UTILIZATION OF OMEGA-3 AND CONJUGATED FATTY ACIDS TO DEVELOP NEW ANTIOBESITY STRATEGIES

Thesis submitted to *Universidade Católica Portuguesa* to attain the degree of PhD in Food Science and Technology and Nutrition

Ana Sofia Nobre Salsinha

January 2024

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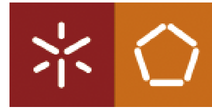
ESCOLA SUPERIOR DE BIOTECNOLOGIA
PORTO



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Ana Sofia Nobre Salsinha

Supervisor: **Professor Maria Manuela Estevez Pintado**

Co-supervisior: **Professor João Bettencourt Relvas and Doctor Luís Miguel Rodríguez-Alcalá**

January 2024

To all my family, especially my parents, sister, grandparents, and Pedro,

To my friends,

And to my furry friends.

You made it easier.

Acknowledgments must be addressed to Universidade Católica Portuguesa and FCT/MCT for the financial support for the CBQF research unit (project UIDB/50016/2020), through national funds and where applicable co-financed by the FEDER, within the PT2020 Partnership Agreement. Ana Sofia Salsinha is also grateful for the financial support from FCT through the fellowship Grants SFRH/BD/136857/2018 and COVID/BD/153213/2023. The authors acknowledge the support of i3S Scientific Platform Advanced Light Microscopy, a member of the national infrastructure PPBI-Portuguese Platform of BioImaging (supported by POCI-01-0145-FEDER-022122) for the support in the work described in Chapter 2.

ABSTRACT

Abstract

According to the World Health Organization's latest data, overweight and obesity prevalence is increasing mostly in urban settings. Diet, particularly the rise of the Western diet, characterized by high-sugar and high-saturated fat consumption, has been pointed out as one important contributor to this trend. Western diet has been shown to detrimentally affect brain function by inducing chronic low-grade inflammation. This neuroinflammation is in part mediated by microglia, so the first part of this study intended to determine how different bioactive fatty acids, namely omega-3 and conjugated fatty acids isomers (conjugated linoleic (CLA) and linolenic acid (CLNA) isomers) modulate microglia activity and if they can suppress the negative effect of obesogenic nutrients (palmitic acid and fructose), intended to mimic in this study the Western diet main ingredients. Importantly, short-time exposure to omega-3, CLA, and CLNA was demonstrated, for the first time, to be sufficient to abolish NF-κB pathway activation, reactive oxygen species production, and the activation of LynSrc in a human microglia cell line, suggesting a potential for neuroprotection. Interestingly, the NF-κB pathway activation was demonstrated for the first time for CLA and CLNA, to be mediated by the GPR120 receptor.

Nevertheless, despite this promising potential one of the most straightforward routes of administration is through oral supplementation and the use of enriched oils has been used as a strategy to achieve therapeutical doses of the mentioned bioactive fatty acids. So, to explore the antiobesity potential of omega-3 and conjugated fatty acids, through oral supplementation, bioaccessibility and bioavailability are important parameters to consider. So, in the second stage of this work, the bioaccessibility of these fatty acids in different matrixes was determined using the INFOGEST static *in vitro* protocol of gastrointestinal tract simulation. After digestion the recovery index for most of the major bioactive fatty acids – punicic acid, the omega-3 EPA, DPA, and DHA fatty acids, and the CLA isomers, rumenic acid, and C18:2 *t*10*c*12 - was very low between 1.90-17.03%, demonstrating the negative effect of gastrointestinal digestion upon these fatty acids in this type of matrix. Besides, using a Caco-2/HT29-MTX co-culture it was demonstrated that a significative percentage of these fatty acids is incorporated in the intestinal cells (8.44-26.71%), which may affect their permeability performance and explain the null apparent permeability values obtained. Moreover, due to this observation, it was demonstrated that in fact, most of them remain in the non-bioaccessible/ colon-available fraction. Despite the drawbacks of this observation in terms of brain bioavailability, the potential of gut microbiota modulation was considered.

Western diet has been considered one important player in the gut microbiota alterations in industrialized societies and a causal role between gut microbiota changes and obesity has been demonstrated. So, the third stage of this work intended to assess the effects of omega-3, CLA, and punicic acid on human gut microbiota modulation using *in vitro* fecal fermentations. Indeed, all the samples demonstrated a positive impact, demonstrated by positive relative difference indexes concerning the negative control (RD>0), in the growth of key bacteria that have been associated with a positive effect on obesity treatment namely *Akkermansia* spp.,

Bifidobacterium spp., and *Roseburia* spp. In addition, most samples (Fish oil, Omega-3, CLA, and CLNA capsules content) were able to increase butyrate concentration, a known anti-inflammatory short-chain fatty acid. Moreover, demonstrating the potential of gut-brain axis modulation and its importance in brain function restoration, the sources of omega-3 (Fish oil and Omega-3 capsules content) were able to increase the concentration of GABA and several amino acids that are known precursors of neurotransmitters. Importantly, this first gut microbiota study demonstrated the potential role of these fatty acids in gut microbiota modulation and a possible prediction of their preventive and therapeutical role in obesity. So, a second study intended to assess if the selected oils enriched in omega-3 (Fish oil) and punicic acid (Pomegranate oil) could restore the gut microbiota changes associated with the Western diet that are frequently linked to conditions like obesity and other metabolic diseases, such as type 2 diabetes mellitus. So, Fish oil (as the selected source of omega-3), Pomegranate oil (as a source of punicic acid), and a mixture of both oils (1:1 w/w) were subjected to *in vitro* fermentations using cecal samples from rats subjected either to a control standard chow diet or to a western diet (high-fat and high-sugar diet). Fish oil+Pomegranate oil was able to increase α-diversity, the relative abundance of the Firmicutes and Bacteroidetes phylum as well as *Akkermansia* and *Blautia*, which were affected by the western diet consumption. All samples were able to increase butyrate and acetate concentration in the western diet group. Once again, demonstrating the great potential of these samples to modulate the gut-brain axis, especially in a metabolic syndrome context, tyrosine, a precursor for dopamine and norepinephrine, and GABA concentrations increase in the Fish oil+Pomegranate oil sample from the western diet group.

Finally, to tentatively uncover some of the molecular mechanisms behind these bioactive fatty acids' effects on gut permeability and adipogenesis, the role of CB1 in both these processes and GPR120 in adipogenesis was studied. Interestingly, it was demonstrated that CB1 may have a role in intestinal permeability and different bioactive polyunsaturated fatty acids may respond differently to this receptor. Moreover, there seems to be an important role of both GPR120 and CB1 (studied separately) in Fish and Pomegranate oil inhibitory adipogenesis effects in the studied cell model (3T3-L1 adipocytes).

In this thesis, it was demonstrated, for the first time, the great potential of using bioactive fatty acids, namely omega-3 and punicic acid to promote a systemic effect in obesity prevention and therapy. Moreover, it was demonstrated a synergistic effect of these bioactive fatty acids, by using a mixture of Fish and Pomegranate oil, to restore the gut microbiota changes associated with western diet consumption. Despite these promising results, further studies using as a first approach animal models (such as rodent models) and afterward a clinical trial using human patients, are required to fully elucidate the bioavailability of such bioactive fatty acids in a dietary context and consequently, their possible beneficial role in brain inflammatory processes either directly or via gut-brain axis modulation.

Keywords: Obesity, Western Diet; Omega-3; Punicic acid; CLA

RESUMO

Resumo

De acordo com os últimos dados da Organização Mundial de Saúde, a prevalência do excesso de peso e da obesidade está a aumentar sobretudo nos meios urbanos. A dieta, em particular a dieta ocidental, caracterizada por um consumo elevado de açúcar e de gordura saturada, tem sido apontada como um importante fator que contribui para esta tendência. Foi demonstrado que a dieta ocidental afeta negativamente a função cerebral ao induzir uma inflamação crónica. Esta neuro inflamação é, em parte, mediada pela microglia. Assim, a primeira parte deste estudo pretende determinar de que forma diferentes ácidos gordos bioativos, nomeadamente ómega-3 e isómeros de ácidos gordos conjugados (isómeros de ácido linoleico conjugado (CLA) e ácido linolénico conjugado (CLNA)) modulam a atividade da microglia e podem suprimir o efeito negativo de nutrientes obesogénicos (ácido palmítico e frutose) destinados a representar neste estudo os principais ingredientes da dieta ocidental. É importante notar que, pela primeira vez, foi demonstrado que a exposição de curta duração a ómega-3, CLA e CLNA, na linha celular da microglia em estudo, é suficiente para abolir a ativação da via do NF-κB, a produção de espécies reativas de oxigénio e a ativação de LynSrc, sugerindo um potencial de neuroprotecção. Neste estudo, a ativação da via do NF-κB foi demonstrada para o CLA e o CLNA, tendo esta sido demonstrada pela primeira vez como sendo mediada pelo recetor GPR120.

Considerando este potencial promissor, uma das vias de administração mais simples destes ácidos gordos é a suplementação oral. Assim, o uso de óleos enriquecidos tem sido utilizado como estratégia para obter doses terapêuticas dos referidos ácidos gordos bioativos. De forma a explorar o potencial anti-obesidade do ómega-3 e dos ácidos gordos conjugados, através da suplementação oral, a bioacessibilidade e a biodisponibilidade são parâmetros importantes a considerar. Deste modo, na segunda fase deste trabalho, foi determinada a bioacessibilidade destes ácidos gordos em diferentes matrizes, recorrendo para tal ao protocolo estático e *in vitro* INFOGEST, que visa simular o trato gastrointestinal. Após a digestão, o índice de recuperação para a maioria dos principais ácidos gordos bioativos é muito baixo, entre 1,90-17,03%, demonstrando o efeito negativo da digestão gastrointestinal nestes ácidos gordos quando incorporados neste tipo de matriz. Além disso, utilizando uma co-cultura Caco-2/HT29-MTX, foi demonstrado que existe uma incorporação significativa destes ácidos gordos nas células intestinais (8,44-26,71%), o que pode afetar o seu desempenho em termos de permeabilidade. Além disso, devido a esta observação, foi demonstrado que, de facto, a maioria deles permanece na fração não bioacessível/disponível no cólon. Apesar dos inconvenientes desta observação visando o sistema nervoso central como o alvo terapêutico, foi considerado o potencial destes ácidos gordos na modulação da microbiota intestinal.

A dieta ocidental tem sido considerada um fator importante nas alterações da microbiota intestinal nas sociedades industrializadas e nos últimos anos tem sido demonstrado um papel causal entre as alterações da microbiota intestinal e a obesidade. Assim, a terceira fase deste trabalho pretendeu avaliar os efeitos do ómega-3, do CLA e do ácido punícico na modulação da

microbiota intestinal humana recorrendo para tal, a fermentações fecais *in vitro*. Todas as amostras demonstraram um impacto positivo no crescimento de bactérias-chave, demonstrado por um valor positivo de diferença relativa, calculada em relação ao controlo negativo. Estas bactérias, nomeadamente *Akkermansia* spp., *Bifidobacterium* spp. e *Roseburia* spp., têm sido associadas a um efeito positivo no tratamento da obesidade. Além disso, a maioria das amostras (Óleo de peixe e o conteúdo das cápsulas de ómega-3, CLA e CLNA) foi capaz de aumentar a concentração de butirato, um ácido gordo de cadeia curta com um potencial anti-inflamatório descrito. Além disso, demonstrando o potencial de modulação do eixo intestino-cérebro e a sua importância na recuperação da função cerebral, que se encontra comprometida na obesidade, as fontes de ómega-3 foram capazes de aumentar a concentração de GABA e de vários aminoácidos que são conhecidos precursores de neurotransmissores. É importante salientar que este primeiro estudo da microbiota intestinal demonstrou o papel potencial destes ácidos gordos na modulação da microbiota intestinal e uma possível previsão do seu papel preventivo e terapêutico na obesidade. Assim, um estudo subsequente pretendeu avaliar se os óleos enriquecidos em ómega-3 e ácido punícico, os ácidos gordos bioativos selecionados tendo em conta os resultados anteriores, poderiam restaurar as alterações da microbiota intestinal que são induzidas pelo consumo de uma dieta ocidental. Estas alterações estão frequentemente associadas à obesidade e a outras doenças metabólicas, como a diabetes mellitus tipo 2. Assim, o Óleo de peixe (como fonte selecionada de ómega-3), o Óleo de romã (como fonte de ácido punícico) e uma mistura de ambos os óleos (1:1 m/m) foram submetidos a fermentações *in vitro* utilizando amostras do ceco de ratos submetidos a uma dieta padrão de controlo e a uma dieta ocidental (dieta rica em gordura e açúcar). A combinação do Óleo de peixe e Óleo de romã foi capaz de aumentar a α-diversidade, a abundância relativa dos filos Firmicutes e Bacteroidetes, bem como de *Akkermansia* e *Blautia*, que tinham sido afetados pelo consumo da dieta ocidental. Todas as amostras foram capazes de aumentar a concentração de butirato e acetato no grupo da dieta ocidental. Mais uma vez, demonstrando o grande potencial destas amostras para modular o eixo intestino-cérebro, especialmente num contexto de síndrome metabólica, as concentrações de tirosina, um precursor da dopamina e da norepinefrina, e de GABA aumentaram na amostra de Óleo de peixe+Óleo de romã do grupo de dieta ocidental.

Finalmente, de forma a elucidar alguns dos mecanismos moleculares envolvidos nos efeitos destes ácidos gordos na permeabilidade intestinal e na adipogénese, foi estudado o papel do CB1 em ambos os processos e do GPR120 no último. Curiosamente, foi demonstrado que o CB1 pode ter um papel importante na modulação da permeabilidade intestinal e ainda, que diferentes ácidos gordos polinsaturados podem responder de forma diferente a este receptor. Além disso, parece haver um papel importante tanto do GPR120 como do CB1 (estudados separadamente) nos efeitos inibitórios exibidos pelo Óleo de peixe e de Romã na adipogénese do modelo celular em estudo (adipócitos 3T3-L1).

Nesta tese foi demonstrado, pela primeira vez, o grande potencial da utilização de ácidos gordos bioativos, nomeadamente ómega-3 e ácido punícico, para promover um efeito sistémico na prevenção e tratamento da obesidade. Além disso, foi demonstrado um efeito sinérgico destes

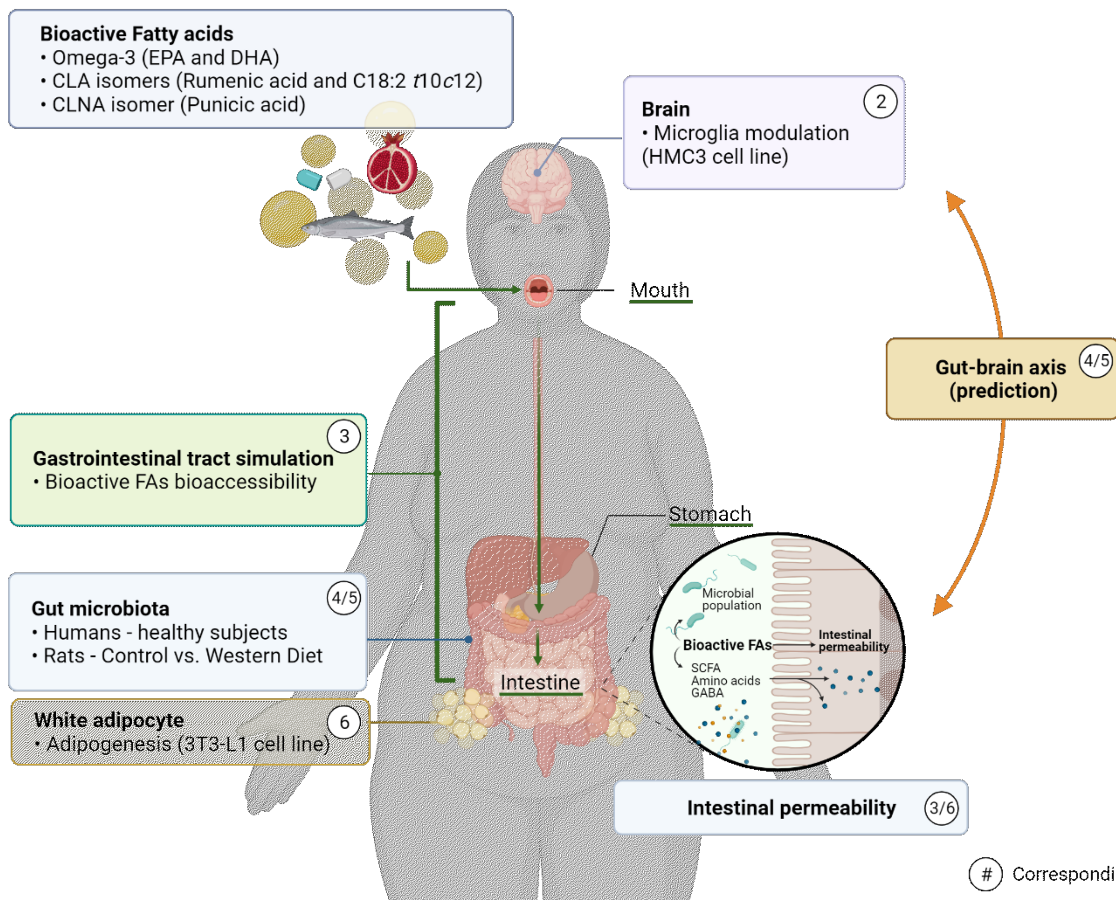
ácidos gordos bioativos, utilizando uma mistura de óleo de peixe e de romã, para restaurar as alterações da microbiota intestinal associadas ao consumo da dieta ocidental. Apesar destes resultados promissores são necessários mais estudos utilizando como primeira abordagem modelos animais (tais como roedores) e posteriormente um ensaio clínico, para elucidar completamente a biodisponibilidade de tais ácidos gordos bioativos num contexto de uma dieta e, consequentemente, o seu possível papel benéfico nos processos inflamatórios cerebrais, seja diretamente ou indiretamente através da modulação do eixo intestino-cérebro.

Palavras-chave: Obesidade; Dieta ocidental; Ómega-3; Ácido punícico; isómeros CLA

Graphical Abstract

Utilization of Omega-3 and Conjugated fatty acids to develop new antiobesity strategies

Can polyunsaturated fatty acids be used to design systemic antiobesity strategies, targeting adipose tissue, brain and gut microbiota?



ACKNOWLEDGEMENTS

Acknowledgments

No journey and no accomplishment are possible and worth celebrating alone.

First, I would like to thank my supervisor Professor Manuela Pintado for giving me this opportunity, for sharing with me all her knowledge, and for supporting my work by being a great leader of an amazing group and an incredible role model to follow. Second, I would like to thank my co-supervisor Doctor Luís Rodríguez-Alcalá for introducing me to the bioactive lipids field demonstrating their great potential, and for allowing me this opportunity. A third appreciation must be made to my co-supervisor Professor João Relvas for receiving me in his *Glial cell biology* group in i3S, and for sharing with me his enthusiasm for science and his great scientific knowledge in the neurobiology field.

An important appreciation to my colleagues and friends *Almoçadeiras* - Ana Lopes, Ana Vilas-Boas, Andreia Fernandes, Carolina Cassoni, Cindy Dias, Diana Castro, Diva Santos, Helena Rodrigues, Nádia Silva, Marta Mendes, Marta Cunha, Rosa Moreira, Sara Cunha, Sara Marçal and Sofia Sousa – for their kindness and constant encouragement throughout these past 6 years. An important recognition to Cindy, Helena, Sara Cunha, and Sara Marçal for their friendship, emotional support, and contribution to my work during these past 4 years. A special thank you to several members of my *Bioactives and Bioproducts* research group namely Tânia Ribeiro, Adma Melo, Marta Coelho, André Cima, Soudabeh Ghalamara, Glenise Voss, Manuela Machado and Ezequiel Coscueta, and the remaining team, for their contribution to my work, help and for making every workday enjoyable, even when everything seems to fail.

I would like to thank the *Glial cell biology* group for receiving me and helping me during my path in i3S. A special thanks to Artur Rodrigues, for all the help and friendship. And to Renato Socodato, for all the support, knowledge, and advice. For Raquel Silva, Joana Moreira, Pedro Melo, Tiago Almeida, Camila Portugal, and the remaining team for their help, availability, and kindness.

A kind appreciation to Doctor Sofia Viana and Professor Flávio Reis from the *Institute of Clinical and Biomedical Research* from the University of Coimbra, for their help in part of this work, and their availability and support. A special thanks to Pedro Ribeiro for his assistance and kindness.

For all my friends who supported me, in so many ways, during the long path that I've been walking on until today and that started when I decided to study Cellular and Molecular Biology, a special recognition. A huge thank you to Mónica Tomé and Catarina Barbosa, my first Porto family. Everything seems simpler if shared with a friend.

But most importantly to my family: my parents, sister, grandparents, and Pedro for always being there, for their love, recognition, and motivation. And to all my remaining family, my uncles, cousins, and my brother-in-law for being an amazing part of my life. Finally, to my furry friends because life is always better if shared with a pet.

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List of abbreviations

	Abbreviation	Definition
A	AA	Arachidonic acid
	ACC	acetyl-CoA Carboxylase
	ACTH	Adrenocorticotropin
	AEA	N-arachidonoylethanolamine
	AgRP	Agouti-related Protein
	2-AG	2-Arachidonoylglycerol
	AI	Atherogenic Index
	α-MSH	α-melanocyte Stimulating Hormone
	ALA	α-linolenic acid
	AMPK	AMP-activated protein kinase
	ARC	Arcuate nucleus
B	BA	Bile Acid
	BMI	Body Mass Index
	BBB	Blood-brain Barrier
	BAT	Brown Adipose Tissue
	BI	Bioaccessibility Index
C	CART	Cocaine and Amphetamine-Regulated Transcript
	CB (1 and 2)	Cannabinoid receptor (1 and 2)
	CBD	Cannabidiol
	CD	Control Diet
	CVD	Cardiovascular Disease
	CFA	Conjugated Fatty Acid
	CHOP	Pro-apoptotic Proteins
	CLA	Conjugated Linoleic Acid
	CLNA	Conjugated Linolenic Acid
	CMA	Chaperone-mediated Autophagy
	CNS	Central Nervous System
	CNTF	Neurocytokine Ciliary Neurotrophic Factor
	CPT1	Carnitine Palmitoyltransferase-1
D	DHA	Docosahexaenoic Acid
	DPA	Docosapentaenoic Acid
E	EMA	European Medicines Agency
	ENS	Enteric Nervous System
	ER	Endoplasmic Reticulum
	EPA	Eicosapentaenoic Acid
F	FA(s)	Fatty Acid(s)
	FAMEs	Fatty Acids Methyl Esters
	FAO	Food And Agriculture Organization of The United Nations
	FAS	Fatty Acid Synthase
	FATPs	Fatty Acid Transport Proteins
	FDA	Food And Drug Administration
	FFAs	Free Fatty Acids
	FRET	Fluorescence Resonance Energy Transfer
G	GABA	Gamma (γ)-aminobutyric Acid
	GHSR1a	Growth Hormone Secretagogue Receptor Type 1a
	GIT	Gastrointestinal Tract

	GPR	G Protein Receptor
H	HFD	High Fat Diet
	HH	Hypocholesterolemic/Hypercholesterolemic Ratio
I	IBD	Inflammatory Bowel Disease
	IBS	Irritable Bowel Syndrome
	Icv	Intracerebroventricular
	IR	Insulin Receptor
	IRS	Insulin Receptor Substrate
L	LA	Linoleic Acid
	LAB	Lactic acid bacteria
	LDL	Low-density Lipoprotein
	LNA	Linolenic Acid
	LPS	Lipopolysaccharide
	LCFA-CoA	Long-chain Fatty Acid-Coenzyme A
	LC-FA	Long-chain Fatty Acid
	LC-PUFA	Long-chain Polyunsaturated Fatty Acid
	LC-SFA	Long-chain Saturated Fatty Acid
	LC-TG	Long-chain Triglyceride
M	MDC	Malonyl-CoA Decarboxylase
	MCR	Melanocortin Receptor
	MCFA	Medium-chain Fatty Acid
	MCSFA	Medium-chain Saturated Fatty acid
	MCTG	Medium-chain Triglyceride
	MRI	Magnetic resonance Imaging
	MUFA	Monounsaturated Fatty Acid
	Myd88	Myeloid Differentiation Primary Response 88
N	NO	Nitric Oxide
	NPY	Neuropeptide Y
	Nrf2	Nuclear Factor Erythroid 2-related factor 2
O	OECD	Organisation For Economic Co-Operation and Development
	OEA	Oleoylethanolamide
	2-OG	Oleoylglycerol
P	PA	Palmitic acid
	PCA	Principal Component Analysis
	PCoA	Principal Coordinates Analysis
	PC2	Pro-convertase 2
	2-PG	Palmitoyl-glycerol
	POMC	Proopiomelanocortin
	PEA	Palmitoylethanolamide
	PPAR	Peroxisome Proliferator-activated Receptor
	PUA	Punicic Acid
	PUFA	Polyunsaturated Fatty Acid
R	RA	Rumenic Acid
	RI	Recovery index
	ROS	Reactive Oxygen Species
	RPS	Reduced Physiological Salt solution
	RT-PCR	Real-time Polymerase Chain Reaction
S	SFAs	Saturated Fatty Acids
T	TAB1	TGF-β activated kinase binding protein 1
	TAG	Triacylglycerol

	TAK1	TGF-β activated kinase 1
	TFAs	Trans Fatty Acids
	THC	Δ^9-Tetrahydrocannabinol
	TI	Thrombogenic Index
	TLR	Toll-like Receptors
	T2DM	Type 2 Diabetes Mellitus
	TG	Triglyceride
	Treg	T regulatory cells
U	UPR	Unfolded Protein Response
	USA	United States of America
W	WAT	White Adipose Tissue
	WHO	World Health Organization
	WD	Western Diet
	WT	Wild type

SCIENTIFIC OUTPUTS

Scientific outputs

A list of all the publications produced during the different tasks of this work is presented in this section.

