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**Efeitos da suplementação de ômega-3 sobre a
microbiota intestinal, aspectos metabólicos e
neurofuncionais em ratos obesos alimentados
com dieta de cafeteria**

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Tese submetida ao Programa de Pós-Graduação em Biociências da Universidade Federal de Ciências da Saúde de Porto Alegre como requisito para a obtenção do grau de Doutor.

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“Demore o tempo que for para decidir o que você quer da vida, e depois que decidir não recue ante nenhum pretexto, porque o mundo tentará te dissuadir”.

Friedrich Nietzsche

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Lista de abreviaturas, símbolos e unidades

5-HT	serotonina
AGCC	ácidos graxos de cadeia curta
AGS	ácidos graxos saturados
ALA	ácido α -linolênico
AP-1	proteína ativadora 1
ARC	núcleo arqueado
ATP	adenosina trifosfato
BHE	barreira hematoencefálica
CAF	dieta de cafeteria
CCK	colecistoquinina
CLDN	claudina
COX-2	ciclooxigenase-2
DHA	ácido docosahexaenoico
DHGNA	doença hepática gordurosa não alcóolica
EPA	ácido eicosapentaenóico
ERK	cinase regulada por sinal extracelular
FFAR	receptor de ácidos graxos livres
GABA	ácido γ -aminobutírico
GLP-1	peptídeo-1 semelhante ao glucagon
HFD	dieta rica em gordura
IFN-γ	interferon-gama
IKK	cinases de IKB
IL-1α	interleucina-1 alfa
IL-1β	interleucina-1beta
IL-6	interleucina-6
iNOS	óxido nítrico induzível
IRAKs	cinases associadas ao receptor de IL-1
JNK	c-Jun N-terminal cinase

LPS	lipopolissacarídeo
MAPK	proteína-cinases ativadas por mitógenos
JAM	moléculas juncionais de adesão
n-3	ácidos graxos poli-insaturados ômega-3
NF-κB	fator nuclear kappa B
NTS	núcleo do trato solitário
OCLDN	occludinas
PAMPs	padrões moleculares associados a patógenos
PKC	proteína cinase C
PPAR-γ	receptor ativado por proliferadores de peroxissoma gama
PYY	peptídeo YY
SNC	sistema nervoso central
TAB	tecido adiposo branco
TLR	receptor do tipo toll
TLR-4	receptor do tipo toll-4
TNF-α	fator de necrose tumoral-alfa
TRAF6	fator 6 associado a receptor de TNF
ZO	zônula occludens
FDA	Food and Drug Administration

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Resumo

Devido ao número crescente de indivíduos obesos, a obesidade tem sido considerada uma verdadeira epidemia mundial, tornando-se um sério problema de saúde pública. A obesidade ocasiona diversas alterações metabólicas, predispondo a secreção crônica de moléculas pró-inflamatórias. Desta forma, a presente tese de doutorado teve como objetivo avaliar o efeito da suplementação de ômega-3 sobre a microbiota intestinal, parâmetros metabólicos e neurofuncionais em ratos machos obesos alimentados com dieta de cafeteria. A dieta foi administrada por 20 semanas, sendo que na 16ª semana, os animais iniciaram a suplementação de ômega-3 por gavagem (500 mg/Kg/dia). Na 20ª semana, os animais foram submetidos ao teste do labirinto em cruz elevado e de memória social, e após foram eutanasiados. Amostras de sangue foram coletadas para as determinações dos níveis de glicose, triglicerídeos, insulina, citocinas e ácidos graxos de cadeia curta e LPS. O SNC foi coletado para as dosagens de citocinas, ácidos graxos saturados e expressão de claudina-5 e TLR-4. O intestino e as fezes do ceco também foram coletados para avaliar a morfologia do intestino e a composição da microbiota intestinal. Nossos resultados demonstraram que a suplementação de ômega-3 diminuiu IL-6 no fígado. No cérebro, diminuiu o TNF- α , porém aumentou os níveis de ácidos graxos saturados (caprílico, palmítico, esteárico, tricosanóico e lignocérico), assim como o ácido miristoleico, que é insaturado, e o ácido linoleico, que é poliinsaturado. Na microbiota, aumentou a razão *Firmicutes/Bacteroidetes*. No plasma, reduziu os níveis de butirato, isobutirato e LPS. Quanto ao comportamento, o ômega-3 diminuiu o comportamento do tipo ansioso nos ratos obesos. A CAF aumentou peso corporal, adiposidade visceral, e os níveis de glicose, insulina e triglicerídeos. Além disso, aumentou o ácido palmítico, a expressão de TLR-4 no córtex cerebral e diminuiu claudina-5 no hipocampo. Os animais alimentados com CAF também apresentaram maior interação social sem efeito do n-3. Desta forma, concluímos que a CAF é um modelo efetivo para provocar obesidade severa, com piora dos parâmetros metabólicos e diminuição da diversidade da microbiota intestinal. Embora, inesperadamente, melhorou a interação social entre os animais. O ômega-3 apresentou limitações para reverter os efeitos nocivos provocados pela exposição crônica a CAF. No entanto, os benefícios em reverter a endotoxemia, parâmetros

neuroinflamatórios e melhora do comportamento ansioso, colocam o ômega-3 como uma alternativa a ser considerada para o tratamento destas condições.

Palavras-chave: obesidade, neuroinflamação, ansiedade, interação social, microbiota intestinal, ômega-3

Abstract

Due to the growing number of obese individuals, obesity has been considered a true worldwide epidemic, becoming a serious public health problem. Obesity causes several metabolic changes, predisposing the chronic secretion of pro-inflammatory molecules. Thus, this study aimed to evaluate the effect of omega-3 supplementation on the intestinal microbiota, metabolic and neurofunctional parameters in obese male rats fed a cafeteria diet. The diet was administered for 20 weeks, and at the 16th week, the animals started omega-3 supplementation by gavage (500 mg/kg/day). In the 20th week, the animals were submitted to the elevated plus maze and social memory test, and after that they were euthanized. Blood samples were collected for determination of levels of glucose, triglycerides, insulin, cytokines, short-chain fatty acids and LPS. The CNS was collected for cytokines, saturated fatty acids and claudin-5 and TLR-4 expression. The intestine and feces of the cecum were also collected to evaluate the morphology of the intestine and the composition of the intestinal microbiota. Our results demonstrated that omega-3 supplementation decreased IL-6 in the liver. In the brain, it decreased TNF- α , but increased levels of saturated fatty acids (caprylic, palmitic, stearic, tricosanoic and lignoceric), as well as myristoleic acid, which is unsaturated, and linoleic acid, which is polyunsaturated. In the microbiota, the Firmicutes/Bacteroidetes ratio increased. In plasma, it reduced the levels of butyrate, isobutyrate and LPS. As for behavior, omega-3 decreased anxious-like behavior in obese rats. CAF increased body weight, visceral adiposity, and levels of glucose, insulin, and triglycerides. In addition, it increased palmitic acid, TLR-4 expression in the cerebral cortex and decreased claudin-5 in the hippocampus. CAF-fed animals also showed greater social interaction without n-3 effect. Thus, we conclude that CAF is an effective model to cause severe obesity, with worsening of metabolic parameters and decrease in intestinal microbiota diversity. Although, unexpectedly, it improved the social interaction between the animals. Omega-3 had limitations in reversing the harmful effects caused by chronic exposure to CAF. However, the benefits in reversing endotoxemia, neuroinflammatory parameters and improving anxious behavior, place omega-3 as an alternative to be considered for the treatment of these conditions.

Keywords: obesity, neuroinflammation, anxiety, social interaction, gut microbiota, omega-3

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1 INTRODUÇÃO

1.1 OBESIDADE

A obesidade é uma doença crônica, recidivante e multifatorial (Bray *et al.*, 2017). Dentre os fatores envolvidos na etiologia da obesidade podem ser destacados: predisposição genética, grau de acesso à saúde, fatores socioculturais e econômicos, nível de exposição a alimentos ultraprocessados, sedentarismo entre outros (Fernández e Shiver, 2004; Maddock, 2004; Allison *et al.*, 2008; Braveman *et al.*, 2010; Cardel *et al.*, 2011; Giskes *et al.*, 2011; Gurka, Filipp e DeBoer, 2018; Park *et al.*, 2020). Apesar de ser um grave problema de saúde, a obesidade ainda é percebida como um fracasso pessoal, e as pessoas com obesidade enfrentam esse estigma, o que conseqüentemente afeta a saúde mental dos indivíduos, podendo impactar no nível educacional e nas oportunidades de emprego dessa população (Sikorski *et al.*, 2011; Tsai *et al.*, 2018).

A obesidade é caracterizada pelo acúmulo excessivo de tecido adiposo, sendo fator de risco significativo para uma série de outras doenças não transmissíveis (DCNT) como: diabetes mellitus tipo 2 (Wentworth *et al.*, 2010), doenças cardiovasculares (Kenchiah, Gaziano e Vasan, 2004; Aghamohammadzadeh e Heagerty, 2012; Powell-Wiley *et al.*, 2021), doença hepática gordurosa não alcoólica (DHGNA) (Fabbrini, Sullivan e Klein, 2010), doenças musculoesqueléticas (Jiang *et al.*, 2011, 2012), doença renal crônica (Singh *et al.*, 2013), transtornos mentais (Anstey *et al.*, 2011; Alford *et al.*, 2018), alguns tipos de câncer (Demark-Wahnefried *et al.*, 2012; Avgerinos *et al.*, 2019). Além disso, a obesidade está associada a maior mortalidade em pacientes acometidos pela COVID-19 (Carneiro *et al.*, 2021; Poly *et al.*, 2021).

Quanto à epidemiologia, nas últimas décadas, a obesidade vem se tornando uma verdadeira epidemia mundial, incluindo um aumento acelerado em países com economias emergentes. Em todo o mundo, o sobrepeso e a obesidade causam mais de 3,4 milhões de mortes a cada ano (GBD 2015 Obesity Collaborators *et al.*, 2017), já atingiu aproximadamente dois bilhões de pessoas na população adulta, o que corresponde a 30% da população mundial (Ng *et al.*, 2014). Na população infantil, com menos de 5 anos de idade, o número é alarmante, 43 milhões já estão com sobrepeso ou obesidade (de Onis,

Blössner e Borghi, 2010). Além disso, estimativas recentes preveem que cerca de 60% das crianças de hoje serão obesas aos 35 anos (Zj *et al.*, 2018). Nos Estados Unidos, 32,8% da população acima de 20 anos tem sobrepeso ou obesidade, gerando um custo de 47% do orçamento total dedicado aos tratamentos de doenças crônicas. O impacto da obesidade na economia norte-americana ultrapassa US \$1,7 trilhão, sendo US \$480,7 bilhões em custos diretos de saúde e US \$1,24 trilhão em perda de produtividade de indivíduos obesos (Waters e Graf, 2018). Atualmente, 53,8% dos brasileiros apresentam sobrepeso enquanto 18,9% da população é obesa (Donnelly *et al.*, 2009). Em 2030, o número de indivíduos com sobrepeso no mundo poderá alcançar 2,16 bilhões, enquanto indivíduos obesos, 1,12 bilhão (Kelly *et al.*, 2008). Diante deste cenário, torna-se premente a busca por novas estratégias terapêuticas.

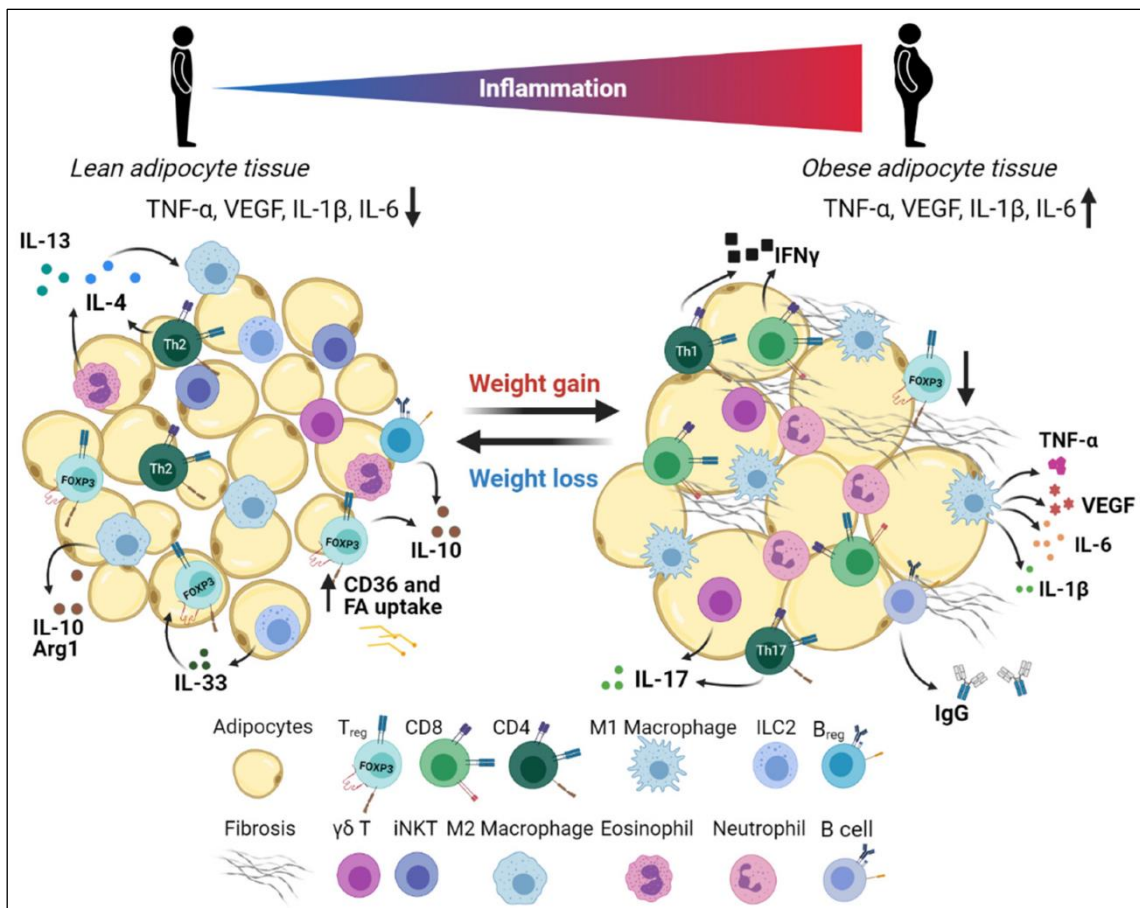
Os tratamentos disponíveis para a obesidade não são eficientes para muitos pacientes, uma vez que são baseados em (1) intervenções no estilo de vida, que geralmente resultam em recidiva; (2) tratamentos farmacológicos, mas a maioria deles não é bem tolerada devido aos efeitos colaterais ou ainda, o acesso é limitado devido ao alto custo das medicações disponíveis; e (3) cirurgia bariátrica, que está associada a vários riscos e deve ser cuidadosamente recomendada (Blüher, 2019).

1.2 INFLAMAÇÃO PERIFÉRICA NAS DISFUNÇÕES METABÓLICAS

O tecido adiposo branco (TAB) é um órgão endócrino, imunológico e regenerativo grande e dinâmico, desempenha um papel importante na regulação de diversos processos, como controle do apetite, metabolismo da glicose, sensibilidade à insulina, inflamação e reparo tecidual (Thompson *et al.*, 2012; Hassan, Greiser e Wang, 2014; Scheja e Heeren, 2019). A obesidade e o acúmulo de TAB causam o aparecimento de adipócitos inflamados e disfuncionais, juntamente com a infiltração de células imunes na região vascular do estroma (Hotamisligil, 2006, 2017). Além disso, para armazenar o excesso de energia e evitar acúmulo ectópico de lipídeos, o tecido adiposo se modifica de duas maneiras: aumentando o tamanho (hipertrofia) e a quantidade de adipócitos (hiperplasia) (Spalding *et al.*, 2008). Entretanto, essas condições prejudicam sua função e favorecem o desenvolvimento de obesidade e outras

doenças metabólicas (O'Rourke, 2018). Os adipócitos inflamados secretam citocinas inflamatórias, tanto local quanto sistemicamente, como: fator de necrose tumoral-alfa (TNF- α), interleucina-1beta (IL-1 β) e interleucina-6 (IL-6), que por sua vez interrompem a função normal do próprio TAB, bem como de órgãos remotos (Hotamisligil, 2006; Cypess, 2022). Uma característica da resposta inflamatória no TAB durante seu processo de amplificação, é a duração e intensidade, ou seja, inflamação persistente e de baixo grau que associada à obesidade e às alterações metabólicas subsequentes é conhecida como metainflamação (Martin-Jiménez *et al.*, 2017; Khazen *et al.*, 2019; Leite e Ribeiro, 2020; Zhao *et al.*, 2020). Esse ambiente inflamatório gera uma reação imunológica envolvendo macrófagos, linfócitos T CD4 e CD8 e linfócitos B, que adquirem um perfil pró-inflamatório (Khazen *et al.*, 2019; Leite e Ribeiro, 2020; Wang *et al.*, 2020). A Figura 1, apresenta as alterações no TAB, que culminam na inflamação periférica. Embora trate-se de uma inflamação de baixo grau, a exposição crônica a esse ambiente é capaz de gerar repercussões sistêmicas, culminando em síndrome metabólica, resistência insulínica e diabetes mellitus tipo 2 (Khazen *et al.*, 2019; Leite e Ribeiro, 2020; Wang *et al.*, 2020). Inúmeras evidências têm associado a inflamação periférica ao desenvolvimento de doenças neurológicas e comportamentais, tais como: ansiedade e depressão (Daumit *et al.*, 2003; Luppino *et al.*, 2010; Anstey *et al.*, 2011; Procaccini *et al.*, 2016; Alonso-Caraballo *et al.*, 2019; Leite e Ribeiro, 2020; Neto *et al.*, 2021) e demência ou doença de Alzheimer (Anstey *et al.*, 2011; Procaccini *et al.*, 2016; Alexaki, 2021). Além disso, algumas áreas cerebrais, como hipocampo e córtex pré-frontal estão envolvidas nessas repercussões (Murphy e Mercer, 2013; Alonso-Caraballo *et al.*, 2019), porém a fisiopatologia envolvida ainda permanece obscura.

Figura 1. O acúmulo de tecido adiposo gera diversas alterações. Hipertrofia dos adipócitos, infiltração de células imunológicas seguida de liberação de citocinas pró-inflamatórias (figura adaptada de (Raja *et al.*, 2021).



1.3 NEUROINFLAMAÇÃO

O sistema nervoso central (SNC) é composto principalmente por dois tipos de células: neurônios e células gliais, que são compostas por astrócitos, oligodendrócitos e microglia (Kwon e Koh, 2020). A microglia são as sentinelas imunes inatas do SNC mais abundantes que residem no parênquima (Prinz e Priller, 2014; You, Baluszek e Kaminska, 2019). Em condições patológicas, a microglia pode ser ativada e desempenhar papéis cruciais na neuroinflamação e diversas doenças do SNC (Karve, Taylor e Crack, 2016; Kim e Cho, 2016; Colonna e Butovsky, 2017). Além disso, a microglia está envolvida no remodelamento sináptico e na resposta aos padrões moleculares associados a patógenos (PAMPs) e padrões moleculares associados a danos (DAMPs). Em circunstâncias normais, a microglia encontra-se envolvida na manutenção da homeostase do SNC e na fagocitose de neurônios em fase apoptótica (Prinz, Jung e Priller, 2019; Cserép *et al.*, 2020; Kwon e Koh, 2020). Os astrócitos são as células mais abundantes do tecido nervoso e possuem um importante papel

no SNC que desempenham um papel importante no crescimento, desenvolvimento, proliferação celular, fornecimento de nutrientes para os neurônios e liberação de neuromoduladores como glutamato, adenosina trifosfato (ATP) e ácido γ -aminobutírico (GABA) (Refolo e Stefanova, 2019). Os astrócitos são parte da unidade neurovascular da barreira hematoencefálica (BHE), atuando como uma interface entre os neurônios e os vasos sanguíneos no encéfalo. Essas células também estão relacionadas com o sistema linfático do encéfalo (Refolo e Stefanova, 2019).

Na neuroinflamação, há grande envolvimento de respostas mediadas pelas células da glia, as quais podem ser estimuladas por elementos advindos da periferia (Kwon e Koh, 2020; Robb *et al.*, 2020). Em consequência, são produzidas citocinas pró-inflamatórias. As células micróglias, por exemplo, secretam citocinas pró-inflamatórias como TNF- α , interferon-gama (IFN- γ) e IL-6. Os astrócitos também secretam interleucina-1 alfa (IL-1 α), TNF- α e complemento C1q (Veerhuis, Nielsen e Tenner, 2011). No encéfalo, o TNF- α desempenha um papel crucial como regulador da inflamação de fase aguda e é um mediador chave na inflamação (Clark e Vissel, 2018). O aumento do TNF- α cerebral desempenha um papel chave na patogênese de doenças neurodegenerativas, o que o torna um importante alvo para se investir em novos tratamentos (Basu *et al.*, 2015; Ramos-Cejudo *et al.*, 2018). A IL-1 β induz uma rápida resposta pró-inflamatória dos astrócitos, levando à expressão de citocinas e moléculas de adesão, quimiocinas e metaloproteinases de matriz (Basu *et al.*, 2015). A IL-1 também desempenha um importante papel na neurodegeneração, induz a produção de IL-6 e estimula a atividade do óxido nítrico induzível (iNOS) nos astrócitos. A IL-6 desempenha múltiplos papéis na neuroinflamação sendo um dos principais mediadores na defesa do hospedeiro. Além disso, provoca ativação microglial e astrogliose e aumenta a produção de proteínas de fase aguda (Rubio-Perez e Morillas-Ruiz, 2012; Hauptmann *et al.*, 2020; Bohmwald, Andrade e Kalergis, 2021).

Outras vias que podem favorecer o estado neuroinflamatório no SNC, é pela via de sinalização do receptor do tipo toll-4 (TLR-4). O TLR-4 é um membro da família TLR que reconhece o lipopolissacarídeo (LPS), um glicolípido presente na membrana externa da maioria das bactérias Gram-negativas (Ran *et al.*, 2020). O TLR-4 é expresso na superfície da micróglio e desempenha um

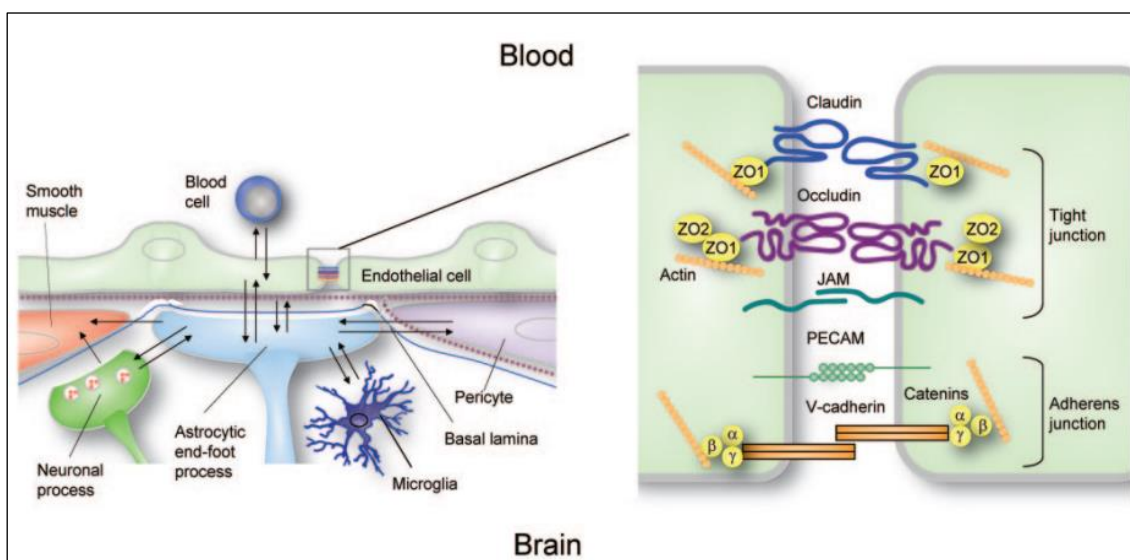
papel crítico na neuroinflamação. A estimulação de TLR-4 induz ativação de cinases associadas ao receptor de IL-1 (IRAKs) e TNF- α , seguido pela ativação do complexo cinases de I κ B (IKK) (Akira, 2007). Essa via ativa o fator nuclear kappa B (NF- κ B), que leva à transcrição de genes que codificam fatores pró-inflamatórios, como TNF- α e IL-1. o fator 6 associado a receptor de TNF (TRAF6) ativa a via de sinalização da proteína cinase ativada por mitógeno (MAPK) que inclui a cinase regulada por sinal extracelular (ERK), c-Jun N-terminal cinase (JNK) e a p38, levando a uma resposta inflamatória. Na via independente de MyD88/dependente de TRIF, TLR-4 coopera com TRIF para induzir interferons (IFNs; por exemplo, IFN- α/β) e NF- κ B (Akira, 2007).

