida. Esse resultado corrobora com diversos estudos em modelos animais que evidenciam o efeito ansiolítico do consumo dessa droga [12, 46, 60, 61]. Considerando que a ansiedade é uma estratégia de defesa em camundongos, uma vez que se associa à aversão por lugares abertos e iluminados que os tornam mais visíveis a possíveis predadores, a diminuição desse comportamento lhes é prejudicial [47, 62]. Neste contexto, esse efeito também é deletério para seres humanos uma vez que é um reforço positivo em situações de elevada ansiedade e até mesmo depressão que pode ser fator chave para o desenvolvimento e manutenção da dependência pela droga [63, 64]. Por isso, várias estratégias são investigadas para bloquear os efeitos ansiolíticos do etanol; por exemplo, Correia e colaboradores (2008) usaram fármacos bloqueadores da atividade catalase que inibiram o metabolismo do etanol no cérebro e conseguiram atuar sobre os efeitos

ansiolíticos do álcool nos animais [65]. Com esse trabalho, os autores concluíram que estratégias psicofarmacológicas que bloqueiam a ação do etanol no cérebro podem ser usadas para diminuir os efeitos da adição por esta droga [65]. Diante disso, o modelo desenvolvido pode ser eficiente no estudo de alterações neurobiológicas e comportamentais relacionadas ao uso do etanol, em especial, para trabalhos que buscam bloquear os efeitos ansiolíticos do álcool em modelos animais

Curiosamente, o teste *Marble Burying* demonstrou maior impulsividade nos animais que tiveram acesso a dieta HSB durante todo o experimento. Tal fato, é comparável com o transtorno obsessivo-compulsivo visto em humanos [44, 66, 67]. Tal resultado é consistente com trabalhos em que dietas hipercalóricas foram associadas à compulsão alimentar [68-70]. Assim, permitindo-nos sugerir que o consumo da dieta HSB pode desencadear um distúrbio do tipo obsessivo-compulsivo nos camundongos e que nosso modelo pode ser aplicado em estudos relacionados a cirurgia bariátrica em humanos, principalmente no que diz respeito a relação deste procedimento com o consumo de álcool. Estudos demonstram que de 6 meses a 3 anos do pós-operatório, cerca de 6% dos pacientes desenvolvem transtornos relacionados ao uso problemático de etanol [71, 72]. De modo geral, acredita-se que há uma transferência da alimentação compulsiva para o alto consumo de etanol, o que potencializa a adição pela substância assim como visto nos grupos troca de dieta (HSB-AIN93G + EtOH, HSB-AIN93G *Il6* KO + EtOH e HSB-AIN93G *Nfat* KO + EtOH) [73, 74].

Considerando ainda os testes comportamentais, observamos que a troca da dieta HSB para AIN93G impactou de forma diferenciada o comportamento dos animais do grupo HSB-AIN93G *Il6* KO + EtOH. Estes animais, demonstraram maior impulsividade em relação aos animais do grupo $H_2O$ que, por sua vez, apresentaram menor latência na caixa claro/escuro dos que animais dos grupos HSB-AIN93G + $H_2O$ e HSB-ANI93G *Nfat* KO + $H_2O$. Apesar desse resultado não ter implicações diretas em relação ao consumo de etanol nos animais *Il6* KO, devem ser considerados em relação a importância da resposta imune e moléculas inflamatórias em alterações comportamentais, já que o sistema imune está implicado no desenvolvimento de diversas doenças psiquiátricas como depressão, transtorno bipolar, esquizofrenia, autismo, ansiedade e até mesmo na desordem pelo uso do álcool [21, 75-77]

De modo geral, sabe-se que a exposição ao etanol ativa a sinalização neuroimune, ocasionando um ambiente altamente inflamatório no cérebro, no qual células da micróglia, infiltrado de macrófagos e monócitos, aumentam a transcrição de marcadores pró-inflamatórios (IL1β, IL6), reduzem a de marcadores anti-inflamatórios (IL10) e elevam a de óxido nítrico sintase (iNOS) [78-80]. Neste contexto, em extensa revisão sobre o assunto, Montesinos e colaboradores (2016) descrevem vias em que o etanol regula a transcrição de