Published works

Papers in international peer-reviewed journals

- Salsinha, A. S., Rodríguez-Alcalá, L. M., Relvas, J. B., Pintado, M. E. (2021). Fatty acids role on obesity induced hypothalamus inflammation: From problem to solution – A review. Trends in Food Science & Technology, 112, 592-607. https://doi.org/10.1016/j.tifs.2021.03.042
- Salsinha, A.S., Socodato, R., Rodrigues, A., Vale-Silva, R., Relvas, J.B., Pintado, M., Rodriguez-Alcalá, L.M. (2023). Potential of omega-3 and conjugated fatty acids to control microglia inflammatory imbalance elicited by obesogenic nutrients. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids, 1868(7):159331. https://doi.org/10.1016/j.bbalip.2023.159331
- Salsinha, A.S., Cunha, S., Machado, M., Rodriguez-Alcalá, L.M., Relvas, J.B., Pintado, M. (2023). Assessment of the bioaccessibility and bioavailability prediction of omega-3 and conjugated fatty acids by in vitro standardized digestion model (INFOGEST) and cell model. Food Bioscience, 53, 102635. https://doi.org/10.1016/j.fbio.2023.102635

Book Editions

- Pintado, M. (ed.), Machado, M. (ed.), Gomes, A. (ed.), Salsinha, A. (ed.) & Rodríguez-Alcalá, L. M. (ed.). 2023. “Bioactive lipids” Cambridge: Academic Press. 426 p. ISBN 978-0-12-824043-4

Book chapters

- Salsinha, A. S., Rodríguez-Alcalá, L. M., Pimentel, L. L. & Pintado, M. 2023. “Role of bioactive lipids in obesity” in “Bioactive lipids”. Pintado, M., Machado, M., Gomes, A. M., Salsinha, A. S. & RodríguezAlcalá, L. M. (eds.). Cambridge: Academic Press, p. 133-167 35 p. https://doi.org/10.1016/B978-0-12-824043-4.00012-9
- Salsinha, A. S., Machado, M., Rodríguez-Alcalá, L. M., Gomes, A. M. & Pintado, M. 2023. ”Bioactive lipids: chemistry, biochemistry, and biological properties” in “Bioactive lipids”. Pintado, M., Machado, M., Gomes, A. M., Salsinha, A. S. & Rodríguez-Alcalá, L. M. (eds.). Cambridge: Academic Press, p. 1-35 35 p. https://doi.org/10.1016/B978-0-12-824043-4.00014-2
- Salsinha, A. S. & Pintado, M. 2023. “Impact of bioactive lipids on gut microbiota in Bioactive lipids” in “Bioactive Lipids”. Pintado, M., Machado, M., Gomes, A. M., Salsinha, A. S. & Rodríguez-Alcalá, L. M. (eds.). Cambridge: Academic Press, p. 191- 207 16 p. https://doi.org/10.1016/B978-0-12-824043-4.00008-7

- Salsinha, A. S., Socodato, R., Relvas, J. B. & Pintado, M., 2023. “The pro- and antiinflammatory activity of fatty acids” in “Bioactive lipids”. Pintado, M., Machado, M., Gomes, A. M., Salsinha, A. S. & Rodríguez-Alcalá, L. M. (eds.). Cambridge: Academic Press, p. 51-75 18 p https://doi.org/10.1016/B978-0-12-824043-4.00002-6

Poster communications

- Salsinha A.S., Rodrigues A., Socodato R., Relvas J.B., Rodríguez-Alcalá L.M., Pintado M.E. (2019, October). Study of inflammation mediated by lipids in a Microglia Cell Model to deepen into brain dysregulation by obesity. 17th Euro Fed Lipid Congress and Expo, Sevilha, Espanha
- Salsinha, A. S., Rodrigues, A., Socodato, R., Relvas, J.B., Rodríguez-Alcalá, L. M., Pintado, M. E. (2020, November). Study of inflammation mediated by fatty acids in a microglia cell model – the obesity perspective. Online posters session in Encontro Ciência 2020, Lisbon, Portugal
- Salsinha, A. S., Rodrigues, A., Socodato, R., Relvas, J.B., Rodríguez-Alcalá, L. M., Pintado, M. E. (2021, June). The role of saturated and polyunsaturated fatty acids in microglia modulation. Online posters session in Encontro Ciência 2021, Lisbon, Portugal
- Salsinha, A.S., Socodato, R., Rodrigues, A., Rodríguez-Alcalá, L.M., Relvas, J.B., Pintado, M.E. (2021, October). CLA and CLNA ameliorate neuroinflammation and cellular oxidation related with western diets. Online poster session and flash poster presentation at 18th Euro Fed Lipids Congress, Leipzig, Germany
- Salsinha, A.S., Cima, A., Araújo-Rodrigues, H., Dias, C., Rodríguez-Alcalá, L.M., Relvas, J.B., Pintado, M. (2023, July). The Use of a Human In Vitro Fermentation Model to Study the Role of Omega-3 and Conjugated Fatty Acids on Gut Microbiota Modulation. Poster presentation session and flash poster communication in SymbNET PhD Summer School on Host-microbe symbiosis, Lisbon, Portugal
- Salsinha, A.S., Cima, A., Araújo-Rodrigues, H., Viana, S., Reis, F., Coscueta, E.R., Rodríguez-Alcalá, L.M., Relvas, J.B., Pintado, M. (2023, October). Assessing the effect of omega-3 and punicic acid in gut microbiota alterations induced by a Western diet using an in vitro fecal fermentation model. Poster presentation in 2nd Microbiome PT Summit, Lisbon, Portugal
- Salsinha, A.S., Cima, A., Araújo-Rodrigues, H., Viana, S., Reis, F., Coscueta, E.R., Rodríguez-Alcalá, L.M., Relvas, J.B., Pintado, M. (2024, January). The use of cecal samples from Wistar rats in *in vitro* fermentation to determine the effect of Fish and Pomegranate oil in gut microbiota alterations induced by a Western diet. Poster presentation at V Congresso da SCPAL, Coimbra, Portugal.

Oral communications

- Salsinha, A.S., Cunha, S.A., Machado, M., Relvas J.B., Rodríguez-Alcalá, L.M., Pintado M. (2022, December). Bioaccessibility determination of omega-3 and conjugated linolenic

acid using an *in vitro* standardized digestion model (INFOGEST) by GC-FID. 12º Encontro Nacional de Cromatografia, Aveiro, Portugal.

- Salsinha, A.S., Relvas J.B., Rodríguez-Alcalá, L.M., Pintado, M. (2023, February). Bioactive lipids role on microbiota modulation' (gut in mouse feces). CBQF day: Community microbiomes & microbiome communities. Internal presentations from CBQF, Universidade Católica Portuguesa. Porto, Portugal

Abstract submission (awaiting decision)

- Salsinha, A.S., Araújo-Rodrigues, H., Cima, A., Machado, M., Rodríguez-Alcalá, L.M., Relvas, J.B., Pintado, M. (2024, April). Unravelling the full therapeutical potential of polyunsaturated fatty acids: a complete study of omega-3 and conjugated fatty acids bioaccessibility, bioavailability prediction and impact in gut microbiota. 8th International Conference on FOOD DIGESTION, Porto, Portugal.

Unpublished works

Papers submitted or awaiting submission to international peer-reviewed journals.

- Salsinha, A.S., Araújo-Rodrigues, H., Dias, C., Cima, A., Rodríguez-Alcalá, Relvas, J.B., Pintado, M. Omega-3 and conjugated fatty acids impact on human microbiota modulation using an in vitro fecal fermentation model. Awaiting submission.
- Salsinha, A.S., Cima, A., Araújo-Rodrigues, H., Viana, S., Reis, F., Coscueta, E.R., Rodríguez-Alcalá, L.M., Relvas, J.B., Pintado, M. The use of an in vitro fecal fermentation model to uncover the beneficial role of omega-3 and punicic acid in gut microbiota alterations induced by a Western diet. Submitted to *Food & Function* Journal
- Salsinha, A.S., Machado, M., Rodrígez-Alcalá, L.M., Relvas, J.B., Pintado, M. Uncovering the role of CB1 and GPR120 in the effect of Fish oil and Pomegranate oil in adipogenesis and Caco-2 permeability. Awaiting submission.

STRUCTURE AND THESIS PLAN

Structure and Thesis Plan

This thesis is divided into 7 chapters, **Chapter 1** corresponds to the Introduction, where the background and the state-of-the-art on the main themes are provided (Chapter 1.1 and 1.2, respectively).

The following chapters are divided into 4 core themes:

1) This theme comprises the assessment of the omega-3 and conjugated fatty acids (CLA and CLNA isomers) in preventing the effects of obesogenic ingredients in a microglia cell line, through GPR120 (**Chapter 2**).
2) Studying the bioaccessibility of bioactive fatty acids (omega-3, CLA, and CLNA isomers) in different enriched oils matrixes (**Chapter 3**).
3) Impact of bioactive fatty acids (omega-3, CLA, and CLNA isomers) in gut microbiota and gut-brain axis modulation prediction (**Chapters 4 and 5**).
4) Brief study about the role of GPR120 and CB1 receptors in adipogenesis and intestinal permeability (**Chapter 6**).

And finally, **Chapter 7** (7.1 and 7.2, respectively) corresponds to the conclusions and future work perspectives.

CHAPTER 1

Introduction

This chapter aims to provide the background on the main themes considered in this thesis and the state-of-the-art on the effect of different fatty acids in obesity development and associated alterations; as well as explore the potential of using polyunsaturated fatty acids as an anti-obesity therapeutical approach.

This chapter was partially published as:

Salsinha, A. S., Rodríguez-Alcalá, L. M., Relvas, J. B., Pintado, M. E. (2021). Fatty acids role on obesity-induced hypothalamus inflammation: From problem to solution – A review. Trends in Food Science & Technology, 112, 592-607. https://doi.org/10.1016/j.tifs.2021.03.042

Salsinha, A. S., Rodríguez-Alcalá, L. M., Pimentel, L. L. & Pintado, M. 2023. "Role of bioactive lipids in obesity" in "Bioactive lipids". Pintado, M., Machado, M., Gomes, A. M., Salsinha, A. S. & Rodríguez-Alcalá, L. M. (eds.). Cambridge: Academic Press, p. 133-167 35 p. https://doi.org/10.1016/B978-0-12-824043-4.00012-9

Salsinha, A. S. & Pintado, M. 2023. "Impact of bioactive lipids on gut microbiota in Bioactive lipids" in "Bioactive Lipids". Pintado, M., Machado, M., Gomes, A. M., Salsinha, A. S. & Rodríguez-Alcalá, L. M. (eds.). Cambridge: Academic Press, p. 191- 207 16 p. https://doi.org/10.1016/B978-0-12-824043-4.00008-7

Chapter 1.1

Background

1. A Worldwide pandemic: the burden of obesity

The World Health Organization (WHO) (1) defines overweight and obesity *"as abnormal or excessive fat accumulation that may impair health"*, which can be triggered by high and imbalanced food intake plus a sedentary lifestyle. Although both are preventable, WHO latest data reported that in 2016 more than 1.9 billion adults (≥18 yrs.; 39% of the world's population) were overweight and over 650 million were obese (13% of the world's population). Recent data about this worldwide pandemic are concerning; according to WHO both obesity and overweight are now on the rise in low- and middle-income countries, particularly in urban settings. Nevertheless, a recent study assessing the trajectory of obesity in children and adolescents has shown that the rising trends in children's and adolescents' body mass index (BMI) have plateaued, since 2000, in many high-income countries (but remaining at high levels). The authors have reported, though, that from 1975 to 2016 children's and adolescents' age-standardized mean BMI increased globally (0.32 kg/m^2 for girls and 0.40 kg/m^2 for boys per decade) (2). Furthermore, regarding children under the age of 5, 38 million were reported to be overweight or obese in 2019, while from ages 5 to 19 the overweight/obesity prevalence reached 340 million in 2016 (1). According to the Organisation for Economic Co-operation and Development (OECD) latest projections, it is expected that obesity rates continue to increase until at least 2030 (3). Moreover, obesity and overweight are recognized as the second metabolic factor, after blood pressure alterations, involved in the development of non-communicable diseases, such as cardiovascular disease (CVD), cancer, chronic respiratory diseases, and diabetes. According to the most recent data, CVD accounts for most non-communicable disease deaths – 17.9 million people annually (representing 31% of all global deaths) (4). Besides, the associated health deficits have high socioeconomic costs: in Europe, CVD is the leading cause of mortality (3.9 million deaths/year) with an estimated burden of €210 billion/year (5). Diabetes is another comorbidity known to be associated with obesity, and it is estimated to affect 60 million people in the European Region. Moreover, diabetes was directly responsible, in 2016, for an estimated 1.5 million deaths and 48% of all deaths due to diabetes occurred before the age of 70 years (6). According to recent projections, the diabetes absolute global economic burden will increase to US$ 2.1 trillion by 2030 (7).

Importantly, obesity is a multifactorial disease, several factors have been attributed to this epidemic, and diet, specifically the Western diet (WD), is pointed out as one of the major contributors to the growing rates of obesity in several countries, including in the United States of America (USA) (8).

2. Western diet: an important player

WD, although not uniform globally, is in general terms characterized by a decrease in the consumption of fruits and vegetables along with an increase in the availability of animal-origin and energy-rich foods, as well as in the level of food processing (9). More specifically, this type of diet is associated with high-glycemic/high-insulinemic carbohydrates, including refined cereals, corn, potatoes, and sugars (particularly sucrose and fructose), dairy products, and considerable amounts of proteins along with a low fiber intake. Moreover, this kind of diet is also characterized by high levels of fat, namely saturated fatty acids (SFAs) and trans fatty acids (TFAs). In contrast, low levels of omega-3 FAs and other long-chain polyunsaturated fatty acids (LC-PUFAs) are present (8,10,11).

According to the Food and Agriculture Organization of the United Nations (FAO), dietary fats include all fats and oils that are edible and produced from plants or animals. Dietary fats consist mainly of triglycerides, which can be split into glycerol and fatty acids (FAs). FAs constitute the main components of these lipids and are required as a source of energy, and for metabolism and structure (12). FAs present a carbon aliphatic chain and a single carboxyl group and are classified as saturated and unsaturated. SFAs contain only single carbon–carbon bonds in the aliphatic chain. They are important as sources of energy and as components of cell membranes. These FAs are not considered essential since the human body can synthesize them. Unsaturated fatty acids (one or more carbon–carbon double bonds), are subdivided into monounsaturated (MUFA) - one carbon–carbon double bond in the aliphatic chain- and polyunsaturated fatty acids (PUFAs) – two or more carbon-carbon double bond in the aliphatic chain (13). The term 'trans-fat' typically refers to edible fats that contain TFAs. These FAs have at least one double bond in the *trans* configuration, as opposed to the more common *cis* configuration (14). Although a high-fat content can lead to obesity, especially in diets rich in SFAs, as is going to be thoroughly discussed in this thesis, the TFAs' effect on obesity development is still controversial. Importantly, several studies have found positive correlations between TFA consumption and obesity and overweight (15–17). On the contrary, effects on body fat mass reduction by TFAs such as conjugated linoleic acid (CLA) isomers were found in several studies (as reviewed by Pipoyan et al. (18)). Similarly, other TFA, vaccenic acid (C18:1 *t*11), a precursor of the C18:2 *c*9*t*11 CLA isomer Rumenic acid (RA), was found to alleviate the features of metabolic syndrome in obese and insulin-resistant JCR: LA-cp rats; 1% supplementation with vaccenic acid for 8 weeks was found to stimulate adipose tissue redistribution, reduced total body fat and decrease adipocyte size in comparison to control without supplementation (19).

Additionally, sugar overconsumption, an important feature of WD, has been widely associated with obesity and some studies related the reduction in sugar consumption to a slowing down of the USA's annual rate of increase in obesity (20). Therefore, since the current WD often provides considerable amounts of sugar and SFA, it may represent a threat to health (21).

3. Limitations of current anti-obesity drugs and therapies

The dimension of obesity worldwide gave rise to the development of several anti-obesity strategies and in the last decades, several drugs and therapies have arisen. Importantly, the use of drugs in obesity treatment is considered to have an efficacy placed between the lifestyle changes efficacy, which accounts for 5 to 10% of weight loss, and bariatric surgery, which is described as the most efficient treatment, with 20 to 30% weight loss. According to the Food and Drug Administration (FDA), for a drug to be considered effective in obesity treatment it should be responsible for a difference in weight as compared to a >5% after 1 year of treatment (as reviewed by Rubio (22)). Data from recent meta-analysis studies showed that the overall placebo-subtracted weight reduction (%) with the use of anti-obesogenic drugs for at least 12 months ranges from 2.9 to 6.8% (23). Although some anti-obesity drugs present promising results, the associated side effects continue to be one of the major drawbacks regarding the available drugs (**Table 1.1**). Indeed, although side effects are widely dependent on the individual, the most common are those associated with increased blood pressure, tachycardia, insomnia, alterations in sexual behavior, malabsorption of nutrients, or carcinogenic effects (24–26). For instance, orlistat, an anti-obesity drug approved by the FDA and European Medicines Agency (EMA), decreases fat absorption by 30% through inhibition of gastric and pancreatic lipases but reported the following side effects (incidence of 5% and at least twice that of placebo): flatulence, oily spotting, fecal urgency, fatty/oily stool, oily defecation, increased defecation and fecal incontinence and other adverse effects such as nephrotoxicity, hepatotoxicity, nephrolithiasis and pancreatitis (26). Moreover, since the adoption by the FDA of stricter regulations and requirements of proof of clinical efficacy, a couple of recently approved anti-obesity drugs have been removed from the USA market for safety concerns: sibutramine (Meridia) was approved between 1997 and 2010. The concerns were related to an elevated risk of CVD events in patients at high risk for CVD when given sibutramine (27). The utilization of Lorcaserin (Belviq) was approved between 2012 and 2020. A re-analysis of a safety clinical trial, from 6 months to 2.5 years, showed an increased incidence of certain cancers. According to the data, a greater number of participants who received lorcaserin compared to placebo were reported with multiple primary cancers (n=20 vs. 8), total cancers (n=520 vs. 470), metastases (n=34 vs. 19), and cancer deaths (n=52 vs. 33) (28). In Europe, for example, the application for lorcaserin was withdrawn in May 2013 after the EMA stated that the weight-loss benefits of lorcaserin did not justify its risks, which included the potential to increase the frequency of psychiatric disorders and valvulopathy (29).

The magnitude of obesity and associated comorbidities as well as the mentioned difficulties with existing weight-loss therapies, emphasizes the need for different approaches and a better and more complete understanding of the problem. Additionally, despite the huge progression made over the last decades, the overweight and obesity prevalence rates continue to increase, suggesting that additional elements must be involved in the pathogenesis of this disease.

Table 1.1 - Summary of the developed drugs for obesity treatment, their mechanisms of action, and associated side effects. (FDA) Food and Drug Administration; (EMA) European Medicines Agency.

Commercial name/ Anti-obesity drug name	Mechanism of action	Side effects	Status	References
Xenical/ Orlistat	Decreases fat absorption by inhibition of gastric and pancreatic lipases.	Flatulence, oily spotting, fecal urgency, fatty/oily stool, oily defecation, increased defecation, fecal incontinence, hepatotoxicity, nephrolithiasis, and pancreatitis.	Approved by the FDA in 1998 and EMA in 1999.	(26)
Sibutramine	It works by preventing the neurotransmitters serotonin and noradrenaline from being taken back up into nerve cells in the brain. The increased levels of neurotransmitters in the brain help patients to feel full after a meal, and this helps to reduce their food intake.	Elevated risk of CVD events in patients at high risk for this disease.	Approved by the FDA between 1997 and 2010. Approved by EMA from 1999 to 2010. Removed from the market.	(27,30)
Belviq/ Lorcaserin	It imitates the effects of serotonin on 5-HT2C receptors, which include an increased sense of fullness after a meal and reduced hunger before meals, thereby reducing food consumption.	Incidence of certain cancers.	Approved by the FDA between 2012 and 2020. It was withdrawn by EMA from the European market in 2013.	(29,31,32)
Fintepla/ Fenfluramine	Used as an anoretic drug. When combined with a norepinephrine stimulant, phentermine, it became part of the anti-obesity medication Fen-phen.	Cardiovascular complications include heart valve disease, pulmonary hypertension, and cardiac fibrosis.	Approved by the FDA in 1973 and withdrawn in 1997.	(33)
Qsymia/ Phentermine-topiramate	Appetite suppression and basal energy expenditure increase.	Dry mouth, insomnia, dizziness, palpitations, constipation, irritability, and mood changes, its use is contraindicated in patients suffering from anxiety, CVD, hyperthyroidism, or glaucoma.	Approved by the FDA in 1959. Restricted to short-term use.	(34,35)
Zimulti/ Rimonabant	Acts as a CB1 receptor inverse agonist (functional antagonist)- anoretic effect.	Severe mood disorders, like anxiety and depression.	Approved by EMA in 2006 and withdrawn in 2009. Withdrawn by the FDA in 2007.	(36)
Saxenda/ Liraglutide 3.0 mg	GLP-1 receptor mono-agonist. Plays a role in the central regulation of feeding through its effects on the arcuate nucleus and nucleus tractus solitarius.	Gastrointestinal adverse events: nausea, diarrhea, constipation, vomiting.	Approved by EMA in 2015 and FDA in 2014 for weight management.	(34,37)
Wegovy/ Semaglutide 2.4 mg	Mimics GLP-1 that targets areas of the brain that regulate appetite and food intake	Nausea, diarrhea, constipation, abdominal pain, headache, and increased heart rate. May increase the chance of developing pancreatitis and it has been found to cause a rare type of thyroid tumor in animal models.	Approved by FDA in 2021 and EMA in 2023 for obesity treatment.	(38)
Amfepramon/ Amfepramone (Diethylpropion)	Indirect-acting sympathomimetic agents act by releasing noradrenaline from presynaptic vesicles in the lateral hypothalamus. The increase in noradrenaline concentration results in the stimulation of β2-adrenergic receptors and consequent inhibition of appetite.	Dizziness, dry mouth, difficulty sleeping, irritability, nausea, vomiting, diarrhea, constipation.	Approved by the FDA in 1959. Restricted to short-term use. Withdrawn from the European market by EMA in 2000.	(39)
Phendimetrazine	Activity similar to amphetamines that stimulates the central nervous system and elevates blood pressure most likely mediated via norepinephrine and dopamine metabolism. Causes stimulation of the hypothalamus. Reduces food intake	Insomnia, dry mouth, constipation, hyperpyrexia, mydriasis, chest pain, arrhythmias, delirium, rhabdomyolysis.	Approved by the FDA in 1959. Restricted to short-term use.	(34,40)
Didrex, Regimex/ Benzphetamine	Sympathomimetic and CNS stimulant. Similar action to amphetamines.	Insomnia, dry mouth, elevation of mood, nausea, vomiting, palpitation.	Approved by the FDA for short-term use.	(34,41)
Contrave, Mysimba/ Naltrexone-bupropion	Naltrexone and bupropion act on the parts of the brain that control food intake and energy balance. Acts also reduce the effect of the part of the brain that controls the pleasure associated with food consumption.	Constipation, diarrhea, dizziness, dry mouth, headache, increased blood pressure, increased heart rate, insomnia, liver damage, nausea, and vomiting. May increase suicidal thoughts or actions.	Approved by the FDA in 2014 and EMA in 2015.	(42,43)
Imcivree/ Setmelanotide	It is a cyclized octapeptide that binds and activates multiple melanocortin receptors–MC4R, MC3R, and MC1R selectively. Specific to patients with obesity caused by genetic conditions: pro-opiomelanocortin (POMC), proprotein subtilisin/kexin type 1 (PCSK1), and leptin receptor (LEPR) deficiency.	Injection site reaction, skin darkening nausea, disturbance in sexual arousal depression, and increase of suicidal thoughts. Risk of serious adverse reactions in neonates and infants with low birthweight.	Approved by the FDA in 2020. Approved by EMA in 2021.	(44)

State-of-the-art

1. The role of fatty acids in obesity development: effect in peripheral tissues

In the last few years, new policy strategies devised to fight obesity have emerged. As already discussed, an important factor that has contributed to the rapid increase in cases of obesity among the world population is the change in dietary patterns of individuals, mainly characterized by increased consumption of energy-dense foods, rich in sugar and SFAs, combined with a sedentary lifestyle (8). Thus, diet, and fat intake specifically, have been widely recognized as playing a central role in both obesity and overweight. In consequence, it is feasible to reduce the risk of obesity through modifications to the daily diet. For instance, high-fat diets (HFDs) are often blamed for increasing obesity rates; however, fats are diverse and respond differently *in vivo* as discussed for TFAs. Considering that lipids have been commonly recognized as important players in obesity, their distinct roles in different organs are going to be further discussed.

1.1. Adipose tissue

In mammals, adipose tissue can be divided into two major types: brown (BAT) and white adipose tissue (WAT). Regarding BAT, in newborns, this tissue is important in the regulation of energy expenditure by thermogenesis. In adults, the amount of BAT is inversely correlated to BMI suggesting a potential role in metabolism (45). On the other hand, WAT considered the main site of energy storage, is currently seen as an active and important participant in regulating physiological and pathological processes, such as immunity and inflammation (46). WAT is considered the largest endocrine organ, and it is composed of adipocytes that are held together by poorly vascularized and innervated connective tissue, where sympathetic innervation has been described (25,47,48). Indeed, macrophages are components of this tissue and important regulators of its activities. Moreover, there is a crosstalk between lymphocytes and adipocytes, which implies immune regulation (49). Adipose tissue also produces a variety of factors, like adipokines, such as leptin, adiponectin, and resistin, as well as pro-inflammatory (*e.g.,* TNF-α and IL-6) and anti-inflammatory cytokines (IL-10 and IL-1) (50). Consequently, it can affect the function of many systems as adipocytes are known to secrete more than 600 bioactive factors – collectively known as adipokines (51) -, in addition to lipids and their metabolites (52).

In the last few years, several studies have demonstrated that consumption of WDs, namely the high presence of SFAs can be considered a pro-inflammatory factor itself and an association between this type of diet and the presence of obesity, hepatic steatosis and type 2 diabetes mellitus (T2DM) have been extensively described (53–57). Thus, in a condition of chronic positive energy balance – like what happens in SFA-induced obesity -, adipose tissue undergoes profound modifications including adipocyte expansion, induction of hypoxia, and

mitochondrial function alteration, which ultimately leads to tissue remodeling, inflammation, and metabolic dysfunction (48,58). Such events result in severe changes in the immune response and consequently, in the development of a pro-inflammatory profile. The meta-inflammation - a chronic low-grade inflammatory state – is growingly associated with adipose tissue in an obesity context and is considered a characteristic feature of metabolic syndrome: there is secretion of inflammatory adipokines mainly from adipose tissue, including leptin, IL-6, and TNF-α. Besides, the meta-inflammation state along with the reduction in the production of adiponectin, a significant predictor of cardiovascular mortality, is associated with impaired fasting glucose, leading to T2DM development, metabolic abnormalities, coronary artery calcification, stroke, and cancer (48,59).

The SFA diet excess itself, characteristic of obesity, is responsible for increasing lipid storage in adipose tissue. Such a process results in an accumulation of lipids in adipocytes. This leads adipocytes to develop larger lipid droplets and therefore to contain more triglycerides (TGs). The increased intracellular TG pool leads to increased leptin secretion by adipocytes (53,56). Besides, this accumulation triggers cellular stress as well as the activation of pro-inflammatory pathways such as JNK and NF-κB (59). This process raises the circulating levels of several acute-phase proteins and inflammatory cytokines resulting in the mentioned chronic low-grade inflammation state (25,58). An increasing number of studies have suggested that lipids, specifically FAs, play an important role in obesity development and the interplay between excessive adiposity and the development of associated comorbidities (previously enumerated) (48,60). Indeed, the type of FAs stored in adipose tissue critically affects tissue functions, since FAs can directly or indirectly modify immune and inflammatory responses, by acting on the cell surface and intracellular receptors, such as TLR4, that control cell signaling and gene expression (61,62). Recent studies have shown that overeating SFAs (*e.g.,* palmitic acid (PA, C16:0) promotes greater visceral fat storage (associated with metabolic disease) when compared to unsaturated fatty acids. The authors showed that there is a link between SFAs in visceral adipose tissue and *HSD11B1*, a gene responsible for the expression of 11-β-hydroxysteroid-dehydrogenase type 1 (11β-hsd1), which is a major regulator of cortisol (important in body fat distribution regulation) activity (63).