Em outro contexto, o TLR-4 é capaz de regular indiretamente a inflamação pela interação com ácidos graxos saturados (AGS), modificando o metabolismo lipídico dos macrófagos. E isso pode estar associado com a microbiota intestinal. A exposição ao excesso de gordura altera a composição da microbiota intestinal e com isso, ocorre um aumento na absorção intestinal de produtos microbianos que por consequência aumentam a produção de LPS circulante (Lancaster *et al.*, 2018). Há estudos que associam AGS a vias de sinalização mediadas pelo TLR-4 em células imunes (Park *et al.*, 2009; Rogero e Calder, 2018). Além disso, pesquisas mostraram que os AGS do tipo ácido palmítico e ácido esteárico induzem um fenótipo microglial reativo e aumentam os níveis de marcadores inflamatórios através do TLR-4, culminando na ativação da via NF- κ B e na expressão da enzima ciclooxygenase-2 (COX-2). Os AGS podem aumentar atividade da via NF- κ B, proteína ativadora 1 (AP-1) e MAPK e elevar a expressão de marcadores inflamatórios. A ativação das proteínas cinases C (PKCs) ativam as vias IKK, MAPK e NADPH oxidase. A ativação de IKK-NF- κ B e MAPKs aumenta a expressão de genes inflamatórios, enquanto a ativação da NADPH oxidase aumenta a produção de ROS, induzindo o recrutamento de TLR, aumento da atividade de PKC e ativação do inflamassoma NLRP3, liberando citocinas pró-inflamatórias (Zhou, Urso e Jadeja, 2020).

1.3.1 Barreira Hematoencefálica

Outro aspecto importante para a homeostase das funções neurais é a existência da BHE. A BHE é formada por uma monocamada de células endoteliais especializadas e compactas ao longo da vasculatura cerebral que são ligadas mecanicamente por complexos proteicos de junção oclusivas que eliminam o espaço paracelular elas (Abbott e Friedman, 2012; Iadecola, 2013; Obermeier, Verma e Ransohoff, 2016). Embora seja chamada de barreira, a BHE é permeável, permitindo a passagem bidirecional de moléculas, porém de forma seletiva mantendo o microambiente do SNC diferenciado (Galea, 2021). A BHE é formada pelas junções oclusivas presentes entre as células endoteliais dos vasos sanguíneos do encéfalo, o que garante o acesso controlado de substâncias circulantes ao SNC. Essas junções são formadas principalmente por três tipos de proteínas: claudinas (CLDN), ocludinas (OCLDN) e zonula ocludens (ZO) (de Fraga *et al.*, 2021). As proteínas juncionais estão conectadas ao citoesqueleto (Galea, 2021). Os pés dos astrócitos são justapostos aos vasos sanguíneos, e também contribuem com a formação da BHE por expressarem transportadores específicos e de forma polarizada (Liu *et al.*, 2018). A estrutura e organização da barreira hematoencefálica pode ser vista na Figura 2.

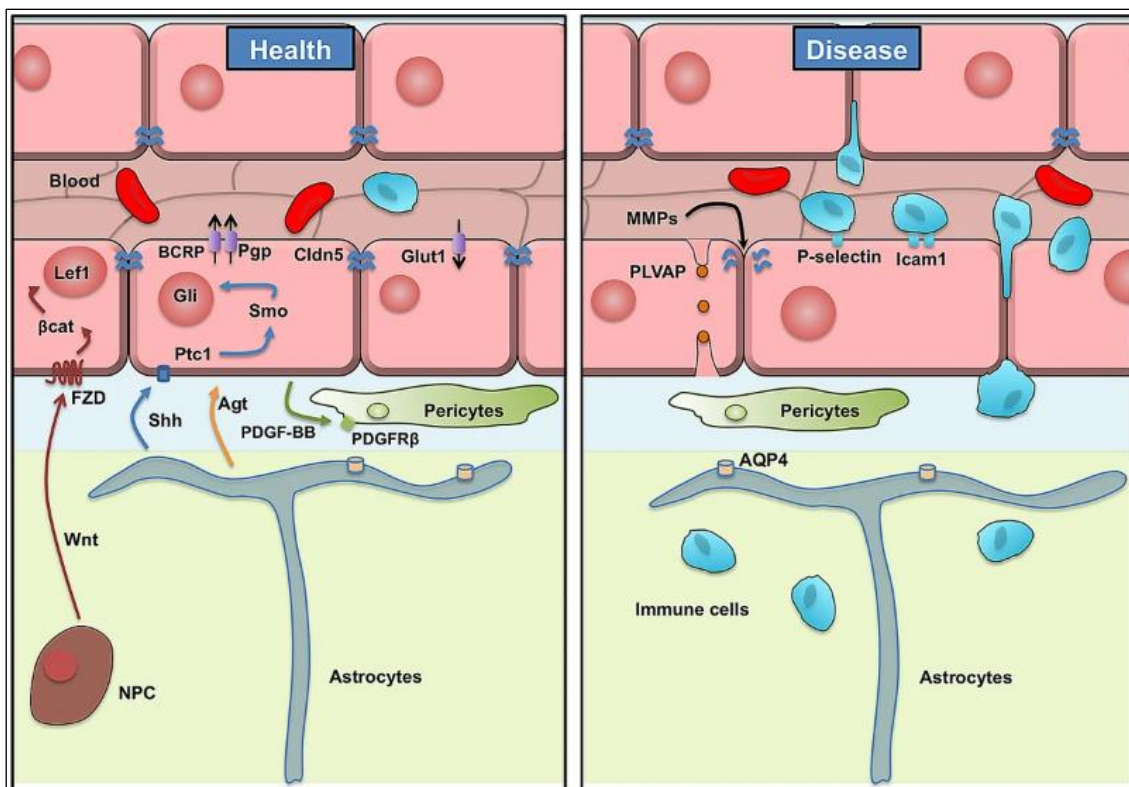
Figura 2. A arquitetura da barreira hematoencefálica, composta pela presença das junções oclusivas e reforçada pelos pés astrocitários, o que garante o ajuste paracelular entre as células endoteliais. Disfunções nesta estrutura culminam em quadros neuroinflamatórios (figura adaptada de (Benarroch, 2012))



Dentre as junções oclusivas, a claudina-5 é considerada a principal proteína que compõe esta estrutura. A claudina-5 e a ocludina são proteínas transmembrana, enquanto a ZO-1 localiza-se no espaço intracelular. A claudina-5, assim como a ocludina, reduz o transporte paracelular, contribuindo para o estreitamento desse espaço (Rossa *et al.*, 2014; Stamatovic *et al.*, 2016). A função da claudina-5 é dependente da sinalização da proteína cinase relacionada ao Rho e da fosforilação da claudina-5 (Lochhead *et al.*, 2020). As ZOs são proteínas associadas ao citoesqueleto e que interagem com claudinas, ocludinas e moléculas de adesão juncional para ancorar essas proteínas, ligando-as ao citoesqueleto de actina (Lochhead *et al.*, 2020).

A inflamação periférica causada pela obesidade, induz uma série de alterações na função e fisiologia da BHE, culminando em mudanças em sua permeabilidade (Hurtado-Alvarado *et al.*, 2016). Essas mudanças facilitam o acesso de leucócitos, assim como de patógenos e toxinas ao SNC, o que também contribui para a perda da integridade da própria BHE, com alterações subsequentes no transporte paracelular, transcelular e na expressão das proteínas das junções oclusivas, que por sua vez estimulam mais respostas inflamatórias, causando um ciclo vicioso. A Figura 3 mostra os eventos associados à perda da integridade da BHE, como a migração de células imunológicas para o SNC (Hurtado-Alvarado *et al.*, 2016; Varatharaj e Galea, 2017; Van Dyken e Lacoste, 2018; Schneider *et al.*, 2019).

Figura 3. Durante os processos neuroinflamatórios há perda da integridade da barreira hematoencefálica com diminuição da expressão das proteínas das junções oclusivas e retração dos pés astrocíticos. Além disso, ocorre invasão de células imunológicas da periferia e moléculas inflamatórias (figura adaptada de (Sohet e Daneman, 2013)).



A microbiota intestinal também interfere na regulação da BHE através de uma variedade de vias, incluindo os nervos simpáticos e vago, os sistemas imunológico e endócrino, e metabólitos microbianos intestinais, como ácidos graxos de cadeia curta (AGCC) e LPS (Bercik *et al.*, 2011; Fessler *et al.*, 2013; Forsythe, Bienenstock e Kunze, 2014; Cani e Knauf, 2016; Powell-Wiley *et al.*, 2021).

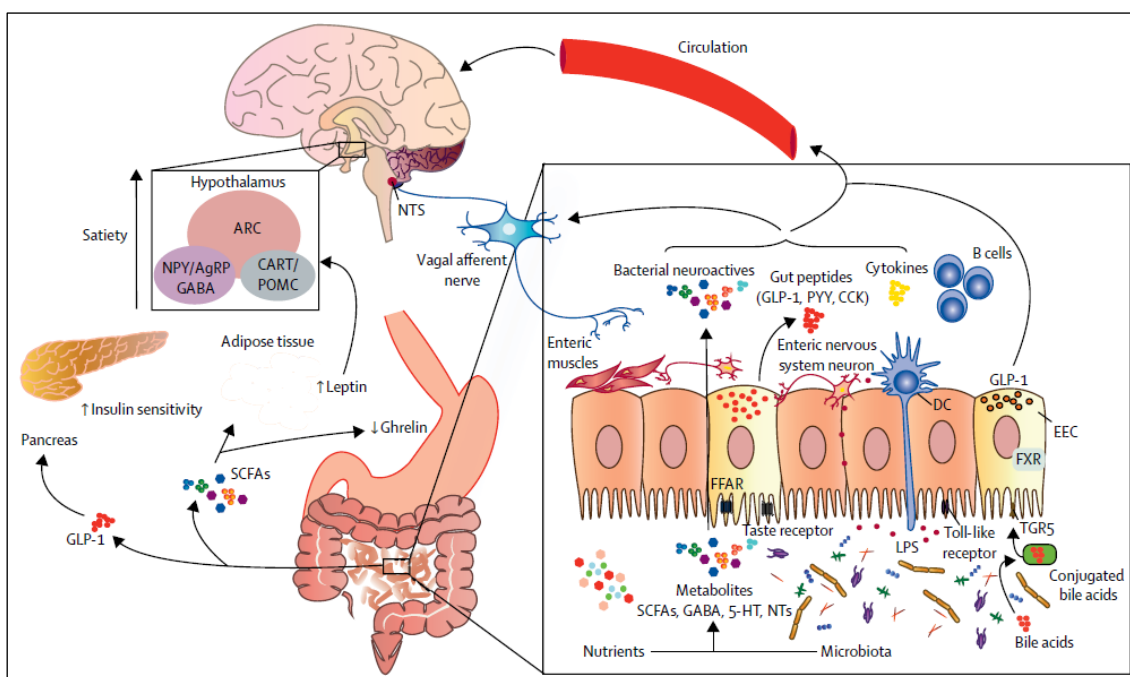
1.4 MICROBIOTA INTESTINAL

A microbiota intestinal é um ecossistema complexo, dinâmico, diversificado, habitado por trilhões de micróbios residentes que interagem entre si e com o hospedeiro, incluindo bactérias, fungos, archae e vírus (Sender, Fuchs e Milo, 2016; Koh e Bäckhed, 2020; Chen, Zhou e Wang, 2021). O microbioma intestinal, codifica mais de 3 milhões de genes, enquanto o genoma humano consiste em aproximadamente 23.000 genes (Fan e Pedersen, 2021). Em indivíduos saudáveis é povoada por cinco filos principais: Firmicutes, Bacteroidetes, Actinobacteria, Verrucomicrobia e Proteobacteria, entretanto cada indivíduo é único nos níveis de gênero e espécie (Thaiss *et al.*, 2016). A microbiota intestinal saudável é caracterizada pela alta diversidade e riqueza de

bactérias benéficas e um menor número de bactérias patogênicas (Rinninella *et al.*, 2019). Foi demonstrado que a composição da microbiota intestinal é influenciada pela dieta, idade, estilo de vida e presença de processos inflamatórios (Shi *et al.*, 2017; Kim e Jazwinski, 2018). Na última década, diversas evidências elucidaram como a microbiota é capaz de controlar e modificar determinados segmentos da fisiologia do hospedeiro, como o sistema imunológico, o sistema digestivo e o cérebro (Morrison e Preston, 2016; Rooks e Garrett, 2016; Dinan e Cryan, 2017). A interação entre o SNC, o intestino e a microbiota acontece através do chamado eixo intestino-cérebro, uma complexa rede de comunicação bidirecional entre o intestino e o SNC (Forsythe, Bienenstock e Kunze, 2014). O eixo intestino-cérebro, envolve diferentes vias, como o sistema nervoso autônomo e as células enteroendócrinas que são ativadas por alguns metabólitos, como os AGCC. Acetato, propionato e butirato são os principais AGCCs produzidos no cólon pela fermentação dos nutrientes advindos da dieta, envolvidos no fornecimento de energia e fatores tróficos (Dalile *et al.*, 2019). Além disso, os AGCCs regulam as colônias de células T reguladoras (Arpaia *et al.*, 2013; Francalacci *et al.*, 2013). Estudos apoiam a influência desses AGCCs na fisiologia de vários órgãos, incluindo o SNC (Stilling *et al.*, 2016; Fung, Olson e Hsiao, 2017; Dalile *et al.*, 2019). Desta forma, a manipulação da microbiota e a administração de AGCC têm sido propostas como alvos de tratamento para doenças neurológicas (Dinan e Cryan, 2017). Além disso, esses AGCC ativam diferentes receptores, como por exemplo, os receptores de ácidos graxos livres (FFAR), levando à produção de hormônios intestinais, como o peptídeo semelhante ao glucagon-1 (GLP-1), peptídeo YY (PYY) e colecistocinina (CCK) (Duvallet *et al.*, 2017; Blacher *et al.*, 2019; Burberry *et al.*, 2020; Harmon, Deng e Breslin, 2021). Esses hormônios intestinais sinalizam do intestino para o núcleo do trato solitário (NTS) através do nervo vago ou pela secreção direta no sistema circulatório (Forsythe, Bienenstock e Kunze, 2014; Mudd *et al.*, 2017). As informações do NTS são distribuídas para o núcleo arqueado (ARC) no hipotálamo, onde o apetite e o balanço energético são regulados. Alguns neurotransmissores, como GABA, serotonina (5-HT), e outros neurotransmissores, que têm diferentes efeitos periféricos e centrais, são capazes de modificar o metabolismo do eixo hipotálamo-hipófise-adrenal e a regulação central do apetite diretamente via estimulação vagal ou indiretamente por meio de mecanismos neuroendócrinos

imunológicos. A complexa comunicação entre o intestino e o cérebro na manutenção do apetite e balanço energético foi esquematizada na Figura 4 (Yano *et al.*, 2015; O'Keefe, 2016).

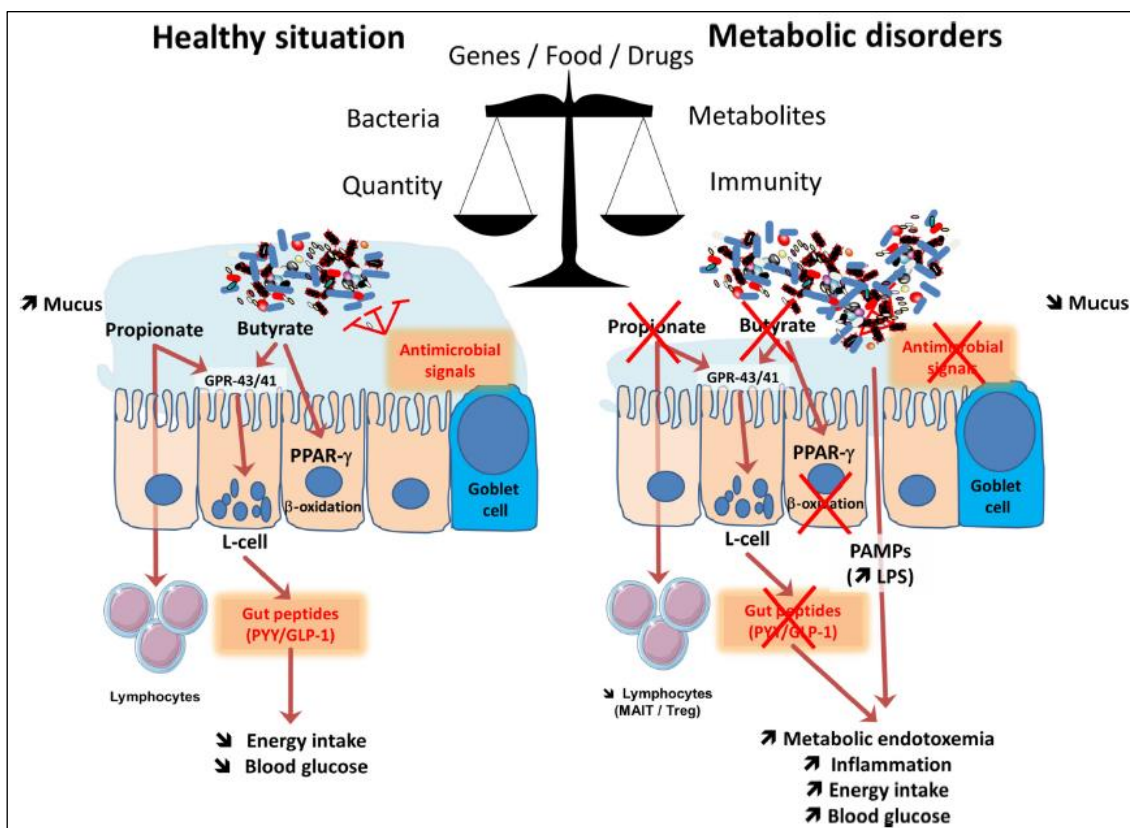
Figura 4. O apetite, a ingestão de alimentos e o balanço energético são controlados pelo SNC por vias neuroendócrinas complexas que medeiam a comunicação bidirecional entre o trato gastrointestinal e o encéfalo (figura adaptada de (Torres-Fuentes *et al.*, 2017)).



Alterações no microambiente da microbiota intestinal podem favorecer a inflamação sistêmica, conforme representado na figura 5. Durante os distúrbios metabólicos, ocorrem modificações na microbiota intestinal, diminuindo a espessura do muco intestinal, a capacidade de defesa antimicrobiana e a produção de butirato e propionato, assim como as células L também secretam menos peptídeos intestinais. A menor ativação do receptor ativado por proliferadores de peroxissoma gama (PPAR- γ) ocasiona maior disponibilidade de oxigênio para a microbiota, aumentando a proliferação de Enterobacteriaceae. Além disso, as concentrações de propionato também diminuem, contribuindo para a menor abundância de células T específicas no intestino. A soma dessas alterações, ambiente microbiano alterado e diminuição dos metabólitos, induzem um extravasamento dos PAMPs, como LPS, que estão

aumentados no sangue, desencadeando uma inflamação de baixo grau (Cani, 2018).

Figura 5. A disbiose intestinal provoca uma sequência de alterações no microambiente intestinal: diminuição da espessura do muco, ausência de sinal antimicrobiano, baixos níveis de propionato e butirato. Essas alterações culminam em inflamação sistêmica (figura adaptada de (Cani, 2018)).



O LPS pode ser derivado da microbiota intestinal, principalmente do filo Proteobacteria, e seus níveis aumentados estão relacionados ao desenvolvimento de doenças relacionadas à inflamação crônica. Os níveis de LPS no plasma sanguíneo são normalmente baixos, mas encontram-se elevados em muitas doenças de caráter inflamatório crônico, como obesidade e diabetes tipo 2 (Hersoug, Møller e Loft, 2016), DHGNA (Fuke *et al.*, 2019), e doenças neurodegenerativas, como doença de Alzheimer, doença de Parkinson e esclerose lateral amiotrófica (Brown, 2019). Da mesma forma, estudos com animais e humanos sustentam que a disbiose da microbiota intestinal tem sido associada a distúrbios neurológicos, como depressão, doença de Alzheimer,

doença de Parkinson e transtorno do espectro do autismo (Jenkins *et al.*, 2016; Vancamelbeke e Vermeire, 2017; Camilleri, 2019; Dalile *et al.*, 2019).

Quanto aos filos predominantes na microbiota intestinal, sabe-se que Firmicutes e Bacteroidetes representam cerca de 90% da composição da microbiota (Hildebrandt *et al.*, 2009). Estudos clínicos e pré-clínicos de obesidade mostraram inicialmente um aumento na abundância do filo Firmicutes e diminuição do filo Bacteroidetes (Ley *et al.*, 2005; Turnbaugh *et al.*, 2009). Entretanto, outras pesquisas não evidenciaram este padrão (Duncan *et al.*, 2008; Schwartz *et al.*, 2010; Jumpertz *et al.*, 2011; Tims *et al.*, 2013; Zhang *et al.*, 2019). Outros estudos com indivíduos obesos mostraram aumento na abundância nos filos Proteobacteria e Actinobacteria, e redução no filo Verrucomicrobia (Turnbaugh *et al.*, 2009; Clarke *et al.*, 2012; Karlsson *et al.*, 2012; Rizzatti *et al.*, 2017). Quanto a famílias e gêneros bacterianos associados com a obesidade, podem ser destacados: *Archaea*, *Metanobacteriales*, *Prevotellaceae*, *Clostridium ramosum*, *Clustridium leptum*, *Bacteroides thetaiotaomicron* e *Methanobrevibacter smithii*, *Lactobacillus spp.* - *Lactobacillus reuteri*, *Ruminococcus*, *Oscillibacter*, *Staphylococcus* (Million *et al.*, 2012; Duca *et al.*, 2014; Woting *et al.*, 2014; Chakraborti, 2015; Gomes, Hoffmann e Mota, 2018). Contudo, existem muitas discrepâncias entre os estudos e ainda não existe um padrão definitivo de microbiota associada aos distúrbios metabólicos como a obesidade.

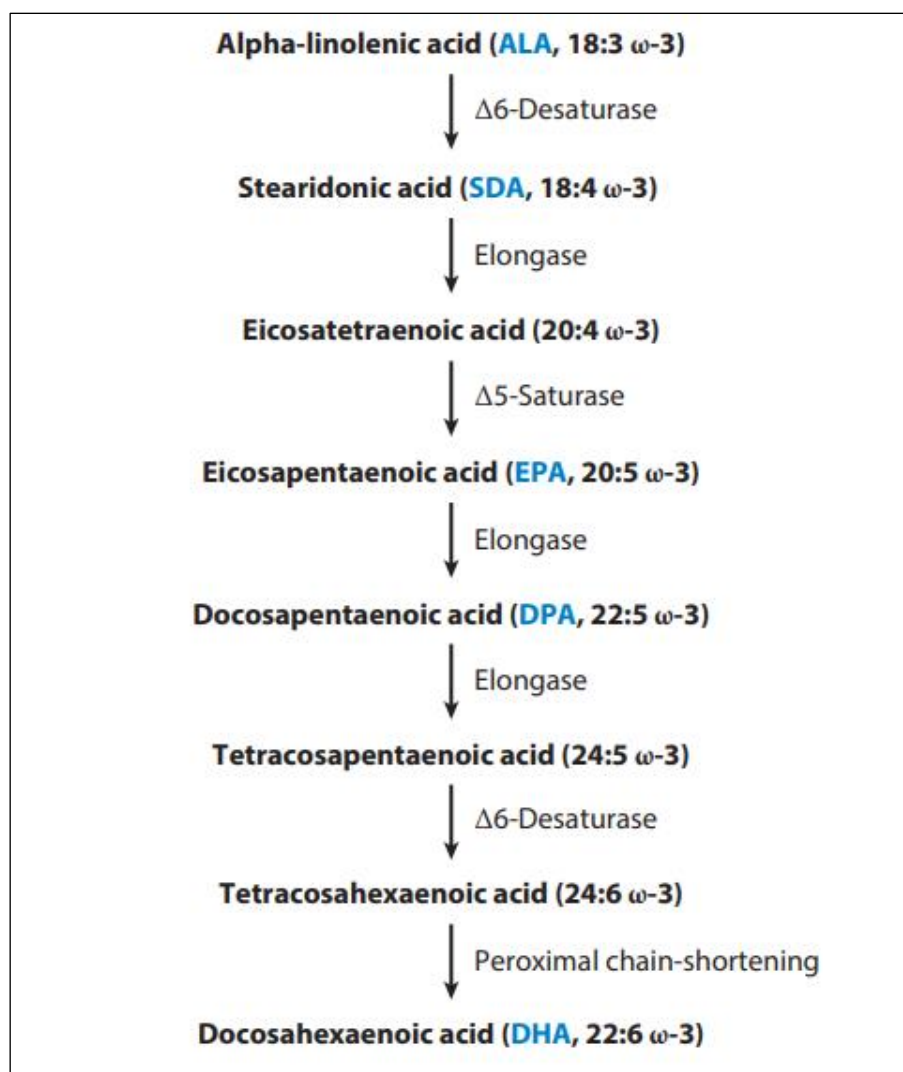
1.5 ÁCIDOS GRAXOS POLI-INSATURADOS ÔMEGA-3

As estratégias para reduzir a obesidade tornaram-se prioridade de saúde pública à medida que a sua prevalência aumentou em todo o mundo. Recentemente, a *Food and Drug Administration* (FDA), agência federal do Departamento de Saúde e Serviços Humanos dos Estados Unidos, aprovou o uso de dois produtos contendo ácidos graxos do tipo ômega-3 para a melhora do perfil lipídico: icosapento de etilo e ésteres etílicos de ômega-3 (Krupa, Fritz e Parmar, 2022). A indicação de uso dessas medicações é para adultos com hipertrigliceridemia (≥ 500 mg/dl), sendo a dose recomendada de ômega-3 entre

2 a 4 g/dia, associado ao controle da dieta e prática de exercício físico (Skulas-Ray *et al.*, 2019).