genes neuroimunes e microgliais e, em especial, citam a ativação de receptores *toll-like* do tipo 4 (TLR4) presentes em células imunes e neurônios por lipopolissacarídeos bacterianos (LPS) que ativam a resposta inflamatória [20]. De fato, em trabalhos anteriores, nosso grupo de pesquisa descreveu alterações na abundância e estrutura da microbiota intestinal e propôs que o aumento da translocação bacteriana para a corrente sanguínea possibilitaria a ativação de TLR4 por LPS bacterianos, o que resultaria em ativação da resposta inflamatória cerebral [39, 80]. No presente estudo, nos grupos HSB-AIN93G (HSB-AIN93G + EtOH, HSB-AIN93G *Il6* KO + EtOH e HSB-AIN93G *Nfat* KO + EtOH), que apresentaram alto consumo e preferência pelo etanol, observamos um aumento de macrófagos (CD45+F4/80+CD11b+) no cérebro dos animais [81, 82]. Funcionalmente, CD11b regula a adesão e migração de leucócitos para mediar a resposta inflamatória [83-85]. Portanto, a possibilidade de uma neuroinflamação impulsionada pela microbiota nesses animais não deve ser desconsiderada. No baço, houve uma diminuição dos macrófagos (CD45+F4/80+CD11b+) dos animais *Il6* KO e *Nfat* KO mostrando que esses genes são importantes para manutenção destas células no órgão frente aos tratamentos dados aos animais.

Especificamente no estriado, região cerebral sensível ao etanol, é observada ativação da sinalização neuroimune com o consumo da droga. Ao expor camundongos ao etanol por 8 semanas, Asatryan e colaboradores (2015) verificaram no estriado uma maior neurodegeneração e transcrição de mediadores pró-inflamatórios [78]. Esses resultados corroboram com nossos achados, uma vez que observamos no estriado uma hiperregulação dos genes relacionados a citocinas pró-inflamatórias *Il6* e *Il1β* e hiporregulação de *Il10* nos camundongos dos grupos com alto consumo de etanol. Curiosamente, observamos hiperregulaçao de *iNOS* nos animais dos grupos HSB-AIN93 + EtOH e HSB-AIN93G *Il6 KO* + EtOH o que faz sentido pela relação inversa com a transcrição de *Il10* já que essa interleucina é conhecida por suprimir a indução de iNOS [86]. Assim, os níveis baixos de *Il10* nos animais que consumiram altas quantidades e tiveram preferência por etanol podem estar contribuindo para o aumento de *iNOS* cuja expressão é resultado de uma resposta inflamatória localizada ou difusa [87, 88]. Paralelamente aos resultados descritos, encontramos uma hiporregulação do gene *Lrrk2* e hiperregulação de *Nfat* nestes grupos, o que faz sentido, considerando seu papel inibidor sobre NFAT [32]. Assim, com a menor transcrição de *Lrrk2* há maiores níveis de NFAT o que pode desencadear a um aumento nas citocinas inflamatórias dependentes desta molécula e que tem papel chave no processo de inflamação. De fato, observamos uma correlação negativa entre esses genes o que nos permite sugerir a possibilidade de que a baixa transcrição de *Lrrk2* está aumentanto a neuroinflamação e direcionando em parte o fenótipo de adição pelo etanol não pode ser desconsiderada.

Neste contexto, nosso grupo de pesquisa identificou, associações do gene *Lrrk2*, até então associado à Doença de Parkinson e a doenças inflamatórias intestinais, no comportamento de preferência e perda de controle pelo etanol em camundongos, Zebrafish e humanos [28, 31, 58, 89-91]. Este gene está envolvido em processos neurais, como a recaptação de componentes sinápticos, plasticidade sináptica e em diversas vias de sinalização por desempenhar funções GTPase e quinase, que influenciam processos como a proliferação e diferenciação celular, apoptose, inflamação e resposta imune [33, 92-97]. Estudos em humanos e camundongos corroboram com os resultado deste trabalho em relação a regulação diferencial com uma hiporregulação de *Lrrk2* em relação ao consumo de etanol. Neste cenário, em humanos foi obsersevado hiporregulação de *LRRK2* no cérebro *post-mortem* de pacientes com AUD e em camundongos observou-se que o álcool diminui a atividade da quinase LRRK2 no estriado e que a deleção do gene em neurônios estriatais que expressam D1 promove o consumo compulsivo fortalecendo a sinalização e a função do receptor D1 [31, 58]. Apesar disso, alguns estudos demonstraram resultados contrários. Em modelo de perda de controle pelo etanol em camundongos, foi observada uma hiperregulação do gene *Lrrk2* no estriado de animais que apresentaram preferência pela substância mesmo após estímulo adverso e a inibição da função quinase deste gene em *Zebrafish* reduziu a preferência por etanol nos animais [90, 98].