Among the mentioned adipokines secreted by WAT, leptin is one of the hormones presenting a direct link to body fat and obesity. In a study aiming to identify the association of leptin gene (*ob*) expression in visceral and subcutaneous adipose tissue with fatty acid intake in adults, it was reported that dietary intake of SFA is positively associated with both subcutaneous and visceral adipose tissue leptin gene expression (64). In peripheral tissues, leptin is highly relevant since it is involved in several physiological processes, such as angiogenesis, hematopoiesis, bone formation, wound healing, immunocompetence, or lipid and carbohydrate metabolism regulation as well as nutrient intestinal absorption (65). Leptin resistance in obesity has been suggested to be initiated by the activation of inflammatory signals. Inflammatory factors, such as TNF-α and IL-1a as well as lipopolysaccharide (LPS), are known to increase circulating leptin concentrations in both rodents and humans (65). Since NPY neurons and leptin interact to

maintain homeostasis to regulate body-fat mass and energy levels at both central nervous system (CNS) and adipocyte levels, early studies showed that leptin is involved in the regulation of lipolysis (65–67) – defined as the hydrolytic cleavage of ester bonds in TGs, resulting in the generation of fatty acids and glycerol (68). In fact, the lipolytic effect observed in adipocytes from lean mice was lower than from *ob/ob* mice (67). Moreover, recent studies have demonstrated that leptin induces intracellular signaling in preadipocytes and adipocytes promoting adipogenesis and modulating the secretion of inflammatory mediators (increasing TNF-α production in 3T3-L1 cells) contributing to the inflammatory profile characteristic of obesity (69). Thus, impaired regulation of leptin response, due to leptin resistance, may lead to the development of more and bigger adipocytes – WAT expansion - and contribute to the accumulation of excessive fat mass found in obese state.

In addition, excessive lipid accumulation in adipose tissue, and ectopic accumulation (defined as steatosis) appear in other tissues like the liver and muscle. These adipocytes release free FAs into the bloodstream through the action of the CD36, the plasmatic fatty acid binding protein (FABPpm), and the fatty acid transport proteins (FATPs). The circulating free FAs are captured by other organs, especially the mentioned ones (liver and muscle) leading to steatosis (53).

1.2. Liver

Besides adipose tissue, the liver plays a major role in homeostasis regulation and is important for the maintenance of nutrient metabolism. Since leptin regulates hepatic gluconeogenesis and insulin sensitivity, defects in leptin action impair hepatic function leading to hyperglycemia, hyperinsulinemia, and hyperlipidemia (65). Morbidly obese patients have a prevalence of more than 90% of changes in liver histology, namely hepatic steatosis (also known as fatty liver disease - which is characterized by the presence of an increased liver due to a higher concentration of TGs in hepatocytes). In fact, non-alcoholic fatty liver disease is a prevalent condition associated with obesity and insulin resistance, which is becoming the most common form of liver disease worldwide (70).

The problem with obesity is that the increase of FAs in hepatocytes leads to a higher synthesis of TGs. Consequently, the liver is not able to export them efficiently being accumulated in the hepatocytes (liver parenchymal cells), which leads to a non-alcoholic steatohepatitis-like phenotype, characterized by hepatic steatosis (71). Besides, this high production of TGs is also associated with ER stress on hepatic cells, which ultimately leads to hepatocyte lipoapoptosis (72). Moreover, the accumulation of LC-SFAs leads to the formation of toxic lipids such as ceramides, known to modulate signaling pathways involved in regulating glucose metabolism, triglyceride synthesis, apoptosis, and fibrosis (73,74). Such lipids induce lipotoxicity, which ultimately leads to ER stress and inflammation (53). Besides, since macrophages are recruited to the adipose tissue, they induce the secretion of other proinflammatory cytokines amplifying the

already existing inflammatory state. Thus, such cytokines are continuously and abundantly released by adipose tissue and reach the liver through the portal vein circulation. Afterward, they stimulate the secretion of C-reactive protein, an important marker of inflammation, the progression of hepatic insulin resistance, and hepatic steatosis in obese individuals. Moreover, the direct contact of visceral fat with the proinflammatory cytokines contributes to the progression of insulin resistance (53,70).

2. The effect of dietary lipids on the brain imbalance triggered by obesity.

It has been suggested that energy balance is maintained by adipose tissue-brain crosstalk, and energy imbalance results in an accumulation of excessive calories in the form of TGs in adipose tissue, leading to overweight and obesity (75). Nonetheless, in the last decade, research focused on the humoral, neuronal, and molecular mechanisms involved in the regulation of hunger and satiety, unraveled the fundamental role performed by CNS, especially by the hypothalamus in coordinating these processes (76). Hypothalamic lesion studies in rats have described specific areas in this region as central regulators of feeding (77–79). Moreover, certain hypothalamic lesions led to the cessation of feeding and subsequent death by starvation (80,81). Indeed, this research demonstrated that obesity is far more complex than initially thought as it is associated with altered brain functions. In a study by Jastreboff et al. (82) using functional Magnetic Resonance Imaging (MRI), it was observed that after drinking a cherry-flavored glucose-sweetened beverage, obese adolescents presented decreased brain perfusion in the prefrontal cortex (associated with decision-making) and increased perfusion in the hypothalamus (homeostatic appetite regions of the brain). The authors have demonstrated that study subjects have impaired prefrontal executive control responses, which seem to be related to glucose and fructose overconsumption, consequently increasing weight gain. Food intake is a complex process that is controlled by several molecules, namely neurotransmitters - dopamine, Gamma (γ)-aminobutyric Acid (GABA), norepinephrine, serotonin -, peptides, and amino acids. Dopamine has attracted increased interest due to its role in regulating food intake. Interestingly, Wang et al. (83) showed that in obese human subjects, the availability of dopamine D2 receptor (D2R) was decreased in proportion to their BMI. The authors suggested that since dopamine modulates motivation and reward circuits, the dopamine deficiency observed in obese individuals may perpetuate their pathological eating since they are less sensitive to reward stimuli. In another study, Johnson and Kenny (84) associated the downregulation of striatal D2R found in obese rats, with drug addiction mechanisms. Indeed, the authors reported that similar changes in reward homeostasis are induced by addiction drugs, such as cocaine or heroin, and excessive food consumption, triggering similar compulsive behaviors. Consequently, common hedonic mechanisms may underlie both obesity and drug addiction. These similarities gave rise to the hypothesis of food addiction. Furthermore, Kuhn et al. (85) showed that in a mice model, the intake of hydrogenated vegetable oil containing 11.72% trans-fat resulted in a higher preference

for amphetamine and it was associated with withdrawal signs, such as anxiety and fear. Although as mentioned, the negative effect of TFA on health is still controversial, the authors proposed that chronic consumption of foods rich in TFAs can modify factors related to drug preference. In addition, TFA presence in diet during the development and growing periods can exacerbate both withdrawal symptoms as well as brain oxidative status. Such alterations were thought to arise from the health condition; however, studies focusing on diet effects demonstrated that it can be fostered by nutrients alone. Feeding trans-α-linolenic acid (ALA) to Wistar rats for up to 21 months decreased the dopamine levels (a neurotransmitter associated with attention, motivation, and emotion) by 95% in the hippocampus. Indeed, it was observed that the isomerization of dietary ALA can induce a “deficiency-like” status that results in modifications of the levels of endogenous dopaminergic neurotransmitters (86). In addition, Wistar rat offspring whose mothers were fed with hydrogenated vegetable oil (rich in TFA) during gestation and lactation showed modification of spatial memory. Although only a slight brain incorporation of dietary TFA was observed, it was enough to modify behavioral and biochemical parameters in the experimental animal model (21). Thus, by focusing on the food addiction problem it is easily understood how most obese people are unable to regulate their food intake and relapse towards their elevated body weight after repeated dieting and exercise attempts. This cycle of overconsumption, dieting, and relapsing has been compared to the cycle of drug intoxication, abstinence, and relapse observed in drug addiction (87). Such a perspective explains, along with all the socioeconomic and lifestyle conditionings, how the present strategies are ineffective and how obesity, although preventable, is on the rise. Considering all the discussed points, perhaps we should start considering the direct effect of diet, precisely nutrients in obesity; not only its role on adipose tissue but also if such nutrients can have a direct (or indirect) impact on the brain.

2.1. Lipid metabolism in the brain and its impact on food intake

Early studies from the 1950s have suggested the existence of neuronal hypothalamic populations able to sense the energy status of the body and respond to this status by controlling hunger/caloric intake and energy expenditure (88–90), suggesting a role of the brain in appetite regulation and therefore in obesity. Later, studies have demonstrated that indeed there are FA membrane receptors that act through other signaling mechanisms to control energy homeostasis (91–93), providing some insights into the action of these molecules on brain mechanisms and clarifying their role in obesity induced-neuroinflammation.

In summary, as reviewed by Dragano and collaborators (88) the brain is rich in PUFAs, but essential FAs are transported into the brain from the circulation. This transport is made through the blood-brain barrier (BBB) and once they arrive at the brain these FAs are converted into Long-chain fatty acid-coenzyme A (LCFA-CoA) and are later either metabolized by β-oxidation or incorporated into phospholipids. The mechanisms through which FAs pass through BBB are not yet fully understood. Nevertheless, some evidence suggests that they can pass by

passive diffusion or be translocated by carrier proteins, the cluster of differentiation CD36 and fatty acid transport proteins (FATP)-1 and 4 are the commonly accepted mechanisms (93–95). Besides its role in FA uptake from BBB, the receptor CD36 is also suggested to be involved in many lipid-sensing responses in hypothalamus neurons (96,97).

After entry into neurons, long-chain fatty acids (LC-FA) are esterified by LCFA-CoA synthase to form LCFA-CoA. Studies have demonstrated that this process is indeed important for the inhibition of food intake during systemic increases in lipid availability since LC-FAs produce an increase in LCFA-CoA levels and generate a metabolic signal of energy surplus (98). Intracellular LCFA-CoAs are translocated into mitochondria via carnitine palmitoyltransferase-1 (CPT1) where they undergo β-oxidation. In neurons of the arcuate nucleus (ARC), mitochondrial CPT1c activity is regulated by the availability of malonyl-CoA. Under normal physiological conditions, CPT1 activity is inhibited by increased malonyl-CoA concentration, and hypothalamic malonyl-CoA levels closely correlate with nutritional status. Thus, increased levels of malonyl-CoA may act as a signal of energy surplus that regulates orexigenic and anorexigenic neuropeptide release to suppress food intake and increase energy expenditure (88). Moreover, malonyl-CoA levels depend on the equilibrium of acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and malonyl-CoA decarboxylase (MDC). Being the activities of ACC and MDC directly regulated by phosphorylation via AMP-activated protein kinase (AMPK); when AMPK is active it phosphorylates and inhibits ACC, decreases FAS mRNA expression, and activates MCD. Thus, AMPK activation reduces malonyl-CoA levels and the flux of substrates through the FA biosynthetic pathway. The decreased malonyl-CoA levels stimulate the CPT1, which promotes access of LCFAs-CoA into the mitochondria and increases FA oxidation (88).

When there is increased and sustained availability of fatty acids, the hypothalamic nutrient-sensing system is dysregulated, the pool of LC-FAs increases and there is an augmented uptake by the brain. This enhanced uptake results in rising LCFA-CoA levels in the hypothalamus, which has a negative impact on the regulation of food intake and energy expenditure (99,100).

2.2. The negative effect of saturated fatty acids in the brain: hypothalamic inflammation

The effect of an HFD in obesity animal models has been largely reported. In comparison with the control diet, HFD presents increasing levels of endoplasmic reticulum (ER) stress proteins (pJNK, pPERK, peIF2α, GPR94, and GPR78), leading to the accumulation of unfolded proteins due to high cellular demands. In response, affected cells activated a complex signaling system known as the unfolded protein response (UPR) to preserve cell integrity. This signaling system induces the production of inflammatory cytokines (TNF-α, IL-1, iNOS, and IL-6) (101,102), resulting in elevated levels of hypothalamic inflammation markers after HFD consumption. Consequently, these results point out the existence in the brain of mechanisms to precisely recognize FAs. Toll-like receptors (TLR) 2 and 4, in the hypothalamus, are recognized binding

sites for LC-SFA. Indeed, studies have shown that diet also significantly increases the expression level of TLR gene expression (102). Most of the studies were focused on TLR4, which is expressed in macrophages, dendritic cells, adipocytes, hepatocytes, muscles, and the hypothalamus. Indeed, Milanski et al. (103) showed that LC-SFA activates predominantly TLR4 resulting in the induction of the mentioned ER stress, which ultimately leads to inflammatory pathways activation in the hypothalamus, by cytokine expression. Furthermore, a loss-of-function mutation and inhibition of TLR4 protects mice from diet-induced obesity. As reviewed by Li et al. (104) TLR4 activation by SFAs has been demonstrated in cultured adipocytes, monocytes/macrophages, hepatocytes, endothelial cells, and skeletal muscle cells. However, the direct binding of SFAs to TLR4 has been challenged (105) and it is thus, possible that SFAs interact with TLR4 indirectly through fetuin-A (106). Although, SFAs, including PA and stearic acid, can activate TLR2 and TLR4-dependent signaling pathways, MUFAs and PUFAs, particularly omega-3 PUFAs, do not and might instead protect against SFA-induced TLR activation (107). Such a hypothesis is going to be further discussed in the next sections.

Besides, it has been reported that HFD elevates the markers of hypothalamic inflammation within 24 hours, in contrast with inflammation in peripheral tissues, which is a slow process that takes weeks or months to develop (108). A recent study has shown that after 3 days of HFD, no changes in inflammation-related proteins were observed, but instead many proteins associated with cellular stress were found to be changed in response to this diet. Thus, such results suggest that oxidative stress in neurons may precede and ultimately cause HFD-induced hypothalamic inflammation (109). In fact, SFAs have not only been associated with inflammatory processes, but they have been shown to promote hypothalamic apoptosis (110,111) or alterations in the cell volume distribution of proopiomelanocortin (POMC), resulting from defective regulation of hypothalamic POMC, and orexin-producing neurons (112,113). These results suggest that, in both human and rodent models, HFD-induced obesity is associated with dysregulation in important brain areas for energy homeostasis. This is highly relevant since CNS energy homeostasis, as depicted in **Figure 1.1**, is largely controlled by the fine balance between the two distinct subpopulations of neurons in the hypothalamus arcuate nucleus (ARC), namely the ones co-expressing orexigenic neuropeptides (agouti-related protein [AgRP] and neuropeptide Y [NPY]), and those producing anorexigenic neuropeptides α-melanocyte stimulating hormone (α-MSH) - a product of POMC precursor protein processing - and cocaine and amphetamine-regulated transcript (CART), whose peptides are neuromodulators involved in feeding, drug reward, stress, cardiovascular function, and bone remodeling (112,114). NPY/AgRP and POMC neurons in the ARC control energy homeostasis and coordinate the response to changes in metabolic status, namely nutrient and hormonal fluctuations (107,112). AgRP and POMC, together with downstream target neurons, which express melanocortin receptors (MCR) 3 and 4, a family of G protein receptors (GPRs), are the core of the melanocortin system. For instance, NPY acts on Y1 and Y5 receptors in the paraventricular nucleus of the hypothalamus to stimulate food intake and reduce basal energy expenditure (112). α-MSH is an endogenous MCR 3 and 4

agonist, consequently generating an anorexigenic output by suppressing appetite and enhancing thermogenesis. On the other hand, AgRP is an antagonist of these receptors counteracting the anoretic effect of α-MSH, namely on food intake and body weight (107,115). Through NPY/AgRP and POMC neurons, the hypothalamus can exert control over other groups of neurons. This action ultimately leads to a regulation of efferent CNS outputs that regulate energy balance and fuel homeostasis in the rest of the body.

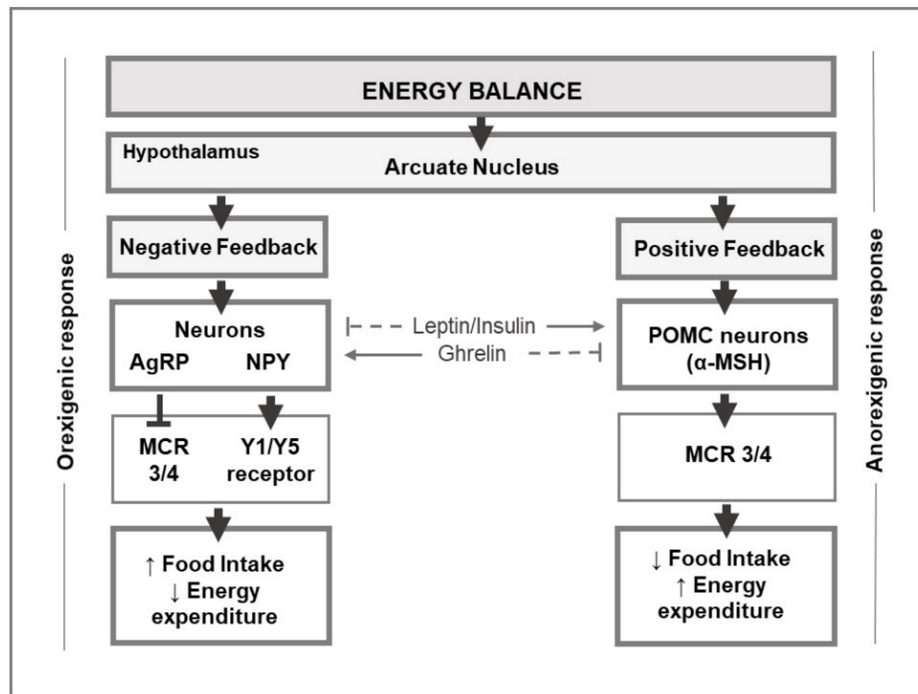

Figure 1.1 - Schematic representation of the normal hypothalamic function. It involves a reciprocal interaction between AgRP/NPY and POMC neurons in the hypothalamus ARC. These neurons respond to signals from leptin, insulin and ghrelin and regulate both food intake and energy expenditure. Leptin and insulin stimulate the activity of anorexigenic POMC neurons while inhibiting AgRP/NPY neurons. Such effect results in increased release of α-MSH and the activation of secondary neurons expressing MCR 3/4 receptors, leading to reduced food intake and increased energy expenditure. Ghrelin exerts its orexigenic effects through AgRP/NPY neurons, enhancing the expression of NPY and AgRP. AgRP acts as MCR 3/4 antagonist, while Y1 and Y5 receptors stimulate orexigenic outputs, increasing food intake and reduce basal energy expenditure (99).

The problem with hypothalamic ER stress-mediated by obesity is related to its possible interference with the proper synthesis and processing of POMC. A defective POMC processing prevents the release of α-MSH in response to leptin, leading to leptin resistance. Additionally, pro-convertase 2 (PC2), responsible for catalyzing the conversion of adrenocorticotropin (ACTH) into α-MSH, was found to be reduced by ER stress, consequently leading to the reduced α-MSH levels in diet-induced obesity (115). Moreover, studies found a direct correlation between HFD feeding, in an obesity context, and POMC neurons apoptosis. Indeed, Moraes et al. (110) reported that an HFD alters the expression of 57% of genes associated with neuronal apoptosis, proving the effect of dietary fats in inducing hypothalamic neuronal cell death. However, HFD administration was shown to reduce the number of POMC neurons, but not NPY neurons, leading to an imbalance in energy homeostasis. The reduced number of POMC neurons was demonstrated not only to be a consequence of obesity but also to be involved in the onset of obesity development. A hypercaloric (high-carbohydrate, high-fat) environment induces mitochondrial stress in POMC

neurons, a consequence of a persistent elevation of microglial (principal resident immune cells of the brain) reactivity which results in TNF-α secretion. This indicates that the HFD-induced inflammation along with ER stress, plays a major role in the functional impairment of hypothalamic POMC neurons and ultimately generates an additional pathogenic drive towards impaired energy homeostasis and obesity (116).

Moreover, an *in vitro* study carried out in embryonic mouse hypothalamic cell lines concluded that palmitate significantly increased mRNA levels of NPY and pro-apoptotic proteins (CHOP) associated with ER stress (117). These changes observed in the ARC neuron population, and the consequent energy homeostasis imbalance seem to be involved in the main challenge of the anti-obesity therapies: patients that rapidly regain weight after finishing treatment. When mice fed a HFD were administered with the neurocytokine ciliary neurotrophic factor (CNTF) the effects were a 20% body weight loss, neurogenesis of both POMC and NPY cells, and increment of pSTAT3, a component of the leptin-activated signaling cascade in leptin receptor–containing cells of the hypothalamus (118). However, if animals were treated with the antimitotic drug Ara-C to prevent the proliferation of neural cells, body weight increased almost immediately after ending the intervention, reaching the starting value in 15 days and surpassing it by 20% by the end of the study (35 days).

In addition, recent research has shown that HFD induces chaperone-mediated autophagy (CMA) in the hypothalamus in short-term feeding. Prolonged exposure to this type of diet leads to CMA impairment (119). CMA is an important process since it regulates protein quality control and other cellular homeostasis mechanisms. In consequence, in the CNS, CMA is responsible for neuron protection against injuries and chronic neurodegeneration. Specifically, palmitate (a SFA) can directly activate CMA in hypothalamic neurons (119). The authors reported that HFD leads to early modifications in CMA machinery in the hypothalamus. Although such a relation has not yet been proved, this is highly relevant since CMA HFD-induced impairment may lead to an absence of misfolded and oxidized protein degradation, resulting in hypothalamus dysfunction and neurodegeneration.

2.2.1. Hypothalamic Insulin Resistance

Insulin executes important roles in the hypothalamus since it suppresses food intake and improves glucose metabolism. When there is a disruption of such effects a condition known as hypothalamic insulin resistance arises. Insulin signaling in some hypothalamic cell types, is stimulated by insulin in the CNS. For instance, in neurons, insulin signaling is initially mediated by insulin receptor (IR) activation, which results in electrophysiological and/or transcriptional changes in neurotransmitters (120). Besides, insulin (through IR) plays an important role in astrocyte glucose transport from peripheral blood to CNS (121,122).

It is recognized that obesity-induced inflammation closely connects obesity to the development of both glucose intolerance and insulin resistance, a central component of T2DM

(123). The HFD-induced low-level hypothalamic inflammation, namely through the expression of several proinflammatory cytokines and inflammatory responsive proteins, is accompanied by increased activation of intracellular serine kinases JNK and NF-κB, a transcription factor that regulates the expression of proinflammatory genes, including cytokines (such as IL-1 and TNF-α), chemokines and adhesion molecules (124). The problem arises since the activation of intracellular kinases such as JNK and IkB kinase β (IKKβ), which act as intermediaries for proinflammatory signaling, induces phosphorylation of insulin receptor substrate (IRS) on its serine residues, inhibiting phosphorylation of its tyrosine residues (125,126). Importantly, the phosphorylation of those IRS-1 tyrosine residues, upon activation of the IR, constitutes a critical step in the transmission of the insulin signal to downstream effectors and biological outcomes. In contrast to tyrosine phosphorylation, the induced serine phosphorylation blunts insulin signaling which promotes insulin resistance. Reactive oxygen species (ROS) have also been associated with insulin resistance. Under normal circumstances, hypothalamic insulin triggers the transient production of ROS to enhance insulin signaling. In obesity and diabetes, ROS overproduction is reported, which will induce inflammation, consequently disrupting insulin signaling (120,127).

2.2.2. Hypothalamic Endoplasmic Reticulum Stress

The ER homeostasis can be altered by strong and prolonged cellular disturbance, leading to the accumulation of potentially toxic unfolded or misfolded proteins in the ER lumen. In such conditions, to restore adequate ER performance, a set of stress-responsive signaling pathways, referred to as the UPR, is activated. If normal ER function is not restored the UPR sustained activation can lead to cell death by the activation of autophagic programs or apoptosis (115).

HFD diet exposure in rodent models has been shown to affect the mRNA expression of hypothalamic ER stress-related UPR markers. Consequently, the UPR markers seemed to be a sensitive sensor of FA availability as well as nutrient load (128). Recently, PA (a SFA) was demonstrated to increase the mRNA expression of the ER stress markers - Chop, Grp78, and the Bax/Bcl2 ratio - along with cellular neuroinflammation markers - mRNA levels of pro-inflammatory cytokines Il6, TNF-α, and Il1b, TLR4 receptor, and the proinflammatory transcription factor NF-κB, in mHypoA-POMC/GFP-2 neurons (POMC expressing neurons) (129). In a different study, other SFAs besides PA, such as lauric and myristic acids, increase ER stress mRNA expression of Atf4, Atf6, Xbp1, Bip and Chop in mHypoE-N43/5 (130). It has been suggested that lipid overload, especially SFAs, causes hypothalamus ER stress by inducing alterations in the ER membrane composition and its biophysical properties. Those variations are sensed by UPR transducers, such as the ER membrane protein Ire1 and PKR-like kinase (PERK), an ER transmembrane kinase (131,132). A close relationship between ER stress and obesity was described for the first time by using dietary (HFD-induced) and genetic models (*ob/ob*) of murine obesity. The authors observed an increased phosphorylation of PERK in the obesity models. Consequently, the phosphorylation of translation initiation factor 2 (eiF2) by PERK, was also

increased (133). Indeed, eiF2 is an important factor for protein translation, and phosphorylation of its serine residue (Ser51) has an inhibitory effect on protein translation. Therefore, the phosphorylation of both PERK and eIF2 is a key indicator of ER stress (134).

Under normal conditions, NF-κB, remains inactive in the cytoplasm through the action of its inhibitor, the IκB inhibitory protein (IκBα). Activation of IKKβ through phosphorylation induces IκBα phosphorylation (its substrate), which results in ubiquitination and ultimately proteasomal degradation. This action releases NF-κB to translocate into the nucleus resulting in the transcription of its target genes (135). Obesity-induced ER stress was found to be both an upstream intracellular mediator and a downstream event of the hypothalamic IKKβ/NF-κB activation. Indeed, the benefits observed by suppressing ER stress in the CNS resemble the ones observed from suppressing IKKβ/NF-κB. IKKβ/NF-κB in the hypothalamic neurons was found to respond to the metabolic signals that are produced by overnutrition and that are a cause of multiple neuronal disease pathways (135). Moreover, the NF-κB pathway activation is mediated by myeloid differentiation primary response 88 (Myd88) activation, through TLR4. In a recent study, astrocyte-specific Myd88 knockout mice fed an HFD or injected with SFAs showed ameliorated hypothalamic reactive gliosis and inflammation. Furthermore, the Myd88 expression in hypothalamic astrocytes was also increased in the mice subjected to the treatment. These results suggested an important role of Myd88 in mediating HFD signals for inflammation (136). This means that in obesity and overnutrition, ER stress and IKKβ/NF-κB in the hypothalamus enhance each other leading to energy imbalance and consequently disease.

In conclusion, excessive nutrients, namely SFA, lead to UPR signaling in the hypothalamus and consequently to inflammation and crosstalk with innate and adaptive immunity. Besides, high levels of SFA cause resistance to the action of key metabolic hormones, such as leptin and ghrelin, which are important players in the neuroendocrine control of energy homeostasis (137). Interestingly, contrary to SFA, studies have shown that the expression of ER stress markers was not affected when cells were treated with MUFA oleic acid (130).