O ômega-3 é um grupo de ácidos graxos poli-insaturados definidos por uma ligação dupla localizada no terceiro carbono a partir do carbono metil terminal da cadeia. Os ácidos graxos da família ômega-3 são considerados essenciais, pelo fato de o organismo não possuir uma dessaturase para adicionar uma ligação dupla no décimo quinto carbono deste ácido graxo de cadeia longa, impedindo sua síntese endógena tanto do ácido α -linolênico quanto do ácido linoleico (Jeromson *et al.*, 2015). Fazem parte desta família, principalmente os ácidos α -linolênico (C18:3n-3) (ALA), o ácido eicosapentanoico (C20:5n-3) (EPA) e o ácido docosahexaenóico (C22:6n-3) (DHA) (Calder, 2013; Shahidi e Ambigaipalan, 2018). A via metabólica do ômega-3, inicia com a ação da enzima Δ 6-dessaturase a partir da dessaturação do ALA, inserindo uma dupla ligação na posição 6, convertendo a ácido estearidônico (SDA). Em seguida, sua cadeia carbônica é alongada a ácido eicosatetraenóico, pela enzima elongase, sendo subsequentemente convertido a EPA pela Δ 5-dessaturase e alongado a ácido docosapentaenóico (DPA). o DPA sofre adição de 2 carbonos formando ácido tetracosapentaenóico, o qual é convertido a ácido tetracosahexaenóico pela ação de uma Δ 6-dessaturase. A conversão a DHA é realizada por meio de etapas adicionais envolvendo reações de alongamento, dessaturação e posterior encurtamento da cadeia nos peroxissomos (Shahidi e Ambigaipalan, 2018). A Figura 6 apresenta de forma resumida a via metabólica da síntese de ácidos graxos poli-insaturados ômega-3 a partir do ALA consumido na dieta.

Figura 6. O ALA ingerido na dieta é sintetizado a SDA, que posteriormente é convertido a EPA, DPA e DHA. A conversão requer enzimas dessaturases (5 e 6), uma elongase do sistema microssomal, e oxidação nos peroxissomos para encurtamento da cadeia (figura adaptada de (Shahidi e Ambigaipalan, 2018)).



Sendo assim, os ácidos graxos ômega-3 devem ser obtidos pela dieta em alimentos como óleos que contêm esses ácidos graxos advindos de fontes vegetais e podem ser encontrados em produtos à base de peixe, sementes, nozes, vegetais de folhas verdes e feijões (Behl e Kotwani, 2017; Shahidi e Ambigaipalan, 2018). Nosso organismo tem uma baixa capacidade de estocar ácidos graxos ômega-3, dessa forma, o seu consumo deve ser contínuo, visto que o ALA é rapidamente catabolizado a dióxido de carbono como fonte energética e apenas uma pequena proporção pode ser convertida tanto a EPA quanto a DHA (entre 0.3 a 8%) (Arterburn, Hall e Oken, 2006). O EPA é o principal precursor de prostaglandinas da série 3 e de leucotrienos da série 5 e o mais abundante ácido graxo poli-insaturado presente no óleo de peixe, enquanto o DHA é um precursor de resolvinas, protectinas e maresinas (Sanak, 2016). Além disso, o DHA é essencial para as funções do SNC, estando presente em aproximadamente 30% a 40% dos fosfolipídios na substância cinzenta do

córtex cerebral, modulando a neurotransmissão, expressão gênica e atividades de enzimas, receptores e canais iônicos, ativando vias de sinalização envolvidos na função sináptica e a sobrevivência neuronal (Farooqui, 2009; Oster e Pillot, 2010).

Os ácidos graxos ômega-3 demonstraram modular várias vias inflamatórias promovendo inibição da quimiotaxia leucocitária, da expressão de moléculas de adesão, da atividade da COX e da subsequente produção de eicosanóides, como leucotrienos e prostaglandinas a partir do ácido araquidônico, inibição de citocinas pró-inflamatórias (TNF- α , IL-1, IL-6), inibição da ativação do NF κ B, ativação do fator de transcrição anti-inflamatório NR1C3, ativação de PPARs, ativação do receptor GPR120 (Calder, 2013; Nabavi *et al.*, 2015; Rogero e Calder, 2018; Shahidi e Ambigaipalan, 2018; Calder e Deckelbaum, 2019; Ishihara, Yoshida e Arita, 2019; Sakamoto *et al.*, 2019; Fazelian *et al.*, 2021). O ômega-3 já apresentou efeitos benéficos em diversas doenças metabólicas, como hipertrigliceridemia (Skulas-Ray *et al.*, 2019), diabetes tipo 2 e DHGNA (Shahidi e Ambigaipalan, 2018). No SNC, já existem diversos estudos mostrando os benefícios do ômega-3 com efeito protetor na função cognitiva e doença de Alzheimer, depressão, transtorno de déficit de atenção e hiperatividade, epilepsia e ansiedade (Bozzatello *et al.*, 2016; Chang *et al.*, 2018; Shahidi e Ambigaipalan, 2018; Neto *et al.*, 2021; Wood, Chappell e Zulyniak, 2022).

A suplementação de ômega-3 demonstrou ser benéfica no tratamento das condições supracitadas; no entanto, ainda existe controvérsia em relação ao seu uso. Desta forma, tornam-se necessárias mais pesquisas para a indicação de seu uso.

1.6 DIETA DE CAFETERIA

Os modelos experimentais com animais desempenham um papel crucial na pesquisa básica e clínica. É fundamental que existam modelos animais válidos para abordar e minimizar os distúrbios humanos (Belzung e Lemoine, 2011). A dieta de cafeteria (CAF) foi utilizada em experimentos pela primeira vez nos anos 70. Entretanto, ganhou força no meio científico na última década. A CAF, baseia-se em oferecer aos animais de laboratório acesso ilimitado a uma

dieta com alimentos consumidos por humanos (Sclafani e Springer, 1976). Nesse modelo, roedores de laboratório comem os mesmos produtos saborosos, ultraprocessados, que não são saudáveis, porém, os humanos consomem. Além disso, é facilmente obtido em supermercados e restaurantes de fast food. Por esse motivo, também é chamada de “dieta de junk food”, “dieta de supermercado” ou “dieta ocidental” (Lalanza e Snoeren, 2021). Portanto, o modelo de dieta CAF imita um padrão de consumo humano prejudicial à saúde.

A dieta de CAF, diferente de outros modelos obesogênicos como a dieta rica em gordura (HFD), se diferencia por suas propriedades hedônicas ao oferecer experiência orosensorial, que ativam o córtex gustativos primário e secundário e também estimulam o sistema de recompensa de forma semelhante à forma como as drogas de abuso ativam o sistema de recompensa (Kenny, 2011; Volkow, Wise e Baler, 2017).

Existem muitas evidências validando a CAF como um modelo obesogênico que melhor induz o ganho de peso, levando à síndrome metabólica, diabetes grave, esteatose hepática e outras alterações metabólicas (Macedo *et al.*, 2012; Lalanza *et al.*, 2014; Mucellini *et al.*, 2014; Romero *et al.*, 2014; Gomez-Smith *et al.*, 2016; Suárez-García *et al.*, 2017; Lewis, Singh e Youssef, 2019). Além disso, quando a CAF foi comparada a HFD, os efeitos da obesidade e as alterações/doenças metabólicas foram mais pronunciadas (Sampey *et al.*, 2011; Zeeni *et al.*, 2015; Buyukdere, Gulec e Akyol, 2019; Gual-Grau *et al.*, 2019; Lalanza e Snoeren, 2021). Além disso, a CAF gera hiperfagia em roedores semelhantes aos evidenciados em humanos (Shafat, Murray e Rumsey, 2009; Goularte, Ferreira e Sanvitto, 2012; South, Westbrook e Morris, 2012). Sendo assim, a CAF é um modelo de grande importância para o estudo das consequências da obesidade induzida por dieta.

2 JUSTIFICATIVA

Nas últimas décadas, a obesidade vem se tornando uma verdadeira epidemia mundial, repercutindo negativamente sobre a saúde da população. A obesidade ocasiona diversas alterações metabólicas causando, secreção crônica de moléculas pró-inflamatórias. A microbiota intestinal e o fígado são diretamente afetados, assim como o sistema nervoso. O estado inflamatório sistêmico ocasiona neuroinflamação, o que predispõe às doenças neurodegenerativas e psiquiátricas. Entretanto, embora a relação entre obesidade, inflamação periférica e risco aumentado à disfunção metabólica e neurológica seja conhecida, intervenções que reduzam esses efeitos deletérios da obesidade são bastante limitadas. Certamente o combate à obesidade é uma estratégia fundamental para a prevenção de seus malefícios, porém, também é importante estabelecer intervenções que minimizem os prejuízos da função neurológica na população obesa. Desta forma, os ácidos graxos da família ômega-3, por seu conhecido potencial anti-inflamatório, podem exercer efeitos benéficos sobre a disbiose intestinal e a neuroinflamação. Dessa forma, o presente projeto propõe avaliar os efeitos da suplementação de ômega-3 sobre a microbiota intestinal, aspectos metabólicos e neurofuncionais em ratos obesos alimentados com dieta de cafeteria, que é um modelo animal que mimetiza a dieta consumida pelos seres humanos.

3 OBJETIVO GERAL

Avaliar o efeito da suplementação de ômega-3 sobre a microbiota intestinal, aspectos metabólicos, comportamentais e neuroinflamatório em ratos obesos alimentados com dieta de cafeteria.

3.1 OBJETIVOS ESPECÍFICOS

- I. Determinar o ganho de peso e a adiposidade visceral.
- II. Verificar o perfil lipídico, a glicemia e os níveis plasmáticos de insulina e resistência à insulina pelo índice de HOMA.
- III. Avaliar o perfil inflamatório sistêmico por meio da dosagem de citocinas pró-inflamatórias TNF- α , IL-6.
- IV. Avaliar o comportamento do tipo ansiedade e a memória social, por meio do teste de labirinto em cruz elevado e teste de memória social de cinco tentativas.
- V. Analisar parâmetros neuroinflamatório como nível de TNF- α no córtex cerebral e a expressão de TLR-4 no córtex cerebral e hipocampo.
- VI. Avaliar a integridade da barreira hematoencefálica por meio da expressão da claudina-5 no córtex cerebral e hipocampo.
- VII. Avaliar a morfologia do cólon intestinal pelo método de coloração hematoxilina-eosina.
- VIII. Caracterizar a microbiota intestinal por sequenciamento de nova geração.
- IX. Avaliar os níveis de ácidos graxos de cadeia curta e LPS no plasma e ácidos graxos saturados no córtex cerebral.

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5 ARTIGO CIENTÍFICO 1

**DHA/EPA SUPPLEMENTATION DECREASES ANXIETY-LIKE BEHAVIOUR,
BUT IT DOES NOT AMELIORATE METABOLIC PROFILE IN OBESE MALE
RATS**

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DHA/EPA supplementation decreases anxiety-like behaviour, but it does not ameliorate metabolic profile in obese male rats

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Abstract

Obesity is a major public health problem that predisposes to several diseases and higher mortality in patients with COVID-19. Obesity also generates neuroinflammation, which predisposes to the development of neuropsychiatric diseases. Since there is a lack of effective treatments for obesity, the search for new strategies to reverse its consequences is urgent. In this perspective, the anti-inflammatory properties of omega-3 polyunsaturated fatty acids such as DHA/EPA might reduce the harmful effects of obesity. Here, we used the cafeteria diet (CAF) model to induce obesity in Wistar rats. Animals received ultra-processed food for 20 weeks, and DHA/EPA supplementation (500 mg/kg per d) was performed between the 16th and the 20th week. At the end of the experiment, it was evaluated: body weight, visceral fat deposition, plasma glucose, insulin and triglycerides, and it was also measured the levels of inflammatory cytokines TNF- α and IL-6 in plasma and liver, and TNF- α in the prefrontal cortex. The elevated plus maze test was performed to analyse anxiety-like behaviour. Our results demonstrated that DHA/EPA could not reverse weight and fat gain and did not modify plasma dosages. However, there was a decrease in IL-6 in the liver (DHA/EPA effect: $P = 0.023$) and TNF- α in the brain (CAF compared with CAF + DHA/EPA, $P < 0.05$). Also, there was a decrease in the anxiety index in CAF + DHA/EPA compared with the CAF group ($P < 0.01$). Thus, DHA/EPA supplementation is helpful to reverse the consequences of obesity in the brain.

Key words: Obesity: Cafeteria diet: Neuroinflammation: Anxiety: *n*-3 PUFA

The global prevalence of obesity has risen over the years representing a major health issue nowadays⁽¹⁾. Obesity increases the risk of developing several chronic diseases such as diabetes mellitus, cardiovascular diseases, some types of cancer and non-alcoholic fatty liver disease^(2,3). More recently, increased pathogenicity to infectious diseases has also been linked to excessive body fat⁽⁴⁾, seen by higher mortality rates in patients with obesity in the COVID-19 pandemic^(5,6).

Treatments available for obesity are not efficient for many patients since they are based on (1) lifestyle interventions, which commonly result in weight regain; (2) pharmacological

treatments, but most of them are not well tolerated because of side effects; and (3) bariatric surgery, which is associated with several risks and it should be carefully recommended⁽¹⁾. Considering the harmful effects of obesity, the search for new therapeutic strategies is urgent. In this perspective, it has been shown that the consumption of omega-3 (*n*-3) polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA) or eicosapentaenoic acid (EPA) is associated with a significant reduction in plasma levels of triglycerides^(7,8) and reduced fasting glucose levels⁽⁹⁾. In this regard, *n*-3 supplementation is recognised for reducing the risk of coronary heart

Abbreviations: CAF, cafeteria diet; CT, control diet; HFD, high-fat diet; IR-HOMA, insulin resistance-homeostasis model assessment.

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disease mortality and related events⁽¹⁰⁾. Its anti-inflammatory properties are demonstrated in several animal models of inflammatory diseases⁽¹¹⁾. Moreover, *n*-3 may be related to anxiolytic and anti-depressant effects⁽¹²⁾, although these results are controversial and need further investigations^(13,14).

It is worth mentioning that obesity is related to a low-grade chronic inflammatory profile originating from adipose tissue's immune signals. These signals activate the NF- κ B signalling pathway in the cells, followed by an increase in the transcription of proinflammatory cytokines such as interleukin 6 (IL-6) and tumour necrosis factor- α (TNF- α)⁽¹⁵⁾. The increased levels of inflammatory mediators appear not only in the periphery but also in the brain, establishing a neuroinflammatory state⁽¹⁶⁾. Pathological brain conditions may be initiated or intensified due to neuroinflammation such as cognitive decline and neurodegenerative diseases, characterised by impaired memory and attention^(17,18), and psychiatric diseases, such as depression and anxiety⁽¹⁹⁾. In this regard, it is shown that obesity and metabolic dysfunction are correlated with cognitive dysfunction⁽²⁰⁾ and anxiety symptoms⁽²¹⁾.

Among different protocols to induce obesity in animal models, the cafeteria diet (CAF) is beneficial since it mimics the human population's Western diet. CAF is composed of ultra-processed foods that are characterised by their high palatability. Therefore, it can lead to a hyperphagia state similar to the human pattern of food consumption⁽²²⁾. Another advantage of animal studies using CAF is the presence of several food additives and the lack of vitamins and minerals⁽²³⁾. Consequently, this diet induces metabolic changes related to obesity such as hepatic steatosis, increased visceral adiposity, glucose intolerance⁽²⁴⁾ and is also capable of inducing neuroinflammation in regions such as the hippocampus⁽²⁵⁾, which is essential for memory consolidation and neurogenesis⁽²⁶⁾.

Although DHA/EPA benefits in metabolic dysfunction are largely studied, there are still controversial findings. In addition, DHA/EPA effects on the brain are also debated. To elucidate the beneficial properties of DHA/EPA in the management of obesity and obesity-related neurological outcomes, we evaluated whether 4 weeks of DHA/EPA supplementation were able to change metabolic and neuroinflammatory parameters and anxiety-like behaviour in rats fed with CAF.

Materials and methods

Animals

Forty-eight adult male Wistar rats were obtained from the animal facility of the Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA) and housed in plastic cages at 23 (SD 1)°C with a 12/12 h of light/dark cycle. This project was approved by the Institutional Animal Care and Use Committee of UFCSPA under the protocol N°. 570/18. The procedures were designed to minimise the number and the suffering of animals following the international laws for laboratory animals' care. Only male rats were employed to avoid female hormonal fluctuations, which may impact the results of this study.

Table 1. Nutritional information of cafeteria diet*

Diet components	Energy (kJ)	Protein (g)	Carbohydrates (g)	Lipids (g)
Salty snacks	2142.2	9.2	56	28
Biscuits	2119.6	6.66	66.66	23.66
Wafer	2259.36	3.33	63.33	30.33
Chocolate	2292.83	3.33	60	33.33
Mortadella	1181.9	12	6	23.33
Sausage	1004.1	12	6	19
Soft drink	207.1	0	12	0

* Values obtained from food labels considering a portion of 100 g of food or 100 ml of soft drink.

Experimental groups and diet

Animals ($n=12$ /group) were randomly allocated into four groups: Control diet (CT); CT + DHA/EPA; CAF; and CAF + DHA/EPA. Animals received normal chow diet Nuvilab® CR-1 (Nuvital®) or normal chow diet plus CAF for 20 weeks with DHA/EPA supplementation starting at the 16th week. Groups supplemented with DHA/EPA received Mega DHA® (Vitafor®) in a high concentration of EPA (10%) and DHA (50%) at the dose of 500 mg/kg every day by gavage. The other groups received water (0.5 ml/rat) instead of DHA/EPA. The sample size was chosen based on previous studies using a similar methodology^(27,28).

Cafeteria groups were fed with three distinct menus interchanged every 2 d to maintain novelty and stimulate consumption. Menus were composed of standard chow plus palatable human food as cookies, wafer, sausage, bologna and white chocolate. They also received orange-based soft drink besides water *ad libitum*. Every component's leftovers were weighed every 2 d, including soft-drink consumption, to determine food intake per cage.

The regular chow diet's total energy content was 14.22 kJ/g (3.4 kcal/g, 63% carbohydrates, 26% protein, 11% fat). The CAF energy content was calculated based on the manufacturer's information and provided 18.8 kJ/g (4.5 kcal/g) distributed in 42% carbohydrates, 16% protein and 42% lipids. Detailed nutritional information on the components of the diet is shown in Table 1. Animals were weighed weekly to determine weight gain.

Elevated plus maze test

Elevated plus maze was used to assess anxiety-like behaviour in rats. The test was conducted on the 20th week of the experiment and was applied as described by ref.⁽²⁹⁾. The apparatus consisted of four arms 50 cm long and 10 cm wide. Two of those are open, and the other two arms are closed with walls (40 cm high) on the sides. The maze was 50 cm above the floor. The rats were placed on the central platform facing an open arm and freely explored the maze for 5 min. The entrance was defined when the four paws touched one of the arms. More time spent in the closed arm and a decrease in risk behaviour (head dipping) could indicate anxious-like behaviour^(30,31).

The following parameters were analysed in the test: number of head dipping, the number of entries in the open and the

closed arms, total time spent in the open and closed arms. Subsequently, it was measured the percentage of open arm entries ($100 \times$ open arms entries/total arms entries; PEOA) and the percentage of time spent in the open arm ($100 \times$ time in the open arms/total time; PTOA)^(30,31). Also, the anxiety index was calculated using the formula: anxiety index = $1 - ((\text{open arm time}/5 \text{ min}) + (\text{open arm entry}/\text{total entry}))/2$ ⁽³²⁾. Results vary from 0 to 1; values closer to 1 indicate high anxiety.

Tissue and blood collection

In the 20th week of the experiment, the rats were euthanised by decapitation after fasting for 6 h. Trunk blood was collected and, after 30 min, it was centrifuged at 3500 rpm for 10 min. Plasma obtained was aliquoted and stored at -80°C for further analysis. Brains were quickly removed. The prefrontal cortex was manually dissected and immediately frozen in liquid nitrogen. Samples were kept at -80°C until further processing.

Plasma metabolic parameters

Plasma levels of glucose and triglycerides were measured using commercial enzymatic colourimetric kits (Labtest©). Insulin levels were assessed by ELISA (Sigma©). Tests were conducted following the manufacturer's instructions.

Insulin resistance-homeostasis model assessment index

Insulin resistance-homeostasis model assessment (IR-HOMA) index was used to evaluate insulin resistance⁽³³⁾. The IR-HOMA index was calculated using the formula: fasting blood glucose levels \times fasting blood insulin levels/22.5⁽³⁴⁾.

Pro-inflammatory cytokines

Levels of inflammatory cytokines in plasma (IL-6 and TNF- α), liver (IL-6 and TNF- α) and prefrontal cortex (TNF- α) were quantified by ELISA (Invitrogen©), following the manufacturer's instructions.

Statistical analysis

Main effects and interactions were analysed with two-way ANOVA and differences between groups with Tukey post-hoc test. The results of two-way ANOVA are shown as the main effects (diet or DHA/EPA supplementation) and the interaction between diet and supplementation. Weight gain per week was analysed by two-way repeated-measures ANOVA. Rout test was used to remove outliers. All statistical analysis was conducted using Graphpad Prism 8 (GraphPad Software, Inc.). The results were expressed as mean values with their standard error of the mean. The results were considered statistically significant at $P < 0.05$.

Results

Weight gain, adiposity and metabolic profile

Weight gain was significantly increased in CAF groups (diet effect: $F_{(1,42)} = 114.2$, $P < 0.0001$) regardless of DHA/EPA supplementation (DHA/EPA effect: $F_{(1,42)} = 3.92$, $P = 0.054$;

interaction: $F_{(1,42)} = 0.05$, $P = 0.81$), as shown in Fig. 1(a). Weight gain was significantly different between CAF and CT groups from the 8th week onwards (Fig. 1(b)). Similarly, the visceral fat deposition was higher in CAF-fed groups (diet effect: $F_{(1,42)} = 140.3$, $P < 0.0001$) with no effect of DHA/EPA (DHA/EPA effect: $F_{(1,42)} = 1.72$, $P = 0.19$; interaction: $F_{(1,42)} = 0.07$, $P = 0.78$) as demonstrated in Fig. 1(c).

Metabolic effects of CAF and DHA/EPA were analysed by measuring plasma levels of glucose, triglycerides, insulin and HOMA-index calculation. A diet effect ($F_{(1,35)} = 26.75$, $P < 0.0001$) was found in plasma glucose, which was increased in CAF groups. Two-way ANOVA did not show a significant effect of DHA/EPA supplementation (DHA/EPA effect: $F_{(1,35)} = 2.6$, $P = 0.11$; interaction: $F_{(1,35)} = 1.8$, $P = 0.18$). In the multiple comparisons, the CAF group was significantly different from the CT and CT + DHA/EPA groups ($P < 0.001$) (Fig. 2(a)). Also, CAF increased triglycerides levels (diet effect: $F_{(1,41)} = 45.39$, $P < 0.0001$) and DHA/EPA supplementation did not mitigate it (DHA/EPA effect: $F_{(1,41)} = 2.35$, $P = 0.13$; interaction: $F_{(1,41)} = 0.35$, $P = 0.55$) (Fig. 2(b)). Following that, insulin levels were also affected by the diet ($F_{(1,38)} = 12.62$, $P = 0.001$) but not by the DHA/EPA treatment (DHA/EPA effect: $F_{(1,38)} = 1.25$, $P = 0.27$; interaction: $F_{(1,38)} = 0.10$, $P = 0.75$) (Fig. 2(c)). Insulin resistance, assessed by the HOMA-index, also showed a CAF effect ($F_{(1,38)} = 12.28$, $P = 0.0012$), although the supplementation did not impact it (DHA/EPA effect: $F_{(1,38)} = 0.49$, $P = 0.48$; interaction: $F_{(1,38)} = 0.0009$, $P = 0.97$). However, Tukey post-hoc test showed a significant difference between CAF and CT + DHA/EPA group ($P < 0.05$) for insulin levels and HOMA-index (Fig. 2(d)).

Inflammatory markers

We also evaluated the levels of inflammatory cytokines TNF- α and IL-6 in plasma and liver, and TNF- α in the prefrontal cerebral cortex. Plasma TNF- α (diet effect: $F_{(1,25)} = 0.014$, $P = 0.9$; DHA/EPA effect: $F_{(1,25)} = 1.21$, $P = 0.28$; interaction: $F_{(1,25)} = 0.14$, $P = 0.70$) and IL-6 (diet effect: $F_{(1,22)} = 0.22$, $P = 0.63$; DHA/EPA effect: $F_{(1,22)} = 0.75$, $P = 0.39$; interaction: $F_{(1,22)} = 0.69$, $P = 0.41$) were affected neither by diet nor by supplementation (Fig. 3(a) and (b), respectively). However, in the liver, TNF- α levels were increased following CAF (diet effect: $F_{(1,24)} = 31.55$, $P < 0.0001$), with no effect of DHA/EPA (DHA/EPA effect: $F_{(1,24)} = 0.19$, $P = 0.66$; interaction: $F_{(1,24)} = 0.93$, $P = 0.34$), as shown in Fig. 3(c). Interestingly, IL-6 in the liver was not affected by diet (diet effect: $F_{(1,21)} = 0.02$, $P = 0.88$; interaction: $F_{(1,21)} = 0.50$, $P = 0.48$), but DHA/EPA supplementation diminished it in both CT and CAF groups (DHA/EPA effect: $F_{(1,21)} = 6.003$, $P = 0.0231$), although post-hoc analysis did not show differences among groups (Fig. 3(d)). In the prefrontal cerebral cortex, TNF- α level showed an interaction between diet and supplementation ($F_{(1,19)} = 6.121$, $P = 0.023$; diet effect: $F_{(1,19)} = 0.74$, $P = 0.39$; DHA/EPA effect: $F_{(1,19)} = 2.56$, $P = 0.12$). Post-hoc test also evidenced that DHA/EPA supplementation decreased TNF- α in the CAF + DHA/EPA compared with CAF (Fig. 3(e)), showing the ability of *n-3* in reducing this pro-inflammatory cytokine in the cerebral cortex of obese animals.