Os resultados conflitantes encontrados nesses trabalhos são de difícil comparação em relação a este estudo, tendo em vista as diferenças existentes quanto aos tratamentos utilizados, tempo de exposição ao etanol, concentração de etanol e modelos experimentais. Assim, mesmo com achados discrepantes, nossos resultados nos permitem propor que alterações no perfil de regulação transcricional deste gene tenham um papel-chave no alto consumo e preferência de etanol. Entretanto, mais estudos devem ser feitos para entender a função desse gene na neurobiologia da adição pelo etanol. Em resumo, os resultados do presente estudo demonstram como o modelo animal de consumo de dieta rica em gordura e ingestão de etanol desenvolvido por nosso grupo é reprodutível e pode ser eficiente no estudo de alterações relacionadas ao consumo de etanol. Com este modelo, mostramos ainda que o consumo de etanol afetou (I) o comportamento dos animais, (II) a neuroinflamação e (III) a regulação da transcrição dos genes *Lrrk2, Nfat* e de citocinas no estriado. Adicionalmente, observamos que nos animais *Il6* KO a transcrição dos genes alvo não foi alterada neste modelo animal e fica como **perspectiva** a avaliação em relação aos animais *Nfat* KO já que o número de animais para avaliação ainda não foi obtido, mas, o experimento já está em andamento para finalização deste estudo.

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# 5 CONCLUSÃO GERAL

# CONCLUSÃO GERAL

Os estudos apresentados nesta tese, organizados em três capítulos, apresentam um corpo de evidências que corroboram com a hipótese de que nossos achados estão implicados no fenótipo de uso problemático de etanol; sustentam, ainda, a eficiência da dieta HSB no desenvolvimento de uma condição semelhante à obesidade nos camundongos.

Haja vista a diferença no ganho de peso dos animais que consomem apenas a dieta HSB, no modelo desenvolvido, investigamos mais a fundo esse fenótipo e sua relação com a dieta. Dessa forma, no capítulo 1, demonstramos que o consumo crônico da dieta HSB afeta o índice de adiposidade, as concentrações de colesterol, de glicose, de triglicerídeos e de leptina, além do comportamento, da regulação de genes relacionados à ingestão alimentar e do microbioma fecal dos animais [139]. Todos esses parâmetros parecem contribuir para um fenótipo semelhante à obesidade nos animais HSB.

Com a utilização de um modelo de consumo de dieta rica em gordura e livre escolha por etanol, no capítulo 2, conseguimos demonstrar que a dieta HSB e o etanol afetaram a estrutura, a composição e a abundância de diversos grupos bacterianos na microbiota intestinal [120]. Adicionalmente, mostramos que a retirada da dieta HSB afeta a preferência pelo etanol e demonstra uma resiliência estrutural no microbioma fecal [120]. No capítulo 3, conseguimos reproduzir os dados do modelo descrito no capítulo 2 e ainda demonstramos que o consumo de etanol afeta o comportamento dos animais, desencadeia uma neuroinflamação associada a uma hiperregulação de citocinas inflamatórias, hiporregulação de citocina anti-inflamatória, hiperregulação de *Nfat* e hiporregulação do gene *Lrrk2* no estriado. Adicionalmente, mostramos que a ausência dos genes *Il6* e Nfat não afeta o consumo de etanol nos animais, apesar de alterar em parte seu comportamento e regulação gênica.

Em conjunto, nossos achados sustentam que a ingestão da dieta HSB leva os animais a desenvolverem comportamentos que os direcionam a consumir compulsivamente a dieta, que, por sua vez, resulta no desenvolvimento de um fenótipo semelhante à obesidade. Ao retirar a dieta HSB e expor os animais ao paradigma da livre escolha por etanol, o comportamento que até então era visto em relação à dieta passa a ser - de mesmo modo - em relação ao etanol. Por isso, o alto consumo e preferência por esta droga nos animais HSB-AIN93G + EtOH. Neste grupo, o consumo abusivo de etanol está associado a alterações no sistema nervoso central, em especial, no sistema de recompensa, conforme relatado em [129]. Além disso, sugerimos: (1) a existência de uma influência da microbiota intestinal e do sistema imune na busca e consumo desta droga; (2) e que há uma associação destes fatores com a regulação transcricional do gene *Lrrk2*, o que direciona a

preferência pelo etanol. Assim, os dados obtidos em nossos estudos contribuem para o escopo de evidências de uma interconexão entre o sistema nervoso central, sistema imune, dieta, microbiota intestinal e regulação do gene *Lrrk2* no consumo abusivo de etanol. Como perspectivas para maior aprofundamento dos resultados apresentados nesta tese, é importante avaliar a expressão de proteínas associadas aos processos aqui avaliados como LRRK2, PKA, TLR4 e NFAT, e fazer uma avaliação metabolômica do modelo a fim de investigar possíveis biomarcadores preditores dos fenótipos desenvolvidos pelos animais. Adicionalmente, um modelo de transferência de microbiota intestinal entre animais dos grupos HSB-AIN93G + $H_2O$ e HSB-AIN93G + EtOH pode ajudar a elucidar a importância desta no consumo e preferência por etanol.

# 6 REFERÊNCIAS – INTRODUÇÃO E CONCLUSÃO GERAL


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