2.2.3. Hypothalamic Leptin Resistance

As previously mentioned, adipose tissue secretes a variety of humoral factors – adipokines -, which regulate nutrient metabolism. Some adipokines, like leptin, serve as signals related to body energy storage and availability to the brain (138,139). The brain, particularly the hypothalamus, senses and integrates these signals and maintains energy homeostasis and body weight by controlling feeding behavior and energy expenditure (75).

The gene product of the obese gene (*ob)* locus was first described by Ingalls et al. (140). Defects in the *ob* gene were later associated with a marked increase in adipose tissue mass as part of a syndrome that resembled morbid obesity in humans (141). The *ob* gene product was finally described as leptin, a 16-kilodalton (kDa) protein produced by the adipose tissue and defined as being able to reduce body fat in mice. Its absence in obese mice leads to a massive

increase in body fat (139,142). Later, Wiesner et al. (143) proposed a release of leptin from the brain into the blood. Such a mechanism suggested an intrinsic brain source of leptin. Leptin expression in the rat hypothalamus was found to be downregulated by prolonged food restriction, like what has been previously observed in WAT. However, this downregulation could not be abolished by refeeding, indicating that leptin expression by prolonged fasting/refeeding is affected in a different way than in adipose tissue (144). Recently, a conditioned place preference test was used to assess the effect of leptin on the preference of leptin-deficient *ob/ob* mice for HFD. In this study conditioned place preference for HFD is higher among *ob/ob* mice than among wild-type mice (WT). Moreover, leptin replacement was shown to decrease the reward value of HFD and sucrose independently of obesity. Such results suggested that leptin reduces food intake by suppressing the hedonic feeding pathway in *ob/ob* mice (145).

Leptin receptor (LepRb) is known to be expressed in various sites in the brain, namely on the choroid plexus, ventral tegmental area, the ARC and paraventricular nuclei, and the ventromedial and dorsolateral hypothalamus. Importantly, mice with hypothalamic deficiency of LepRb developed early-onset obesity (146). These results indicate that leptin can act directly on LepRb in the CNS, informing various parts of the brain about the amount of peripherally stored energy (147,148). Through the binding and activation of its brain receptor - LepRb -, leptin can decrease food intake while increasing energy expenditure. One of the most surprising features regarding leptin in obese individuals was that its circulating levels are abnormally high in these situations. This situation corresponds to a state defined as hyperleptinemia. Studies in both humans and mice showed that indeed brain leptin transport is impaired in those subjects (149–151). It has been speculated that hyperleptinemia leads to leptin resistance (152). Indeed, leptin resistance is defined by the reduced ability of leptin to suppress appetite and weight gain (75). However, over the past 10 years and as reviewed by Zhou and Rui (75), other mechanisms besides hyperleptinemia have been proposed to explain leptin resistance, including impairment in leptin transport, leptin signaling, and the leptin-targeted neuronal circuits. Thus, defects in LepRb signaling cascade components, such as reduction of LepRb cell surface levels, and upregulation and downregulation of negative and positive regulators, respectively, result in leptin resistance (153,154). Although the core biological mechanisms behind leptin resistance and how it can be induced by overnutrition are currently unknown, HFD-related inflammation has been reported to have a role in leptin resistance (155). On one hand, activation of the hypothalamic IKKβ/NF-κB pathways was shown to induce leptin resistance, as summarized in **Figure 1.2**, whereas inhibition of hypothalamic IKKβ protects against obesity in mice. A recent review by (156), discussed the role of cytokine signaling 3 (SOCS3), a core inhibitor of insulin and leptin signaling, as an important mechanism involved in the appearance of leptin resistance in hyperleptinemic states. Indeed, in HFD-fed mice SOCS3 inhibitory pathway is altered and its expression basal levels were higher than in control groups and were no longer responsive to leptin injection (157). The involved molecular mechanisms may include the control of IKKβ/NF-κB over SOCS3 (135). On the other hand, and regarding ER stress, Ozcan et al. (158) demonstrated

that deletion of an important regulator of ER homeostasis, neuronal X-box binding protein 1 (XBP-1), leads to hypothalamic ER stress and consequently to leptin resistance in the XBP-1 knockout mice (*XNKO* mice). The leptin levels in XNKO mice dramatically increased (not proportionally to the body weight) in the early phases of HFD feeding. Therefore, these results might suggest that contrary to what was initially thought, in HFD, the induced ER stress and/or IKKβ/NF-κB activation could be a reason for the development of leptin resistance, independently of prolonged leptin action and even before the onset of adiposity and hyperleptinemia.

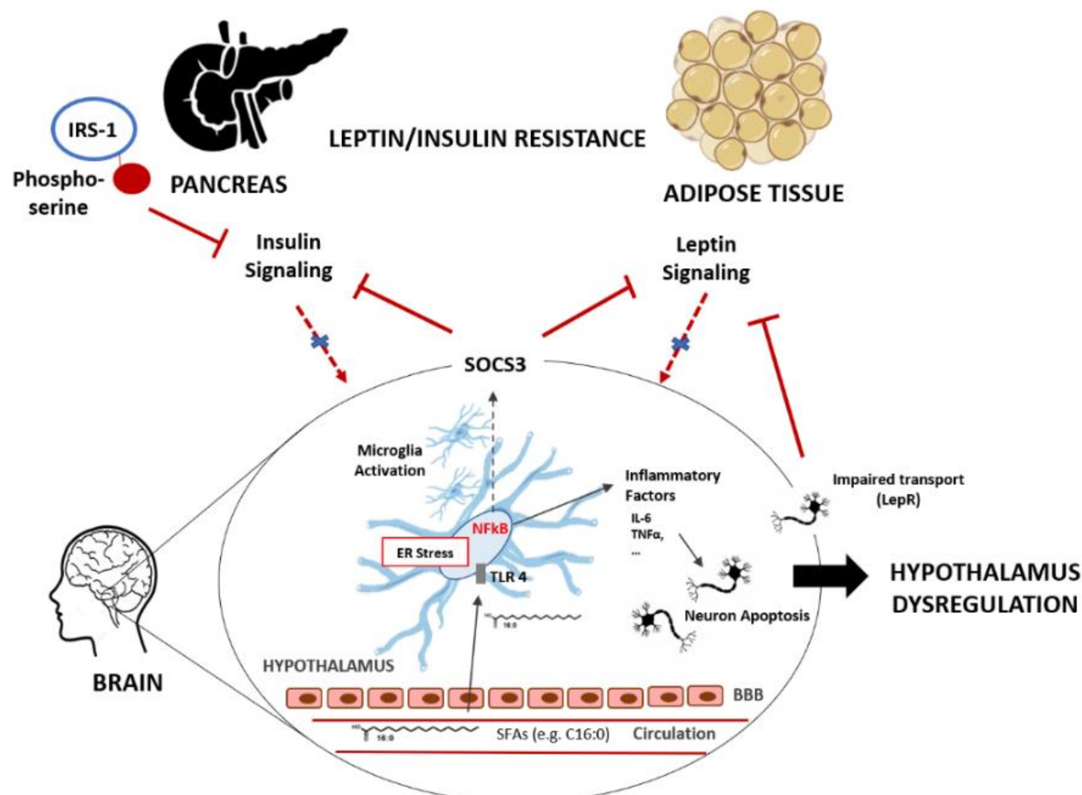

Figure 1.2 - Schematic representation of the hypothalamus dysregulation caused by Saturated Fatty acids (SFAs), e.g., Palmitic acid (C16:0), through TLR4 activation in microglia. The microglia activation and accumulation, as well as the activation of NFkB, results in the production of inflammatory factors, inducing neuron apoptosis and the consequent disruption of the neuronal network. The induced overactivation of NFkB, caused by ER stress, includes the control over suppressor of cytokine signaling 3 (SOCS3), a core inhibitor of insulin and leptin signaling. In addition, the serine residue phosphorylation of insulin receptor (IRS-1) inhibits the insulin signaling in pancreas. In neuronal cells, the leptin receptor (LepR) inhibition, a consequence of an HFD, impairs the leptin transport to hypothalamus and consequently its normal CNS signaling pathway. Those mechanisms result in insulin and leptin resistance (207,352).

2.2.4. Hypothalamic Ghrelin Resistance

Ghrelin is a 28 amino acid peptide hormone acting on the brain to stimulate appetite. It is secreted by endocrine X/A-like cells present in the stomach mucosa, intestinal mucosa, ARC of the hypothalamus, the pituitary, and other tissues. Besides, it is produced in the pancreatic islets, acting as an autocrine/ paracrine growth factor (159–161). It was first discovered by Kojima (162) as the ligand of growth hormone secretagogue receptor type 1a (GHSR1a). Acylation of ghrelin is required for its binding to GHSR and its endocrine, metabolic, and orexigenic actions. Ghrelin binds to GHSR, and the starvation signals to the brain, are transmitted to NPY and AgRP neurons

of the ARC via the vagal afferent pathway, therefore stimulating appetite (137). Thus, when nutrient availability is low, levels of ghrelin increase, and after consumption of a meal, ghrelin levels are decreased. Some investigations point out that ghrelin can be also produced in the hypothalamus, specifically in the ARC (163). GHSR is highly expressed in the hypothalamic cell populations that regulate feeding and body weight, such as ARC AgRP- and NPY-expressing neurons and VMH neurons expressing AMP-activated protein kinase (AMPK) (164–167).

As commented above, obesity is associated with alterations in the hypothalamic neuron population, low physical activity, and ghrelin resistance. Unexpectedly for an orexigenic hormone, a positive energy balance induces ghrelin resistance in humans and rodent models, and obesity is associated with reduced secretion and plasma levels of ghrelin (168–171). Nonetheless, diet-induced hypothalamic inflammation results in the reduction of *Ghsr* expression in the nodose ganglion and hypothalamus of mice, causing impaired transmission of gastric-derived ghrelin signals to the hypothalamus (172). Ghrelin resistance has been demonstrated to be reversible following the reversal of HFD-induced inflammation and obesity phenotypes (173). The diet-induced hypothalamic inflammation is thus demonstrated to have a high impact on ghrelin resistance. The HFD-induced ghrelin resistance mechanisms may also involve ER stress, the AMPK pathway, or targeting rapamycin (mTOR) and k-opioid receptor (KOR) (137). Perez-Tilve et al. (174) demonstrated that ghrelin resistance in response to HFD can occur rapidly and is almost independent of the length of the nutritional intervention. Short-term exposure (12 hours) to HFD is enough to alter the orexigenic effects of ghrelin. Nonetheless, the capacity of ghrelin to modulate lipogenesis in WAT is unaffected by HFD, indicating that different neuronal circuitries mediate ghrelin-specific regulation of food intake and lipid metabolism (137). However, contradictory results were demonstrated by Briggs et al. (175): ghrelin resistance in NPY/AgRP neurons occurs within 3 weeks of HFD feeding in mice. These authors observed that leptin-deficient genetically obese mice (*ob/ob*) are still ghrelin sensitive but become ghrelin resistant when leptin is administered through an intracerebroventricular (icv) injection. These results indicated that ghrelin resistance occurs because of increased plasma leptin (hyperleptinemia) associated with weight gain, instead of acute exposure to an HFD. Nevertheless, the exact mechanism by which leptin prevents ghrelin's effects on NPY neurons remains unknown. These observations reinforce the need for further studies to completely understand the HFD's role in ghrelin resistance.

3. Polyunsaturated fatty acids as a promising anti-obesity therapy: a multi-organ approach

Briefly, PUFAs are unsaturated fatty acids with two or more double bonds. They can be classified into two groups, omega-3 and omega-6, according to the position of the first double bound relative to the methyl-end group. Omega-3 fatty acids have the double bound 3 carbon atoms away from the terminal methyl group and omega-6 have their first double bound 6 carbons away. Omega-3 and omega-6 fatty acids are synthesized from the essential FAs ALA (C18:3 *c*9*c*12*c*15) and LA (C18:2 *c*9*c*12), respectively. These precursors, ALA and LA, cannot be synthesized in the human body and have to be obtained through diet (13). Several *in vivo* studies using rodent models have shown that substitution or supplementation of an HFD rich in SFA by fish oil (rich in PUFAs, specifically omega-3 FAs) has several beneficial effects in both adipose tissue and the hypothalamus. Indeed, omega-3 PUFAs are essential nutrients derived from either marine or vegetable sources. The most relevant omega-3 are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which have a marine origin since they can be found in oily fish, such as salmon, tuna, mackerel, anchovy, and sardines (**Table 1.2**). Although ALA (the vegetable derivative) can be converted into EPA and DHA, the conversion rate is not enough, and a dietary intake of omega-3 PUFAs from their marine sources is needed (176). EFSA has recognized the beneficial effects of omega-3 fatty acids and it recommends dietary intakes of 250 and 500 mg/day of EPA and DHA for European adults based on CVD risk considerations. This is highly relevant considering the role of obesity in the development of comorbidities, such as CVD (177). Their important role in obesity-induced effects is going to be discussed in the next sections.

On the other hand, conjugated fatty acids (CFAs) represent PUFAs with conjugated double bonds, usually found in a mixture of positional and geometric isomers (178). Dietary CFAs triggered great interest in the last decades with isomers of conjugated linoleic acid (CLA) and conjugated linolenic acid (CLNA) being the target of numerous studies due to their bioactive potential (179,180). CLA corresponds to a group of positional and geometric isomers of linoleic acid (LA; *cis*-9 *cis*-12-octadecadienoic acid; C18:2 *c*9*c*12). Although several CLA isomers are found in food, the primary research focus is on the two main isomers: C18:2 *c*9*t*11 and C18:2 *t*10*c*12. In fact, naturally occurring CLA primarily consists of the *c*9*t*11 isomer (>80%) present in food, such as beef, milk, and dairy products, since it is produced by rumen bacteria from LA (181). CLNA isomers refer to a mixture of different linoleic acid (LNA) conjugated isomers occurring naturally in milk fat and meat of ruminants but are mostly found in vegetable oils. Punicic acid (PUA) (C18:3 *c*9*t*11*c*13) is mostly found in pomegranate (*Punica granatum*) seed oil (≈70g of PUA/100g of fat) (182). CLNA isomers share similarities with CLA, such as carbon composition, atomic arrangement, and the number of carbon double bonds (183), and some works suggest that they can exert similar effects to CLA at lower doses. An effective dose of CLA in humans is 3 g/day (184) while for CLNA is 2-3 g/day (185).

The main dietary sources of the mentioned PUFAs – LA, ALA, EPA and DHA, CLA, and CLNA isomers - are described in **Table 1.2**.

Table 1.2 - Polyunsaturated fatty acids (PUFA) main dietary sources (mg of fatty acid/ 100 g of fat).*The fatty acid content of the mentioned fish and ruminant meat products greatly depends on the species, geographical location, food supply, and time of the year, thus the values presented in this table intend to be demonstrative. Table from Salsinha, A. S., Machado, M., Rodríguez-Alcalá, L. M., Gomes, A. M. & Pintado, M. 2023. "Bioactive lipids: chemistry, biochemistry, and biological properties" in "Bioactive lipids". Pintado, M., Machado, M., Gomes, A. M., Salsinha, A. S. & Rodríguez-Alcalá, L. M. (eds.). Cambridge: Academic Press, p. 1-35 35 p. https://doi.org/10.1016/B978-0-12-824043-4.00014-2.

Lipid ID	Main Dietary Sources (≈ g fatty acid/100 g of fat)		References
Linoleic acid (LA -omega 6 fatty acid) and alpha-linolenic acid (ALA-omega-3 fatty acid)	Sunflower oil	54.6-65.7 total PUFA → 54.5-65.7 LA	(186,187)
	Corn oil	54.7 total PUFA → 53.2 LA	(186)
	Soybean oil	57.3 total PUFA → 50.1 LA +7.8 ALA	(186,188)
	Grapeseed	63.6 total PUFA → 63.3 LA	(187)
	Canola oil	26.9 total PUFA → 21 LA + 11 ALA	(189)
	Flaxseed oil	52 total PUFA → 39.9-53 ALA + 12.25-17 LA	(190)
	Walnut	68-69 total PUFA → 56-57.3 LA; 10.8-12.1 ALA	(187)
	Rapeseed oil	35 total PUFA → 23.8 LA+ 11.3 ALA	(191)
Omega-3 PUFA (EPA+DHA)	Mackerel (12-20% fat)*	45.4-48.2 total PUFA → omega-3 PUFA 30.1-43.6 → 7.41% EPA+DHA	(192,193)
	Herring-bloater (13.9-20% fat)*	40.8 total PUFA → omega-3 PUFA 30.2 → 8.63 EPA+13 DHA	(192)
	Sprat (≈17% fat)*	26-37 total PUFA → omega-3 PUFA 22-25.6 → 6.3 EPA+10 DHA	(192,194)
	Salmon (10-13% fat)*	13.5-24.2 total PUFA; n-3 PUFA 0.53-2.4 (0.65 EPA+ 1.8 DHA)	(195–197)
	Sardine (≈14% fat)*	30-49 total PUFA → n-3 PUFA 21-36 → 8.6-18.9 EPA+10.7-32.5 DHA	(198–200)
	Anchovy (8-16% fat)*	29.6-36 total PUFA → omega-3 PUFA 25.7-31.2 → 9.2-11.6 EPA+ 14.7-19 DHA	(201,202)
Conjugated linoleic acid (CLA)	Milk	0.7-1.03 total CLA → *c*9*t*11 isomer 75-90% total CLA content	(203–206)
	Ruminant meat*	0.07-2.97 total CLA	
	Cheese	0.06-1.42 total CLA → 0.14-0.73 *c*9*t*11 isomer →78-84% total CLA content	
Conjugated linolenic acid (CLNA)	Pomegranate seed	≥70 of PUA isomer	(182)
	White mahaleb	40 of α-eleostearic acid isomer	
	Snake gourd (*Trichosanthes kirilowii*)	40 of PUA isomer	
	Milk (bovine)	0.03-0.39 rumelenic acid isomer (C18:3 *c*9*t*11*c*15) + 0.02-0.06 C18:3 *c*9*t*11*t*15 isomer	
	Ruminant meat	0.08-0.28 fat rumelenic isomer (C18:3 *c*9*t*11*c*15) + 0.02-0.03 C18:3 *c*9*t*11*t*15 isomer	

3.1. Acting in the brain (hypothalamus)

In the brain, PUFAs are largely esterified to the phospholipid cell membranes of neurons, glial cells, and endothelial cells (207). As reviewed by Ouyang et al. (208) PUFAs account for 35% of total lipids in the adult brain. Arachidonic acid (AA) (an omega-6 fatty acid) and DHA, which make up 50% and 40% of brain PUFAs, respectively, are important to brain development and maintenance of brain structure and function. In healthy humans, PUFAs can cross the BBB at physiological concentrations by passive diffusion or via a selective protein-mediated transport process by FA transport proteins and FA binding proteins, and cross the plasma membrane, followed by intracellular transport.

3.1.1. Omega-3

Considering the SFA hypothalamic effect presented here and framed within the homeostatic processes, it may be expected that other FAs could be also recognized by specific receptors. In fact, G protein receptor 120 (GPR120), highly expressed in adipocytes and macrophages, is known to bind some FAs. Indeed, some PUFAs, such as the omega-3 fatty acids, ALA, DHA, and EPA are established activators of GPR120 (209). Regarding obesity-induced hypothalamus inflammation, Pimentel et al., (210) demonstrated that fish oil supplementation decreased the levels of hypothalamic pro-inflammatory mediators (*i.e.,* TNF-α and IL-6) and higher levels of anti-inflammatory cytokine IL-1. Besides, the fish supplementation led to normal leptin levels, and improved blood lipid profile. Further studies aiming to assess the effect of lard substitution by fish oil in the feeding of Wistar rats, reported both the reduction of inflammation and apoptosis markers. Besides, the use of fish oil was also associated with lower body weight gain (211). Such relevant anti-inflammatory effects on the hypothalamus are thought to be mediated through the GPR120 receptor. As mentioned, some PUFAs, mostly omega-3 fatty acids - ALA, DHA, and EPA – are proven activators of GPR120 (209). It has been suggested that activation of GPR120 by these omega-3 fatty acids leads to the recruitment of β-arrestin 2 – ubiquitously expressed proteins known for being a canonical G-protein coupled receptor (GPCR) signaling partner. A GPR120- β-arrestin 2 complex is formed and is internalized. Such complex interacts with TGF-β activated kinase binding protein 1 (TAB1), inhibiting its interaction with another protein, the TGF-β activated kinase 1 (TAK1). This inhibition is highly relevant since their interaction mediates downstream inflammatory processes by activating NF-κB and JNK pathways (**Figure 1.3**). Thus, GPR120 activation by omega-3 fatty acids inhibits pro-inflammatory pathways activation, reverting the inflammatory action of SFAs via the TLR4 receptor (212). Furthermore, Oh Da et al. (209) reported that DHA stimulation of GPR120 inhibits both TLR 2/3/4 and the TNF-α proinflammatory cascade. Moreover, Wellhauser and Belsham (213) studied the gene expression levels of proinflammatory cytokines in rHypoE-7 hypothalamic neuronal cells, upon exposure to TNF-α treatment in the presence or absence of DHA. Those authors concluded that

translational and transcriptional inflammatory response triggered by TNF-α exposure resulted in abundant GPR120 expression levels since it is functionally responsive to DHA. Nevertheless, the inflammatory state was prevented by DHA pretreatment, since GPR120 was activated thereby reducing the inflammatory response to TNF-α. The positive effects of omega-3 fatty acids on both weight management and their anti-inflammatory potential have attracted great interest in obesity therapy development. Recently, male C57BL/6J mice were used as the model to determine the beneficial central effects and mechanism of DHA (by icv injection) in HFD-fed mice. The authors reported that DHA administration reduced both energy intake and body weight gain. Moreover, it ameliorated the HFD-induced hypothalamic inflammation and improved the central leptin's action in regulating hepatic lipid metabolism (214). Using the same *in vivo* model, another study observed that fish oil supplementation also protects mice against the anxiogenic and depressive-like effects of HFD (215). Importantly, besides reversing the changes in the inflammatory state, omega-3 treatments are also able to reverse the oxidative damage parameters and attenuate the alteration in the antioxidant defense and energy metabolism (216). Some *in vitro* studies have also reported relevant results in microglia cell lines: DHA was able to reverse LPS inflammatory effects in N9 microglia cells (217,218) and is responsible for a reduction in ROS production (oxidative stress) in BV-2 microglia cells (219). All these results have shown that omega-3 fatty acids, especially EPA and DHA, present a complete action tackling the obesity-induced effects on the hypothalamus, addressing both hypothalamic inflammation and the neuronal damage in key brain areas for body weight control. In conclusion, the PUFA's beneficial effect on obesity-induced hypothalamic inflammation is thought to be closely related to their role in inhibiting the IKKβ/NF-κB pathway by GPR120 activation.

Recently, omega-3 has also demonstrated neurogenesis activities (preferentially in POMC neurons) when administered as FAs (icv injection) or assayed in diets using Swiss mice (220). Indeed, PUFAs were observed to increase hypothalamic neurogenesis to levels like or even higher than the ones induced by the brain-derived neurotrophic factor (BDNF), a well-described factor responsible for inducing hypothalamic neurogenesis (220). Interestingly authors associated the neurogenic activities to GPR40 and not to GPR120, as previously discussed. In fact, GPR120 was found to be expressed predominantly in microglial whereas GPR40 was in POMC and NPY neurons. Indeed, in a study performed by Dragano et al. (221) the use of GPR120 and GPR40 agonists showed that while the first acted predominantly by reducing hypothalamic inflammation, the latter acted by reducing body mass and increasing POMC expression. Such results suggest that the combined activation of both receptors in the hypothalamus may result in better metabolic outcomes.

Moreover, lard substitution by fish oil (rich in omega-3 FAs) in the feeding of male Wistar rats resulted in the reduction of inflammation and apoptosis markers. In this work, the utilization of fish oil led to lower body weight gain compared with lard, as well as decreased phosphorylation of AMPK (decreased activation) (211). Indeed, AMPK, a serine/threonine kinase activated by phosphorylation, acts as a central nutrient sensor involved in glucose uptake and lipogenesis

among other metabolic functions, contributing to homeostasis (222). In another study by Pimentel et al. (210), HFD enriched with either soy (rich in omega-6 FAs) or fish oil was compared by evaluating insulin hypophagia and hypothalamic signaling after insulin injection. In contrast to what was found in the soy-rich diet, fish oil supplementation showed a decrease in the TRAF6, TNF-α, and IL-6 hypothalamic proinflammatory mediators' levels, while showing increased anti-inflammatory cytokine IL-1 levels. Besides, the fish group showed normal fat pad weight and leptin levels, as well as improved blood lipid profile. In addition, reduced levels of corticosterone, a feature that is known to favor insulin sensitivity, were detected in the fish group. In a recent study, evaluating the potential of fish oil in the reversion of a depression-induced state in rats, Dang et al. (223) observed that interestingly, fish oil supplementation attenuated the induced abnormal behavior and brain inflammatory response. Furthermore, fish oil supplementation also restored the neurochemical disturbance associated with induced depression. It suppressed the expression of proinflammatory mediators and oxidative stress by inhibiting NF-κB and the inducible isoform of Nitric oxide synthases (iNOS). Thus, the well-studied anti-inflammatory effect of omega-3 FAs of marine origin EPA and DHA is thought to involve the inhibition of the phosphorylation of the inhibitory subunit of NF-κB – IκB – trapping, as a result, NF-κB in its active trimeric state on the cytosol. These effects are thought to be mediated by membrane-bound GPR120 (224). **Table 1.3 (1.3a and 1.3b)** summarizes results found in several studies that reinforce the possible beneficial effect of dietary omega-3 fatty acids on neuroinflammatory diseases, such as obesity.

Table 1.3a - Summary of the most recent (last decade) and relevant studies on the biological action of omega-3 fatty acids (mainly EPA and DHA) in obesity and brain-associated mechanisms.