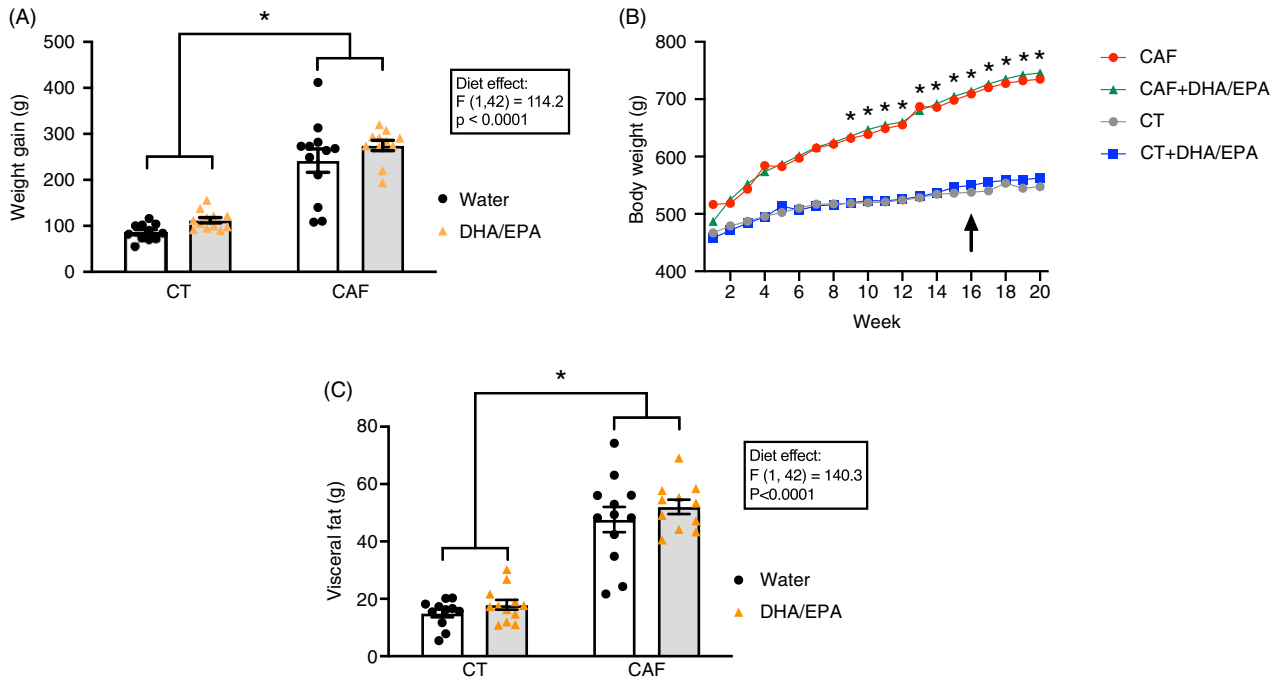


Fig. 1. DHA/EPA supplementation did not change weight gain or visceral adiposity in cafeteria diet (CAF)-fed rats. (a) Weight gain at the end of the experiment. (b) Body weight over time. (c) Visceral fat mass at the end of the experiment. Arrow indicates the beginning of DHA/EPA supplementation. Significant differences showed by two-way ANOVA regarding effects of diet (CAF and CAF + DHA/EPA v. CT and CT + DHA/EPA), EPA/DHA treatment (CAF and CT v. CAF + DHA/EPA and CT + DHA/EPA) and diet \times DHA/EPA interactions are indicated in the text boxes. Multiple comparisons were performed by Tukey post-hoc test and are indicated as follows: * $P < 0.0001$ comparing CAF v. CT or CT + DHA/EPA; CAF + DHA/EPA v. CT or CT + DHA/EPA. $n = 11-12$ animals/group.

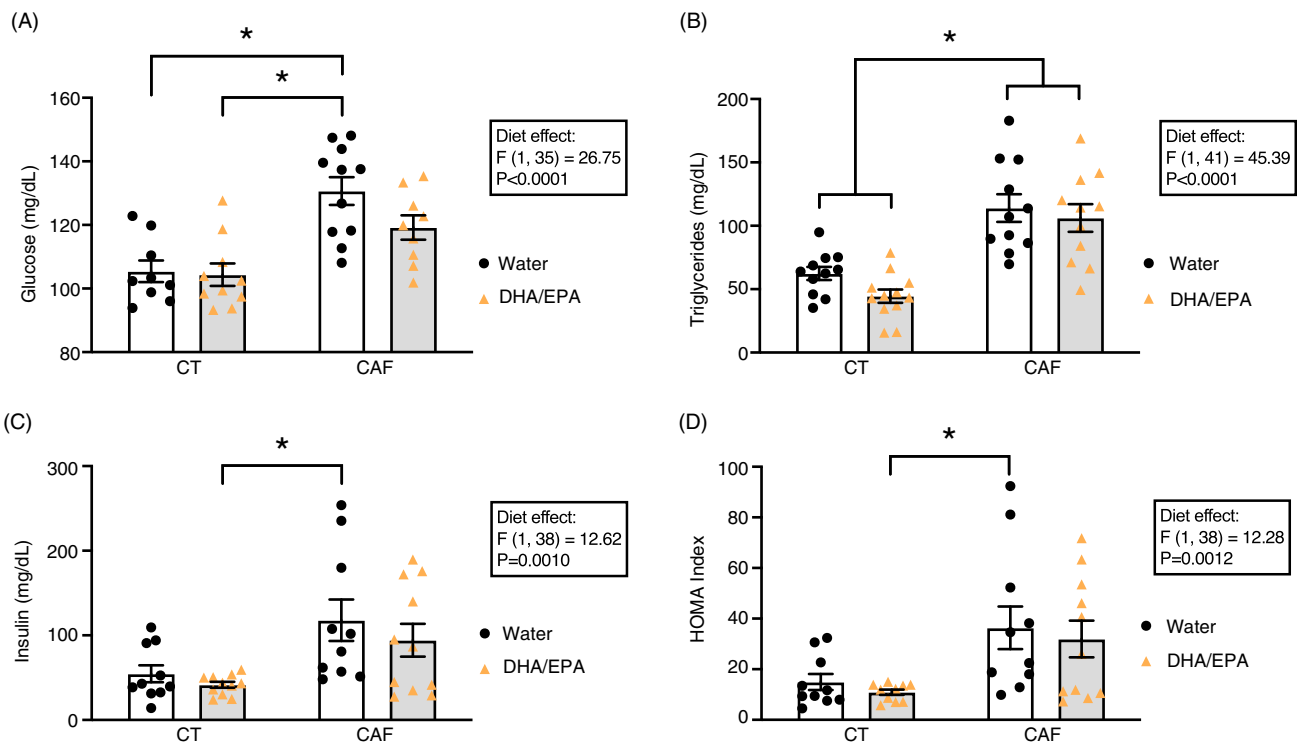


Fig. 2. DHA/EPA supplementation did not change glycaemic control and triglycerides levels following cafeteria diet (CAF). (a) Fasting plasma glucose. (b) Triglycerides levels. (c) Plasma insulin levels. (d) HOMA-Index. Significant differences showed by two-way ANOVA regarding effects of diet (CAF and CAF + DHA/EPA v. CT and CT + DHA/EPA), EPA/DHA treatment (CAF and CT v. CAF + DHA/EPA & CT + DHA/EPA) and diet \times DHA/EPA interactions are indicated in the text boxes. Multiple comparisons were performed by Tukey post-hoc test and are indicated as follows: glucose: * $P < 0.001$ comparing CAF v. CT or CT + DHA/EPA; triglycerides: * $P < 0.01$ comparing CAF v. CT or CT + DHA/EPA; insulin, HOMA: # $P < 0.05$ comparing CAF v. CT + DHA/EPA. $n = 9-12$ animals/group.

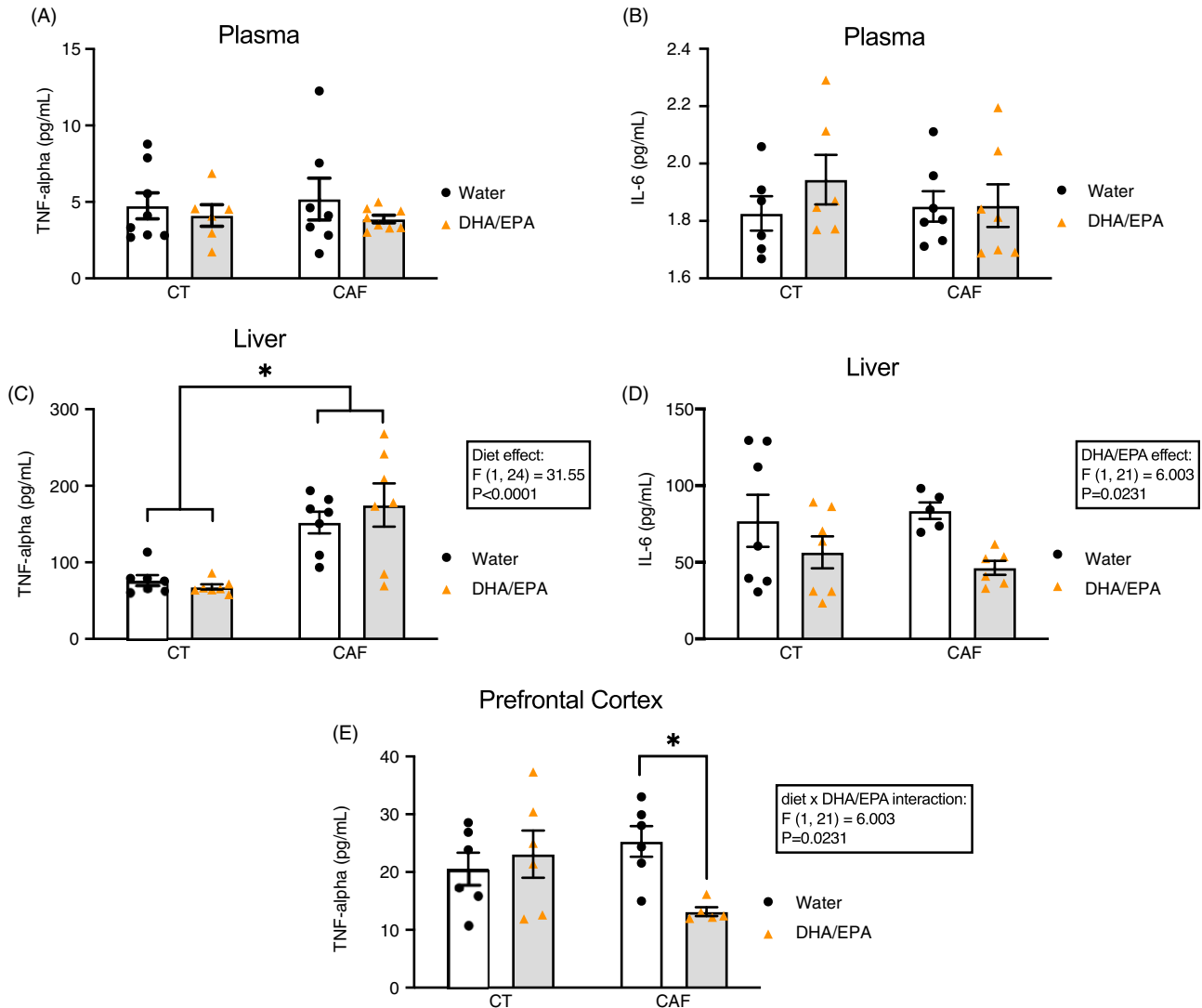


Fig. 3. DHA/EPA supplementation did not modify TNF- α and IL-6 levels in the plasma and liver, but it reduced TNF- α in the prefrontal cortex of cafeteria diet (CAF)-fed rats. (a) Plasma levels of TNF- α . (b) Plasma levels of IL-6. (c) TNF- α levels in the liver. (d) IL-6 levels in the liver. (e) TNF- α levels in the prefrontal cerebral cortex. Significant differences showed by two-way ANOVA regarding effects of diet (CAF and CAF + DHA/EPA v. CT and CT + DHA/EPA), DHA/EPA treatment (CAF and CT v. CAF + DHA/EPA and CT + DHA/EPA) and diet x DHA/EPA interactions are indicated in the text boxes. Multiple comparisons were performed by Tukey post-hoc test and are indicated as follows: TNF- α liver: * $P < 0.0001$ comparing CAF v. CT or CT + DHA/EPA; CAF + DHA/EPA v. CT or CT + DHA/EPA. TNF- α cortex: * $P < 0.05$ comparing CAF v. CAF + DHA/EPA. $n = 5-8$ animals/group.

Evaluation of anxiety-like behaviour

Figure 4 shows results regarding the elevated plus maze task, which was chosen to evaluate anxiety-like behaviour. The number of head dipping (NHD), which assesses risk behaviour, was measured every time the rats inclined the head towards the lower arm; its increase is related to an anxiolytic manifestation⁽³¹⁾. Regarding NHD, we found a significant interaction between diet and supplementation (interaction: $F_{(1,33)} = 5.544$, $P = 0.024$; diet effect: $F_{(1,33)} = 0.2620$, $P = 0.61$; DHA/EPA effect: $F_{(1,33)} = 1.316$, $P = 0.25$), although Tukey post-hoc test did not show any difference among groups (Fig. 4(a)). Considering the number of entries in the open arms (NEOA, Fig. 4(b)) and the number of entries in the closed arms (NECA, Fig. 4(c)), there was a diet effect in both parameters, showing that rats fed with CAF had a reduced number of entries (NEOA – diet effect: $F_{(1,29)} = 6.96$, $P = 0.013$; interaction: $F_{(1,29)} = 0.83$, $P = 0.36$;

DHA/EPA effect: $F_{(1,29)} = 0.83$, $P = 0.36$; NECA – diet effect: $F_{(1,32)} = 6.13$, $P = 0.018$; interaction: $F_{(1,32)} = 0.32$, $P = 0.57$; DHA/EPA effect: $F_{(1,32)} = 0.32$, $P = 0.57$). Furthermore, considering the percentage of entries in the open arms (PEOA, Fig. 4(d)), we found a significant interaction between diet and supplementation (interaction: $F_{(1,32)} = 6.27$, $P = 0.017$; diet effect: $F_{(1,32)} = 0.63$, $P = 0.43$; DHA/EPA effect: $F_{(1,32)} = 0.004$, $P = 0.94$), although Tukey post-hoc did not show differences. Another analysis we conducted was the time spent in the open arms (TSOA, Fig. 4(e)) and time spent in the closed arms (TSCA, Fig. 4(f)), which were altered by DHA/EPA supplementation (TSOA – DHA/EPA effect: $F_{(1,33)} = 6.51$, $P = 0.015$; interaction: $F_{(1,33)} = 3.89$, $P = 0.056$; diet effect: $F_{(1,33)} = 0.15$, $P = 0.69$; TSCA – DHA/EPA effect: $F_{(1,33)} = 6.51$, $P = 0.015$; interaction: $F_{(1,33)} = 3.89$, $P = 0.056$; diet effect: $F_{(1,33)} = 0.15$, $P = 0.69$). Also, Tukey post-hoc test indicated that CAF-fed animals showed

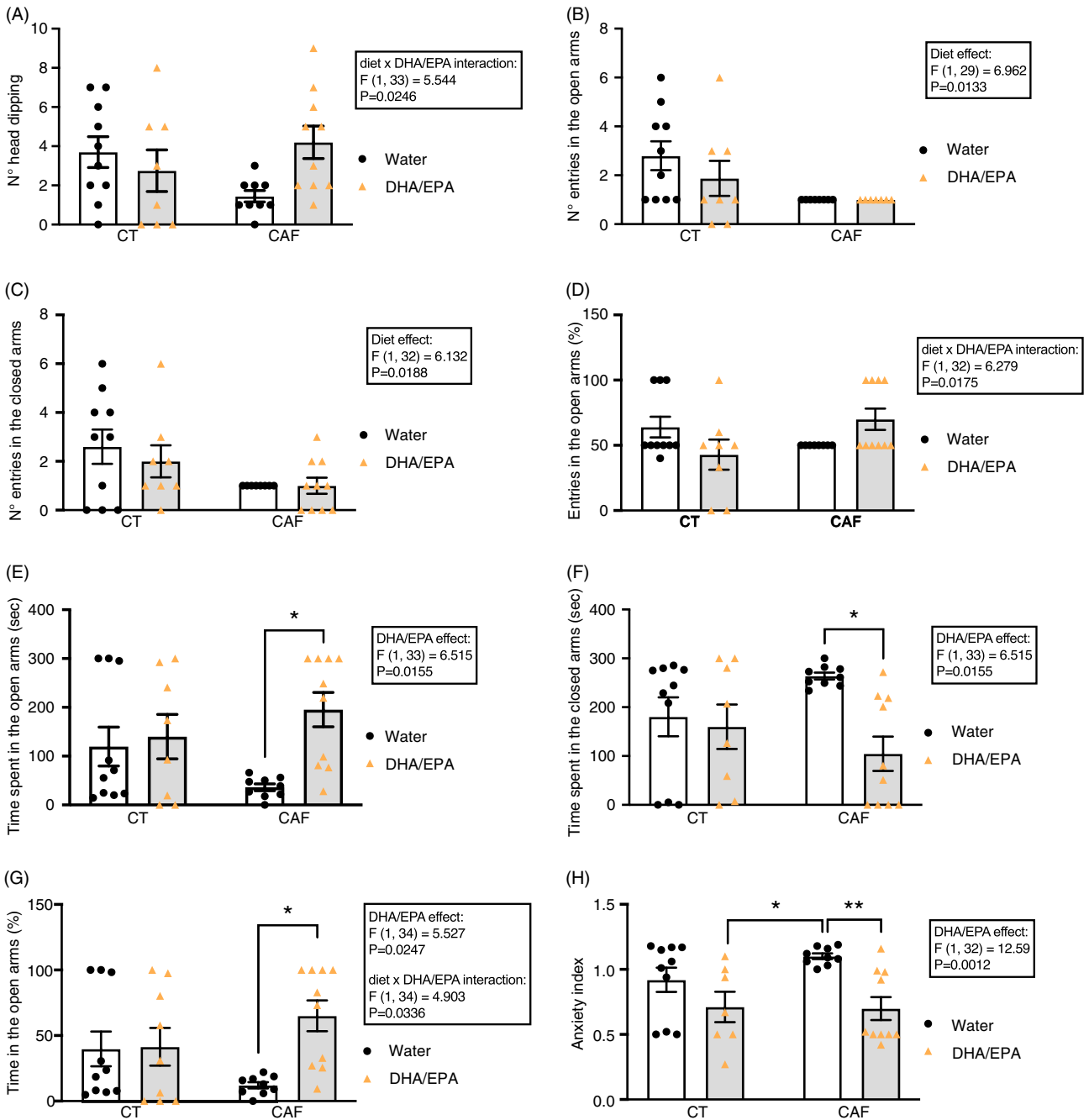


Fig. 4. DHA/EPA decreased anxiety-like behaviour in cafeteria diet (CAF)-fed rats. (a) Number of head dipping. (b) Number of entries in the open arms (NEOA). (c) Number of entries in the closed arms (NECA). (d) Percentage of entries in the open arms (PEOA). (e) Time spent in the open arms (TSOA). (f) Time spent in the closed arms (TSCA). (g) Percentage of time in the open arms (PTOA). (h) Anxiety index. Significant differences showed by two-way ANOVA regarding effects of diet (CAF and CAF + DHA/EPA v. CT and CT + DHA/EPA), DHA/EPA treatment (CAF and CT v. CAF + DHA/EPA and CT + DHA/EPA) and diet x DHA/EPA interactions are indicated in the text boxes. Multiple comparisons were performed by Tukey post-hoc test and are indicated as follows: CT, control group. TSOA, TSCA, PTOA: * $P < 0.05$ comparing CAF v. CAF + DHA/EPA; anxiety index: * $P < 0.05$ comparing CAF v. CT + DHA/EPA, ** $P < 0.01$ comparing CAF v. CAF + DHA/EPA. $n = 7-10$ animals/group.

a significant reduction in TSOA ($P = 0.013$) and, consequently, increased TSCA ($P = 0.013$) in comparison with the CAF + DHA/EPA group. These findings point to a decrease in anxiety-like behaviour in obese rats supplemented with DHA/EPA since higher time spent in the open arms indicates reduced anxiety-like behaviour. These results were also seen in the percentage of time in the open arms (PTOA, Fig. 4(g)), which,

besides the effect of DHA/EPA, we also found an interaction between diet and supplementation (DHA/EPA effect: $F_{(1,34)} = 5.52$, $P = 0.024$; interaction: $F_{(1,34)} = 4.90$, $P = 0.033$; diet effect: $F_{(1,34)} = 0.031$, $P = 0.86$). These findings were corroborated by the post-hoc test, which demonstrated a significant reduction of PTOA in the CAF group compared with the CAF + DHA/EPA group ($P = 0.014$). In addition, the anxiety

index was calculated (Fig. 4(h)). Two-way ANOVA showed an effect of DHA/EPA supplementation (DHA/EPA effect: $F_{(1,32)} = 12.59$, $P = 0.0012$; interaction: $F_{(1,32)} = 1.26$, $P = 0.26$; diet effect: $F_{(1,32)} = 0.96$, $P = 0.33$), and post-hoc test showed a higher score in CAF compared with the CAF + DHA/EPA ($P = 0.0088$) and CT + DHA/EPA ($P = 0.0238$) groups, showing a higher anxiety level in obese rats. Taken together, these results corroborate the effect of DHA/EPA in reducing anxiety-like behaviour.

Discussion

The effects of *n*-3 PUFA in brain function have been extensively studied^(35,36); however, the results are controversial. Here, we showed an anti-inflammatory effect of DHA/EPA supplementation in the brain in an experimental model of obesity, the CAF. In addition, we showed the ability of DHA/EPA in decreasing anxiety-like behaviour in obese rats. Otherwise, despite the evidence showing the role of *n*-3 in the improvement of metabolic profile^(37,38), we found that DHA/EPA was not able to revert weight gain, adiposity, lipidic profile and hepatic levels of TNF- α in CAF-fed rats. Thus, using CAF, a highly obesogenic diet, we are showing for the first time that DHA/EPA beneficial effects might depend on the severity of the obesity. On the other hand, DHA/EPA still provides neuroprotection, irrespective of the metabolic dysfunction.

DHA/EPA does not ameliorate metabolic dysfunction and inflammation in severe obesity

A CAF leads to a more pronounced obesity phenotype in rodents than other diet protocols such as a high-fat diet (HFD)^(27,39,40). It has been shown that CAF is more efficient in inducing hyperglycaemia, glucose intolerance and insulin resistance compared with HFD^(27,41). We tested whether DHA/EPA would abrogate the metabolic disruption triggered by CAF. Our study demonstrated that 20 weeks of CAF increased weight gain, adiposity, blood glucose, triglycerides and insulin levels, and insulin resistance, providing an efficient model to mimic Western diet-associated obesity in Wistar rats. However, 4 weeks of DHA/EPA administration (500 mg/kg) did not ameliorate any of these parameters. In a previous study, 4 weeks of *n*-3 supplementation (400 mg/kg) also failed to decrease weight gain after 6 weeks of HFD in mice, but it reduced adipose tissue storage⁽⁴²⁾. Although it was an interesting finding, 6 weeks of HFD may be a short period to investigate chronic manifestations of obesity. In another study, 16 weeks of HFD lead to increased weight and adipose tissue deposition with no effect of fish oil supplementation at a dose of 0.7 mg/kg for 5 weeks⁽²⁸⁾. Thus, results about the effects of DHA/EPA supplementation are controversial. However, we can suppose that in cases of more severe obesity, it may not be sufficient to revert metabolic dysregulation, as shown in the present study.

Most of the studies about the effects of PUFA in metabolism are conducted in males. When sexual differences were investigated following CAF and a HFD, no differences were found in fatty acid metabolism regarding sex, including depletion of EPA content in male and female rats⁽⁴³⁾. In addition, the effects

of DHA/EPA supplementation have already been evidenced in female rats in different experimental models. In female rats with polycystic ovary syndrome, DHA/EPA reduced levels of triglycerides, insulin, blood glucose and weight gain⁽⁴⁴⁾. The present study used only male rats, but it would be important to include females to evaluate differential responses to DHA/EPA in obesity.

In obesity, the accumulation and expansion of adipocytes lead to increased inflammatory cytokine secretion, promoting a sustained inflammatory state related to the onset of other chronic diseases⁽⁴⁵⁾. Here, we assessed the systemic inflammation caused by CAF by measuring IL-6 and TNF- α levels in the plasma and liver. Although we did not find diet or supplementation effects on circulating levels of IL-6 and TNF- α , evidence has shown that DHA/EPA may play an anti-inflammatory role in the periphery. Souza *et al.* showed that 8 weeks of *n*-3 supplementation decreased IL-6 and TNF- α levels after HFD⁽⁴⁶⁾. Also, Candido *et al.* showed a protective effect of *n*-3 in decreasing IL-6 in rats fed with HFD after 2 months of supplementation⁽⁴⁷⁾. In the liver, obesity increases inflammatory markers, driving hepatic dysfunction such as non-alcoholic fatty liver disease and hepatocellular carcinoma⁽⁴⁸⁾. In the present study, we found increased hepatic levels of TNF- α after CAF, which was already expected. However, we did not find the same increase in IL-6 levels. Nonetheless, in obesity, the increase in TNF- α leads to the production of IL-6 in the liver⁽⁴⁸⁾, suggesting that this could be a subsequent event as the disease develops. Importantly, we found a statistically significant effect of DHA/EPA in reducing IL-6 hepatic basal levels, although it could not mitigate the TNF- α increase. Worthwhile to note, we started supplementation after 16 weeks of CAF, while most studies start this intervention simultaneously with the diet protocol. Thus, we may speculate that the time point for the beginning of the supplementation may be a determining factor in the protective effect of *n*-3, at least in the liver. This idea is corroborated by a study in which supplementation of *n*-3 decreased TNF- α levels, but, in this case, the supplementation started concomitantly with the high-fat and high-sucrose diet⁽⁴⁹⁾. Lionetti *et al.* also found lower levels of TNF- α after 6 weeks of HFD rich in fish oil, demonstrating a protective effect of *n*-3 in the liver⁽⁵⁰⁾. Although previous studies demonstrate a protective role of *n*-3 on hepatic TNF- α levels, it seems that the beneficial effect may not be seen under chronic conditions. Regarding the effects of *n*-3 in reducing hepatic IL-6, Schmöcker *et al.* found a significant decrease in IL-6 mRNA in mice treated with *n*-3 in a model of hepatitis, contributing to the reduction of inflammation⁽⁵¹⁾. It was already demonstrated that EPA and DHA interact with peroxisome proliferator-activated receptor alpha (PPAR- α), preventing NF- κ B activation⁽⁵²⁾. Nevertheless, it is suggested that the anti-inflammatory properties of *n*-3 may depend on the pathogenesis and the strength of the inflammation⁽⁵³⁾. Thus, our protocol of CAF may have elicited such intense liver inflammation that DHA/EPA could not completely reverse.

There is a close relationship between systemic inflammation triggered by obesity and neuroinflammation^(54,55). In the neuro-inflammatory process, microglia and astrocytes carry out an inflammatory response that can result in psychiatric

manifestations^(56,57). Here, we showed a decrease in TNF- α levels in the prefrontal cortex of CAF-fed rats supplemented with DHA/EPA compared with the CAF group with no supplementation, suggesting a neuroprotective effect of DHA/EPA. In a previous study, we demonstrated that DHA/EPA supplementation decreased IL-6 and TNF- α in the prefrontal cortex of obese rats after HFD⁽⁵⁸⁾. The anti-inflammatory effect of DHA/EPA in the central nervous system may be mediated by inhibiting the activation of intracellular phospholipase A2, an enzyme that cleaves plasma membrane phospholipids to become available for metabolism to lipid mediators⁽⁵⁹⁾. In this context, these PUFA can also compete with arachidonate for the enzymes 5-LOX and COX2, blocking the formation of pro-inflammatory products^(60,61). Moreover, in an *in vitro* study, DHA administration to hypothalamic neurons reduced NF- κ B pathway activation and TNF- α production upon inflammatory challenge in a G protein-coupled receptor 120 (GPR120)-dependent way⁽⁶²⁾.

DHA/EPA influences brain function in obesity by decreasing anxiety-like behaviours

Obesity is considered a risk factor for anxiety disorders, but the pathophysiological mechanisms linking these conditions are still unclear. There is evidence showing a stronger association between severe obesity (defined as a BMI \geq 35) and anxiety disorders compared with moderate obesity (BMI 30–35)^(63,64). Besides, chronic conditions related to obesity can increase anxiety risk^(65,66). On the other hand, anxiety disorders may lead to weight gain by deregulation of the hypothalamic–pituitary–adrenal axis that alters the appetite and leads to subsequent weight gain in stressed individuals^(67,68). The symptoms of anxiety disorders can increase appetite and stimulate the desire for foods rich in sugar and fat^(67,69,70). To better clarify the relationship between obesity and anxiety, Garipey *et al.* performed a systematic review with meta-analysis, including sixteen studies with a total of 346289 individuals, suggesting (moderate evidence) that obesity is positively associated with anxiety disorders in adults⁽⁷¹⁾.

Since neuroinflammatory processes are directly linked to the aetiology of behavioural disorders, *n*-3 supplementation benefits emotional states through its anti-inflammatory actions in the central nervous system^(72,73). It was shown elsewhere that CAF drives an anxiety-like behaviour⁽⁷⁴⁾, while *n*-3 may be efficient to improve mood⁽⁷⁵⁾ and attenuate anxiety symptoms in humans⁽⁷⁶⁾. Studies also demonstrated that an adequate supply of *n*-3 could protect against cognitive decline and neurodegenerative diseases development by supporting adequate synaptic function and plasticity^(77,78). These findings are in agreement with our results, which also show a neuroprotective DHA/EPA effect in CAF-fed rats. In female rats, *n*-3 fatty acids also showed an anxiolytic effect^(79,80). However, to our knowledge, no study addressed the behavioural effects of DHA/EPA in obese female animals, which would be essential to investigate.

Here, we assessed anxiety-like behaviour using the elevated plus maze test. The consumption of a CAF for 20 weeks was sufficient to trigger anxiety-like behaviour in our study. CAF-fed rats supplemented with DHA/EPA showed a consistent reduction of anxiety parameters evaluated in the elevated plus maze test. It

was shown elsewhere that chronic activation of GPR120 through an intracerebroventricular infusion of its agonist had an anxiolytic effect but failed to affect energy balance in HFD mice⁽⁸¹⁾. *n*-3 fatty acids are also GPR120 ligands and therefore were demonstrated to inhibit cytokine production in cultured neurons upon inflammation, evidencing its anti-inflammatory properties regarding central nervous system cells⁽⁶²⁾. In the HFD model, a study found that fish oil enriched with DHA/EPA (0.7 mg/kg) could protect against behaviour abnormalities, glial activation and increased neuroinflammation compared with the non-treated obese counterparts⁽²⁸⁾. In agreement with our current data, Demers *et al.* also demonstrated that *n*-3 could improve behaviour even without changing body weight⁽²⁸⁾. However, we could speculate that although supplemented rats in our study did not benefit from significant metabolic improvements, DHA/EPA was efficient to protect obese rats against the anxiogenic manifestations observed in the obese non-treated group. This is a relevant finding considering the potent effect of CAF in inducing obesity, and even in this condition, DHA/EPA was able to improve the behaviour. Since psychiatric symptoms such as anxiety are prevalent in patients with obesity, *n*-3 may be indicated as a strategy for this population. It is worth mentioning that *n*-3 supplementation is a low-cost treatment, and it does not have any side effects, reinforcing the importance of its usage.

Limitations and conclusions

Here we showed that DHA/EPA supplementation decreased anxiety-like behaviour in a preclinical model of obesity-induced after a CAF. It is worth mentioning that the dose of 500 mg/d used in rats in the present study is higher than *n*-3 consumed by humans, even by supplementation. In human studies, the dose of DHA/EPA ranges from 1 to 4 g/d⁽⁸²⁾. Thus, it can be a limitation to translate our findings to the human population. However, our findings are important to highlight the potential of these PUFA in exerting neuroprotection. Based on these beneficial results, more studies should be encouraged to develop new formulations of DHA/EPA with alternative routes to facilitate brain delivery, such as intranasal. On the other hand, the absence of robust metabolic effects indicates that DHA/EPA supplementation might not be a useful treatment in severe obesity, which was induced in the present study by CAF. Thus, DHA/EPA supplementation may exert an important neuroprotective effect in obesity, but further investigations are needed to elucidate effective doses of DHA/EPA.

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Authors Contributions

JN and JJ, investigation, formal analysis, writing - original draft; SO, MFB, LFSC and BFD, investigation; JCFM, MG and MP, formal analysis, writing - review & editing; RPG, conceptualization,

funding acquisition, supervision, writing - review & editing. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest.

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6 ARTIGO CIENTÍFICO 2

**IMPACT OF CAFETERIA DIET AND N3 SUPPLEMENTATION ON THE
INTESTINAL MICROBIOTA, FATTY ACIDS LEVELS,
NEUROINFLAMMATORY MARKERS AND SOCIAL MEMORY IN MALE
RATS**

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Impact of cafeteria diet and n3 supplementation on the intestinal microbiota, fatty acids levels, neuroinflammatory markers and social memory in male rats

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ABSTRACT

Objective: To assess the effects of omega-3 (n3) supplementation on intestinal microbiota, fatty acids profile, neuroinflammation, and social memory of cafeteria diet (CAF)-fed rats.

Methods: Male Wistar rats were fed with CAF for 20 weeks. Omega-3 (500 mg/kg/day) was supplemented between the 16th and 20th week. Colon morphology, intestinal microbiota composition, short-chain fatty acids (SCFA) and lipopolysaccharide (LPS) in the plasma, fatty acids profile, TLR-4 and claudin-5 expressions in the brain, and social memory were investigated.

Results: CAF reduced colon length, crypts' depth, and microbiota diversity, while n3 increased the Firmicutes/Bacteroidetes ratio. CAF increased SCFA plasma levels, but n3 reduced butyrate and isobutyrate in obese rats. LPS was increased in CAF-fed rats, and n3 decreased its levels. In the cerebral cortex, n3 increased caprylic, palmitic, stearic, tricosanoic, lignoceric, myristoleic, and linoleic acids. CAF increased palmitic acid and TLR-4 expression in the cerebral cortex while decreasing claudin-5 in the hippocampus. In the social memory test, CAF-fed animals showed greater social interaction with no effect of n3.

Conclusions: The lack of n3 effect in some of the evaluated parameters may be due to the severity of the obesity caused by CAF. However, n3 reduced LPS levels, suggesting its ability to reverse endotoxemia.

1. Introduction

The consumption of a high-calorie diet is one of the main contributing factors to obesity development, mainly when associated with ultra-processed foods high in sugars and fats. In addition to energetic, metabolic, hormonal, and inflammatory imbalances, the urban Western diet also culminates in disturbances in the intestinal microbiota, promoting dysbiosis that cause chronic inflammation and hamper energy homeostasis even further [1,2].

The diversity and content of the gut microbiota are influenced by multiple factors such as the birth delivery method, dietary habits, use of

antibiotics and other medications, aging, and other environmental factors [3]. In humans, the composition of the gut microbiota is mainly subdivided into five phyla: Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria and Verrucomicrobia, with 90% contained in the Firmicutes and Bacteroidetes phyla [4]. A healthy intestinal microbiota means a positive proportion between certain beneficial bacterial species and other harmful ones, which is related to the improvement of the health and well-being of the host [5,6]. On the other hand, an imbalance favoring pathogenic species can not only support the development of diseases, such as obesity, but also aggravate and perpetuate already established pathological conditions [7].

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An important role of microbiota in the host's health is due to the production of short-chain fatty acids (SCFA). The most investigated SCFAs are butyrate, propionate, and acetate, which are involved in the maintenance of the gut epithelial barrier integrity [8,9], mainly by their interaction with G protein-coupled receptors (GPRs) such as GPR-41 and GPR-43 [10]. Dysbiosis may decrease SCFA production [11] and disturb barrier permeability. Also, lipopolysaccharide (LPS), a product mainly derived from gram-negative bacteria in the gut, can reach the blood and trigger low-grade inflammation [12,13]. Both decreases in SCFA production and LPS invasion are mechanisms proposed to affect the gut-brain axis, which is implicated in neuroinflammatory responses [14].

The interaction between the periphery and the central nervous system (CNS) parenchyma is primarily regulated by the blood-brain barrier (BBB), composed of capillaries highly connected through tight junctions, in which claudin-5 is one of the most important proteins that prevent the passage of transmembrane solutes as shown in mice [15]. Peripheral inflammation resulting from obesity can disturb the integrity of the BBB, which allows the entry of pro-inflammatory molecules such as LPS. LPS can interact with the toll-like receptor-4 (TLR-4) in both mice and rats, exacerbating inflammation in nervous tissue and triggering a pro-inflammatory phenotype of cells such as astrocytes and microglia, essential for maintaining neuronal homeostasis [16,17].

Persistent neuroinflammation may interfere with proper brain functioning and lead to behavioral impairments, such as memory loss, as seen in several cognitive disorders associated with elevated brain levels of pro-inflammatory markers in rodents [18,19]. One of the many behavioral aspects perturbed in obesity is social memory and sociability. However, there is a lack of knowledge about the relationship between changes in the gut microbiota and the impact on social memory in obesity study models using mice and rats [20,21].

Omega-3 (n3) polyunsaturated fatty acids (PUFAs) have shown evidence of beneficial effects on the inflammatory response in several diseases [22-26]. The two main bioactive forms are eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6). Therefore, the consumption of foods that offer n3 is usually low, which has made EPA and DHA widely used as dietary supplements. Thus, the main aim of this study was to investigate the effects of n3 on the intestinal microbiota, and BBB of rats fed a cafeteria diet (CAF), which is composed of high-energy industrialized foods, mimicking the human consumption of the Western diet. In addition, differently from other types of behaviors [27-30], the impact of obesity and n3 supplementation on social memory is not well comprehended. Thus, here, we also performed the five-trial social memory test to assess social recognition memory and interaction. In addition, we aimed to evaluate the TLR-4 and claudin-5 expression in the cerebral cortex and hippocampus since these regions are highly susceptible to metabolic dysfunctions.

2. Material and methods

2.1. Animals

Forty-six male Wistar rats aged 4 months old were obtained from the animal facility of the Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSA). During the experiment, the animals were kept in pairs in plastic cages at $23 \pm 1^\circ\text{C}$ with a light/dark cycle of 12/12 h. This project was approved by the Institutional Animal Care and Use Committee of UFCSA under protocol No. 570/18 and all procedures were designed to minimize the number and suffering of animals in compliance with international laboratory and animal care laws.

2.2. Experimental groups and diet

We divided the animals into four experimental groups: CT, control diet + water by oral gavage (n=11); CT+n3, control diet + n3 supplementation (n=12); CAF, cafeteria diet + water by oral gavage (n=11);

and CAF+n3, cafeteria diet + n3 supplementation (n=12).

Information about the experimental groups, diet and supplementation are shown in Fig. 1.

All groups received the standard chow Nuvilab® CR-1 (Nuvital®, Curitiba, PR, Brazil). The omega-3 was purchased from Mega DHA® (Vitafor®, Araçoiaba da Serra, SP, Brazil), which had a high concentration of EPA (10%) and DHA (50%). In addition to the standard chow, cafeteria groups were fed with three different menus interchanged every two days to maintain novelty and stimulate consumption. Menus comprised commercially available cookies, sausage, bologna, and white chocolate. They also received an orange-based soft drink besides water *ad libitum*. The leftovers of every component were weighed every two days, including soft-drink consumption, to determine food intake per cage. Animals were weighed weekly to determine weight gain. Omega-3 (500 mg/Kg/day, 10% EPA, and 50% DHA) or water was administered by oral gavage. We used a curved stainless steel gavage needle with a ball tip. The gavage was performed by a trained researcher. Supplementation was performed daily for 4 weeks, from the first day of week 16 to the last day of week 19. The 20th week was used for the behavioral evaluation.

2.3. Tissue, plasma and feces collection

In the 20th week of the experiment, the rats were euthanized by decapitation. The animals were fasted for 6 h at the time of euthanasia. The brain and intestine were quickly removed. The cerebral cortex and hippocampus were manually dissected and immediately frozen in liquid nitrogen. Samples from the proximal colon, approximately 3 cm above the cecum, were used for morphological analysis and were fixed in 10% formaldehyde solution until the day of the preparation of histological slides. Plasma was separated from whole blood by centrifugation and stored at -80°C . The feces used for the Next-Generation Sequencing were collected from the intestinal cecum. Samples were kept at -80°C until further processing.

2.4. Histology

Colon samples were fixed in 10% formaldehyde solution, dehydrated in a graded ethanol series, and embedded in paraffin. Subsequently, serial sections of $3\mu\text{m}$ of the transverse plane of the colon were performed in a microtome (Leica) and then stained with hematoxylin and eosin (HE). Five sections per sample were analyzed at 100 x magnification (EVOS microscope, Thermo Fisher Scientific). Five to seven animals per group were used for crypts depth evaluation. For each animal, 3-5 fields were analyzed per section, and a mean of the 5 deepest crypts was used for the statistical analysis. Crypt depth was manually measured using ImageJ software, considering the distance from the lumen to the base of the crypts.

2.5. Next-generation sequencing in gut microbiota

2.5.1. Sample processing

Stool samples were collected in sterile tubes and immediately frozen (-20°C). The genomic material was obtained from approximately 200 mg of fecal sample with an appropriate commercial kit (Microbiome DNA Purification kit, Invitrogen, U.S.A) following the manufacturer's instructions. At the end of the DNA extraction, an average of 5-10 μg of genomic material was obtained.

2.5.2. Amplification and next-generation sequencing

The hypervariable V4 region from rRNA gene was amplified through PCR using genomic DNA (approximately 50 ng per reaction) and the following primer pair: 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). In order to pool different samples in the same reaction, we used the primer-fusion method and each sample had a distinct barcode attached to the corresponding PCR

EXPERIMENTAL DESIGN

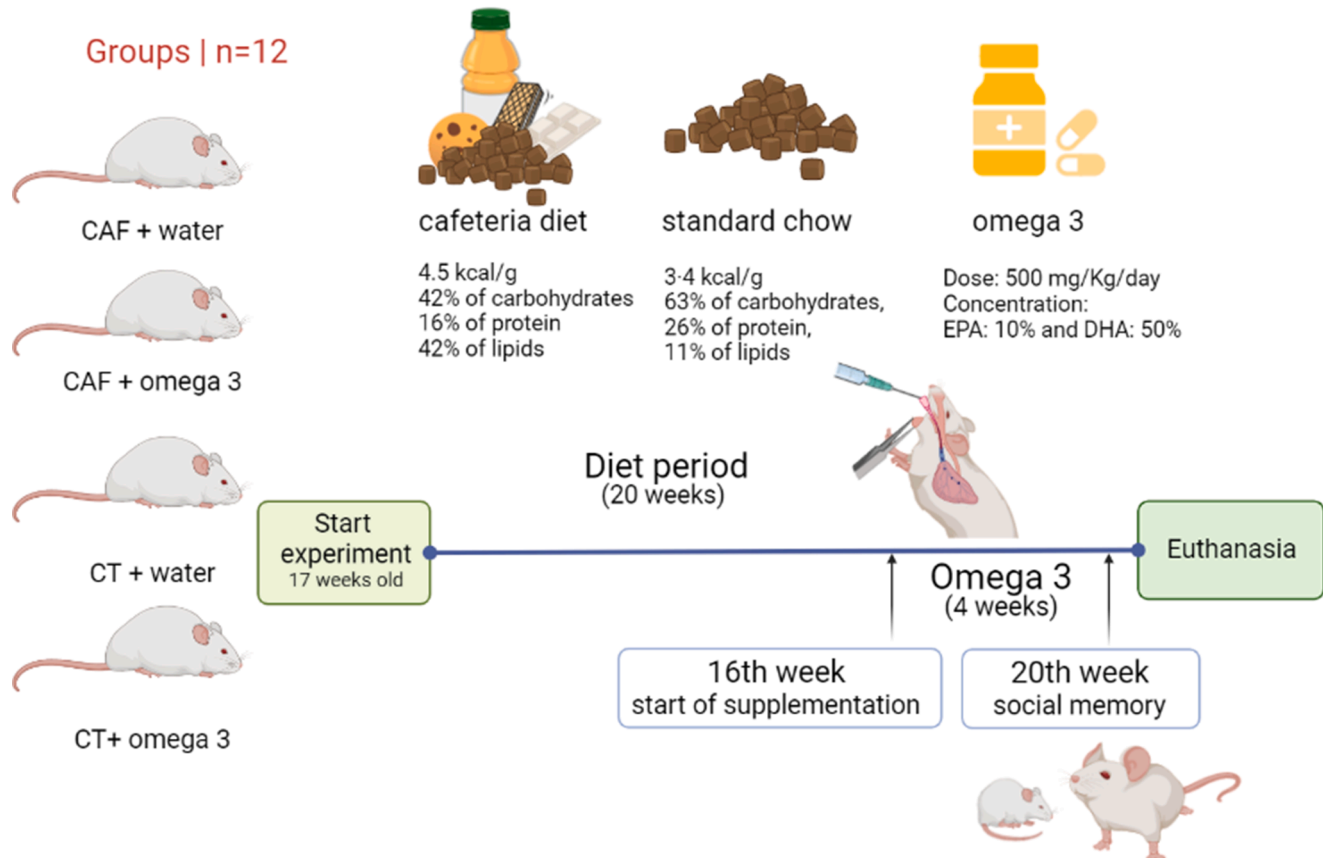


Fig. 1. Experimental groups. CT, control diet; CAF, cafeteria diet.

product. The amplification was performed using Platinum™ PCR SuperMix High Fidelity (Invitrogen). The products were verified through electrophoresis in an agarose gel, purified with AMPure XP PCR Purification Kit (Beckman Coulter), quantified using Qubit™ dsDNA HS Assay Kit (Invitrogen) and subjected to emulsion PCR using Ion PGM™ Hi-Q™ View OT2 Kit (Thermo Fisher Scientific). After, the resulting enriched beads were sequenced in a NGS machine (Ion Torrent PGM, Life Technologies) using Ion PGM™ Hi-Q™ View Sequencing Kit (Thermo Fisher Scientific).

The 16S rDNA reads generated by high-throughput sequencing were submitted to a quality control analysis that retained sequences with a minimum length of 100 base pairs and trimmed the sequences to remove low quality bases to obtain a minimum Phred score of 30, using PRINSEQ [31] (Schmieder & Edwards, 2011). The remaining sequences were dereplicated and sorted by decreasing read abundance and filtered to exclude singletons using USEARCH v7.0.1090. Clusters were assembled using a minimum identity of 99% and chimeras were removed using the Ribosomal Database Project (RDP) reference database [32] (Cole et al., 2014).

The taxonomic assignment was obtained using QIIME v1.7 [33] (Caporaso et al., 2010). Operational taxonomical units (OTUs) were selected based on 97% sequence similarity and taxonomic data were obtained using a classification algorithm with the 97% OTUs version of GreenGenes 13.8 [34] (DeSantis et al., 2006). Diversity index was calculated utilizing Vegan:ecological diversity package in R.

2.6. Determination of short-chain fatty acids in plasma by liquid chromatography coupled to mass spectrometry

Quantification of short-chain fatty acids in plasma was performed by

LC-MS/MS. Aliquots of 50 μ L of plasma and 20 μ L of 2M NaOH were added to plastic microtubes, followed by the addition of 175 μ L of 2M HCl. The sample was vortexed for 30 s, centrifuged for 6 min at 12,000 g and 175 μ L of the supernatant was collected and transferred to a glass vial. 25 μ L of 2,4-Dinitrophenylhydrazine (DNPH) were added to promote fatty acid derivatization. The flask was closed and incubated for 30 min at 40°C. Then, 100 μ L of the mixture was added to a vial and 100 μ L of acetonitrile was added. A 50 μ L aliquot was transferred to a new Eppendorf tube and 150 μ L of acetonitrile was added to the tube. The sample was vortexed for 30 s and centrifuged for 6 min at 12,000 g. Then 150 μ L were transferred to an insert and vial and an aliquot of 10 μ L was injected into the analytical system equipment.

The LC-MS/MS equipment consisted of a chromatographic system Nexera UFLC (Shimadzu, Japan) equipped with two binary pumps (LC-30AD), a column oven (CTO-30A), a diode array absorbance detector (SPD-M20A) and an automatic injector (SIL-30AMP) coupled with a quadrupole mass spectrometer model LCMS-8045 (Shimadzu, Japan). The results obtained were processed and evaluated using LabSolutions software (Shimadzu, Japan).

2.7. Determination of LPS in plasma by liquid chromatography coupled to mass spectrometry

Plasma LPS measurement was adapted from Teixeira et al. (2021) [35]. In sum, 150 μ L of plasma were aliquoted and hydrolyzed with 75 μ L of NaCl 150 mM and 300 μ L of HCl 8 mol and then incubated for 4 h at 90°C. Afterwards, 3 mL of hexane were added and samples were centrifuged at 3500 rpm for 10 min. 1,5mL of supernatant was collected and then dried in a sample concentrator apparatus until the formation of a pallet. After that, 50 μ L of methanol were used for resuspension and

then injected in the analytical system.

For the analysis, we used the Nexera UFLC system coupled to a LCMS-8040 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan). The electrospray parameters were set in the negative ion mode ($[M-H]^-$) as follows: capillary voltage, 3000 V; desolvation line temperature, 250°C; heating block temperature, 500°C; drying gas, 18/L min; and nebulizing gas, 2 L/min. Collision-induced dissociation was obtained with 230 kPa argon pressure. Analyses were carried out with multiple reaction monitoring (MRM) by using the following fragmentation: m/z 243.1 \rightarrow m/z 59.0. The chromatographic separation was conducted with an Acquity UPLC® C18 column (2.1 \times 50 mm, 1.7 μ m particle size) (Waters Corporation, Ireland). The analyses were performed in gradient elution mode with a flow rate of 0.4 mL/min and the gradient mobile phase system consisted of water (solvent A) and acetonitrile (solvent B) both fortified with 0.2% acetic acid as follow: 0–1.5 min, 75–100% of B; 1.5–2.0 min, 100% of B; 2.0–2.1 min, 100–75% of B; 2.1–5.5 min, 75% B. The column oven was kept at 50°C. The data were processed using LabSolutions software (Shimadzu, Kyoto, Japan). Calibration curves were constructed at intervals of 0.2–1000 ng/mL.

2.8. Determination of medium and long chain fatty acids profile in the brain by gas chromatography coupled to mass spectrometry

Quantification of cerebral cortex medium and long chain fatty acids was adapted from previously described methods [36]. Firstly, 950 μ L of a mixture of chloroform and methanol at a ratio of 2:1 (v/v) was added to 50 μ L of tissue homogenate (100mg of tissue in 1mL of water). Then, 50 μ L of HCl and 200 μ L of water were added to cause phase separation. The lower phase was collected and dried in a sample concentrator apparatus until the formation of a pallet. After that, 30 μ L of methanol were used for resuspension.