Studied FA/Source	Experimental model	Study Objective	Main Results	Reference
Saturated FAs - Lauric acid (C12:0), Palmitic acid (C16:0) and Stearic acid (C18:0)- and polyunsaturated FAs -DHA (C22:6(n-3)).	Primary astrocytes derived from Sprague-Dawley rats	Effect of long-chain fatty acids on inflammatory signaling in cultured astrocytes.	Palmitic acid, lauric acid and stearic acid, trigger the release of TNF-α and IL-6 from astrocytes. DHA acts in a dose-dependent manner to prevent the actions of palmitic acid on inflammatory signaling.	(225)
Flax seed oil (rich in C18:3), Olive oil (rich in C18:1) and omega-3 and omega-9 fatty acids	Male Wistar rats and Male Swiss mice	Evaluate the effects of unsaturated fatty acids on hypothalamic inflammation in obesity.	Unsaturated fatty acids can act either as nutrients or directly in the hypothalamus, reverting diet induced inflammation and reducing body adiposity. omega-3 and omega 9 fatty acids activate signal transduction through GPR120.	(226)
Soy oil (omega-6 PUFAs) and fish oil (omega-3 PUFAs)	Male Wistar rats	Effect of high-fat PUFA diets on the expression of proteins involved in inflammatory pathways in hypothalamus and other organs (muscle and tissue).	Soy diet induced local stimulation of the NF-κB pathway. Fish diet diminished hypothalamic levels of TRAF6 and of the inflammatory cytokines TNF-α and IL-6, along with enhanced anti-inflammatory IL-10 cytokine levels.	(227)
Soybean oil (omega-6 PUFAs), fish oil (omega-3 PUFAs) and hydrogenated vegetable fat (saturated and *trans* FA)	Female Wistar rats	Influence of soybean oil, fish oil and hydrogenated vegetable fat on preference parameters for amphetamine.	Only fish oil did not show any anxiety-like symptoms or increased locomotion; it was related to lower oxidative damages to proteins and increased catalase activity in striatum and hippocampus.	(85)
DHA	rHypoE-7 rat hypothalamus cells	GPR120 activation at the level of individual neurons upon exposure to TNF-α in the presence or absence of DHA.	DHA pretreatment prevents the inflammatory state and this effect was inhibited by the reduction of endogenous GPR120 levels.	(228)
Omega-3 FAs	Sprague-Dawley rats and hypothalamic cells from obese rats	Effect of omega-3 fatty acids on brain derived neurotrophic factor (BDNF) gene expression.	Omega-3 FAs in vivo assays showed to reverse the negative effect that obesity has on BDNF gene expression: decreased in serum total cholesterol and TAG. In vitro omega-3 FAs present an increase in BDNF expression.	(229)
DHA	Male Swiss albinus mice	Potential of omega-3 PUFAs, in diet or by local injection, to induce hypothalamic neurogenesis.	Omega-3 PUFAs increase neurogenesis in the hypothalamus, accompanied by reduction of apoptosis markers, increased responsiveness to leptin, and reduced body mass gain.	(220)
Fish oil (omega-3 PUFAs)	Male Wistar rats	The potential of PUFA beneficial effects being mediated by AMPK in the hypothalamus	Substitution of saturated by unsaturated fatty acids diet (omega-3) has beneficial effects on modulation of hypothalamic inflammation and function in obesity.	(211)
	Male Spraque-Dawley rats	Effects of fish oil supplementation on induced behavioral changes, inflammatory cytokine expression and oxidative reactions in frontal cortex and hippocampus.	Fish oil supplementation attenuated the induced abnormal behavior, the brain inflammatory response, the induced oxidative reactions and neural apoptosis. It restored the neurochemical disturbance.	(223)

Table 1.3b - Summary of the most recent (last decade) and relevant studies on the biological action of omega-3 fatty acids (mainly EPA and DHA) in obesity and brain-associated mechanisms.

Studied FA/Source	Experimental model	Study Objective	Main Results	Reference
Krill oil (omega-3 PUFAs) and Buttermilk fat globule membranes (BMFC)	Wistar rats	Effect of dietary bioactive phospholipid concentrates of krill oil and/or BFMC on insulin signaling, mitochondrial activity and biogenesis, and synaptic signaling in the hippocampus and cortex.	Dietary supplementation with krill oil and BFMC improves peripheral and central insulin resistance, and the energy state within neurons, and facilitates both mitochondrial and protein synthesis, necessary for synaptic plasticity.	(230)
Fish oil	Male Swiss mice	Evaluate the effects of omega-3 on inflammation, oxidative stress, and energy metabolism parameters in the brain of mice subjected to HFD-induced obesity model.	Omega-3 treatment partially reversed the changes in the inflammatory and oxidative damage parameters and attenuated the alterations in the antioxidant defense and energy metabolism.	(216)
DHA	Male C57BL/6J mice	Determine the beneficial central effects and mechanism of DHA (by icv injection) in HFD-fed mice.	Icv administration of DHA reduced energy intake body weight gain and HFD-induced hypothalamic inflammation. Improved leptin JAK2 and Akt signaling pathways in the hypothalamus. Central leptin's action in regulating hepatic lipid metabolism can be improved by central DHA administration.	(214)
Fish oil (equal amounts of DHA and EPA)	Male C57Bl/6J mice	Study the effects of omega-3 PUFA supplementation on energy homeostasis, anxiodepressive behavior, brain lipid composition, and gliosis in diet-induced obesity.	Fish oil supplementation also defended against the anxiogenic and depressive-like effects of HFD. Brain lipids, particularly anti-inflammatory PUFA, were diminished by HFD, whereas FO restored levels beyond control values.	(215)
DHA	Male C57BL/6J mice	Determine the beneficial central effects and mechanism of icv injection of DHA in HFD-fed mice.	Icv administration of DHA reduced energy intake and body weight gain and corrected the HF diet-induced hypothalamic inflammation: decreased leptin signaling inhibitor SOCS3 improved the leptin JAK2-Akt signaling pathways in the hypothalamus.	(231)
DHA-TG and DHA-ethyl ester	Male C57BL/6J mice, male *ob*/*ob* mice (C57BL/6J background) and wild-type (WT) mice	Investigate if triacylglycerol (TAG) rich in DHA (DHA-TG) can regulate appetite in mice fed with a high-fat and high-sugar diet and describe the mechanisms behind this process.	DHA-TG can reduce food intake and regulate neuropeptides (POMC, AgRP, and NPY) expression in the experimental model, by ameliorating leptin secretion and attenuating central leptin resistance. It prevents damage to the intestinal epithelial barrier by improving leptin sensitivity and protecting intestinal endocrine function. However, it was ineffective in repressing appetite and improving gut leakage in leptin-deficient mice (*ob*/*ob* mice).	(232)
Fish oil (omega-3 source) and Sunflower oil (omega-6 source)	Female Pitman-Moore miniature pigs	Investigate the effect of central consequences of the substitution of saturated fatty acids (lard diet) with omega-3 or omega-6 PUFA in obesogenic diets	BBB permeability was higher in the lard group. Brain connectivity analysis and voxel-based comparisons show regional differences between groups. BBB's decreased permeability in omega-3 and omega-6 PUFAs groups was protective against an obesity-driven damaged BBB	(233)

3.1.2. Conjugated Linoleic Acid (CLA)

As reviewed by Rodríguez-Alcalá et al. (206) several biological activities have been attributed to CLA: it has been shown to reduce cancer in several animal models; to reduce atherosclerotic lesions in rabbits and hamsters; and to reduce total cholesterol, triacylglycerides, low-density lipoprotein-cholesterol (LDL-cholesterol) and increased high-density lipoprotein-cholesterol (HDL-cholesterol) in several animal models. Furthermore, the anti-obesity effects of CLA are well-studied in different animal models since it has been shown to increase lean body mass and reduce body fat mass. Although its effect on peripheral tissues, such as adipocyte tissue has been widely assessed as it is going to be discussed in **Section 3.2.2** of this Chapter, and its ability to reduce body fat in animals was first reported in 1995 (234), several other beneficial health effects have been attributed to it. In a study aimed at assessing the effect of CLA on ameliorating colitis, it was found that CLA exerted anti-inflammatory properties by repressing TNF-α expression and NF-κB activation while inducing the expression of the immunoregulatory cytokine transforming growth factor β1 (TGF-β1). The anti-inflammatory CLA action was reported to be mediated by PPAR (Peroxisome proliferator-activated receptor) γ and δ induction. Indeed, the loss of the PPAR γ gene in the colon was found to cancel the beneficial effects of CLA in induced colitis (235). PPAR (α, β/δ, and γ) are nuclear receptors that translate nutritional and/or pharmacologic stimuli into changes in gene expression. They were originally described as components of adipocyte gene expression differentially regulating lipid homeostasis (236,237). In further studies, PPARs were shown to be involved in the regulation of inflammation, immunity, and epithelial cell differentiation (235,238–242). Some *in vitro* studies concluded that dietary PUFA and their metabolites are endogenous PPAR γ ligands (243). CLA has been previously demonstrated as being able to activate PPAR γ eliciting *in vivo* effects consistent with PPAR γ activation, namely on the reduction of the inflammatory response (244,245).

Although the beneficial effect of CLA on several peripheral tissues is well documented, namely on reducing body fat (246,247), CLA isomers incorporation in the brain has been detected in a few cases at very low concentrations (248,249). Specifically, C18:2 *c*9*t*11 and C18:2 *t*10*c*12 were demonstrated to be actively incorporated in rat brains and *in vitro* astrocyte cultures (250). After icv administration of CLA, Cao et al. (252) reported that food intake was inhibited in rats. This effect was shown to be related to decreased mRNA expression of NPY and AgRP. Besides, promising results have been shown regarding decreased serum leptin levels in rats following CLA treatment (253,254). Acute and chronic activation of CNS PPAR γ led to positive energy balance and restored leptin sensitivity in HFD-fed rats (255). Recently, CLA was demonstrated to bind to PPAR α, a nuclear receptor key regulator of FA metabolism and inflammatory responses. Thus, it was suggested that after their incorporation, CLA isomers are metabolized into brain tissue (mouse brain) where they induce the biosynthesis of endogenous PPAR α ligands palmitoylethanolamide (PEA) and oleoylethanolamide (OEA), possibly through positive feedback (251). As reviewed by Murru et al. (251) OEA and PEA are natural ethanolamides of oleic acid

and PA, respectively. OEA reduces food intake and body weight gain in obese rats, stimulates lipolysis and fatty acid oxidation, and reduces the content of TGs in both liver and adipose tissue. Since PPARs, specifically PPAR α, are important regulators of inflammatory responses, CLA anti-inflammatory actions in CNS are possibly connected to the activation of such factors. Indeed, PPAR α anti-inflammatory action is mediated through its repressive action on many activated transcription factors, such as NF-κB, among others. Moreover, CLA may also be able to ameliorate oxidative stress by increasing peroxisomal β-oxidation acting as well as an antioxidative factor. Despite the promising results that have emerged over the last few years regarding a potential beneficial effect of CLA in the brain, few studies have specifically targeted the anti-obesogenic effect of CLA isomers on CNS, especially on hypothalamic inflammation. Since it is known the presence of PPARs or GPRs (GPR120 and GPR40) in different brain areas, the beneficial effect of CLA could be achieved through specific PPAR-mediated differentiation pathways or as reported for omega-3 through GPR120 action.

The above-discussed anti-inflammatory and anti-proliferative activities linked to CLA could impact positively neurological diseases, including obesity, where inflammatory response contributes to the pathogenesis. Fa et al. (250) have found that indeed, CLA isomers C18:2 *c*9*t*11 and C18:2 *t*10*c*12 are actively incorporated and metabolized in rat brains and in *in vitro* astrocyte cultures. Since it is known the presence of PPARs in different brain areas, the beneficial effect of CLA could be achieved through specific PPAR-mediated differentiation pathways. Moreover, after icv administration of CLA, Cao, and collaborators reported that food intake was inhibited in rats (252). This effect was shown to be related to decreased mRNA expression of NPY and AgRP. Importantly, such inhibition was not repeated by other unsaturated FA, indicating a CLA-specific action. Besides, promising results have been shown regarding decreased serum leptin levels in rats following CLA treatment (253,254). Acute and chronic activation of CNS PPAR γ led to positive energy balance and restored leptin sensitivity in HFD-fed rats (255). Nevertheless, as demonstrated in **Table 1.4**, despite the promising results that have emerged over the last few years regarding a potential beneficial effect of CLA in the brain, few studies have specifically targeted the antiobesogenic effect of CLA isomers on CNS, especially on hypothalamic inflammation. Some contradictory results have been reported: when C18:2 *t*10*c*12 CLA was added to cell cultures it increased PPAR γ gene expression and activation, NF-κB activation, and expression of TNF mRNA. Although there was an upregulation of PPAR γ, the studied CLA isomer acted in a proinflammatory manner (224). However, in the presence of lipopolysaccharide (LPS), the NF-κB activation was decreased and so were the TNF mRNA levels. In the presence of such inflammatory stimulus, C18:2 *t*10*c*12 CLA acted in an anti-inflammatory manner (256). These results suggest that indeed FAs may present different actions depending on the exact conditions that prevail. In a study performed by Wargent and collaborators (257) treatment of leptin-deficient genetically obese mice (*ob/ob*) with CLA showed to initially decrease but subsequently increase insulin sensitivity, suggesting that in the short term another mechanism, namely the reduction of adipocyte number, and consequently plasma adiponectin concentration, may decrease insulin

sensitivity. Therefore, although recent research has evaluated the role of some CLA isomers, there is still a need to better understand the isomer-specific effects of CLA. Besides, some safety concerns and contradictory results regarding the use of CLA in humans increase the need for further investigations (247). A summary of the available studies on the CLA isomers' action in the brain is illustrated in **Table 1.4**.

Table 1.4 - Summary of the available studies on the biological action of conjugated linoleic acid (CLA) isomers in the brain.

Studied FA	Experimental model	Study Objective	Main Results	Reference
***c*9*t*11 and t10c12 CLA isomers**	Female Sprague-Dawley rats and in vitro culture of astrocytes	Accumulation and metabolism of CLA in the brain.	CLA isomers were incorporated and metabolized in rat brain	(250)
	Sprague-Dawley rats	Hypothalamic effect of CLA by icv injections.	CLA can inhibit food intake by decreasing the expression of NPY and AgRP.	(252)
	Female Long Evans rats	Effect of oral administered CLA on CNS vasculature.	CLA administration significantly reduces angiogenesis in the cerebellum by decreasing pro-angiogenic growth factors and their receptors.	(258)
	Male mice Jcl: ICR strain	Effect of CLA Triacylglycerol oil on brain endocannabinoid content (EC).	CLA influences brain EC system by reducing the amount of 2-AG in the cerebral cortex.	(259)
	Male ICR mice	The effect of *t*10, *c*12 CLA on energy intake and body weight composition is confounded by dietary fat concentration and involves hypothalamic appetite-controlling mechanisms.	The Hypothalamic proopiomelanocortin (POMC) and AMP-activated protein kinase α2 elevated mRNA expression was suppressed due to CLA treatment.	(260)
	Male ICR mice	Effect of dietary CLA brain lipids incorporation in appetite-regulation neuropeptide expression and reductions in feed intake and body fat.	No CLA isomer was detected in the brain nor was any change in the brain lipid profile	(261)
	Mice	Effects of dietary CLA intake on CNS autoimmunity.	CLA supplementation suppresses CNS autoimmunity and reduced CNS inflammation	(262,263)
CLA isomers not specified	Neural Stem cells	Effect of CLA on neural stem cell differentiation.	CLA promotes neuronal cell differentiation and arrests the cell cycle by activating cyclin-dependent kinase inhibitors.	(264)
***c*9*t*11 CLA isomer**	Male C57bl/6 mice	Effect of supplementation with cheese enriched with CLA and omega-3 ALA on the metabolism and lipid profiles. The analysis was conducted also in brain tissues.	Supplementing cheese enriched in CLA and ALA reduced the level of SFA and increased the content of CLA and ALA in all tissues (liver, muscle and adipose tissue), except for the brain. But there is the reduction of inflammatory genes, including genes (*CERK*) involved in neurodegenerative diseases.	(265)

3.1.3. Conjugated Linolenic Acid (CLNA)

Similar biological activities to CLA have been attributed to CLNA: some studies reported a cytotoxic effect of CLNA isomers on different human tumor cell lines and antioxidant activity since a reduction in lipid peroxidation was observed. Moreover, CLNA isomers have also been described to exert positive effects on body weight (182).

Regarding CLNA action on CNS, specifically on hypothalamus inflammation, very few studies have addressed such a possibility. Research work determined that CLA is converted into CLNA in rat brains (250). The neuroprotective potential of pomegranate seed oil (a known source of PUA) in HFD-obese mice was reported as shown in **Table 1.5** (266). Indeed, in a study aiming to assess the effect of pomegranate seed oil (source of PUA) on BV-2 microglial cell activation, the authors demonstrated that the pomegranate seed oil did not suppress the intracellular oxidant generation and did not influence the intracellular distribution of cholesterol. However, the morphology of activated cells was affected. The authors suggested that pomegranate seed oil may have an immunomodulation and cytoprotecting potential in BV-2 cells comparable to omega-3 PUFAs (267). However, considering that the effect of CLNA isomers on the brain is largely unknown and although some promising results have emerged regarding assays on adipocyte cells showing a potential antiobesogenic role, there is the need for further investigations, specifically on human subjects, as well as the potential adverse health effects. The described

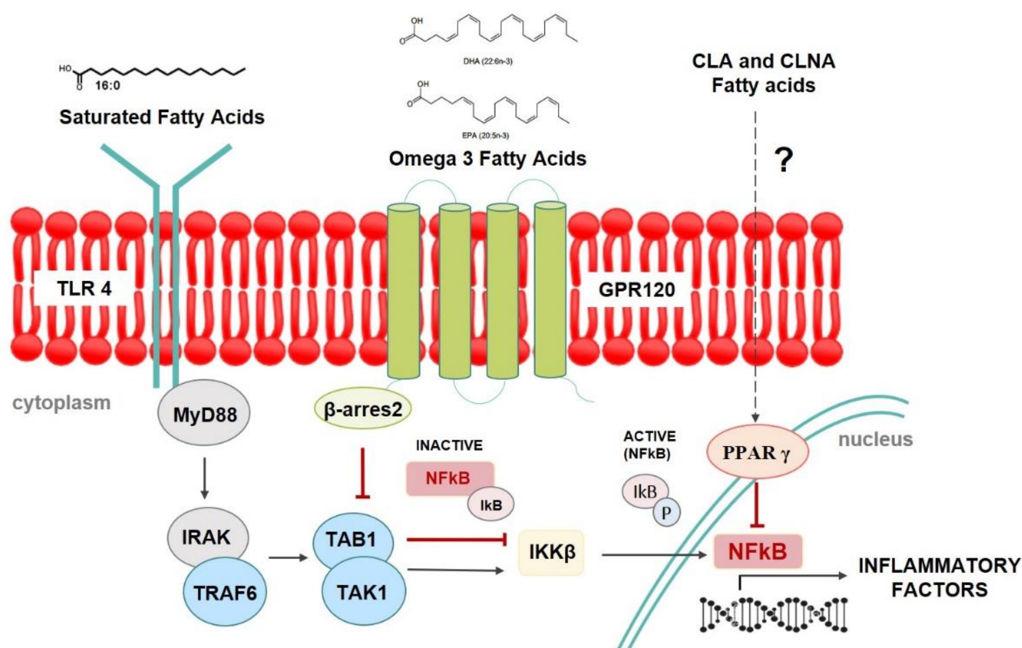

Figure 1.3- Molecular mechanisms behind SFA induced inflammation and anti-inflammatory effect of PUFAs. Stimulation of -proinflammatory signaling pathways by SFAs is a result of TLR4 activation. The activation of Myd88 leads to TAK1 activation and consequent interaction with TAB1 resulting in NF-κB activation. Omega-3 fatty acids, such as EPA and DHA, stimulate GPR120 inhibiting TLR4-proinflammatory cascade. GPR120 stimulation specifically inhibits TAK1 phosphorylation and activation, by interacting with TAB1 (109). Regarding both CLA and CLNA isomers, several studies have reported their anti-inflammatory potential in adipose tissue, which is mediated by PPARs. Nevertheless, regarding the obesity-induced hypothalamic inflammation process, despite some promising results, there is still the need to further clarify CLA, and especially CLNA isomers' anti-inflammatory actions in the hypothalamus. Since it is known the presence of PPARs in different brain areas, the beneficial effect of those fatty acids in the hypothalamus, specifically, is hypothesized as being modulated through specific PPAR-mediated differentiation pathways.

molecular mechanisms behind SFAs' action in the hypothalamus as well as the anti-inflammatory mechanisms presented by omega-3 and CFAs are described in **Figure 1.3**.

Table 1.5 - Summary of the available studies on the biological action of pomegranate seed oil (PUA source) in the brain.

Studied FA/Source	Experimental model	Study Objective	Main Results	Reference
Pomegranate seed oil (omega-5 fatty acids source)	BV-2 microglial cells	Effect of Pomegranate seed oil (omega-5 rich oil, including PUA) in immunomodulation and cytoprotection of BV-2 microglia cells.	No notable suppression of the intracellular oxidant generation and no influence on the intracellular distribution of cholesterol. But affected the morphology of activated cells.	(267)
Pomegranate extracts	High-fat-high fructose diet induced-obese rat	Investigate the beneficial effects of Pomegranate seed oil, leaves, juice, and peel on brain cholinesterase activity, brain oxidative stress, and lipid profile.	Neuroprotective effects of pomegranate extracts by inhibition of cholinesterase and the stimulation of antioxidant capacity	(266)

3.2. Modulation of adipose tissue

3.2.1. Omega-3

In the last decades, several studies have reported that omega-3 PUFAs can significantly decrease body weight and fat mass. Such results are extremely relevant from an obesity perspective. Nevertheless, others reported that a significant action on body weight cannot be found. Instead, omega-3 fatty acids were suggested to only act by reducing fat depots. Contradictory results have been reported: some studies described no change in body weight or fat mass (268). Despite these contradictory results regarding omega-3-specific action in body weight and fat mass results, promising results have been reported. Relevantly, omega-3 can present a positive effect in ameliorating the negative effects associated with obesity. Accordingly, a high dose of omega-3 PUFA supplementation (4 g/day) was provided for 3 months to insulin resistance patients with obesity. The omega-3 supplementation was able to modulate significant changes in plasma fatty acid profile, adipose tissue, and systemic inflammation. Moreover, significant improvement in insulin-stimulated glucose disposal was also reported (269). Besides, a wide range of studies have reported a TG-lowering property which is highly supported by human trials (268). Furthermore, favorable effects on glucose metabolism and insulin sensitivity (268) have also been reported.

Moreover, the effects of omega-3 PUFAs specifically on adipose tissue have been documented regarding the regulation of adipocyte inflammation, differentiation, and apoptosis, as well as effects on lipid storage and mobilization, mitochondrial biogenesis, and adipose tissue browning and adipokines production (268). Similarly, to what happens in hypothalamus cellular models, EPA and DHA attenuate inflammatory activation of *in vitro* human adipocytes (270). Some studies reported that EPA and DHA were shown to be able to regulate adipocyte differentiation by inhibiting its differentiation and proliferation processes (271). Besides, a direct relation with *Ppar γ* gene regulation has been suggested and it was demonstrated that at least part of the action mediated by these fatty acids occurs through PPAR γ (272). Another well-known effect of omega-3 fatty acids on adipose tissue is related to their effect on lipid storage and mobilization. It is important to consider that the accumulation of TGs in adipocytes is a result of a balance between lipolysis - hydrolytic cleavage of ester bonds in TGs, resulting in the release of fatty acids and glycerol (68) - and fatty acid oxidation and lipogenesis - *de novo* lipogenesis is the process by which carbon precursors of acetyl-CoA are synthesized into fatty acids (273). The TG storage in adipocytes can be a result of dietary fatty acid uptake or *de novo* fatty acid biosynthesis (268). Omega-3 supplementation can modulate hepatic *de novo* lipogenesis. Omega-3 fatty acids (EPA and DHA) both *in vivo* and *in vitro* have been shown to decrease hepatic lipogenesis and increase fatty acid oxidation and plasma glucose concentration (274). In WAT, omega-3 PUFAs' action on lipogenesis has been related to their modulatory action on specific lipogenic enzymes. Indeed, in 3T3-L1 adipocytes there was a suppression of lipid droplet formation in the presence of EPA when compared to either SFA or MUFA. EPA was demonstrated to suppress PPAR γ, Cidea – a protein

highly localized in lipid droplets important for fatty acid esterification and lipid mobilization - and D9D/SCD1 – a desaturase required to convert SFA to MUFA that participates in lipid metabolism in adipocytes - gene expressions, while maintaining the expression of lipolytic genes: LPL and HSL (275). Another study, assessing the impact of omega-3 EPA, docosapentaenoic acid (DPA), and DHA on lipid droplet formation in 3T3-L1 adipocytes reported that all three PUFAs significantly reduced lipid droplet formation and the metabolic disorder marker, SCD1. DHA significantly increased lipolysis and ATGL gene and protein expression but reduced the gene expression of three proteins that are related to lipid droplet formation: the mentioned Cidea, Perilipin-A, and Caveolin-1 (276). *In vivo,* studies using rats reported that fish oil supplementation for 2 weeks in rats fed with a high sucrose diet suppresses FAS (involved in the *de novo* biosynthesis of FAs) mRNA levels in BAT (277). These studies support the importance of omega-3 PUFAs in downregulating lipogenic gene expression and consequently decreasing lipogenesis and fat accumulation.

As discussed in previous sections, insulin resistance is strictly linked to inflammatory pathways. This inflammatory state is intimately associated with ER stress, ROS production, and mitochondrial function impairment (278). Importantly, the association between adipose tissue mitochondrial dysfunction and the progression of obesity and T2DM has been proved. Indeed, the lipid oversupply from chronic overfeeding, characteristic of obesity, has been linked to a negative effect on several organelles, namely ER and mitochondria. There is a reduction in the abundance of adipocyte mitochondrial number and an impaired mitochondrial function which leads to reduced fatty acid β-oxidation and therefore fat accumulation (268). Moreover, since mitochondrial dysfunction is intrinsically connected to ER stress a role in insulin resistance has also been suggested. Mitochondrial morphology is highly variable, and it is maintained through a dynamic balance between fusion – regulated by mitofusins 1 and 2 (Mfn1 and Mfn2) - and fission processes, which allow mitochondria to redistribute in a cell, exchange contents, and repair damaged mitochondria. Omega-3 PUFA presents a positive modulatory effect on Mfn2, which may be related to the induction of fusion processes linked to amelioration of mitochondrial function (279). Even when compared to oleic acid, DHA maintained a healthy mitochondrial structure under induced inflammation on primary adipocytes while oleic acid led to elongated mitochondria with thin thread-like structures in adipocytes exposed to LPS (280). In summary, omega-3 fatty acids, opposing to SFAs, stimulate mitochondrial function and fusion processes reducing ROS production, they are also able to attenuate ER stress. Moreover, they present a positive modulatory effect on Mfn2 which may explain the induction of fusion processes that are linked to the amelioration of mitochondrial function and maintenance of mitochondria-associated ER membrane (MAM) integrity, which is responsible for efficient communication between these organelles exchanging calcium ions, lipids, and other metabolites to maintain cellular metabolism and integrity. This is highly relevant since both processes are important for insulin sensitivity (279). Besides, several pieces of evidence suggest that omega-3 PUFAs can counteract the adipokine dysregulation that occurs in obesity (268).