Fatty acids were analyzed as their methyl esters after a direct transesterification with methanol on an analytical system consisting of a GC-2010 A PLUS gas chromatograph coupled to the QP-2010 Ultra mass spectrometer (Shimadzu, Japan). The spectrometer was operated in scan mode (50 – 700 m/z) and the results obtained were verified by comparing the findings with those described in the mass spectral reference library of the National Institute of Standards and Technology (NIST).

2.9. Western blot

To quantify the protein expression of the TLR-4 and claudin-5 in the cerebral cortex and hippocampus, tissues were first homogenized in a lysis buffer with a protease inhibitor cocktail. Samples were centrifuged for 10 min at 8000 rpm, and the concentration of proteins was determined using the Bradford protein assay. Laemmli buffer was mixed with 30 μ g of proteins and heated to 100°C for 3 min. After that, proteins were loaded on a 10% SDS-PAGE gel at 140 V for 2 h. Proteins were transferred into nitrocellulose membranes and blocked overnight at 4°C with 5% nonfat milk in Tris-buffered solution (TBS) with tween. Membranes were then washed with distilled water and incubated overnight with primary antibody against, claudin-5 (1:1000, Cat# ABT45, RRID: AB_11205041; MilliporeSigma, Burlington, Massachusetts), TLR-4 (1:200, Cat# sc-293072, RRID:AB_10611320; Santa Cruz Biotechnology, Dallas, Texas), and β -actin (Cat# sc-47778 horseradish peroxidase, RRID:AB_2714189; Santa Cruz Biotechnology). The next day, membranes were washed with TBS and then incubated for 2 h with a horseradish peroxidase-conjugated secondary antibody. A chemiluminescent signal was visualized in a Chemi-Doc MP Imaging System (Bio-Rad Laboratories, Hercules, California) after the exposition of membranes to electrochemiluminescence solution (Bio-Rad Laboratories).

2.10. Five-trial social memory test

To assess social recognition memory and interaction, we used the Five-Trial Social Memory Test as described in previous studies [37,38,39]. We conducted the behavioral analyses in the last week of the experiment, in the 20th week. This behavioral task assesses cognition and social memory through the ability to recognize familiar conspecifics and distinguish different individuals [40]. The test consists of 4 consecutive 5-min exposures between the subject animal and an intruder (Juvenile 1) with 5 min interval between sessions. 5 min after the fourth trial, an unfamiliar intruder (Juvenile 2) is exposed to the subject animal for a single trial and the exploration time between the familiar intruder and the novel intruder is compared. As intruders, male juvenile rats (21 days old) were used, once the older male is less likely to present aggressive behavior towards younger males than others with similar age.

The time of investigation by the subject rat was scored with a stopwatch by an observer blinded to experimental groups. The sniffing of intruders was defined as the measure of social exploration and interaction. A reduction in exploration between the subject and Juvenile 1 from the 1st to the 4th trial was considered an indication of social recognition. An increase in social interaction between the subject and juvenile 2 on the 5th trial compared to the 4th trial indicated the ability to distinguish different individuals. Animals that showed freezing or aggressive behaviors were excluded from the analysis.

2.11. Statistical analysis

The differences between the four groups were analyzed by two-way ANOVA. The two factors evaluated were diet (CT or CAF) and treatment (water or n3). Multiple comparisons were performed by Bonferroni *post hoc* test (CT versus CT+n3 and CAF versus CAF+n3). For the social memory test and caloric intake, we used repeated measures ANOVA and Tukey's multiple comparisons test. The results were expressed as mean \pm standard error of the mean. Graphpad Prism 9 (GraphPad Software, Inc.) was used for statistical analysis. The results were considered statistically significant at $p < 0.05$.

3. Results

Data on weight gain, visceral adiposity, and metabolic parameters of the experimental groups used in the present study were already published [28]. CAF-fed rats showed an increased caloric intake compared to CT diet-fed rats ($F_{33, 627}=104.1$, $p < 0.01$) from the first to the last experimental week (Supplementary Fig. 1).

3.1. Colon morphology

We measured the total length of the colon and the crypt depth (Fig. 2). CAF-fed animals showed a decrease in the total length of the colon (diet effect $F_{1,39}=31.27$, $p < 0.01$) (Fig. 2A). CAF also promoted a decrease in crypt depth (diet effect: $F_{1,20}=22.95$, $p < 0.01$), along with an interaction between the diet and n3 supplementation in the crypt depth measurement ($F_{1,20}=6.76$, $p=0.01$). In multiple comparisons, we showed that crypt depth was increased in the CT+n3 group compared to CT ($p=0.01$) (Fig. 2B).

3.2. Composition of the intestinal microbiota

The composition of intestinal microbiota is shown in Fig. 3. The number of reads of the main phyla and genera found by 16S rRNA V4 region sequencing is shown in the heatmap (Fig. 3A). Two-way ANOVA was performed to search for diet and supplementation interaction and main effects, and multiple comparisons were also analyzed. The main findings of the statistical analysis are described below.

We found an effect of diet on the phylum Firmicutes ($F_{1,19}=4.70$, $p <$

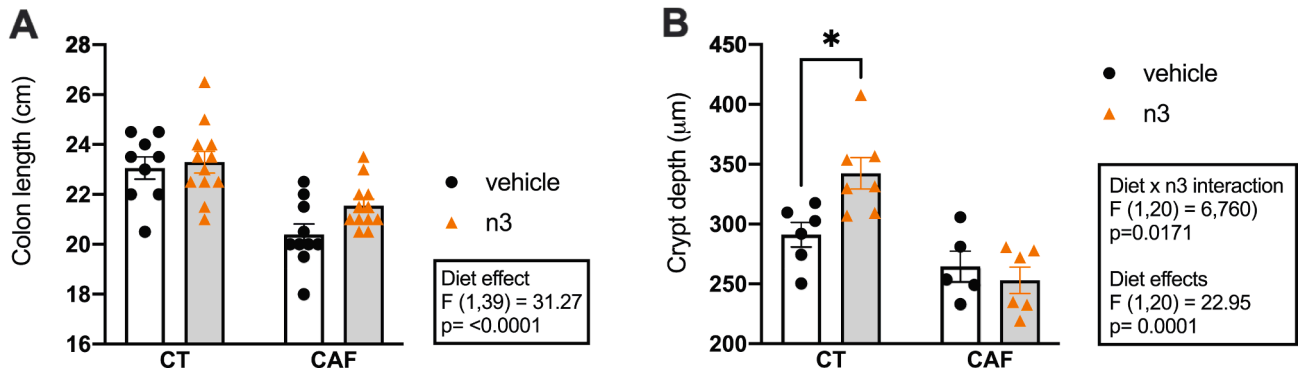


Fig. 2. Colon morphology in control (CT) and cafeteria diet (CAF)-fed rats. A) Colon length. B) Crypt depth. Significant differences showed by two-way ANOVA regarding effects of diet (CAF and CAF+n3 vs CT and CT+n3), n3 treatment (CAF and CT vs CAF+n3 and CT+n3) and diet × n3 interactions are indicated in the text boxes. Multiple comparisons were performed by Bonferroni post-hoc test and are indicated by the asterisk (*p < 0.05). n=9–12 animals/group for colon length; n=5–7 animals/group for crypt depth.

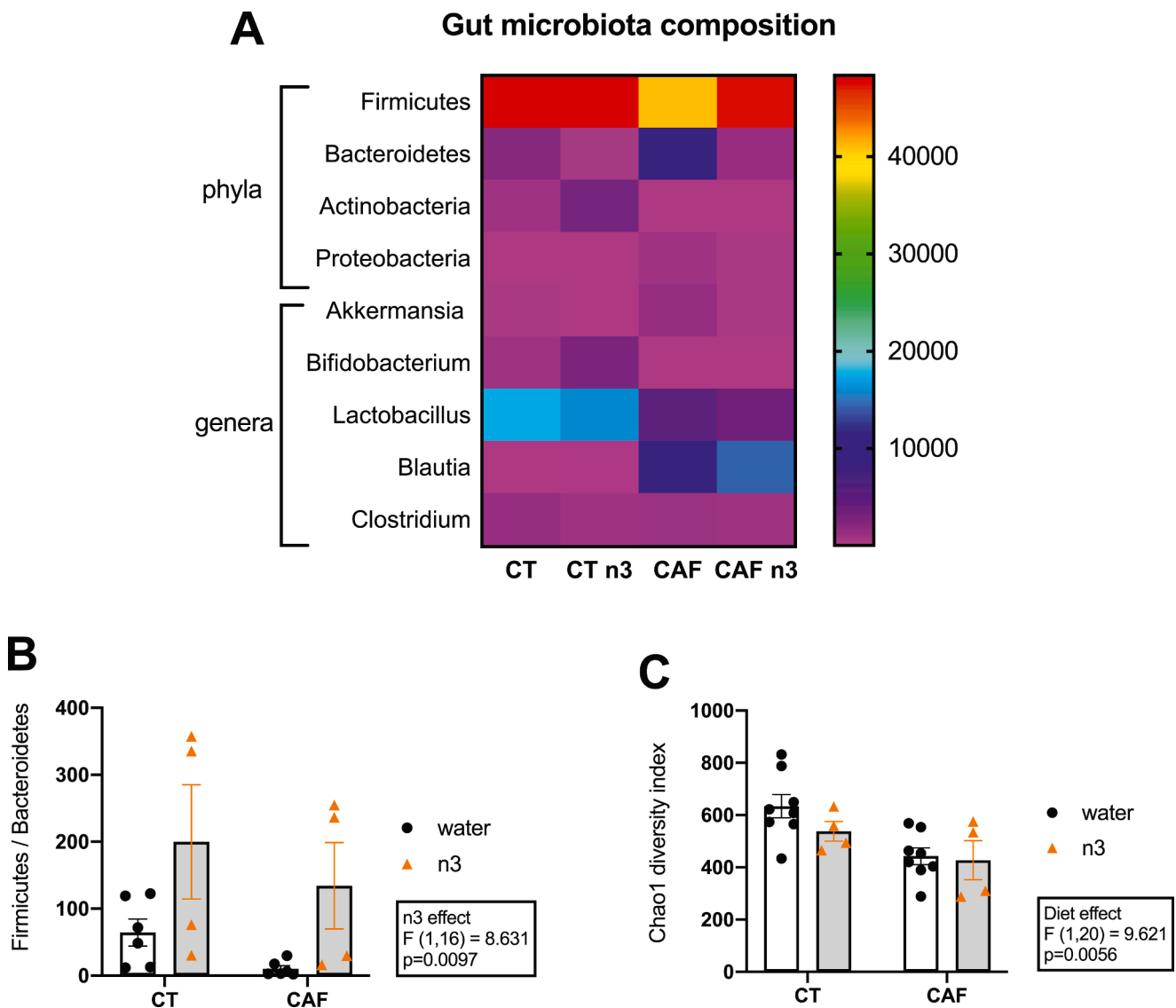


Fig. 3. Gut microbiota composition of control (CT) and cafeteria diet (CAF)-fed rats. A) Heatmap showing the phylum and genus levels of the main bacterial community found in the samples. A range of colors, from pink to red, indicates the abundance of each phylum or genus per group. B) Firmicutes/Bacteroidetes ratio. C) Chao 1 diversity index. Significant differences showed by two-way ANOVA regarding effects of diet (CAF and CAF+n3 vs CT and CT+n3), n3 treatment (CAF and CT vs CAF+n3 and CT+n3) and diet × n3 interactions are indicated in the text boxes. n=4–8 animals/group.

0.05), which was decreased in CAF-fed rats. Nevertheless, CAF+n3 animals had a slight increase in the population of this phylum in comparison to CAF ($p < 0.05$). The phylum Bacteroidetes showed a diet ($F_{1,17}=5.0$, $p=0.03$) and a n3 supplementation ($F_{1,17}=8.41$, $p=0.01$) effects. Still, the CAF+n3 group showed a decrease in the abundance of this phylum compared to CAF ($p < 0.01$). The Firmicutes/Bacteroidetes ratio was increased in n3-supplemented rats ($F_{1,16}=8.63$, $p < 0.01$), demonstrating that n3 could revert the Firmicutes/Bacteroidetes colonization following CAF (Fig. 3B).

The presence of the phylum Actinobacteria was also different among groups, being lower in CAF-fed rats (diet effect: $F_{1,20}=5.73$, $p < 0.01$) with no effect of n3. However, for the phylum Proteobacteria we found an interaction between diet and n3 ($F_{1,18}=5.67$, $p < 0.01$), a diet effect ($F_{1,18}=13.68$, $p < 0.01$) and a n3 effect ($F_{1,18}=5.08$, $p < 0.05$). The CAF group showed an increase in Proteobacteria colonization compared to CAF+n3 ($p=0.01$).

Regarding genera, we analyzed *Akkermansia*, *Bifidobacterium*, *Lactobacillus*, *Blautia* and *Clostridium*. Supplementation with n3 induced a general reduction in *Akkermansia* ($F_{1,15}=5.48$, $p < 0.05$). Furthermore, there was a decrease in *Bifidobacterium* (diet effect: $F_{1,20}=5.52$, $p < 0.05$) and *Lactobacillus* (diet effect: $F_{1,17}=42.38$, $p < 0.01$) following CAF, with no effect of n3. The abundance of *Blautia* showed an increase in CAF-fed animals (diet effect: $F_{1,17}=20.24$, $p < 0.01$). *Clostridium* composition did not change among groups.

When analyzing the diversity of microbiota composition by the Chao 1 index, we found a reduction in this parameter following CAF (diet effect: $F_{1,20}=9.62$, $p < 0.01$), which reinforces the detrimental effect of this type of diet on the microbiota. Omega-3 supplementation did not exert influence on the Chao 1 index (Fig. 3C).

3.3. Plasma levels of SCFAs

Plasma levels of SCFAs were quantified to analyze changes in the integrity of the intestinal barrier after diet and supplementation (Fig. 4). Acetate plasma levels were increased following CAF ($F_{1,41}=35.36$, $p < 0.01$) with no effect of n3 supplementation (Fig. 4A). However, isobutyrate levels showed an interaction between diet and n3 ($F_{1,39}=10.25$, $p < 0.01$), a diet effect ($F_{1,39}=98.14$, $p < 0.01$) and also a n3 effect ($F_{1,39}=14.43$, $p < 0.01$). The CAF group also showed a higher concentration of isobutyrate compared to the CAF+n3 group ($p < 0.01$) (Fig. 4B). Butyrate concentration was similar to isobutyrate: there was an interaction between diet and n3 ($F_{1,40}=22.51$, $p < 0.01$), a diet effect ($F_{1,40}=130.9$, $p < 0.01$) and a n3 effect ($F_{1,40}=26.38$, $p < 0.01$). Again, the CAF group showed an increased butyrate concentration in comparison to CAF+n3 ($p < 0.01$) (Fig. 4C). Isocaproate plasma levels showed a diet effect ($F_{1,41}=57.1$, $p < 0.01$) with increased concentration in CAF-fed groups (Fig. 4D).

3.4. Plasma levels of LPS

Plasma levels of LPS were quantified to analyze the relationship between obesity, n3 supplementation and endotoxemia (Fig. 5). Our results showed that CAF triggered an increase in LPS levels in the plasma of CAF-fed animals ($F_{1,28}=37.33$, $p < 0.01$). Interestingly, we also found an effect of n3 supplementation ($F_{1,28}=5.04$, $p < 0.05$) since CAF+n3 had reduced plasma LPS compared to the CAF group ($p=0.02$).

3.5. Medium and long chain fatty acids profile in the brain

Table 1 shows the concentration of several fatty acids that were

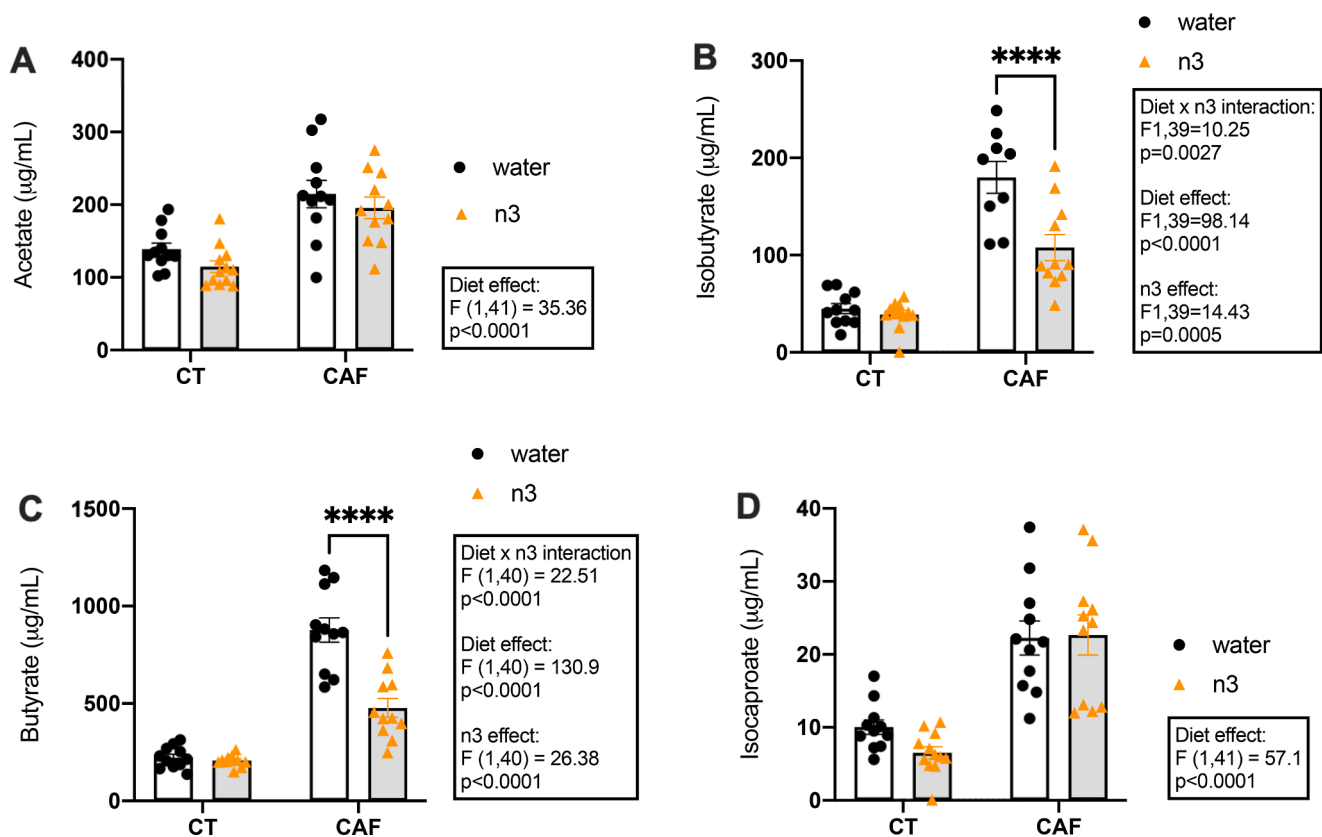


Fig. 4. Short-chain fatty acids concentration in the plasma of control (CT) and cafeteria diet (CAF)-fed rats. Acetate (A), isobutyrate (B), butyrate (C) and isocaproate (D) plasma concentrations. Significant differences showed by two-way ANOVA regarding effects of diet (CAF and CAF+n3 vs CT and CT+n3), n3 treatment (CAF and CT vs CAF+n3 and CT+n3) and diet × n3 interactions are indicated in the text boxes. Multiple comparisons were performed by Bonferroni post-hoc test and are indicated by the asterisk (**** $p < 0.0001$). n=9–12 animals/group.

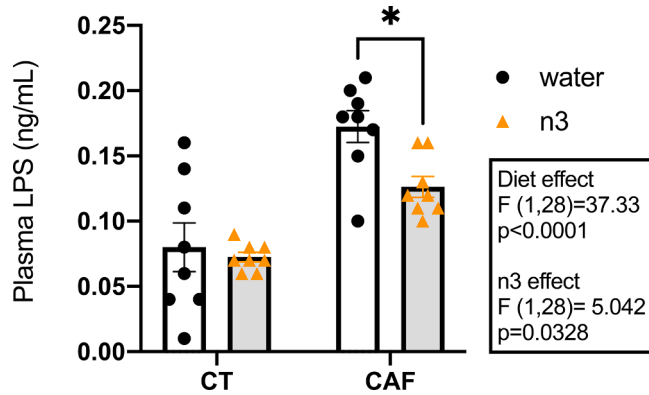


Fig. 5. Lipopolysaccharide (LPS) concentration in the plasma of control (CT) and cafeteria diet (CAF)-fed rats. Significant differences showed by two-way ANOVA regarding effects of diet (CAF and CAF+n3 vs CT and CT+n3), n3 treatment (CAF and CT vs CAF+n3 and CT+n3) and diet × n3 interactions are indicated in the text boxes. Multiple comparisons were performed by Bonferroni post-hoc test and are indicated by the asterisk (**p* < 0.05). *n*=8 animals/group.

measured in the cerebral cortex. We found an interaction of diet and n3 supplementation for caprylic acid concentration ($F_{1,36}=7.63$, $p<0.01$), which also showed a significant effect of n3 ($F_{1,36}=4.11$, $p < 0.05$). Bonferroni post hoc test also showed a significant difference between CT and CT+n3 group ($p < 0.01$). These findings showed that caprylic acid is increased in response to both CAF and n3 supplementation.

Similarly, myristoleic acid, an unsaturated fatty acid, showed an interaction between diet and n3 supplementation ($F_{1,32}=9.05$, $p < 0.01$)

and a n3 effect ($F_{1,32}=9.98$, $p < 0.01$). Again, both CAF and n3 seemed to increase myristoleic acid levels.

Palmitic acid showed a significant interaction ($F_{1,29}=83.12$, $p < 0.01$), diet ($F_{1,29}=42.06$, $p < 0.01$) and n3 ($F_{1,29}=34.16$, $p < 0.01$) effects. In multiple comparisons, we found a difference between CT and CT+n3 ($p < 0.01$). These results show that palmitic acid also increased in response to CAF and to n3.

Stearic acid increased in n3-supplemented rats (n3 effect: $F_{1,36}=8.65$, $p < 0.01$). In addition, CT+n3 group was significantly higher than CT ($p=0.03$). Linoleic acid concentration also increased following n3 supplementation (n3 effect: $F_{1,35}=5.68$, $p=0.02$), but no differences were found in multiple comparisons tests.

Tricosanoic acid showed an interaction between diet and n3 supplementation ($F_{1,33}=14.23$, $p < 0.01$) and also a n3 effect ($F_{1,33}=4.26$, $p < 0.05$). CT+n3 group was significantly higher than CT ($p<0.01$).

Lignoceric acid increased in n3 supplemented rats (n3 effect: $F_{1,28}=14.56$, $p < 0.01$). CT+n3 group was significantly higher than CT ($p < 0.01$).

3.6. Claudin-5 and TLR-4

Fig. 6 shows TLR-4 and claudin-5 expression in the cerebral cortex and hippocampus. TLR-4 showed a significant interaction between diet and n3 supplementation in the cerebral cortex ($F_{1,17}=5.08$, $p=0.04$) and also an increased expression in CAF-fed rats (diet effect: $F_{1,17}=13.52$, $p < 0.01$) (**Fig. 6A**). Intriguingly, we did not find differences in TLR-4 expression in the hippocampus (**Fig. 6C**). In the cerebral cortex, claudin-5 did not change (**Fig. 6B**), but in the hippocampus, we found a reduction in its expression following CAF (diet effect: $F_{1,17}=5.80$, $p=0.03$) (**Fig. 6D**).

Table 1
Fatty acids concentration (ng/mg) in the cerebral cortex.