3.2.2. Conjugated linoleic acid (CLA)

The ability of CLA isomers to reduce body fat mass in *in vivo* models was first reported in 1995 (234) and later confirmed by several studies (246,247). As reviewed by Shen and McIntosh (281) the C18:2 *t*10*c*12 CLA isomer is the major responsible for CLA's antiobesity effects and its antiobesity mechanisms are thought to include decreased adipogenesis and lipogenesis, increased lipolysis and fatty acid oxidation, inflammatory signaling, adipocyte apoptosis, increased energy expenditure and browning. Moreover, maternal supplementation 10 days before mating and throughout pregnancy/lactation of CLA to an HFD showed beneficial effects in adult male offspring namely on physiological, metabolic, and adipogenic markers. Interestingly, maternal CLA supplementation was shown to be sufficient to prevent the programmed obesity and metabolic impairment induced by HFD (282). In addition, maternal supplementation of CLA is also responsible for a reduction in TG levels related to a reduction of FAS, acetyl-CoA carboxylase (ACC), and glucose-6-phosphate dehydrogenase enzyme activities. A reduction of lipogenesis was also found in the liver of the offspring. Such results reinforce the positive role of CLA on obesity-induced effects, namely the programming effect of CLA on the lipid metabolic pathways leading to a preventive effect on the TGs accretion in adipose tissue and liver of male rat offspring (283). These effects were partially related to a decrease in adipocyte size and cell number by alteration of transcription of key adipogenic genes and adipose cellularity in adipocytes isolated from specific pathogen-free chicken. Indeed, the C18:2 *c*9*t*11 CLA isomer was shown to downregulate the expression of LPL – a fat metabolism-related gene - and acyl-coenzyme A binding domain containing 5 (ACBD 5) genes (284).

These beneficial effects attributed to CLA isomers are intrinsically linked to their anti-inflammatory potential. For instance, the ameliorating effect of CLA on colitis was found to be related to its anti-inflammatory action on TNF-α and NF-κB pathways. Recently, the C18:2 *t*10*c*12 isomer was the one showing a homogenous reduction of the studied pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β), which suggests a more balanced and efficient physiological activity and possibly a better protective potential (285). The anti-inflammatory CLA action was reported to be mediated by PPAR γ and δ induction (285,286). CLA has been previously demonstrated as being able to activate PPAR γ eliciting *in vivo* effects consistent with PPAR γ activation, namely on the reduction of the inflammatory response (244,245). Besides PPARs, some *in vitro* studies have reported that CLA isomers may activate some GPR receptors, namely GPR120 and GPR40, and thus such receptors may mediate some of their intracellular action in WAT (281).

Nevertheless, caution is necessary when assessing the possible anti-obesogenic role of CLA isomers since contradictory results have been reported. For instance, in a study where C18:2 *t*10*c*12 CLA was added to cell cultures although it increased PPAR γ gene expression it acted in a proinflammatory manner since it upregulated NF-κB and TNF gene expression (224). However other studies reported that when in the presence of an inflammatory stimulus, such as LPS, the same CLA isomers acted in an anti-inflammatory manner (256). Moreover, in *in vivo* studies in

genetic leptin-deficient obese mice, CLA increased insulin sensitivity (257). Thus, further studies are required to fully characterize the anti-obesogenic effects of CLA isomers, especially their role in hypothalamus obesity-induced inflammation.

3.2.3. Conjugated linolenic acid (CLNA)

An antiobesogenic role has also been discussed for CLNA isomers, specifically PUA, in adipose tissue. Vroegrijk and colleagues (287) reported that PUA can improve peripheral insulin sensitivity without affecting liver insulin. Moreover, using a commercial source of PUA, Xanthigen®, 3T3-L1 adipocyte differentiation and lipid accumulation were suppressed due to a decrease in PPAR γ expression levels. The authors hypothesized that PPAR γ being a regulator of adipogenesis and being necessary for differentiation, a decrease in its expression is beneficial in adipocyte cells (288). Nevertheless, other studies have reported that PUA specifically activates both PPAR α and γ in WAT in mice. Such activation is responsible for the improvement of glucose homeostasis and suppression of inflammation, namely NF-κB activation and TNF-α expression (289). Supplementation of the diet with 1% pomegranate seed oil (with PUA) was shown to not affect abdominal WAT and serum lipid levels compared with the control diet. Nevertheless, this supplementation was sufficient to decrease the hepatic TG accumulation in obese, hyperlipidemic rats. The authors attributed this suppression, at least in part, to the suppression of Δ-9 desaturation, a key step in the membrane-bound stearoyl-CoA desaturase synthesis of MUFA from SFA (290). Furthermore, a reduction in LDL cholesterol (40% reduction) and total cholesterol (24% reduction) as well as TG reduction was reported by pomegranate seed oil oral supplementation in rats (291).

It is important to consider that the naturally occurring agonists of PPARs remain largely unknown. Due to the similarities found between PUA, omega-5 octadecatrienoic acid, and the mentioned C18:2 *c*9*t*11 CLA isomer, the possibility of PUA being also a PPAR activator was hypothesized. Indeed, PUA specifically activates PPAR α and γ in adipocyte cells in a dose-dependent manner (292). Moreover, dietary PUA was found to decrease fasting plasma glucose concentrations, improve the glucose-normalizing ability, suppress NF-κB activation, TNF-α expression and upregulate PPAR α and γ responsive genes in both skeletal muscle and WAT in mice. In addition, loss of PPAR γ impaired the ability of dietary PUA to improve glucose homeostasis and suppress inflammation (292). Moreover, PUA was found to ameliorate HFD-induced obesity and insulin resistance in mice, by improving peripheral insulin sensitivity without affecting liver insulin (293). In a similar way to what was found for CLA, the beneficial effect of PUA is known for the peripheral tissues, namely adipose tissue. A commercial source of PUA - Xanthigen® - was able to significantly suppress 3T3-L1 adipocyte differentiation and lipid accumulation. This effect was attributed to a decrease in PPAR γ expression levels (288). This is relevant since PPAR γ is a regulator of adipogenesis and it is necessary for adipocyte differentiation (237,294).

Although some promising results have emerged regarding assays on adipocyte cells showing a potential anti-obesogenic role, there is a need for further investigations of CLNA effects on both peripheral tissues and the brain, specifically on human subjects, as well as the potential adverse health effects. The most important effects of both CLA and CLNA isomers in Brain and adipose tissue are summarized in **Figure 1.4**.

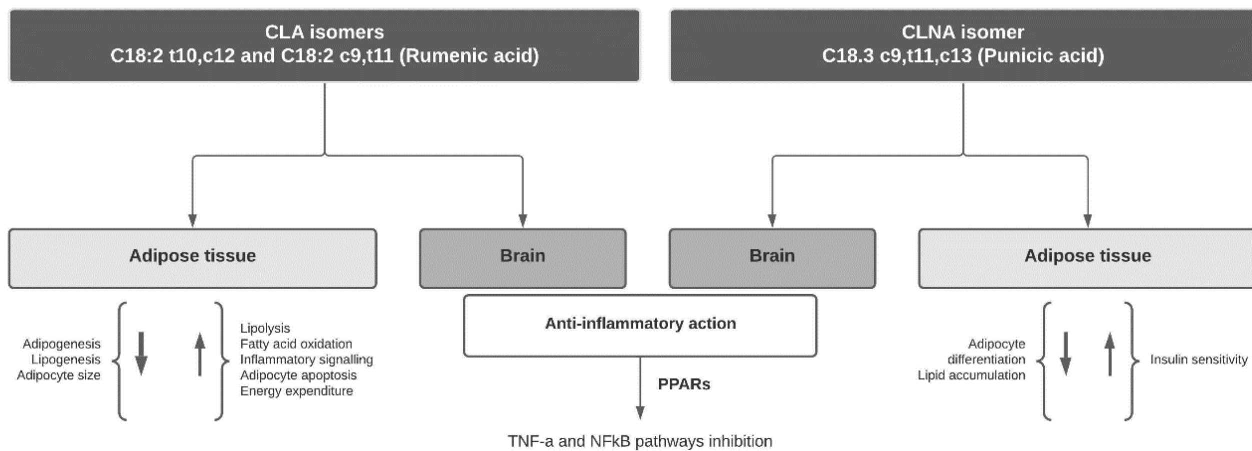

Figure 1.4 - Conjugated fatty acids role on obesity. Schematic representation of CLA and CLNA antiobesity properties in both peripheral tissues, specifically adipose tissue, and brain.

4. A promising (micro) approach: the role of fatty acids in gut microbiota modulation and their impact on obesity

The total number of bacteria in a standard adult man of 70 kg is estimated to be 3.8×10^{13}, which comprises 99.9% of the human microbiome, being the number and diversity of microorganisms greatly modulated by physiological and environmental factors (295,296). According to the last estimations, most of the human microbiome, corresponding to 95%, is estimated to be in the intestine. This gut microbiome is considered an essential component of the gastrointestinal tract (GIT) but also the largest immune organ (297). It is important to clarify two important concepts: microbiota and microbiome. While microbiota describes the living organisms that are found in a specific environment, such as gut microbiota. Microbiome, on the other hand, refers to the collection of genomes from all the microorganisms in the environment, including not only the community of organisms itself but also the microbial structural elements, metabolites, and environmental conditions (298). Although the gut microbiota also includes archaea, viruses, and fungi, more than 99% of the microbial genes detected in the gut are bacterial genes (299).

Importantly, throughout the years, the evolution of *Homo sapiens* has been closely connected to mutualistic collaboration among the diversity of bacteria that cohabit the different sites of the human body, with a special focus on the GIT. In the gut, most bacteria are adherent to mucus, important to form the outer and inner physical barrier. More than 90% of the bacterial species comprising the human microbiome belong to 4 major phyla: Firmicutes, which account for 65% of gut microorganisms, Bacteroidetes (16%), Actinobacteria (9%) and Proteobacteria (5%). The Firmicutes phylum is mainly comprised of bacteria from Bacilli (*e.g.*, *Lactobacillus*) and Clostridia classes, which are Gram-positive bacteria. While Bacilli are obligate or facultative aerobes, Clostridia are anaerobic. On the other hand, the Bacteroidetes phylum corresponds to Gram-negative, non-spore-forming, anaerobic bacteria which can tolerate the presence of oxygen but cannot use it for growth. Regarding Actinobacteria phylum, comprising for example the *Bifidobacterium* genus, they are Gram-positive, with multiple branching rods, non-motile, non-spore-forming, and anaerobic bacteria. Lastly, Proteobacteria, *e.g., Escherichia, Klebsiella, and Enterobacter* genus, are aerobic or facultatively anaerobic, Gram-negative, non-spore-forming rods bacteria known to be present in the intestinal tract of all vertebrates. Importantly, such organisms are transmitted to babies mainly via the mother's milk, which is rich in *Bifidobacterium* and *Lactobacillus* (as reviewed by Belizário et al. (295)). Indeed, the establishment of the gut microbiota occurs early in life, starting from a low level of diversity, increasing throughout the years, and reaching a complexity comparable to the adult microbiota by 3 to 5 years of age (299). Later, in adults, most bacteria belong to the genera *Bacteroides* (Bacteroidetes phylum), *Parabacteroides* (Bacteroidetes phylum), and *Clostridium* (Firmicutes phylum). Nevertheless, each site of the gastrointestinal tract presents a distinctive microbiota (295). In fact, the diversification and composition of the microbiota are influenced by several factors, such as perinatal features (mode of delivery: caesarian section or vaginal delivery), nutrition, and weaning.

Therefore, environmental and host-specific factors, such as genotype, age, and gender, as well as habitat and most importantly diet, are determinants to define the host's microbiome (295,299).

4.1. Importance of Gut microbiota on host's homeostasis

Gut microbiota's importance lies in its role in maintaining the host's normal homeostasis through three major functions: protection against pathogen colonization, achieved through nutrient competition and production of antimicrobial agents (*e.g.,* hydrogen peroxide, acidophylin, acidolin, lactallin, etc.); stimulation of innate immunity and restriction of toxins production and penetration of pathogenic microorganisms into gut tissues; promotion of nutrient absorption through indigestible dietary fibers or tri/tetrasaccharides metabolization to monosaccharides ultimately producing B-group vitamins (300). Besides, the gut microbiota also interacts with enteroendocrine cells and produces vitamins, steroid hormones, and neurotransmitters such as GABA and serotonin (311). Specifically, some species, namely *Akkermansia muciniphila* and *Bifidobacterium* spp. are believed to be especially relevant since they mediate multiple interactions between microorganisms (299).

Considering the importance of gut microbiota in the host's homeostasis, the development and use of prebiotics and probiotics have been widely assessed throughout the years. They are relevant since they can change the composition of the gut microbiota consequently being able to induce beneficial effects. Prebiotics are nondigestible food ingredients that stimulate the growth and/or activity of gut bacteria, benefiting the health of the host. On the other hand, probiotics are live microorganisms that confer a health benefit on the host when administered in adequate amounts (299). As mentioned, the importance of gut microbiota in the host's homeostasis has been widely assessed and its fundamental role in the development and therapeutical approach of several diseases has been determined. For instance, key roles for the gut microbiota and its metabolites have been attributed to glucose metabolism. Indeed, by using prebiotics and/ or probiotics microbiota remodeling has been associated with improved glucose metabolism in subjects with T2DM (299,301–303). Considering this, several beneficial effects have been associated with microbiota modulation. Microbiota dysregulation has been related to several pathologies and the important role of bioactive lipids in such processes has been unraveled, as is going to be discussed in **Section 4.3** of this Chapter.

4.1.1. Gut dysbiosis and disease development

Gut dysbiosis is a persistent imbalance of gut microbial species abundance, caused by a loss of microbe species richness and increased interindividual variability. Such alteration results in impaired gut barrier function as well as inflammatory cell activation. Therefore, gut dysbiosis has been associated with the onset of several pathological diseases not only involving the GIT but also other organs. Among the diseases associated with such problems, there is inflammatory

bowel disease (IBD), irritable bowel syndrome (IBS), T2DM, obesity, cancer, rastelli CVD, and CNS disorders, namely neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease and Multiple sclerosis (295,304). Shortly, in several of the mentioned diseases where gut dysbiosis occurs, a strong inflammatory component has been associated with it. In general terms, in healthy individuals, anti-inflammatory species - *e.g., Faecalibacterium prausnitzii* - are predominantly present. In contrast, in IBD, Parkinson's disease, Crohn's disease, and obesity, for example, there is the presence of potential proinflammatory bacteria such as *Bacteroides, Ruminococcus gnavus, Verrucomicrobia,* associated with the production of inflammatory factors (295,305–307). Nevertheless, it has been suggested lately that it is not the specific modulation of a single bacterium the source of pathology development instead, general alterations to the ecosystems seem to be responsible.

Several factors have been associated with drastic changes in human gut microbiota and diet has been suggested as one of the major ones. Indeed, food additives, such as sweeteners and emulsifiers, found in processed foods have been shown to affect negatively gut homeostasis and thus, the gut microbiota. Such additives have been connected to metabolic syndrome and chronic inflammatory diseases (299) suggesting a direct link between diet, namely WD, obesity, and microbiota dysregulation. Although obesity is a multifactorial disease and both poor diet and low physical activity are well-known contributors, other factors need to be taken into consideration. Indeed, alterations in the gut microbiome, such as the alterations induced by antibiotics usage, have been associated with increased risk for a variety of diseases including obesity, T2DM, IBD, celiac disease, allergies, and asthma (308). Considering this, the changes in human gut microbiota associated with WD consumption and ultimately obesity are going to be discussed as well as the importance of bioactive lipids in modulating such changes.

4.2. Bioactive lipid's role on gut microbiota function

Lipids are the major constituents of cell membranes thus, being essential elements in gut permeability. Due to this role, they are important in the modulation of the gut microbiome. Besides their structural role, lipids regulate multiple cell functions through intercellular and intracellular signaling mediators present both in the brain and the enteric system. These lipids are known as bioactive lipids. These molecules are released into the bloodstream, migrating to distant organs through the gut-brain axis. Importantly, multiple bioactive lipids exert either pro- or anti-inflammatory actions on the gut microbiome, influencing several important processes such as immune regulation, inflammation, and homeostasis (as reviewed by Baptista et al. (304)). Other relevant roles of bioactive lipids in microbiota modulation and consequently in pathology development have been reported. Thus, in the next sections, the more relevant bioactive lipids in the context of gut microbiota function and modulation and disease progression are going to be presented (**Figure 1.5**).

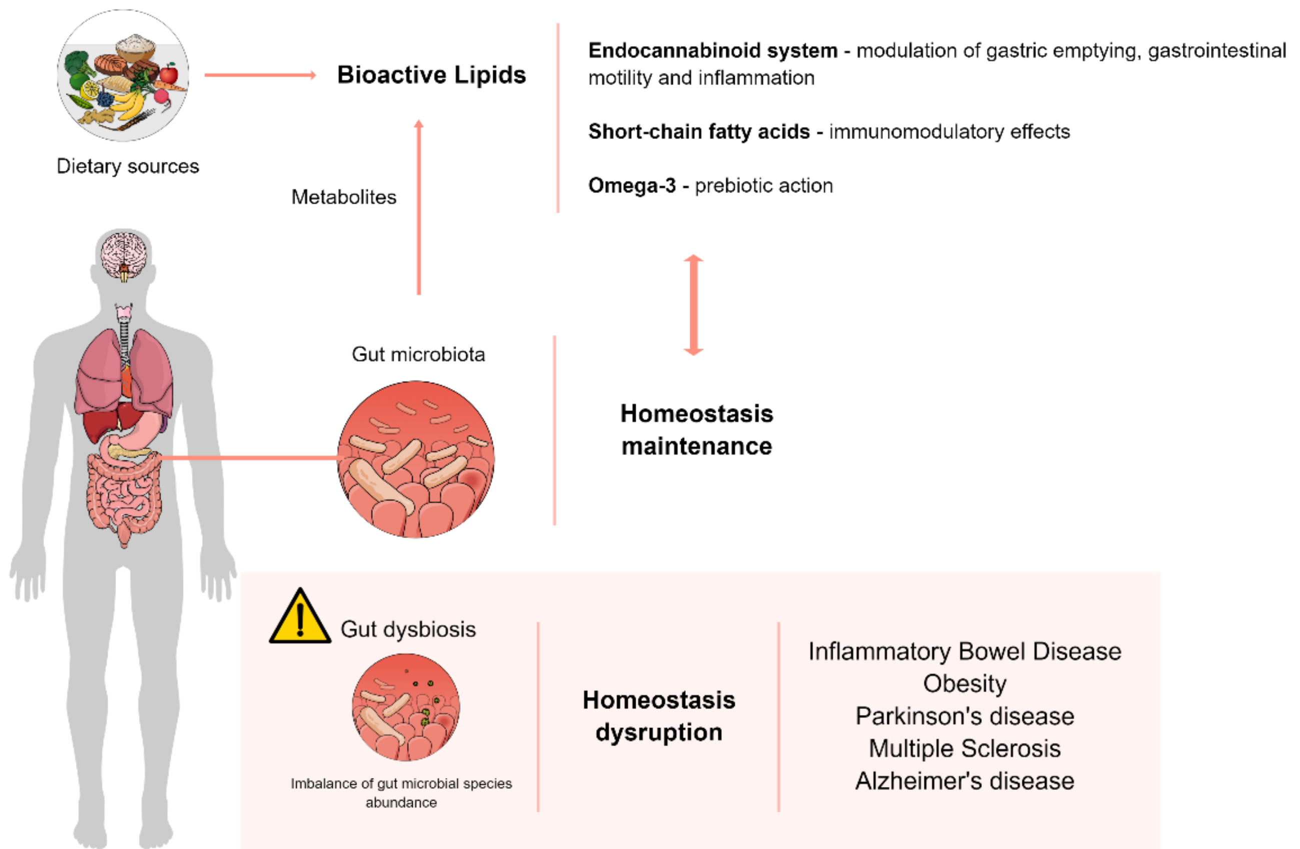

Figure 1.5 - Gut microbiota importantly maintains the host's homeostasis. When there is an imbalance of gut microbial species richness, defined as gut dysbiosis, there is the development of several pathologies like IBD, obesity, and CNS disorders – Parkinson's disease, Alzheimer's disease and Multiple Sclerosis. The gut microbiota homeostasis is closely connected with bioactive lipids action. Indeed, the endocannabinoid system maintains gut physiology through the modulation of gastric emptying, gastrointestinal motility and inflammation. SCFAs are metabolites produced by bacterial fermentation of non-digestible carbohydrates such as dietary fibers and resistant starch, that present immunomodulatory effects. Several of the mentioned diseases are associated with impairment ofSCFAs-producing species. Omega-3 fatty acids present anti-inflammatory effects and help restore dysbiosis, functioning as prebiotics.

4.2.1. Endocannabinoid system

N-arachidonoylethanolamine (AEA) is a member of the N-acylethanolamine (NAE) family, a large group of bioactive lipids. 2-Arachidonoylglycerol (2-AG) is another member of the same endocannabinoid family, which was identified in the intestine. Although further members of the endocannabinoid family have been identified and other AA derivatives have been shown to interact with endocannabinoid receptors, AEA and 2-AG are the most studied. AEA and 2-AG are synthesized from AA through enzymatic activation by multiple pathways in the membrane of different cell types such as neurons, adipocytes, and skeletal muscle cells, in response to increased intracellular Ca^{2+} concentration, membrane depolarization, and/or receptor stimulation (309). These bioactive lipids comprise the endocannabinoid system. Such a system is widely known to be mediated by cannabinoid receptor 1 (CB1) and CB2, which are GPCR receptors. Besides, endocannabinoids also interact with PPAR-α and PPAR-γ as well as with GPR55. In addition, molecules that structurally resemble the mentioned endocannabinoids -

endocannabinoid analogs or related bioactive lipids- can mediate or interfere with the endocannabinoid response without activating the endocannabinoid receptors, including lipids that belong to the acylglycerol family such as palmitoyl-glycerol (2-PG) and oleoylglycerol (2-OG). Indeed, endocannabinoids are greatly produced in organs that contribute to the regulation of energy homeostasis, such as the brain, liver, adipose tissue, muscles, and pancreas. Furthermore, the endocannabinoid system is involved in gut physiology through modulation of gastric emptying, gastrointestinal motility, and inflammation. Interestingly, among the factors involved in energy balance, the gut microbiota has a crucial role. Evidence suggests that the gut microbiota contributes to host metabolism by communicating with adipose tissue, primarily by regulating fat storage (299). Such observations suggest *a priori* link between microbiota and the endocannabinoid system. Indeed, a study has demonstrated that oral administration of a *Lactobacillus acidophilus* strain induced the expression of cannabinoid and μ-opioid receptors - previously demonstrated to have anti-inflammatory functions in several experimental models of colitis - in intestinal cells. The oral administration of *L. acidophilus* also mediated analgesic functions in the gut by reducing abdominal pain in a rat model of chronic colonic hypersensitivity, mimicking IBS (310). Moreover, the endocannabinoid system has been suggested as a possible link between microbiota and adipose tissue, developing an important role in obesity (299).

4.2.2. Short-chain fatty acids

SCFAs are carboxylic acids containing 2 to 5 carbon atoms and are considered metabolites that are produced by bacterial fermentation of non-digestible carbohydrates such as dietary fibers and resistant starch, in the proximal colon. Importantly, they have been shown to have immunomodulatory effects, as they are known to promote anti-inflammatory effects. Interestingly, the type and number of fibers consumed affect the composition of the gut microbiota and consequently, the type and amount of SCFAs produced. The most abundant SCFAs are acetate (C2), propionate (C3), and butyrate (C4) being produced by bacterial species within the phyla Firmicutes, Bacteroidetes, and Actinobacteria. SCFA's importance lies in the fact that they are a source of energy to the colonic epithelium and maintain the integrity of the epithelial barrier by regulating mucus production and tight junction expression (as reviewed by Melbye et al. (311)). Importantly, SCFAs also interact with the BBB being able to cross it and consequently modulate brain function. It is suggested that SCFAs trigger mucin production and tight junction synthesis, which strengthen the intestinal epithelial barrier ultimately leading to a reduced passage of toxic and proinflammatory substances across the epithelial barrier. Importantly, the presence of SCFAs also modulates T-cell differentiation towards regulatory subtypes (*i.e.* T regulatory cells (Treg)) and suppresses proinflammatory Th17 and Th1 cells. It is the balance between such cells that drives pathogenic or protective responses in other tissues, such as the CNS and modulates autoimmunity. Especially the generation of Th17 cells and their cytokines have been closely associated with gut microbiota. Both Th1 and Th17 cells can cross the BBB and cause CNS

inflammation after activation through microglia modulation. Proteins claudin-5 and occludin are tight junction proteins important in BBB permeability. Increased BBB permeability has been associated with reduced expression of these proteins along with changes in the gut microbiota of mice. Importantly, butyrate (C4) was shown to upregulate these proteins, thereby restoring the BBB permeability in mice (312). In fact, SCFAs' gastrointestinal levels are associated with CNS disorders, namely AD, suggesting their important role in gut-brain communication (309). Moreover, in inflammatory diseases, such as IBD and obesity, SCFAs develop an important role since they can also act as signaling molecules to activate GPR receptors - GPR41 and GPR43 - activating signaling cascades that control immune functions (313,314).

Furthermore, gut microbiota also seems to play a crucial role in the development and functionality of the CNS immune system, specifically in microglia modulation. Interestingly, microglia from specific pathogen-free mice show normal maturation and function, while non-colonized young germ-free mice exhibit stunted microglia under homeostatic conditions, suggesting an important role of gut microbiota in this process. Moreover, the oral application of SCFAs (a mixture of acetate, propionate, and butyrate) was found sufficient to induce the maturation of microglia in germ-free mice (315). Nevertheless, the mechanisms involved in this modulation remain unknown.

Additionally, SCFAs improve gut health by maintaining intestinal barrier integrity, mucus production, and protection against inflammation. As mentioned, it is known that SCFAs bind to GPRs, namely GPR43, GPR41, GPR109a/HCAR2 (hydrocarboxylic acid receptor), and GPR164, present in cells from the intestinal mucosa, immune and nervous system. The binding of SCFAs to their receptors on enteroendocrine cells, results in stimulated secretion of glucagon-like peptide 1 and peptide YY, while signaling in β-pancreatic cells leads to increased insulin secretion. Effects on brown adipose tissue activation, regulation of liver mitochondrial function, whole-body energy homeostasis, and control of appetite and sleep have also been attributed to SCFAs (315).

4.2.3. Polyunsaturated fatty acids: the omega-3 example

PUFAs, specifically omega-3 EPA and DHA, are important regulators of inflammatory processes, being recognized as having an anti-inflammatory role. Thus, they possess health benefits against different chronic degenerative diseases, such as CVD, rheumatoid arthritis, IBD, cognitive disorders, depression, cancer, and obesity (320). Omega-3 and omega-6 PUFAs are suggested to have antagonistic roles in inflammatory response: omega-6 FAs have a pro-inflammatory role, by acting as a precursor to inflammatory eicosanoids; omega-3 FAs present anti-inflammatory actions competing with the same enzymatic pathway. Similarly, these PUFAs seem to have opposing effects on intestinal homeostasis (316, 317).

Contrary to SCFAs, the impact of omega-3 on the human gut microbiota is still overlooked. Very few studies were performed on humans, nevertheless, they showed promising results as there were some common changes in the gut microbiota after omega-3 PUFA

supplementation: a decrease in *Faecalibacterium*, which is often associated with an increase in Bacteroidetes and butyrate-producing bacteria. Recently, a 6-week dietary intervention study aiming to compare the daily supplementation effect of omega-3 (500 mg) with a well-characterized prebiotic, inulin (20 g) demonstrated that omega-3 can alter gut microbiota composition and through the produced gut microbial products may have a relevant effect on cardiovascular health, presenting in this way a relevant prebiotic effect. More specifically, the authors reported that omega-3 supplementation resulted in significant increases in *Coprococcus* spp. and *Bacteroides* spp., and significantly decreased *Collinsella* spp. which is associated with fatty-liver condition. Moreover, similarly to inulin, omega-3 supplementation resulted in a significant increase in iso-butyrate and iso-valerate and although not statistically significant increases in butyrate (318). In addition, in a randomized cross-over trial with 8 weeks of mixed EPA/DHA (4 g) supplementation using two different formulations (soft-gel capsules and Smartfish drinks) it was observed that there is a reversibly increased abundance of several genera, including *Bifidobacterium*, *Roseburia* and *Lactobacillus* (319). Moreover, some *in vivo* studies using rodent models have shown that the interplay between gut microbiota, omega-3 fatty acids, and immune response allows the maintenance of intestinal wall integrity and interaction with host immune cells (320). Indeed, omega-3 supplementation (60 and 90 mg) of BALB/c mice after ceftriaxone sodium administration (used to establish an intestinal microbiota dysbiosis model) significantly increases bacterial richness and diversity. Indeed, a higher percentage of *Lactobacillus*, *Helicobacter,* and *Ruminococcus* and a lower percentage of *Bacteroides*, *Clostridium,* and *Prevotella* were observed. Besides and highly relevantly, the expression of the tight junctions' proteins zonula occludens 3 (ZO3) and occluding of the ileal wall increased compared with the natural recovery group. Moreover, the secretion of the intestinal mucosa immunoglobulin SIgA and serum IL-10 levels were also increased while the LPS, IL-1β, and TNF-α levels were decreased when compared to the dysbiosis group (321). In addition, omega-3's potential action in gut microbiota lies in restoring the dysbiosis that is encountered in several pathologies. For instance, the dysbiosis of the Firmicutes/Bacteroidetes ratio is associated with several conditions, such as weight gain and obesity, insulin resistance, HFD consumption, gut permeability, IBD, and depression. Omega-3 can reverse this condition by restoring the Firmicutes/Bacteroidetes ratio and increasing Lachnospiraceae bacteria, both are importantly associated with increased production of the anti-inflammatory SCFA butyrate. In addition, omega-3 PUFAs increase LPS-suppressing bacteria, *Bifidobacterium,* and decrease LPS-producing bacteria, Enterobacteria, suppressing endotoxemia responsible for a low-grade systemic inflammation (as reviewed by Costantini et al. (320)). Thus, omega-3 PUFAs are considered prebiotics in some pathologies.

In conclusion, the positive effects of omega-3 fatty acids in gut microbiota and associated pathologies are closely connected to their anti-inflammatory capacity, nonetheless, despite promising results, there is still a lack of knowledge regarding this theme, and further studies are warranted.

Besides, there are few studies specifically addressing the effect of CFAs in gut microbiota modulation and most of these studies are specifically focused on an obesity perspective. So, the effect of these PUFAs is going to be further discussed in **Section 4.3.1** of this Chapter.

4.3. Gut microbiota effect in obesity development and the role of bioactive lipids

According to the Human Microbiome Project Consortium, a "healthy" human gut microbiome is often characterized by being mainly composed of *Firmicutes*, *Bacteroides*, *Proteus*, *Actinomycetes*, *Fusobacteria,* and *Verrucomicrobia*. As mentioned before and in summary, its core functions include the biodegradation of polysaccharides, the production of SCFAs, the enrichment of specific LPS, and the production of vitamins and essential amino acids. Moreover, a "healthy" gut microbiota is generally highly diverse and conversely, the relative lack of diversity in the gut microbiota leads to diseases such as obesity (322–325). Indeed, several studies in the last decade have uncovered a causal correlation between disruption/alteration of the gut microbiota with obesity. Importantly, exposure to antibiotics at particularly vulnerable stages, such as during early postnatal life, and the consequent disruption of the gut microbiota have been associated with an increased risk of overweight, by altering both host metabolism and central adiposity in both mice models (326,327) and humans (328). Considering this, the influence of gut microbiota in obesity onset and development has been one important area that has been gaining attention over the recent decades.

Obesity has been associated, in both human and animal studies, with low levels of Bacteroidetes and significant overgrowth of bacteria from the Acidaminococcaceae family (300). Moreover, the intestinal microbiota and the endocannabinoid system have been suggested to interact during the development of obesity. Studies focusing on obesity have shown that on one hand modulation of the endocannabinoid system is associated with changes in gut microbiota, on the other hand, modulation of gut microbiota using probiotics, antibiotics, or germ-free mice, affects endocannabinoid system signaling (309). Indeed, CB1 overactivity is considered a key contributor to the development of obesity. When a mouse model of diet-induced obesity is treated with a CB1 antagonist, microbiota changes are observed: *A. muciniphila* (Verrucomicrobia phylum), a mucin-degrading bacterium that resides in the mucus layer, increases and Lachnospiraceae and Erysipelotrichaceae levels decrease. Although Lachnospiraceae are SCFAs producers, different taxa of Lachnospiraceae are also associated with different intra- and extraintestinal diseases (329). Regarding Erysipelotrichaceae, members of this bacterial family are described as highly immunogenic and are positively correlated with TNF levels (330). Interestingly, *A. muciniphila* increases its abundance after OEA supplementation. Moreover, *A. muciniphila* supplementation can prevent the reduction of 2-PG levels in human patients and increase the production of 2-PG, 2-OG, and 2-AG in obese mice, suggesting an important connection between these important bacteria and the endocannabinoid system. On the other hand, mice treated with antibiotics selectively upregulated the expression of CB2 and exhibited

altered microbiota profile as luminal counts of *Lactobacillus* and Enterobacteriaceae were increased, whereas the *Clostridium* and the Verrucobacteria groups were reduced (as reviewed by Bisogno et al. (309)). Recently, a high-fat, high-sucrose diet, mimicking a WD, induced microbiome disturbances as well as modifications of intestinal and circulating endocannabinoidome mediators, ultimately resulting in glucose intolerance, obesity, and hyperinsulinemia in mice models. Moreover, the authors identified associations of specific bacterial genera - *Barnesiella, Adlercreutzia, Parasutterella, Propionibacterium, Enterococcus, and Methylobacterium* - in the small intestine and cecum with increasing local and circulating levels of endocannabinoidome mediators, AEA (299,331). As described before, administration of *A. muciniphila* to HFD-fed mice led to an increase in intestinal levels of 2-AG, 2-OG, and 2-PG, along with improved gut barrier function and decreased metabolic endotoxemia - a systemic, low-level elevation of gut-derived endotoxin (LPS). In addition, the administration of *A. muciniphila* limits the fat mass gained in HFD-fed mice and improves adipose tissue metabolism (299,332). For instance, *A. muciniphila* can produce acetate and propionate (SCFAs) during this degradation process. Importantly, SCFAs may play a role in T-cell differentiation towards a more anti-inflammatory response. Indeed, these SCFAs are inversely associated with diet-induced obesity, adipose tissue inflammation, and insulin resistance (311). In addition, the role of SCFAs as signaling molecules shows promising effects on obesity management: they can activate GPR41 and GPR43 thus, promoting leptin secretion by adipocytes, peptide YY, and glucagon-like peptide 1 by enteroendocrine cells important to regulate host satiety (314).

Although there is no clear evidence of a specific connection system between gut microbial-host signals and the onset or the progression of metabolic alterations associated with HFD, some studies have shown that intestinal epithelial MyD88 is a primary sensor involved in the cross-talk between nutrients, gut microbes, and host during diet-induced obesity. The intestinal epithelial cell-specific deletion of MyD88 partially protects against diet-induced fat storage, inflammation, and diabetes via mechanisms directly involving the gut microbiota. Importantly, AEA was decreased in the knockout mice whereas 2-AG and 2-OG were increased specifically during HFD feeding. Importantly, 2-OG binds to the GPR119 receptor, stimulating the release of gut peptides such as glucagon-like peptide-1 and glucagon-like peptide-2 involved in glucose homeostasis and gut barrier function, respectively. Such results suggest that the improved glucose homeostasis, the reduced metabolic endotoxemia, and the low-grade inflammatory state observed in the knockout mice are associated with the regulation of the intestinal endocannabinoid system. Moreover, the MyD88 deletion induced changes in the gut microbial community, as expected. The authors stated that among the differences the genus *Allobaculum* was decreased in WT HFD mice compared with WT control mice but was significantly increased in the MyD88 knockout HFD mice compared with the WT ones. Moreover, the authors demonstrated that transplanting gut microbes from the MyD88-knockout mice into germ-free mice replicated the protection against diet-induced metabolic diseases (333). MyD88 can be a mediator

of endocannabinoid system interaction with gut microbiota in obesity models and explain some of the inflammatory responses.

4.3.1. Polyunsaturated fatty acids (omega-3 and conjugated fatty acids)

Interestingly, Robertson and collaborators recently showed that murine maternal endogenous omega-3 PUFA production during gestation or lactation significantly reduces weight gain and markers of metabolic disruption in male offspring fed an HFD. Such effects appeared to be mediated by the restructuring of gut microbiota composition. Reduced maternal omega-3 PUFA exposure led to significantly depleted *Epsilonproteobacteria*, *Bacteroides*, and *Akkermansia* and a higher relative abundance of *Clostridia* (334). Indeed, several studies in the last decade have uncovered a causal correlation between disruption/alteration of the gut microbiota with obesity. Importantly, exposure to antibiotics at particularly vulnerable stages, such as during early postnatal life, and the consequent disruption of the gut microbiota have been associated with an increased risk of overweight, by altering both host metabolism and central adiposity in both mice models (326,327) and humans (328). Considering this, the influence of gut microbiota in obesity onset and development has been one important area that has been gaining attention over the recent decades. Issues related to the gut microbiome, such as its composition, diversity index, relative levels, and functional pathways have been pointed out as predisposing factors for adults towards obesity (335). Indeed, it has been speculated that certain groups of bacteria are efficient in absorbing nutrients and energy through the rapid metabolism of nutrients, boosting calories absorbed and leading to an increase in BMI (336). Moreover, the overgrowth of the phylum Firmicutes and reduction of the bacterium from the phylum Bacteroidetes and consequently an increased Firmicutes/Bacteroidetes ratio has been recognized as a characteristic of obese mice and humans (325,335). In a study using obese mice, it was found that indeed, Firmicutes/Bacteroidetes ratio is increased in these models and that their microbiota presents a higher capacity to harvest energy from the diet (337). Nevertheless, recent studies have not confirmed the Firmicutes/Bacteroidetes ratio differences. Thus, other mechanisms such as anorexigenic gut glucagon-like peptide 1 (GLP-1) and dysregulation of bile acids (BA) signaling have been proposed. Indeed, the transformation of primary BA into secondary BA is due to bile salt hydrolase enzymes that are mainly present in the phyla Firmicutes and Bacteroidetes, especially in the genera Clostridium (as reviewed by Sarmiento-Andrade (335)). When the enzymatic action of the microbiota is altered, the composition of BA is affected resulting in a decrease in secondary BA and an increase in primary ones, which facilitates the absorption of fat and causes obesity (338). In addition, the diversity of gut microbiota is another important parameter that needs to be taken into consideration; several studies have found that the diversity and richness of the gut microbiome are reduced in obese subjects (325). Moreover, the dysbiosis of the gut microbiota often observed in obesity and other diseases, increases the release of LPS which alters the intestinal mucosal barrier and activates inflammatory pathways (339). On the

other hand, Christensenellaceae and the genera *Methanobacteriales*, *Lactobacillus*, *Bifidobacterium,* and *Akkermansia* relative abundance are usually inversely associated with obesity. Importantly, it has been demonstrated that obesity-related microorganisms are species-specific and that bacteria within the same genus may present opposite effects (325).

Regarding CFAs, very few studies have specifically addressed the potential modulatory role of these PUFAs in gut microbiota in an obesity context. But some studies on C18:2 *t*10*c*12 CLA isomer showed that in mice fed a diet high in SFAs and sucrose, an obese mouse model with features of human metabolic syndrome, and in a model in which weight and fat loss were more prominent, resulted in significant gut microbial changes between both models. These observations were accompanied by significant increases in butyrate concentrations in the feces and acetate in plasma and enrichment in species of *Butyrivibrio*, *Roseburia,* and *Lactobacillus* in the CLA-treated group (340,341). Moreover, in a study where *ob/ob* mice were treated with a 1% CLA mixture (not specified), it was found a higher abundance of beneficial bacteria, namely *Lachnolostridium*, *Roseburia*, *Dubosiella*, *Oscillibacter* and *Anaerostipes* compared to the *ob/ob* group without treatment, along with a lower abundance of pro-inflammatory bacteria, such as Tyzzerella and Alistipes. Moreover, the authors found a correlation between gut microbiota and liver inflammation, intestinal permeability, and hepatic FA composition (342). Another study reported that CLA supplementation exerted a prebiotic action on both Bacteroidetes, *Prevotella,* and *A. muciniphila*, but was not able to override the negative effects of an HFD on *Bifidobacterium* spp. (343). Moreover, in the case of PUA, a CLNA isomer, supplementation with this PUFA was able to restore the Firmicutes/Bacteroidetes ratio that is increased due to HFD. Moreover, PUA supplementation can counteract the upregulation of Desulfovibrionaceae (positively related to epididymal and perirenal fat and TGs and total cholesterol levels) and Helicobacteraceae (positively related to epididymal and perirenal fat) as well as the downregulation in Muribaculaceae (negatively related to epididymal and perirenal fat and TGs and total cholesterol levels) and Bacteroidaceae which is induced by HFD. Similarly to what was described for CLA, PUA was also able to restore the increase in *Anaerotruncus*, *Faecalibaculim*, *Mucispirillum* and the decrease in *Lactobacillus*, *Roseburia* and *Oscillibacter* (344). All these results show a great potential for CFAs to, at least in part, ameliorate obesity in rodent models by regulating gut microbiota.

The beneficial role of omega-3 and CFAs in obesity treatment is well documented, although such an effect is thought to be mediated by a direct anti-inflammatory effect, an indirect effect on microbiota modulation must be considered, but further studies are required.

4.4. The role of fatty acids in gut-brain-axis modulation

The CNS and the GIT are known to be in constant communication through a bidirectional pathway, recognized as the gut-brain axis. The gut-brain axis includes the CNS (brain and spinal cord), the autonomic and the enteric nervous system (ENS), and the vagus nerve establishing an

interdependency relationship between host-microbe and environment. Thus, microbiota and their metabolites can target the brain either directly via vagal stimulation or indirectly through immune-neuroendocrine mechanisms. The neuroendocrine system of the brain is comprised of the hypothalamus-pituitary-adrenal axis and immunological pathways. Although the role of the neuroendocrine system in the gut-brain axis is not fully understood, it has been attributed to the modulation of hormonal secretion or the production of bacterial metabolites, such as SCFAs, neurotransmitters, and tryptophan. This interaction influences both brain function and other distant organs since it can modulate the host's immune response and host cell proliferation and vascularization by regulating endocrine functions and neurological signaling (309,345,346). It has been suggested that there is a direct interaction between gut microbiota and the ENS. Indeed, the gut microbiome can impact brain functions by influencing both host metabolism and through biological active mediators' synthesis which ultimately reduces gut and BBB permeability and blocks microglia and astrocytes activation (inflammation mediators in CNS) triggering gut and brain homeostasis. They can also communicate by the production of neurotransmitters and SCFAs. Unfortunately, despite promising results suggesting a crosstalk between the gut microbiome and the endocannabinoid system, the connection between the gut-brain axis and the mentioned system in neurodegenerative diseases has never been deeply investigated (309).

Microbiota can regulate metabolism, adiposity, homeostasis, and energy balance, as well as central appetite and food reward which play important roles in obesity. Thus, several preclinical studies that have been arising in the last few years have supported the existence of bidirectional signaling within the gut-brain axis in the pathophysiology of obesity. Indeed, the gut can interact with the CNS to transfer information on nutritional status through enteroendocrine cells, the vagus nerve, and the ENS. Interestingly, microbial-produced metabolites can modulate these signals (347). So, this is a process mediated by metabolic, endocrine, neural, and immune system mechanisms. It is easily understood how the gut-brain axis may have a role in obesity if considering that the autonomic nervous system and the hypothalamic-pituitary-adrenal axis influence many gastrointestinal processes such as transit, mucus, fluid secretion, immune activation, intestinal permeability, and relative gut microbial abundance. So, through these pathways gut microbiota interacts with the host and affects glucose metabolism and liver function. In fact, during feeding enteroendocrine cells, which are located throughout the epithelium of the gut, respond to nutrient and mechanical stimuli by secreting hormones and neurotransmitters, such as the mentioned leptin, ghrelin, peptide YY, and GLP-1. These molecules mediate their effects in insulin, gastric acid, and bile acid secretion as well as in gut motility and food intake through ENS, for example. Indeed, the neural connection via the vagus nerve also presents a key role in regulating eating behaviors and appetite. Importantly, the vagus nerves send signals from the gut receptors to the CNS, and these gut receptors are strongly influenced by the gut microbiota (346,347). Besides, metabolites that are produced by the gut bacteria population such as SCFAs are also sensed by chemoreceptors (enteroendocrine cells) which leads to calcium signaling mechanisms that may be transmitted to vagus nervus in the gut epithelium. The vagus

nerve is then responsible for downward signals from the CNS to visceral organs and tissues (346). In addition to this direct effect, the immune system also plays a relevant role in the effect of the gut-brain axis in obesity: HFD and the increased permeability of the gut barrier lead to LPS absorption which results in endotoxemia, which in turn can result in peripheral and neuroinflammation.

As mentioned, SCFAs can also modulate the gut-brain axis and or directly cause alterations in the brain (e.g., butyric acid and propionic acid). SCFAs that are derived from dietary fibers are signaling molecules that can bind to GPR43 and GPR41 (348). These molecules can affect different metabolic pathways, namely glucose metabolism and immunological pathways such as microglia maturation indicating that besides playing a role in satiety and increasing energy expenditure, they may also have a role in CNS inflammation (349,350). SCFAs can directly induce GLP-1, affect indirectly the release of ghrelin, and consequently, affect eating behaviors like satiety, hunger, and appetite. Indeed, acetate can suppress appetite via central hypothalamus mechanisms. Besides, gut microbiota can also produce many neurotransmitters, e.g., catecholamines, GABA, serotonin, dopamine, and tryptophan which can have a direct or indirect effect on the hypothalamus and change neuroendocrine functions. (346).

Omega-3 and omega-6 PUFAs, particularly AA and DHA, are important present in CNS and are crucial for infant brain development. They are widely recognized to improve neurological outcomes and as discussed; they have demonstrated a beneficial impact on the gut microbiota ameliorating inflammatory responses. Recently, it has been suggested that the neuroprotective effect of omega-3 is not only mediated by direct incorporation into neural tissues but indirectly through their beneficial effects on gut microbiota (316). Nonetheless, further investigations are required regarding this theme. Additionally, a possible role of CLA isomers on the gut-brain axis has also been suggested: mice receiving CLA supplementation showed a reduction of CNS inflammation in a mouse model of CNS autoimmunity. This reduction was achieved through regulatory immune responses, such as modification of the T cell metabolism along with an alteration of gut microbiota composition (262). Another study has reported that dietary supplementation with a mixture of CLA isomers (0.9% CLA; isomers C18:2 *c*9*t*11 and *t*10*c*12) ameliorates CNS autoimmunity in a spontaneous mouse model of multiple sclerosis. This was accompanied by an attenuation of intestinal barrier dysfunction and inflammation and an increase in intestinal myeloid-derived suppressor-like cells. Although the authors reported that the protective effects of CLA were not abrogated upon microbiota eradication, direct anti-inflammatory effects were observed in myeloid cells, namely increased IL-10 production and T-cell proliferation suppression. In a further study of human patients with multiple sclerosis, using CLA supplementation for 6 months similar results were observed regarding the anti-inflammatory profile of circulating myeloid cells. The authors suggested that all these results demonstrated that CLA is a potent modulator of the gut-brain (CNS) axis by specifically targeting myeloid cells in the intestine which in turn control T-cell responses (351). Altogether these results suggest a possible

role of gut-brain axis modulation by PUFAs, specifically omega-3 and CLA, and possibly other CFAs.

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OBJECTIVES

Objectives

Since some investigations have associated obesity with metabolic and neuronal impairment, this research proposal aims to combine food and health sciences, by exploring the use of enriched oil matrixes with high concentrations of omega-3 /Conjugated fatty acids (CLA and CLNA isomers). The effect of these bioactive fatty acids on the brain, adipose tissue, and gut microbiota will be explored, aiming to revert/prevent the biochemical impairment caused by Western diet consumption (high-sugar and high-fat). Thus, the objectives of this thesis are:

I. **Assessment of obesogenic western-diet nutrients effect on central nervous system inflammation, using a human microglia cell line.** Previous studies were solely focused on testing high-fat diets and their effect on the hypothalamus. There is no available information related to microglia activation and modulation.

II. **Description, for the first time, of the preventive effects on CNS neuroinflammation, specifically on microglial cells, of CLA and CLNA isomers in inflammation markers induced by WD-like nutrients.** Comparison of the mentioned CLNA and CLA preventive effects with those exerted by omega-3 fatty acids since the latter have been successfully tested in brain biochemistry recovery.

III. **Description of the role of GPR120 receptor in omega-3, CLA, and CLNA fatty acids effects in microglia cells.**

IV. **Assessment of the effect of the gastrointestinal tract on the bioaccessibility and bioavailability of the mentioned bioactive fatty acids present in different enriched oil matrixes.**

V. Omega-3 has been described to present beneficial modulatory effects in gut microbiota alterations associated with Western diet consumption, and metabolic diseases such as obesity and type 2 diabetes mellitus. Thus, this thesis will establish **the preventive effects of omega-3, CLA, and CLNA in the gut microbiota of human subjects.**

VI. Description of the effect of the most promising bioactive sources (in the study) in **reversing the gut microbiota alterations induced by Western diet consumption** using cecal samples from a rodent model.

VII. **Prediction of the potential of gut-brain axis modulation by the bioactive fatty acids in the study**.

VIII. **Evaluation of the role of CB1 and GPR120 receptors in gut permeability and adipogenesis.**

CHAPTER 2

Omega-3 and conjugated fatty acids brain effect.

This chapter intends to understand the possible protective role of both omega-3 and conjugated fatty acids in the CNS, namely in the hypothalamus, by using a microglia cell line.

This chapter was published as follows and is presented in this thesis with slight modifications:

Salsinha, A.S., Socodato, R., Rodrigues, A., Vale-Silva, R., Relvas, J.B., Pintado, M., Rodriguez-Alcalá, L.M. (2023). Potential of omega-3 and conjugated fatty acids to control microglia inflammatory imbalance elicited by obesogenic nutrients. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids, 1868(7):159331. https://doi.org/10.1016/j.bbalip.2023.159331

Chapter 2

Chapter 2

Preface

Are other PUFAs besides omega-3, such as CLA and CLNA isomers, able to prevent the neuroinflammatory effects of obesogenic nutrients (WD-like)?

Despite several efforts, the prevalence of obesity continues to rise worldwide. Most of the treatments available are not effective enough or are associated with severe health side effects. One of the reasons behind such failures may be the lack of complete knowledge of obesity. Indeed, most studies and treatments throughout the years have been focused on the effect of obesity in adipose tissue and overlooked the role of the brain in the onset and development of this condition. It is easily understood how the brain, specifically CNS, plays an important role in obesity since the hypothalamus has a fundamental role in feeding regulation and satiety. Moreover, the high-sugar high-fat consumption associated with WD may have a significant impact on CNS. Indeed, FAs (*e.g.,* SFAs) can cross the BBB and can reach the hypothalamus with the potential to modulate, for example, microglial activity and initiate pro-inflammatory responses that ultimately result in dysregulation of important brain areas related to energy homeostasis. These harmful effects can be potentially reversed by other FAs, specifically PUFAs. While omega-3 has been greatly studied due to its important systemic benefits in reversing the HFD-associated negative effects even in the brain, the role of other PUFAs, such as CFAs is still overlooked, despite their promising health benefits and anti-inflammatory potential.

Considering this existing gap in the field, this study aims to evaluate, for the first time, the potential of different PUFAs – omega-3 EPA and DHA, CLA isomers RA and C18:2*t*10*c*12 and CLNA isomer PUA – to prevent the negative effects induced by WD obesogenic nutrients – PA (an SFA) and fructose.

This first study was designed as a preliminary study to fully understand the potential of using these PUFAs as anti-obesity strategies not only targeting the peripheral tissues – which is already extensively described - but also having a more systemic approach by preventing the neuroinflammatory response often associated with obesity.

Microglial cells are highly responsive to foreign stimuli, and in this study, we intended to describe the modulatory action of the described bioactive FAs' action in these cells. Consequently, to achieve this goal, we used analytical standards to exclude possible interferents present in the enriched oils that are going to be presented in the next studies and that we are evaluating as possible matrixes for bioactive FAs oral delivery.

Chapter 2

The potential of omega-3 and conjugated fatty acids to control microglia inflammatory imbalance elicited by obesogenic nutrients.

Salsinha AS[a,b,c], Socodato R[b,c], Rodrigues A[b,c], Vale-Silva, R[b,c,d], Relvas JB[b,c], Pintado M[a]* and Rodríguez-Alcalá LM[a]*

a. Universidade Católica Portuguesa, CBQF - Centro de Biotecnologia e Química Fina –Laboratório Associado, Escola Superior de Biotecnologia, Rua de Diogo Botelho, 1327, 4169-005 Porto, Portugal

b. Department of Neurobiology and Neurological Disease, Glial Cell Biology Laboratory, Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto, 4200-135 Porto, Portugal.

c. Department of Neurobiology and Neurological Disease, Glial Cell Biology Laboratory, Instituto de Investigação e Inovação em Saúde (i3S), Universidade do Porto, 4200-135 Porto, Portugal.

d. Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Universidade do Porto, 4050-313 Porto, Portugal

Abstract

High-fat diet-induced obesity detrimentally affects brain function by inducing chronic low-grade inflammation. This neuroinflammation is, at least in part, likely to be mediated by microglia, which are the main immune cell population in the brain. Microglia express a wide range of lipid-sensitive receptors, and their activity can be modulated by fatty acids that cross the blood-brain barrier. Here, by combining live cell imaging and FRET technology we assessed how different fatty acids modulate microglia activity. We demonstrate that the combined action of fructose and palmitic acid induces Ikβα degradation and nuclear translocation of the p65 subunit nuclear factor-κB (NF-κB) in HCM3 human microglia. Such obesogenic nutrients also lead to reactive oxygen species production and LynSrc activation (critical regulators of microglia inflammation). Importantly, short-time exposure to omega-3 (EPA and DHA), CLA, and CLNA are sufficient to abolish NF-κB pathway activation, suggesting a potential neuroprotective role. Omega-3 and CLA also show an antioxidant potential by inhibiting reactive oxygen species production, and the activation of LynSrc in microglia. Furthermore, using chemical agonists (TUG-891) and antagonists (AH7614) of GPR120/FFA4, we demonstrated that omega-3, CLA, and CLNA inhibition of the NF-κB pathway is mediated by this receptor, while omega-3 and CLA antioxidant potential occurs through different signaling mechanisms.

Keywords: Microglia activation; omega-3 fatty acids; conjugated fatty acids; obesity, palmitic acid; fructose.