Fatty acid	Type of fatty acid	CT	CT + n3	CAF	CAF + n3	Two-way ANOVA results			Bonferroni pos hoc test
						Interaction	Diet effect	n3 effect	
Caprylic	saturated	4.6 ± 0.6 (10)	7.7 ± 0.5 (10)*	6.3 ± 07 (8)	5.9 ± 0.7 (12)	0.0090	ns	0.0499	CT vs CT+n3 (p=0.0031)
Capric	saturated	16.9 ± 1.2 (10)	20.6 ± 1.6 (12)	19.4 ± 1.8 (8)	19.9 ± 2.0 (12)	ns	ns	ns	ns
Hendecanoic	saturated	237.1 ± 29.1 (7)	291 ± 36.3 (11)	207.4 ± 30.4 (8)	280 ± 45.9 (12)	ns	ns	ns	ns
Lauric	saturated	42.8 ± 8.4 (10)	50.3 ± 4.9 (12)	44.1 ± 16.5 (7)	39.2 ± 6.0 (12)	ns	ns	ns	ns
Tridecanoic	saturated	139 ± 20.5 (10)	102.3 ± 15.6 (11)	95.0 ± 17.1 (8)	114.7 ± 16.8 (12)	ns	ns	ns	ns
Myristic	saturated	680.4 ± 94.7 (10)	520.5 ± 43.0 (12)	582.8 ± 160.7 (8)	578.9 ± 116.5 (12)	ns	ns	ns	ns
Myristoleic	unsaturated	331.3 ± 85.9 (8)	844.4 ± 69.8 (12)	564.6 ± 103.8 * (6)	577.12 ± 73.5 (10)	0.0051	ns	0.0034	ns
Pentadecanoic	saturated	302.4 ± 58.9 (6)	215.9 ± 18.2 (11)	310.5 ± 57.6 (6)	341.1 ± 74.1 (9)	ns	ns	ns	ns
Palmitic	saturated	693.6 ± 130.8 (6)	2351.9 ± 99.6 (11)	2423 ± 95.00 (8)	2060 ± 111.7 (8)	<0.0001	<0.0001	<0.0001	CT vs CT+n3 (p<0.0001)
Margaric	saturated	609.6 ± 117.2 (10)	460.2 ± 56.2 (11)	459.9 ± 110.7 (8)	414.7 ± 60.8 (9)	ns	ns	ns	ns
Stearic	saturated	7377 ± 1289 (10)	10314 ± 628 (12)	8827 ± 640 (8)	10940 ± 625 (10)	ns	ns	0.0057	CT vs CT+n3 (p=0,0302)
Elaidic acid	Unsaturated trans	7806 ± 1427 (10)	9213 ± 1026 (12)	8520 ± 1550 (6)	8558 ± 1422 (8)	ns	ns	ns	ns
Linoleic	polyunsaturated	652.9 ± 112 (8)	1243.4 ± 143 (11)	894.1 ± 176 (8)	1354.8 ± 293 (12)	ns	ns	0.0228	ns
Heneicosanoic	saturated	451.8 ± 113 (9)	616.3 ± 55.2 (11)	494.7 ± 123.4 (8)	675.1 ± 112.6 (10)	ns	ns	ns	ns
Behenic	saturated	500.8 ± 142.4 (5)	505.6 ± 69.2 (10)	439.8 ± 105.7 (8)	311.2 ± 43.8 (7)	ns	ns	ns	ns
Tricosanoic	saturated	1042.3 ± 77.1 (8)	2278 ± 218.6 (12)	1565.1 ± 314.7 (6)	1203.8 ± 167.5 (11)	0.0006	ns	0.0468	CT vs CT+n3 (p=0.0002)
Lignoceric	saturated	1127.4 ± 263.7 (7)	3133.4 ± 469.1 (10)	1495.7 ± 128 (6)	3038.4 ± 568.5 (9)	ns	ns	0.0007	CT vs CT+n3 (p=0.0076)

Values are expressed as mean±SEM. *p* values are described when significant differences were found. ns= non significant. CAF, cafeteria diet; CT, control diet; n3, omega-3.

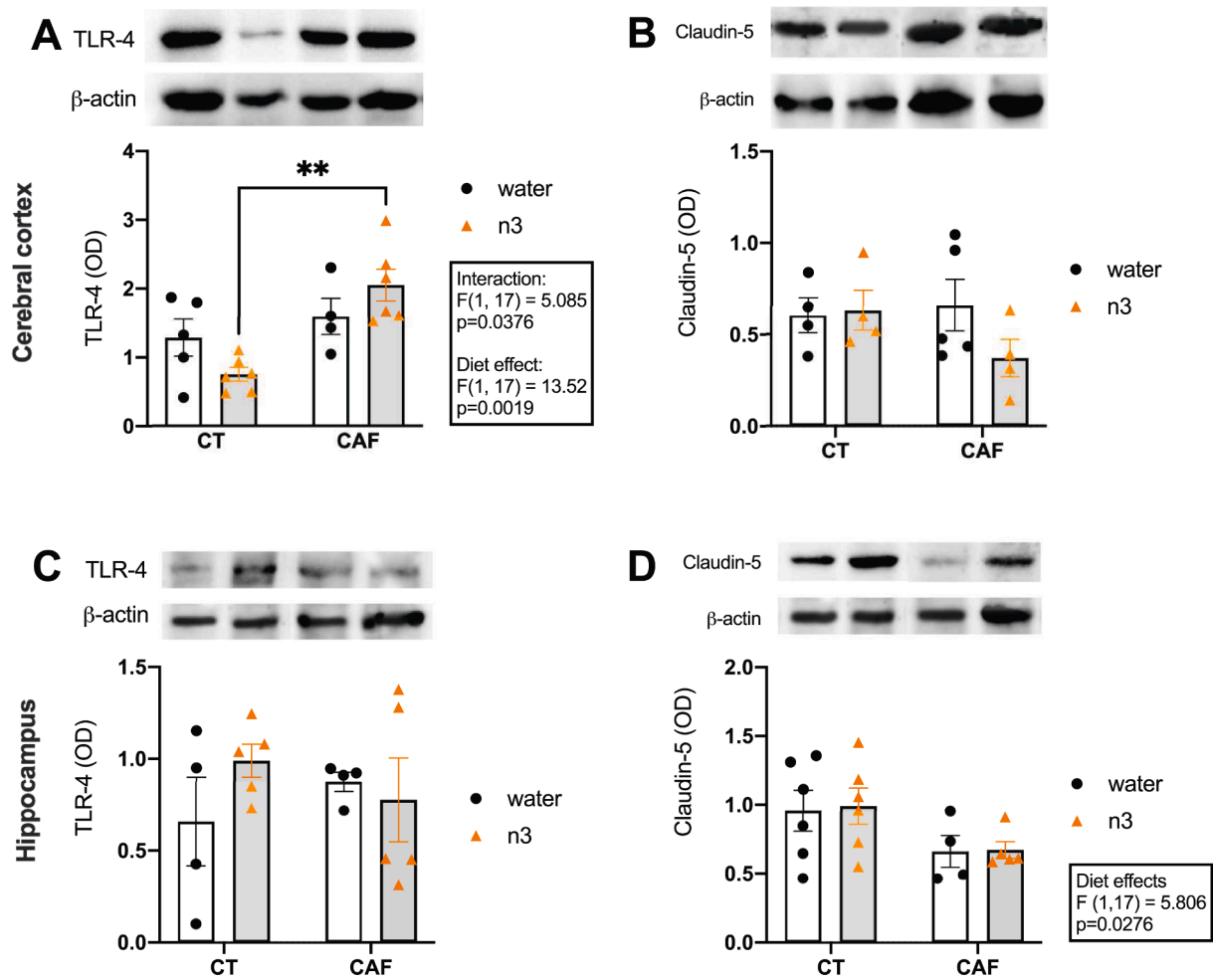


Fig. 6. Protein expression of TLR-4 and claudin-5 in the cerebral cortex (A, B) and in the hippocampus (C, D). Representative bands of each group are shown on the top of the graphs. β-actin was used as a loading control. Significant differences showed by two-way ANOVA regarding effects of diet (CAF and CAF+n3 vs CT and CT+n3), n3 treatment (CAF and CT vs CAF+n3 and CT+n3) and diet × n3 interactions are indicated in the text boxes. CT, control group; CAF, cafeteria diet; n3, omega-3; OD, optical density. n=4-6 animals per group.

3.7. Social interaction and memory

We evaluated social interaction and recognition memory through the five-trial social recognition test (Fig. 7). Fig. 7A presents the time of exploration of each group during each of the 4 exposures to the first intruder (J1) and the time exploring the unfamiliar intruder (J2). There was a significant decrease in the exploration time from the 1st to the 4th exposition of Juvenile 1 for all groups ($F_{3,49}, 136.1=33.82, p < 0.01$), demonstrating that animals were able to recognize the familiar conspecific. On the other hand, when comparing the exploration time of the 4th exposure to the familiar juvenile (4J1) and the exploration time of the novel juvenile (1J2), we found no difference in CT ($t=0.62, df=7, p=0.55$) and CT+n3 ($t=2.13, df=10, p=0.06$) groups (Fig. 7B and 7C), but a significant increase in the exploration of the novel juvenile in CAF ($t=2.76, df=8, p=0.02$) and CAF+n3 ($t=2.78, df=11, p=0.02$) groups (Fig. 7D and 7E).

4. Discussion

The present study assessed whether n3 could mitigate intestinal and cerebral disturbances caused by a cafeteria diet (CAF). The proposed diet-induced obesity model was already shown to induce metabolic disorders, neuroinflammation, and behavioral dysfunctions in rats [41, 28,29]. Indeed, it has been demonstrated that CAF generates more

severe obesity in rodents than other diet protocols [42,43,44]. Furthermore, when compared to other types of hypercaloric diets, such as high-fat diet (HFD) and high-fat high-sucrose (HFS), CAF induces a hyperphagic behavior due to its composition with highly palatable and energetically dense foods [1].

The harmful effects of obesity on metabolic organs such as the liver and adipose tissue are widely known [45]. However, the literature has recently demonstrated that the intestine may also play a key role in the etiology of the chronic inflammation related to obesity, possibly contributing to neuroinflammation [46,47,48]. Here, we measured the colon length and crypts depth, and both decreased following CAF. These findings regarding intestinal morphology in obesity are controversial. While some studies evidenced an increase in the villi height or crypt depth [49,50], others have already shown the opposite of these morphological features in the gut of middle-aged females fed HFD [51] and leptin-deficient transgenic mice [52]. Therefore, we showed that adult rats fed a diet rich in ultra-processed foods are susceptible to these changes. However, the correlation between intestinal and villi length in humans is scarce and contradictory [53,54,55,56]. The decrease in villi and the total length of the intestine was already related to a dysfunction in the progenitor cells, which in response to HFD may assume stem-cell-like features, affecting remodeling and regenerative properties of the intestinal epithelium and thus predisposing to increased tumor incidence [57]. Thus, the decrease in crypt depth may be related to an

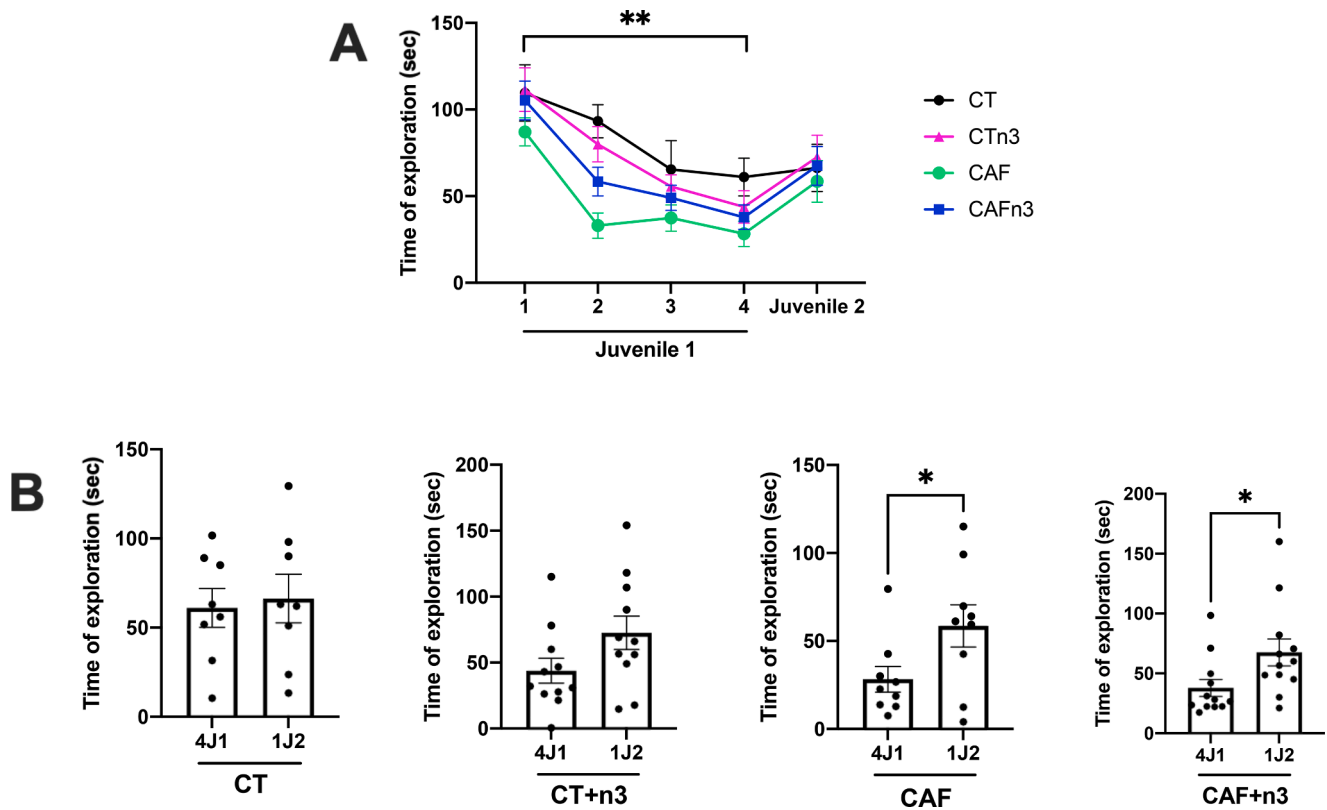


Fig. 7. Five-trial social memory test. A) Time of exploration of control (CT) and cafeteria diet (CAF)-fed rats to the same juvenile rat (juvenile 1, sessions 1-4) and to a different one (juvenile 2, session 5). B) Comparisons of the exploration time of the 4th exposure to the familiar juvenile (4J1) and the exploration time of the novel juvenile (1J2) for each group. * $p < 0.05$; ** $p < 0.01$. $n=8-12$ animals/group.

increased turnover of colonocytes, predisposing intestinal disease development. Our findings showed that n3 supplementation increased the crypt depth only in rats fed a standard diet but not in obese animals. This effect may be related to the preventive role of n3 in protecting intestinal integrity, but it is not sufficient to revert the harmful impact of CAF.

Regarding the intestinal microbiota, in the present study, it has been demonstrated that CAF induces a more robust dysbiosis than other experimental models of obesity [58]. Here, we found that obese animals had a reduced Firmicutes and Actinobacteria phyla population, while Bacteroidetes and Proteobacteria increased after CAF. Firmicutes and Bacteroidetes represent around 90% of microbiota composition [59]. High-fat diet consumption has previously been shown in humans and animals to increase the number of Firmicutes while decreasing the presence of the Bacteroidetes [60,61]. Therefore, a high Firmicutes/Bacteroidetes ratio was already considered a biomarker of obesity, but more recently, this view has been changed [62]. Several factors may be attributed to discrepancies regarding the Firmicutes/Bacteroidetes ratio in obesity, such as differences in sample processing, data analysis, number of subjects in the studies, and other variables that also interfere in microbiota composition, such as the type of nutrients ingested and not only the amount of calories [63]. Also, the classical view of the decrease in the abundance of Bacteroidetes in obesity generates a paradigm since this phylum is the main group of gram-negative bacteria in the intestinal microbiota, from which LPS molecules are derived, responsible for causing metabolic endotoxemia [64]. Here, we found the opposite effect, a decrease in Firmicutes and an increase in Bacteroidetes community following CAF, and an increase in Firmicutes/Bacteroidetes ratio in response to n3 supplementation. A similar finding was also shown elsewhere following 10 weeks of CAF [1].

Concerning the Actinobacteria phylum, our study showed that CAF reduced its phylum with no effect of n3. However, this finding contrasts

with the profile usually related to obesity [65]. We can speculate that the microbiota profile in obesity is not robustly reproducible, as discussed above. In addition, the composition of the menu in the CAF model is variable in different studies, which may cause discrepancies in the microbiota composition [1]. Therefore, the interpretation of these findings must be carefully evaluated since accumulated evidence corroborates that it is not just the change in one bacterial population that influences pathophysiological responses in obesity but the imbalance of all of them.

Proteobacteria phylum has been shown to increase in previous studies with animal models and humans with obesity [66,67]. Also, the administration of *Enterobacter*, a member of this phylum, to germ-free mice resulted in obesity and insulin resistance [68,67]. In our study, CAF also promoted an increase in the Proteobacteria phylum, and n3 was able to significantly decrease it following CAF, suggesting a protective role of supplementation in obese animals regarding this phylum. Also, this finding agrees with plasma levels of LPS, in which we showed increased levels after CAF compared to control groups, and also a beneficial effect of n3 to reduce it. Therefore, we showed that n3 in obese animals could modulate the microbiota in favor of lowering endotoxemia by decreasing the abundance of the Proteobacteria along with LPS.

At the genus level, we found a decrease in the *Akkermansia* community following n3 supplementation. Phylum Verrucomicrobia, composed mainly of *Akkermansia*, was already shown elsewhere to be more abundant in rats fed CAF for 14 weeks [65]. Also, *Akkermansia* is highly responsive to diet change. Its community increased in a fiber-free diet, contributing to intestinal mucus degradation and barrier dysfunction [69]. Therefore, the effect of n3 in reducing *Akkermansia* may contribute to maintaining the intestinal barrier [70]. Furthermore, studies show that *Akkermansia* is abundant in patients with Alzheimer's [52,71] and Parkinson's disease [72,73] in both cases, correlating

disease progression and dysbiosis.

Lactobacilli and *Bifidobacteria* are considered health-promoting bacteria that are reduced in metabolic diseases, including obesity, diabetes, and cardiovascular disease [7]. Omega-3 PUFAs can modulate the gut microbiota composition [74] by increasing the number of *Bifidobacterium*, which decreases intestinal permeability [75]. We found a reduction of *Bifidobacterium* followed by CAF, which is in accordance with previous studies showing a negative correlation with intestinal barrier function [75,76]. CAF also significantly reduced the amount of *Lactobacilli*, but n3 did not show any effect in this case. Studies with humans and animals that have tested n3 supplementation and demonstrated an increase in the abundance of *Lactobacilli* [77,78,79,80]. However, we did not find studies that tested n3 intake to reverse the effects of prolonged exposure to a high-calorie diet, such as CAF.

Blautia belongs to the phylum Firmicutes. We found an increased abundance of this genus following CAF, which agrees with previous studies with obesogenic diets [81,82]. On the other hand, most of the properties of *Blautia* are linked to potential probiotic function, and the causal relationship between its abundance and disease is still unclear [83].

Despite the different findings on intestinal bacteria predominance in obesity studies, CAF is consistently associated with a decrease in microbiota diversity [84]. However, other models of diet-induced obesity in rodents, such as HFD, do not show the same reduction in microbiota diversity [58,1]. Dysbiosis is associated with neurological and neurodegenerative diseases such as Parkinson's disease [85], dementia [86], and multiple sclerosis [87]. Thus, it cannot be affirmed that there is a robust characterization of phyla or genera related to specific diseases. However, intestinal dysbiosis seems to be strongly related to neurological dysfunctions.

One of the mechanisms through which intestinal microbiota can affect the host's physiology may be SCFA signaling [88,89]. SCFA can activate GPRs, inhibit histone deacetylase (HDAC), and be used as an energy substrate, thus affecting the physiological processes of the host [89]. In our research, the CAF-fed groups showed higher plasma SCFA levels. Evidence to date supports that these metabolites provide beneficial rather than harmful effects on obesity and its complications [90,91]. However, a high-carbohydrate diet and obesity can attenuate the binding of SCFAs to GPRs, leading to increased intestinal energy uptake and hepatic lipogenesis [92,93,94]. In addition, it was already shown that rats fed with HFD for four weeks increased acetate levels, stimulating hyperphagia and raising insulin secretion, favoring the augmentation of energy storage and the development of metabolic syndrome [95]. The role of butyrate in obesity is also debated. While evidence points to the beneficial effects of butyrate on intestinal physiology by showing anti-inflammatory properties, its role in obesity is controversial since it also increases lipid synthesis and may facilitate the development of insulin resistance [96,97]. Since the main source of SCFA is the intestinal microbiota, the increased levels of SCFA found here in obese rats may be due to the changes in the microbiota profile described above. Altogether, these alterations might intensify the obese phenotype of CAF-fed animals. In this regard, n3 might exert partial protection since its supplementation decreased butyrate and isobutyrate levels in CAF-fed rats.

By analyzing the profile of fatty acids in the cerebral cortex, we found an effect of n3 supplementation in increasing the saturated fatty acids: caprylic, palmitic, stearic, tricosanoic, and lignoceric. The unsaturated myristoleic acid and the polyunsaturated linoleic acid were also increased. A diet effect was found only for palmitic acid, which increased following CAF. Mason et al. 2017 investigated the fatty acid content, oxidation products, and biological effects of three major n3 dietary supplements available in the United States and found that they contained more than 30 fatty acids, including significant levels of saturated fat (36% of total fatty acids), in addition to the desirable n3 [98]. These data indicate that the levels of saturated fat and oxidized lipids found in commonly available dietary supplements may interfere

with their biological benefits. In addition, other studies have already demonstrated the presence of contaminants, cholesterol, oxidation products, and variable EPA and/or DHA content inconsistent with amounts reported on the label, which may compromise the beneficial effects of n3 [99,100,101]. In our study, the amount of saturated fatty acids informed by the manufacturer is 0.1%, which is expected not to exert any detrimental effect. However, a more detailed evaluation of the purity of this supplement should be performed to discard the presence of other molecules that might interfere with its expected effect.

Increased content of palmitic acid, stearic acid, heneicosanoic acid, and tetracosanoic acid has already been shown in the brain of mice fed a high-fat and high-carbohydrate diet [102]. Saturated fatty acids, such as palmitic and stearic acids, promote a pro-inflammatory state of microglia, act as a ligand for TLR-4 receptors mimicking LPS, activating the NF- κ B pathway, generating microglial activation with increased production of pro-inflammatory cytokines [103,104,9,105]. However, fish oil supplementation attenuated microgliosis and astrogliosis in the brain [36]. In the same direction, we did not find a pro-inflammatory effect of n3 supplementation in our experiment, even though n3 increased saturated fatty acids in the cerebral cortex. Instead, we have previously found that obese mice supplemented with n3 showed a decrease in TNF- α levels [28].

Here, we also evaluated whether CAF increased TLR-4 expression in the cerebral cortex and hippocampus and the effect of n3 on it. To assess blood-brain barrier integrity, we also analyzed claudin-5 expression. Our results showed an increase in TLR-4 expression in the cerebral cortex of CAF-fed rats, consistent with previous studies [106,29]. However, n3 could not reverse the increase in TLR-4 induced by the diet. Still, there was an interaction between n3 and diet effects, suggesting that the severity of obesity caused by CAF prevented the ability of n3 to inhibit this pathway. There is robust evidence that n3 can inhibit the TLR-4 pathway through the receptor GPR120. Activation of GPR120 by DHA suppresses the NF- κ B and JNK pathways, blocking the release of pro-inflammatory cytokines [107,108]. However, in our study, we did not find an effect of n3 on TLR-4 expression in the cerebral cortex, suggesting that chronic exposure to CAF is a potent stimulus to TLR-4 expression in this brain region. Surprisingly, in the hippocampus, we did not find an increase in the expression of TLR-4 following CAF. A similar finding following CAF was already shown in a previous study, in which neuroinflammatory markers were increased in the hippocampus, but TLR-4 expression did not change [109].

Regarding claudin-5 expression, CAF reduced it in the hippocampus but not in the cerebral cortex, and n3 did not affect claudin-5 expression. A similar result was already shown following a hypercaloric diet, with alteration of claudin-5 in the hippocampus but not in the cerebral cortex [110]. Interestingly, in a study with rodents submitted to HFD containing n3, an increase in occludin expression, another tight junction protein, was observed in different brain regions, indicating that the effect of n3 is generalized throughout the brain. In the same study, there was no difference in the expression of claudin-5 in the hypothalamus [111]. Furthermore, n3 treatment for 4 weeks remarkably increased zonula occludens-1 (ZO-1) expression and significantly inhibited glial activation and NF- κ B activation in APP/PS1 mice, a model of Alzheimer's disease [51]. Nevertheless, to the best of our knowledge, no studies show a beneficial effect of n3 on the BBB markers in severe obesity as mimicked by the CAF model.

Another important outcome of the impact of CAF and n3 on the brain is the modification of behavior. Here, we evaluated social memory through the five-trial social memory test. Our results showed that CAF animals had increased social memory as compared to CT animals, but this finding must be carefully analyzed. Although many studies show that obesity is capable of causing a reduction in social interaction [112,113], this result is controversial. Obese animals may have greater sociability than lean counterparts, not because they remember the animal they first met, but because they socialize more with the second animal they come into contact with [21]. Although obese animals showed

increased sociability, they could not discriminate between novel and already known intruders since the animals were exposed to the known and new animals simultaneously [21]. In our study, exposure to the new animal was isolated, and the time of exploration of this animal was compared between groups. We can speculate that for this reason, the result we found indicates that CAF-fed animals have increased sociability rather than improved social memory. It is also discussed that the increased sociability may be due to epigenetic alterations in hypothalamic nuclei related to male-male interaction [114,115,21].

Another important point to address is that the microbiota can modulate the behavior of animals, as well as social behavior and social recognition [116]. CAF can result in different microbiota profiles compared to other diet protocols [58]. One of the interesting findings we obtained was the increase in SCFA measured in animals treated with CAF. Although this has also been found in experimental animal models and obese humans [117,95,118], none of these studies have evaluated the effect of this increase on social memory. Therefore, to our knowledge, this is the first work to show that a CAF may be related to increased SCFA and improved social memory. However, we could not affirm that the dysbiosis caused by CAF was the cause of the increased sociability.

5. Conclusion

Animal models of obesity that use ultra-processed foods cause robust weight gain and metabolic changes. On the other hand, the effect of these diets on gut microbiota and behavioral disturbances is still unclear. Studies using CAF are heterogeneous, which can be hard to be interpreted since there is low reproducibility among studies. Likewise, treatments that can alleviate many harmful impacts of ultra-processed food consumption must be carefully evaluated. In this study, omega-3 supplementation was able to reverse some changes in the microbiota composition. However, it did not improve bacterial diversity, which implies that its benefits on intestinal microbiota are scarce or null. On the other hand, n3 decreased the metabolic endotoxemia caused by the CAF, indicating an advantageous effect of its use in treating obesity-related metabolic dysfunctions.

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CRediT authorship contribution statement

JN, JJ, FR performed experiments, analyzed data, and wrote the manuscript. SS, SE, TFO, AKS, performed experiments. JCFM, MG, MP contributed to data analysis and editing of the manuscript. RPG designed the study, interpreted the results and wrote the manuscript. All authors read and approved the final manuscript.

Supplementary Fig. 1. Caloric intake of control (CT) and cafeteria diet (CAF)-fed rats. CAF-fed rats showed an increased caloric intake compared to CT diet-fed rats from the first to the last experimental week. * $p < 0.05$. $n=11-12$ animals/group.

Declaration of Competing Interest

The authors declared no conflict of interest.

Data availability

Data will be made available on request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.physbeh.2022.114068.

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7 CONCLUSÃO

Nesta tese de doutorado, estudamos o impacto de um modelo animal de obesidade induzida por dieta, a dieta de cafeteria (CAF), sobre parâmetros metabólicos e comportamentais, e ainda, o efeito da suplementação de ômega-3 sobre estes parâmetros.

Os resultados apresentados no artigo 1, intitulado: “DHA/EPA supplementation decreases anxiety-like behaviour, but it does not ameliorate metabolic profile in obese male rats” corroboraram com diversos estudos sobre a capacidade da CAF em gerar ganho de peso, adiposidade visceral e alterações no perfil glicêmico e nos níveis de triglicérides. No entanto, a suplementação com o ômega-3 não foi capaz de reverter esses efeitos, mas apresentou resultados positivos, reduzindo os níveis de citocinas pró-inflamatórias no fígado e no sistema nervoso central, e também o comportamento do tipo ansioso nos animais obesos.

No artigo 2, intitulado “Impact of cafeteria diet and n3 supplementation on the intestinal microbiota, fatty acids levels, neuroinflammatory markers and social memory in male rats” mostramos que a CAF alterou a morfologia e a microbiota intestinais, e de forma inesperada, aumentou a interação dos ratos no teste de memória social. A suplementação com ômega-3 por sua vez, foi capaz de modificar a composição da microbiota intestinal e reduzir a endotoxemia metabólica nos animais alimentados com CAF, o que indica um efeito promissor da indicação deste tratamento no contexto da obesidade.

Os modelos de obesidade induzida por dieta geram ganho de peso e alterações metabólicas robustas, fornecendo ferramentas úteis para o estudo da obesidade e de suas repercussões sobre diversos sistemas, incluindo o sistema nervoso. Por outro lado, os efeitos produzidos por essas dietas são variados, devido a diferenças de composição entre os estudos, o que pode dificultar a interpretação dos achados.

Embora os efeitos benéficos do ômega-3 sobre a saúde, especialmente seu potencial anti-inflamatório, sejam amplamente demonstrados, deve-se ter cautela na sua indicação para o tratamento das disfunções provocadas pela obesidade. Observamos que os efeitos do ômega-3 são parciais, não produzindo benefícios robustos na obesidade severa, a qual é induzida no modelo da CAF.

Contudo, a ação ansiolítica observada merece destaque, já que ansiedade e depressão são condições frequentes na população obesa.

Outro aspecto importante para a prescrição de suplementação de ômega-3 é em relação à qualidade dos produtos disponíveis comercialmente. Quando não há um controle rígido em relação a composição dos produtos, os suplementos podem conter compostos que prejudicam as ações esperadas do ômega-3. Neste trabalho, não testamos a pureza do suplemento empregado, o que é uma limitação do estudo. Contudo, o protocolo de suplementação foi iniciado após a obesidade ter sido induzida, o que certamente limita os efeitos do ômega-3, já que muitos estudos fazem a suplementação concomitante à administração das dietas obesogênicas, ou ainda de forma preventiva.

Assim, concluímos que a indicação de ômega-3 não é suficiente para reverter os efeitos nocivos da obesidade severa. Contudo, sua suplementação exerce benefícios parciais quanto aos efeitos inflamatórios da obesidade, e também é capaz de modular a microbiota intestinal e atenuar o comportamento do tipo ansioso.

APÊNDICE – CURRÍCULO LATTES



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Produção

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Artigos completos publicados em periódicos

- 1.**  [DOI](#) NETO, JOÃO; JANTSCH, JEFERSON; RODRIGUES, FERNANDA; SQUIZANI, SAMIA; ELLER, SARAH; OLIVEIRA, TIAGO FRANCO; SILVEIRA, ALEXANDRE KLEBER; MOREIRA, JOSÉ CLÁUDIO FONSECA; GIOVENARDI, MARCIA; PORAWSKI, MARILENE; GUEDES, RENATA PADILHA Impact of cafeteria diet and n3 supplementation on the intestinal microbiota, fatty acids levels, neuroinflammatory markers and social memory in male rats. *PHYSIOLOGY & BEHAVIOR*. [JCR](#), v.260, p.114068 - , 2023.
- 2.**  [DOI](#) NETO, JOÃO; JANTSCH, JEFERSON; DE OLIVEIRA, SIMONE; BRAGA, MATHEUS FILIPE; DE CASTRO, LUÍS FELIPE DOS SANTOS; DINIZ, BRUNA FERRARY; MOREIRA, JOSÉ CLAUDIO FONSECA; GIOVENARDI, MARCIA; PORAWSKI, MARILENE; GUEDES, RENATA PADILHA DHAVEPA SUPPLEMENTATION DECREASES ANXIETY-LIKE BEHAVIOUR, BUT IT DOES NOT AMELIORATE METABOLIC PROFILE IN OBESE MALE RATS. *BRITISH JOURNAL OF NUTRITION*. [JCR](#), v.2021, p.1 - 25, 2021.
- 3.**  [DOI](#) OLIVEIRA, SIMONE; FEIJÓ, GRACE DOS SANTOS; NETO, JOÃO; JANTSCH, JEFERSON; BRAGA, MATHEUS FILIPE; CASTRO, LUÍS FELIPE DOS SANTOS; GIOVENARDI, MÁRCIA; PORAWSKI, MARILENE; GUEDES, RENATA PADILHA Zinc Supplementation Decreases Obesity-Related Neuroinflammation and Improves Metabolic Function and Memory in Rats. *Obesity*. [JCR](#), v.29, p.116 - 124, 2021.
- 4.**  [DOI](#) DOS SANTOS, CASSIA GONÇALVES; DA ROSA, DANIELLY BRULLINGER; MARTINS, GUSTAVO ANDRADE; DA ROSA, EDUARDO FREITAS; PEREIRA NETO, JOÃO Fisioterapia e qualidade de vida na osteoartrite de joelho. *FISIOTERAPIA BRASIL* , v.21, p.86 - , 2020.
- 5.**  [DOI](#) NETO, J.P.; DA SILVA, D. SANTOS; DA SILVA, I.R. VITAL; THILL, J.; POCHMANN, D.; BRITO, C.; ELSNER, V.R. Relationship between levels of physical activity and brain derived neurotrophic factor in peripheral blood of monozygotic twins. *Comparative Exercise Physiology*. [JCR](#), v.16, p.319 - 324, 2020.
- 6.**  [DOI](#) DA ROSA, EDUARDO FREITAS; CANTERGI, DEBORA; PEREIRA NETO, JOÃO; MOREM, BRUNO GILBERTO DIAS; LOSS, JEFFERSON FAGUNDES Comparação da atividade eletromiográfica em músculos do powerhouse no roll up e roll back no solo e no Cadillac do método Pilates. *FISIOTERAPIA BRASIL* , v.19, p.183 - 189, 2018.

Apresentação de trabalho e palestra

1. NETO, J.P
Escoliose em Gêmeos Monozigóticos e Epigenética: Uma experiência científica, 2017. (Conferência ou palestra,Apresentação de Trabalho)
2. NETO, J.P
Workshop Pilates Solo, 2017. (Conferência ou palestra,Apresentação de Trabalho)
3. PEREIRA NETO, J.
Avaliação Postural, 2016. (Conferência ou palestra,Apresentação de Trabalho)
4. PEREIRA NETO, J.
Biofotogrametria, 2016. (Conferência ou palestra,Apresentação de Trabalho)
5. PEREIRA NETO, J.
Biomecânica da Marcha, 2016. (Conferência ou palestra,Apresentação de Trabalho)
6. PEREIRA NETO, J.
Desvendando as Escolioses, 2016. (Conferência ou palestra,Apresentação de Trabalho)
7. PEREIRA NETO, J.
Dicas para Realizar um TCC com Sucesso, 2016. (Conferência ou palestra,Apresentação de Trabalho)
8. PEREIRA NETO, J.
Dicas para Realizar um TCC com Sucesso, 2016. (Conferência ou palestra,Apresentação de Trabalho)
9. NETO, J.P
Escoliose Na Infância e na Adolescência, 2016. (Conferência ou palestra,Apresentação de Trabalho)
10. PEREIRA NETO, J.
Importância e Benefícios de Uma Boa Postura Corporal no Trabalho, 2016. (Conferência ou palestra,Apresentação de Trabalho)
11. PEREIRA NETO, J.; ELSNER, V. R.; BRITO, C. I. B.; GUERRA, H. M.; REICHET, I.; POCHMANN, D.; THILL, J. F.
Influências de Marcadores Epigenéticos Periféricos em Gêmeos Monozigóticos com Escoliose idiopática, 2016. (Simpósio,Apresentação de Trabalho)
12. PEREIRA NETO, J.
Pilates nas Disfunções da Coluna Vertebral, 2016. (Conferência ou palestra,Apresentação de Trabalho)
13. PEREIRA NETO, J.
Postural Corporal e Doenças Osteomusculares, 2016. (Conferência ou palestra,Apresentação de Trabalho)
14. PEREIRA NETO, J.
Avaliação Postural, 2015. (Conferência ou palestra,Apresentação de Trabalho)
15. PEREIRA NETO, J.
Biofotogrametria, 2015. (Conferência ou palestra,Apresentação de Trabalho)
16. PEREIRA NETO, J.; BRITO, C. I. B.
Influências Fenotípicas e Genotípicas Nas Alterações Posturais em Gêmeos, 2015. (Congresso,Apresentação de Trabalho)
17. BRITO, C. I. B.; PEREIRA NETO, J.
Influências Genotípicas e Fenotípicas nas Alterações Posturais em Gêmeos, 2015. (Conferência ou palestra,Apresentação de Trabalho)

Produção técnica

Demais produções técnicas

1. NETO, JOÃO
Disfunções da Coluna Vertebral, 2021. (Extensão, Curso de curta duração ministrado)
2. NETO, JOÃO
Dry Needling, 2021. (Extensão, Curso de curta duração ministrado)
3. NETO, JOÃO
Dry Needling, 2021. (Extensão, Curso de curta duração ministrado)
4. NETO, JOÃO
Dry Needling, 2021. (Extensão, Curso de curta duração ministrado)
5. NETO, JOÃO
Dry Needling, 2021. (Extensão, Curso de curta duração ministrado)
6. NETO, JOÃO
Dry Needling, 2021. (Extensão, Curso de curta duração ministrado)
7. NETO, JOÃO
Dry Needling, 2021. (Extensão, Curso de curta duração ministrado)
8. NETO, JOÃO
Avaliação Postural Por Fotogrametria, 2020. (Especialização, Curso de curta duração ministrado)
9. NETO, JOÃO
Dry Needling, 2020. (Extensão, Curso de curta duração ministrado)
10. NETO, JOÃO
Dry Needling, 2020. (Extensão, Curso de curta duração ministrado)
11. NETO, JOÃO
Dry Needling, 2020. (Extensão, Curso de curta duração ministrado)
12. NETO, JOÃO
Dry Needling, 2020. (Extensão, Curso de curta duração ministrado)
13. NETO, JOÃO
Dry Needling, 2020. (Extensão, Curso de curta duração ministrado)
14. NETO, JOÃO
Dry Needling, 2020. (Extensão, Curso de curta duração ministrado)
15. NETO, JOÃO
Dry Needling, 2020. (Extensão, Curso de curta duração ministrado)
16. NETO, JOÃO
Dry Needling, 2020. (Extensão, Curso de curta duração ministrado)
17. NETO, JOÃO
Dry Needling, 2020. (Extensão, Curso de curta duração ministrado)
18. NETO, JOÃO
Dry Needling, 2020. (Extensão, Curso de curta duração ministrado)
19. NETO, JOÃO
Dry Needling, 2020. (Extensão, Curso de curta duração ministrado)

20. NETO, JOÃO
Disfunções da Coluna Vertebral, 2019. (Extensão, Curso de curta duração ministrado)
21. NETO, JOÃO
Disfunções da Coluna Vertebral, 2019. (Extensão, Curso de curta duração ministrado)
22. NETO, JOÃO
Dry Needling, 2019. (Extensão, Curso de curta duração ministrado)
23. NETO, JOÃO
Dry Needling, 2019. (Extensão, Curso de curta duração ministrado)
24. NETO, JOÃO
Dry Needling, 2019. (Extensão, Curso de curta duração ministrado)
25. NETO, JOÃO
Dry Needling, 2019. (Extensão, Curso de curta duração ministrado)
26. NETO, JOÃO
Dry Needling, 2019. (Extensão, Curso de curta duração ministrado)
27. NETO, JOÃO
Dry Needling, 2019. (Extensão, Curso de curta duração ministrado)
28. NETO, JOÃO
Dry Needling, 2019. (Extensão, Curso de curta duração ministrado)
29. NETO, JOÃO
Dry Needling, 2019. (Extensão, Curso de curta duração ministrado)
30. NETO, JOÃO
Dry Needling, 2019. (Extensão, Curso de curta duração ministrado)
31. NETO, JOÃO
Minicurso - Dry Needling aplicado à coluna vertebral, 2019. (Extensão, Curso de curta duração ministrado)
32. NETO, JOÃO
Workshop de Dry Needling, 2019. (Extensão, Curso de curta duração ministrado)
33. NETO, JOÃO
Workshop de Dry Needling, 2019. (Extensão, Curso de curta duração ministrado)
34. NETO, JOÃO
Workshop de Dry Needling, 2019. (Extensão, Curso de curta duração ministrado)
35. NETO, JOÃO
Workshop de Dry Needling, 2019. (Extensão, Curso de curta duração ministrado)
36. NETO, JOÃO
Avaliação Postural Por Fotogrametria, 2018. (Extensão, Curso de curta duração ministrado)
37. NETO, JOÃO
Disfunções da Coluna Vertebral, 2018. (Extensão, Curso de curta duração ministrado)
38. NETO, JOÃO
Disfunções da Coluna Vertebral, 2018. (Extensão, Curso de curta duração ministrado)
39. NETO, JOÃO
Disfunções da Coluna Vertebral, 2018. (Especialização, Curso de curta duração ministrado)
40. PEREIRA NETO, J.
Avaliação Postural por Fotogrametria, 2017. (Extensão, Curso de curta duração ministrado)
41. PEREIRA NETO, J.
Disfunções da Coluna Vertebral, 2017. (Extensão, Curso de curta duração ministrado)
42. PEREIRA NETO, J.; FERNANDES, C.; MOUSALLE, M. M.; FATTORI, R.
Atuação do Fisioterapeuta no Manejo da Dor, 2016. (Extensão, Curso de curta duração ministrado)
43. PEREIRA NETO, J.
Avaliação Postural, 2016. (Extensão, Curso de curta duração ministrado)
44. PEREIRA NETO, J.
Disfunções da Coluna Vertebral, 2016. (Extensão, Curso de curta duração ministrado)
45. PEREIRA NETO, J.
Disfunções da Coluna Vertebral, 2016. (Extensão, Curso de curta duração ministrado)
46. PEREIRA NETO, J.
Formação em Pilates - Módulo I, 2016. (Extensão, Curso de curta duração ministrado)
47. PEREIRA NETO, J.
Formação em Pilates - Módulo I, 2016. (Extensão, Curso de curta duração ministrado)
48. PEREIRA NETO, J.
Formação em Pilates - Módulo I, 2016. (Extensão, Curso de curta duração ministrado)
49. PEREIRA NETO, J.
Monitor - Formação em Pilates Módulo I, 2016. (Extensão, Curso de curta duração ministrado)
50. PEREIRA NETO, J.
Monitor - Formação em Pilates Módulo I, 2016. (Extensão, Curso de curta duração ministrado)
51. PEREIRA NETO, J.
Monitor - Formação em Pilates Módulo I e II, 2016. (Extensão, Curso de curta duração ministrado)
52. PEREIRA NETO, J.
Monitor - Formação em Pilates Módulo II, 2016. (Extensão, Curso de curta duração ministrado)
53. PEREIRA NETO, J.
Monitor - Formação em Pilates Módulo III, 2016. (Extensão, Curso de curta duração ministrado)
54. PEREIRA NETO, J.
Monitor - Formação em Pilates Módulo IV, 2016. (Extensão, Curso de curta duração ministrado)
55. PEREIRA NETO, J.
Avaliação Postural Por Fotogrametria, 2015. (Extensão, Curso de curta duração ministrado)

Orientações e Supervisões

Orientações e supervisões

Orientações e supervisões concluídas




Monografias de conclusão de curso de aperfeiçoamento/especialização

1. Fernanda Hackenhaar e Júlia Posoco Agostini. **Dry Needling e seu Efeito na Síndrome da Dor miofascial: Artigo de revisão**. 2018. Monografia (Pós-Graduação em Fisioterapia Traumato-Ortopédica e

Esportiva) - Faculdade de Tecnologia Ibrate

2. Carine Leal Nascimento Corneli e Suellen Ribeiro de Oliveira. **Fisioterapia nas Lesões de Jiu Jitsu: Um Estudo de Revisão**. 2018. Monografia (Pós-Graduação em Fisioterapia Traumatológica e Esportiva) - Faculdade de Tecnologia Ibrate
3. Cristiani Seberino. **Índices que Influenciam na Capacidade Funcional em Idosos Praticantes de Atividade Física**. 2018. Monografia (Pós-Graduação em Fisioterapia Traumatológica e Esportiva) - Faculdade de Tecnologia Ibrate
4. Alice Birk Preissler e Luana Bruxel Vieira. **Prevalência de Escoliose Idiopática do Adolescente: Uma revisão da Literatura**. 2018. Monografia (Pós-Graduação em Fisioterapia Traumatológica e Esportiva) - Faculdade de Tecnologia Ibrate
5. Tatiana Urbano e Taína Barela. **Relação entre Pisada e o Risco de Lesões em Corredores de Rua**. 2018. Monografia (Pós-Graduação em Fisioterapia Traumatológica e Esportiva) - Faculdade de Tecnologia Ibrate
6. Cassia Gonçalves dos Santos e Danielly Brullinger. **Tratamento Fisioterapêutico em Indivíduos com Osteoartrite de Joelho: Estudo de revisão**. 2018. Monografia (Pós-Graduação em Fisioterapia Traumatológica e Esportiva) - Faculdade de Tecnologia Ibrate

Trabalhos de conclusão de curso de graduação

1.  Felipe Jacomeli Machado. **Comparação das Técnicas de Fotogrametria Computadorizada e Goniometria na Avaliação do Joelho no Plano Sagital**. 2018. Curso (Fisioterapia) - Centro Universitário Metodista
2.  Carolina Hoese Cardoso. **A confiabilidade da Fotogrametria Computadorizada na Angulação dos Joelhos no Plano Sagital utilizando Diferentes avaliadores**. 2017. Curso (Fisioterapia) - Centro Universitário Metodista
3.  Juliane Thill Ferreira da Silvs. **A Confiabilidade Interexatidão da Angulação dos Joelhos no Plano Sagital utilizando a Fotogrametria Computadorizada na Análise da**. 2017. Curso (Fisioterapia) - Centro Universitário Metodista

Eventos

Eventos

Participação em eventos

1. **Convenção 50 anos da Fisioterapia: O Futuro da Profissão**, 2019. (Congresso)
Escoliose Idiopática.
2. **II Jornada de Fisioterapia da UNICENEC/ Osório**, 2018. (Seminário)
Escoliose Idiopática.
3. **Semana Acadêmica da Saúde IPA**, 2017. (Encontro)
Escoliose em Gêmeos Monozigóticos e Epigenética: Uma experiência científica.
4. **Semana Acadêmica da Saúde IPA**, 2017. (Encontro)
Workshop Pilates Solo.
5. **VI Mostra de Pós-graduação IPA**, 2017. (Encontro)
O Estado de Acetilação Global de Histonas Não está Associado Com a Discórdância no Diagnóstico de Escoliose Idiopática em Gêmeos Monozigóticos Adultos.
6. **XI Salão de Iniciação Científica e Extensão | V Mostra da Pós-graduação | III Salão de Iniciação Tecnológica**, 2016. (Seminário)
Influências de marcadores Epigenéticos Periféricos em Gêmeos Monozigóticos com Escoliose Idiopática.
7. Apresentação de Poster / Painel no(a) **I Congresso Brasileiro ABRAFITO**, 2015. (Congresso)
Influências Fenotípicas e Genotípicas Nas Alterações Posturais de Gêmeos.
8. **I Encontro Regional ABRAFIN-RS - CIF: Da Ciência à Assistência**, 2015. (Encontro)
9. **II Jornada Gaúcha de Dor: Abordagem Interdisciplinar da Dor Neuropática**, 2015. (Seminário)
10. **A Formação de Uma Cultura Doadora de Órgãos e Tecidos**, 2014. (Encontro)
11. **Aplicação do Pilates na Prática Fisioterapêutica**, 2014. (Encontro)
12. **Avaliação e Diagnóstico Clínico Postural**, 2014. (Encontro)
13. **Desvendando os Projetos do Curso de Fisioterapia**, 2014. (Encontro)
14. Apresentação de Poster / Painel no(a) **I Congresso Sul Brasileiro de Reeducação da Postura e do Movimento**, 2014. (Congresso)
Influências Fenotípicas Sobre as Alterações Posturais de Gêmeos..
15. **O Que é Sistema BodyTalk?**, 2014. (Encontro)
16. **Partindo do Início: A Importância da Avaliação Fisioterapêutica**, 2014. (Encontro)
17. **SIPAT IPA/MEC: Segurança do Trânsito**, 2014. (Encontro)
18. **Semana Acadêmica da Saúde Do IPA**, 2014. (Encontro)
Influências Fenotípicas sobre a escoliose Idiopática em Gêmeos.
19. **Semana de Arte e Cultura - Colegiado das Ciências da Saúde**, 2014. (Encontro)
20. **Termografia Aplicada à Fisioterapia**, 2014. (Encontro)
21. **A Atuação do Fisioterapeuta no SUS: Experiências Transformadoras**, 2013. (Encontro)
22. **A Escoliose e o Tratamento Conservador**, 2013. (Encontro)
23. **Comemoração do Dia do Fisioterapeuta: Fisioterapia ontem, hoje e sempre!**, 2013. (Encontro)
24. **Disfunções da Articulação Temporomandibular - Uma Abordagem Interdisciplinar**, 2013. (Encontro)

25. FREEWALK: Sistema de EMG Wireless com Sensor Inercial e Câmera de Vídeo digital para análise de movimento, 2013. (Oficina)
26. Reabilitação Pulmonar na Criança Asmática no III Ciclo de Palestras do Curso de Fisioterapia, 2013. (Encontro)
27. SIPAT IPA/MEC: Valorizando a Qualidade de Vida dos Colaboradores, 2013. (Encontro)
28. Semana Acadêmica da Saúde, 2013. (Encontro)
29. Apresentação de Poster / Paineis no(a) VII Encontro de Reabilitação e IV Encontro dos EX-Residentes na Área de Ortopedia e Traumatologia, 2012. (Encontro)
Estabilização Central como Estudo Piloto na Lombar de Trabalhadores Hospitalares.
30. Workshop Saúde da Mulher, 2012. (Seminário)
31. II Jornada Científica da Clínica pública de Fisioterapia Esportiva, 2011. (Seminário)
32. Semana Acadêmica do Curso de Fisioterapia, 2011. (Seminário)

Organização de evento

1. NETO, JOÃO; DA ROSA, EDUARDO FREITAS
Convenção 50 anos da Fisioterapia: O Futuro da Profissão, 2019. (Concurso, Organização de evento)
2. NETO, JOÃO; ROSA, E. F.
I Jornada Gaúcha de Terapia Manual, 2019. (Congresso, Organização de evento)
3. NETO, J.P.; ROSA, E. F.
Congresso Brasileiro de Reabilitação Traumatológica e esportiva, 2018. (Congresso, Organização de evento)
4. PEREIRA NETO, J.; BRITO, C. I. B.; ROSA, E. F.
1ª Jornada Gaúcha de Coluna Vertebral, 2017. (Congresso, Organização de evento)
5. NETO, J.P.
Atuação do Fisioterapeuta No manejo da Dor, 2016. (Outro, Organização de evento)
6. PEREIRA NETO, J.; BRITO, C. I. B.; ROSA, E. F.; GONTJO, K.
II Congresso Sul Brasileiro de Reeducação da Postura e do Movimento, 2016. (Congresso, Organização de evento)
7. PEREIRA NETO, J.
I Congresso Sul Brasileiro de reeducação da Postura e do Movimento e do I Seminário sobre Dança., 2014. (Congresso, Organização de evento)

Bancas

Bancas

Participação em banca de comissões julgadoras

Outra

1. Comissão Científica da Convenção 50 anos da Fisioterapia: O Futuro da profissão., 2019
Instituto Golden Pilates e Fisioterapia
2. Comissão científica da I Jornada Gaúcha de Terapia Manual, 2019
Instituto Golden Pilates e Fisioterapia
3. Sessão Bolsista FAPERGS IC no XIII Salão de Iniciação Científica e de Extensão, VII Mostra da pós-graduação e V salão de Iniciação Tecnológica, 2018
Centro Universitário Metodista
4. Sessão Fisioterapia III no XIII Salão de Iniciação Científica e de Extensão, VII Mostra da pós-Graduação e V Salão de iniciação Tecnológica, 2018
Centro Universitário Metodista

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