Graphical Abstract

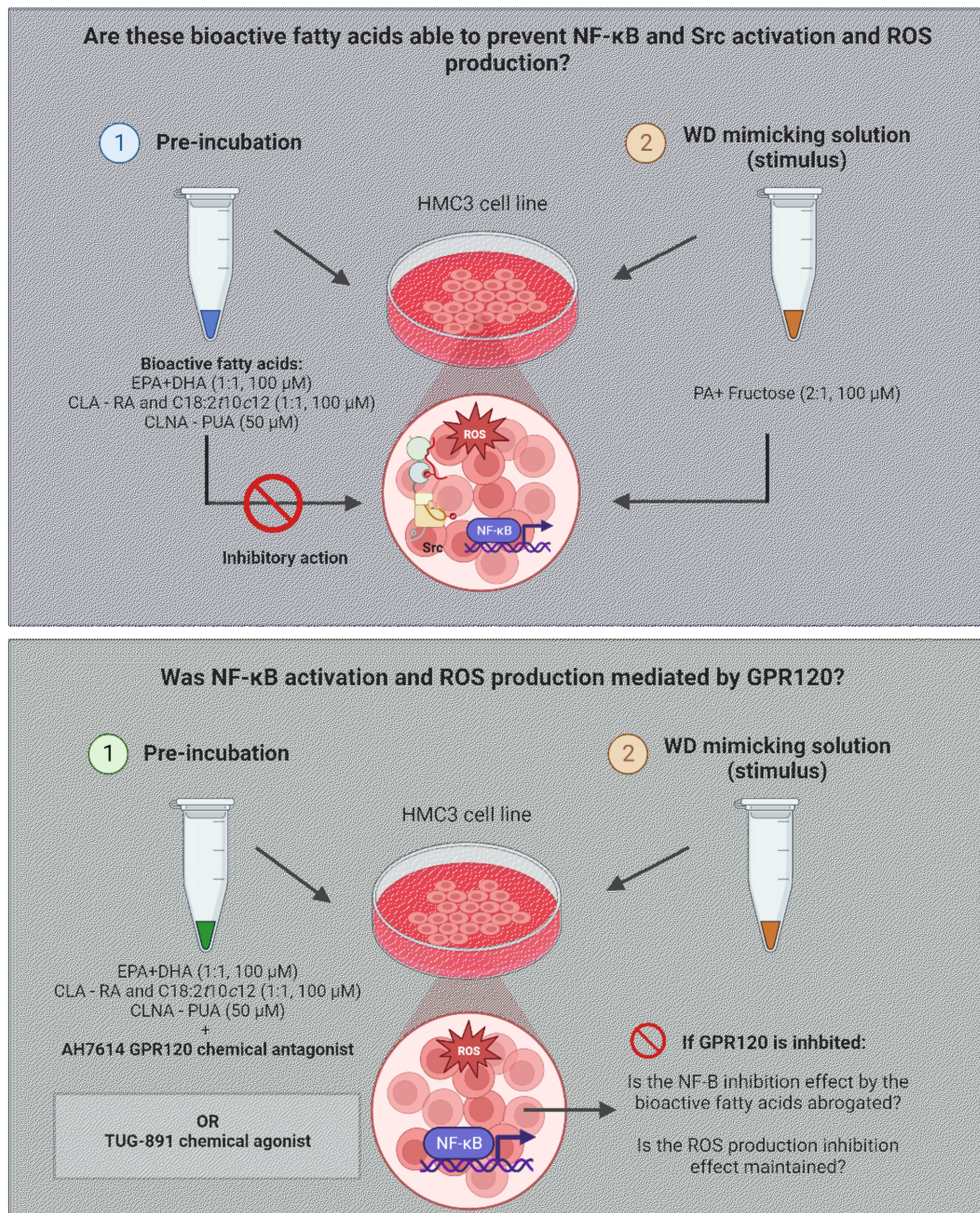

Figure 2.1 – Graphical abstract. Assessment of omega-3 EPA and DHA, CLA isomers, RA and C18:2*t*10*c*12, and CLNA isomer PUA preventive action on the WD-induced effects: NF-κB and Src activation and ROS production. The second aim is to understand if such effects are mediated by GPR120, using a chemical antagonist and agonist of this receptor.

1. Introduction

The prevalence of obesity and overweight is increasing worldwide, having almost tripled since 1975. Even though new strategies devised to fight obesity emerged in the last few years, data from the Organization for Economic Co-operation and Development (OECD) show a steady increase in obesity rates until 2030 (1). Indeed, epidemiologic studies have identified obesity (high body mass index, BMI>30) as a risk factor for cardiovascular disease (2,3), diabetes mellitus, chronic kidney disease (2), cancer (4), and several musculoskeletal disorders. Thus, a growing number of resources are being applied to develop anti-obesity drugs (*e.g.,* orlistat, Sibutramine, Lorcaserin/Belviq, etc.). However, such efforts have been associated with systematic failure as most of the treatments are not effective and the sufficiently efficient ones are related to severe health side effects, being the most common one flatulence, oily spotting, fecal urgency, fatty/oily stool, oily defecation, increased defecation, and fecal incontinence and other adverse effects such as nephrotoxicity, hepatotoxicity, nephrolithiasis, and pancreatitis (5). One reason behind such failures might be the lack of knowledge of how obesity impacts other organs, besides adipose tissue, especially the brain (6), in which the hypothalamus has a fundamental role in regulating hunger and satiety. Indeed, lesions in specific regions of the rat's hypothalamus led to cessation of feeding and subsequent death by starvation (7–9). Thus, there is a need to better understand the effects of obesity, namely HFDs on the hypothalamus.

Processed foods, rich in saturated fats, are a significant part of the diet in Western countries. Such a diet – the WD – is considered a relevant factor for the progression of obesity (10,11). Besides the consumption of high-glycemic/high-insulinemic carbohydrates, including sugars (mainly sucrose and fructose), WD is also characterized by high-fat levels, primarily SFAs and trans fats (10,12). Several *in vivo* studies show that HFD-induced obesity detrimentally affects brain function, including synaptic plasticity and cognitive performance. In models of diet-induced obesity, the consumption of an HFD increased cytokine expression, c-Jun N-terminal kinases (JNK), and inhibitor of kappa B kinase (IKK) activation in both the liver and hypothalamus (13,14). Besides, obesity-induced oxidative stress causes inflammatory reactions resulting in abnormalities in protein, lipid, DNA function, brain aging, and cognitive impairment. Indeed, HFD disrupts intracellular cascades involved in synaptic plasticity and insulin signaling/glucose homeostasis, increases corticosterone levels, and activates the innate immune system (15). As a result, HFD is commonly associated with chronic, low-grade inflammation in the adipose tissue and in the CNS (16–18). High-fructose feeding has been used as an efficient method to establish metabolic syndrome. Indeed, in rodent models, high fructose-fed rats often exhibit hypertension, insulin resistance, impaired glucose tolerance, dyslipidemia, and obesity. Interestingly, compared to glucose, fructose-fed rats show worse features of metabolic syndrome (19,20). Identical results are observed in overweight humans where the administration of fructose-containing beverages causes more visceral obesity and insulin resistance compared to glucose groups (21). Moreover, fructose has been shown to increase TNF-α concentration and to activate cellular stress

responses and ROS in animal models (22,23). Interestingly, in contrast to glucose, CNS delivery of fructose in rodent models promotes feeding behavior and it seems to be an endogenous production of fructose from glucose in CNS, suggesting that fructose effects in CNS may extend beyond its direct dietary consumption (24). Moreover, it has been reported that a potential action of fructose on the brain can be manifested, specifically, in alterations in neuronal and glial cells (25) and one study has reported that short-term consumption elevated GLUT5 levels in the hippocampus (26).

Importantly, microglia are the principal immune resident cells of the CNS, representing 5 to 10% of the total brain cells (27). In response to external stimuli, microglia can initiate a neuroinflammatory response which, like peripheral inflammation, includes the production of cytokines such as TNF-α and IL-1β, and several chemokines. Bioactive fatty acids resulting from nutrition can cross the BBB and reach the CNS, specifically the hypothalamus, with the potential to modulate microglial activity (28,29). Microglia directly respond to LC-SFAs (*e.g.*, palmitic acid, PA), functioning as a sensor capable of initiating inflammation in response to increased SFAs in the hypothalamus (18,30,31). Accordingly, microglia express a wide range of lipid metabolism-related genes and lipid-sensitive receptors, including TLRs (32). Fatty acids act predominantly through TLR4 (30) and hippocampal TLR4 expression is increased under chronic HFD exposure (15).

The harmful effects presented by SFAs may be reversed by other fatty acids, including PUFAs. Recent studies showed that supplementing rats' diet with fish oil has beneficial effects by modulating hypothalamic inflammation and attenuating SFA-induced abnormal behavior, inflammatory response, oxidative reactions, and neuronal apoptosis (33–35). Moreover, the intracerebroventricular administration of DHA, reduces energy intake, body weight gain, and HFD-induced hypothalamic inflammation (36). Several receptors, including GPCR GPR40/FFA1 and GPR120/FFA4 (from now on simply mentioned as GPR120), can be activated by free fatty acids (FFAs), namely LC-PUFAs (37,38). By signaling through GPR120, omega-3 fatty acids, DHA, and EPA produce anti-inflammatory effects (33,38–43). Attention has been given to other PUFAs that may induce similar effects. For instance, the dietary fatty acids CLA and CLNA can decrease the production of several pro-inflammatory agents, including TNF-α, PGE2, nitric oxide (NO), IL-1, and IL-6 in adipose tissue (44,45).

CLA, mostly C18:2 *c*9*t*11 and C18:2 *t*10*c*12, and CLNA (punicic acid - PUA- C18:3 *c*9*t*11*c*13) isomers, have anti-inflammatory properties mediated, at least in part, by the nuclear hormone receptor PPAR-γ in adipocytes (45–49). PPARs are members of the nuclear receptor superfamily of ligand-dependent transcription factors, and PPAR-γ is highly expressed in adipose tissue, adrenal gland, colon, and macrophages (50). Despite the increasing knowledge of CFA's beneficial effects, little is known about their mechanism of action. Although several studies show evidence supporting the anti-inflammatory properties of CLA and CLNA, it remains elusive how they affect the immune system, and very few studies have specifically addressed their role in hypothalamic inflammation (46,51,52).

As stated, FAs, namely SFAs and omega-3 FAs, can exert pro- and anti-inflammatory effects in the hypothalamus, respectively. Nevertheless, the exact mechanisms behind such effects are incomplete and the role of other fatty acids, such as CFAs is still elusive. In this work, using a human microglia cell model, through live cell imaging and Fluorescence resonance energy transfer (FRET) technology, we studied the potential role of omega-3 fatty acids and CFAs - C18:2 *c*9*t*11 and C18:2 *t*10*c*12 CLA isomers and C18:3 *c*9*t*11*c*13 CLNA isomer - in modulating microglia inflammation triggered by obesogenic nutrients (SFAs and fructose).

2. Material and methods

2.1. Reagents

PA, D-(-)-Fructose and RA (C18:2 *c*9*t*11) were purchased from Sigma-Aldrich (Missouri, USA); EPA, DHA, the C18:2 *t*10*c*12 CLA isomer and PUA (C18:3 *c*9*t*11*c*13) analytical standards were purchased from Larodan AB (Solna, Sweden). The cell culture reagents: Dulbecco's modified Eagle's medium (DMEM) + GlutaMAX^{TM}-I, Fetal bovine serum (FBS), Penicillin, Streptomycin, and HBSS supplemented with $CaCl_2$ and $MgCl_2$ and the PrestoBlue reagent were purchased from Thermo Fisher Scientific (Massachusetts, USA). The transfection reagent jetPRIME® was purchased from Polyplus-Transfection SA (Illkirch, France). The Ethanol absolute anhydrous, used as the FAs vehicle, was purchased from Carlo-Erba (Barcelona, Spain). Both GPR120 agonist, TUG-891 (*ortho*-biphenyl ligand 4-{[4-fluoro-4′-methyl(1,1′-biphenyl)-2-yl]methoxy}-benzenepropanoic acid), and antagonist, AH7614 (4-methylN-9H-xanthen-9-yl-benzenesulfonamide), were purchased from Sigma-Aldrich (Missouri, USA). Regarding the western-blot reagents, both RIPA buffer (R0278), Protease inhibitor cocktail (P8340), and Ponceau S dye (78376) were from Sigma-Aldrich. The primary antibodies used were as follows: GPR120 (extracellular) Polyclonal Antibody (PA5-111778) from Sigma-Aldrich and Anti-GLUT5 antibody (ab279363) from Abcam. The 0.45 µm Nitrocellulose blotting membranes (10600002) were purchased from Amersham Protran. The SuperSignal West Pico Plus Chemiluminescent Substrate (34580) was from ThermoFisher. Dimethyl-sulfoxide (DMSO) and PrestoBlue (A13261) used for the cytotoxicity were from Sigma-Aldrich.

2.2. Human microglia clone 3 cell line

The human microglia clone 3 (HMC3) cell line (ATCC CRL-3304) was obtained from primary cultures of human embryonic microglial cells and immortalized through transfection with a plasmid encoding for the large T antigen of SV40 (53). These cells were cultivated with DMEM + GlutaMAX^{TM}-I and supplemented with 10% FBS, 100U/mL penicillin, and 100 µg/mL streptomycin. The cells were maintained at 37 °C, 95% air, and 5% CO_2 in a humidified incubator (54–56).

2.3. Fatty acids solutions

The fatty acids stock solutions (PA and Fructose, RA, EPA, DHA, the C18:2 *t*10*c*12 CLA isomer, and PUA) were prepared at 100 mM and dissolved in ethanol absolute anhydrous. The stock solutions were maintained for up to a month at -20 °C and were stored under a nitrogen atmosphere. The work solutions were freshly prepared according to **Table 2.1** in HBSS with $CaCl_2$ and $MgCl_2$ and added to the cells as a final concentration of 100 µM (except for PUA which was 50 µM). The selected fatty acids' concentrations were based on previous *in vitro* studies. For

instance, a 100 µM PA concentration has been reported by several studies, as inducing the pro-inflammatory responses without presenting cell toxicity (31,32,57–60). Regarding PUFAs, 50-200 µM has been reported as a bioactive concentration for omega-3 fatty acids, specifically EPA and DHA (38,41,59,61,62), for CLNA isomers (C18:3 *c*9*t*11*c*15 and C18:3 *c*9*t*13*c*15 isomers) (63) and for CLA isomers (including C18:2 *c*9*t*11 and C18:2 *t*10*c*12 isomers) (45). Thus, 100 µM was selected as the studied concentration for both PA and PUFAs (omega-3 and CLA) and 50 µM for the CLNA isomer. Fructose has been reported with no cytotoxic effects in higher doses (5-50 mM) in several different cell lines: caco-2 cells (64), cholangiocyte and cholangiocarcinoma cell lines (65) and macrophages (66). In microglia, there are reports of the use of glucose in similar doses (17.5 or 25 mM) (67).

Table 2.1- Fatty acids solutions composition.

Solution ID	Fatty acids	Other Components	Final Proportion	Final concentration added to the cells
Stimulus solution (western pattern diet)	Palmitic Acid (C16:0) (≥99% purity)	D-(-)-Fructose (≥99%)	2:1	100 µM
Omega-3	EPA (C20:5 n-3) (90% purity)	-	1:1	
	DHA (C22:6 n-3) (≥99% purity)	-		
CLA	Rumenic acid (C18:2 *c*9*t*11) (≥96.0% purity)	-	1:1	
	C18:2 *t*10*c*12 CLA isomer (>98% purity)	-		
CLNA	Punicic Acid C18:3 *c*9*t*11*c*13 (>98% purity)	-	-	50 µM

2.4. Cytotoxicity analysis

The cytotoxicity of the FAs solutions (PA and Fructose, RA, EPA, DHA, the C18:2 *t*10*c*12 CLA isomer, and PUA) was evaluated using the PrestoBlue reagent, according to the manufacturer's instructions. The cells were seeded at a concentration of 1×10^4 cells/well in 96-well plates for 24 h. Afterward, cells were exposed to the FAs solutions according to the concentrations presented in **Table 2.1**. Since our experiment for FRET analysis has a 45 min duration, for the cytotoxicity assay we exposed the cells for 5 and 24 h to the study fatty acids. Cells treated with 10% DMSO were used as a negative control. After the two incubation periods (5 and 24 h), the FA solution was removed from the cells and PrestoBlue reagent was added to the medium and it was incubated for 1 h in the dark. The fluorescence signal was read in a Synergy H1 microplate reader.

2.5. Western blot to confirm the expression of GPR120 and GLUT5 membrane receptors in microglia cell line

To confirm the expression of both GPR120 and GLUT5 transmembrane receptors in the study cell line, HMC3 cell lysates from 3 independent cultures were collected in RIPA Buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) containing 1:1000 of a phosphatase inhibitor and DTT (Dithiothreitol – Promega, V3151, 1M) and 1:100 of a protease inhibitor. Protein concentrations were determined using a BCA kit (Pierce BCA Protein Assay Kit, Thermo Scientific). The samples were stored at -80 ºC until use. The cell lysates (10 µg) were processed using SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and western Blot. For the SDS-PAGE, 1% DTT and 1X GLB (Gel loading buffer, 150mM Trizma Base – Fisher Scientific, BP152 –, 6% SDS – Sigma Aldrich, L4390 –, 0.05% Bromophenol Blue – Alfa Aesar, A18469 –, 30% glycerol – Merck, 1.04092.1000 – and 6nM EDTA pH 8.8 – Merck, 1.08452.1000) were added to each cell lysate and a 10 min 95 ºC denaturation process was performed, before the addition of the samples to a 10% polyacrylamide electrophoresis gel. After, the proteins were blotted from the gel into a nitrocellulose blotting membrane by a semidry transfer process, using the Trans-Blot Turbo System (Biorad). The protein transfer was confirmed by Ponceau S staining. The membranes were blocked with 5% milk and were further incubated overnight with the GPR120 and GLUT-5 primary antibodies. After incubation with appropriate secondary antibodies, the immunodetection was performed with a Chemiluminescent Substrate solution and using a ChemiDoc XRS+ (Biorad).

2.6. Live cell imaging and FRET

HMC3 human microglia were plated on plastic bottom culture dishes (µ-Dish 35 mm, iBidi, Gräfelding, Germany) at a density of 20,000 cells/dish with DMEM + Glutamax® (supplemented and maintained as previously described). Cells were transfected with the different biosensors (miRFP703-Ikβα, GFP-p65, HSP, and LynSrc, previously described in **Section 2.7** from this chapter) using the jetPRIME® DNA transfection reagent according to the manufacturer's instructions: in a proportion of 2 µL of reagent per 1µg of DNA. The total medium was changed 4 h after transfection. Imaging was performed 48 h post-transfection using a Leica DMI6000B inverted microscope as previously described (54,55,76,77). The cell preparation and the experimental protocol were specifically designed for each experiment as described in the following sections.

Live cell imaging was performed using a fully monitorized DMI6000B inverted microscope (Leica Microsystems, Wetzlar, Germany) equipped with high-speed low vibration external excitation/emission filter wheels equipped with filter cubes for cyan fluorescent protein (CFP) (BP427/10) and yellow fluorescent protein (YFP) (BP504/12) working with specific dichroic (CG1 440-520 nm) mounted into a microscope filter carrousel (Leica fast filter wheels). The excitation light source was a mercury metal halide bulb, integrated with an EL6000 light alternator. Microglia

cells were observed with a PlanApo 63x 1.3 NA glycerol immersion objective with a correction ring. Images were acquired with a 2x2 binning with an exposure of 200ms using a digital CMOS camera (ORCA-Flash 4.0 v2, Hamamatsu Photonics, Japan). The LAS X software (Leica Microsystems Wetzlar, Germany) controlled all microscope parameters. For FRET biosensors and at each time-point, CFP and FRET images were sequentially acquired using different filter combinations: CFP excitation plus CFP emission (CFP channel), and CFP excitation plus YFP emission (FRET channel). A digital small-stage incubation (iBidi, Gräfelding, Germany) was used for real-time monitoring of temperature. For quantification purposes, images were exported as 16-bit tiff files and processed in Fiji software. The background was dynamically subtracted from all frames from both channels. For FRET biosensors, segmentation (on a pixel-by-pixel basis) and generation of 32-bit ratiometric images were achieved using the precision FRET (pFRET) plugin for Image J. A whole-cell/subcellular domain analysis was performed and the mean grey intensity for each time point was extracted. Values were normalized to the control untreated group (ethanol or ethanol+DMSO) and plotted.

2.7. GPR120 receptor chemical activator and inhibitor

TUG-891 (*ortho*-biphenyl ligand 4-{[4-fluoro-4′-methyl(1,1′-biphenyl)-2-yl]methoxy}-benzenepropanoic acid) was the chosen selective agonist for the long-chain FFA receptor GPR120. The stock solution was prepared at 20 mg/ml (≈55mM) in DMSO. It was added to the cells as a final concentration of 30 µM, considering previous studies (68–70). AH7614 (4-methylN-9H-xanthen-9-yl-benzenesulfonamide) was the selected negative allosteric modulator of the long-chain FFA receptor GPR120 (71). The stock solution was prepared at 100 mM in DMSO, and it was added to the cells at a final concentration of 100 µM. Indeed, *in vitro* studies (including neuronal cells) reported AH7614 GPR120 antagonistic effect on concentrations ranging from 10 to 100 µM (59,70,72). The stock solutions were maintained at -20 °C.

2.8. Biosensors

2.8.1. Cytosolic ROS production:

The generation of cytosolic ROS, mainly superoxide radical and hydrogen peroxide, was detected in microglia using the pFRET-HSP33 cys biosensor (mentioned in this paper as HSP biosensor; addgene plasmid #16076). This is a FRET probe consisting of CFP/YFP proteins linked by a 69 amino acid cysteine-containing regulatory domain from the redox-regulated heat-shock protein HSP-33 (73). Since the oxidation of the CFP and YFP fluorophores increases the CFP/YFP HSP-FRET ratio, an increase in donor-to-FRET fluorescence ratio is translated into the increased generation of ROS.

2.8.2. Src tyrosine kinase activation sensor:

To measure the specific activity of Src at the plasma membrane of microglia, the LynSrc (WT) YPet FRET probe was used (herein mentioned as LynSrc biosensor). This Src reporter is composed of a CFP protein, the SH2 domain, a flexible linker, a Src substrate peptide – derived from a primary *in vivo* c-Src substrate molecule - and a YFP protein (74). An increase in the activation of Src is detected by an increase in donor to FRET fluorescence ratio.

LynSrc and HSP biosensors were previously validated in microglia cells giving reliable FRET and donor signals within the dynamic range of each probe (55,75–77).

2.8.3. Ikβα reporter for the canonical activation of NF-κB pathway:

The canonical activation of the NF-κB pathway was studied using a monomeric near-infrared fluorescent protein probe: the miRFP703-Ikβα reporter (excitation 673 nm, emission 703 nm; addgene plasmid #80005). Ikβα is a known member of a family of proteins that inhibit the NF-κB transcription factor. Canonical activation of NF-κB therefore, depends on induced phosphorylation Ikβα degradation. In resting cells, Ikβα sequesters NF-κB dimers in the cytoplasm. After a certain stimulus, the IKK kinase is activated and phosphorylates Ikβα marking it for degradation, and as a result, NF-κB is released to the nucleus (78). Consequently, the activation of NF-κB is detected by a decrease in the fluorescent signal of Ikβα.

2.8.4. Nuclear translocation of the GFP-p65 subunit for NF-κB pathway activation:

The measurement of the nuclear accumulation of the p65 subunit of NF-κB as a functional indicator of NF-κB activation was achieved by using the NF-κB GFP-tagged p65 (here defined as GFP-p65; addgene plasmid #23255) (79). The nuclear accumulation of NF-κB was assessed by an increased nuclear fluorescence of the p65-GFP construct.

To correctly analyze the live cell imaging of the NF-κB's nuclear translocation, the GFP-p65 probe was co-transfected (1:1) with the mneptune2-H2B-6 (addgene plasmid #56146), which is specific for the nucleus/histones and thus allows the cellular nucleus staining.

2.9. Omega-3, CLA and CLNA effect on microglia activation

To assess the preventive effect of the studied fatty acids' solutions (omega-3- EPA and DHA-, CLA (C18:2 *c*9*t*11, and C18:2 *t*10*c*12) and CLNA (PUA- C18:3 *c*9*t*11*c*13) the cells were transfected with miRFP703-Ikβα reporter, GFP-p65, HSP and LynSrc probes. A pre-incubation with the mentioned fatty acids (testing solutions) was performed as described in **Figure 2.2**. An

ethanol control (0.2% (v/v)) was used as the untreated control since ethanol was used as the FAs' vehicle. In summary, the cells were recorded for 5 min (baseline reading) in the presence of the testing solutions or ethanol. Then all cell groups were recorded in the presence of the stimulus solution (PA+Fructose) or ethanol (negative control) for 15 min (stimulation period). During the assay, cells were kept under 37 ºC in HBSS with $CaCl_2$ and $MgCl_2$.

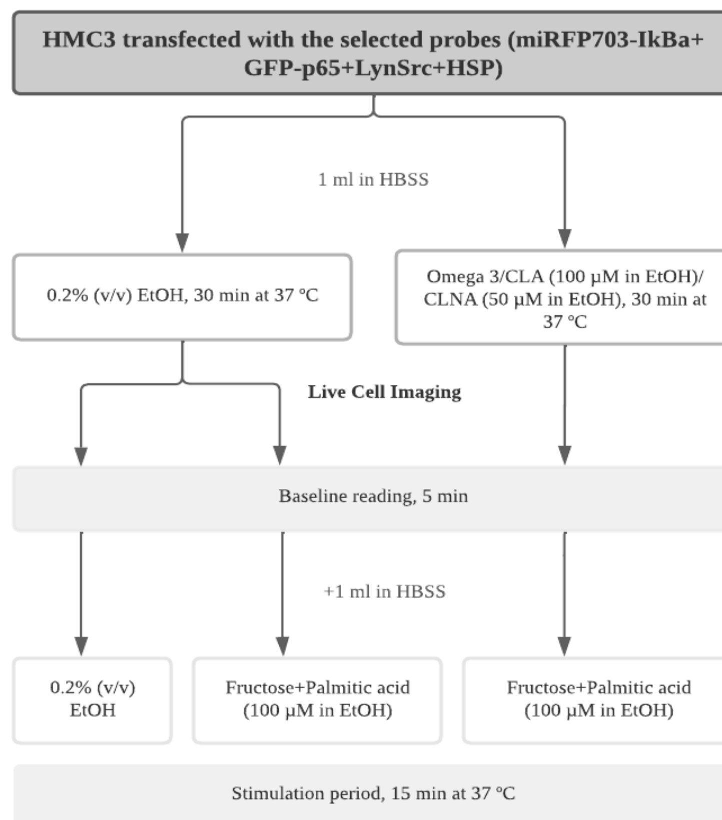

Figure 2.2- Schematic representation of the experimental protocol for the evaluation of omega-3 and conjugated fatty acids (CLA and CLNA) effect on microglia activation.

2.10. GPR120 as fatty acids receptor in microglia

To elucidate the role of GPR120 as an HMC3 cellular receptor/sensor of omega-3, CLA, and CLNA, a chemical agonist (TUG-891) and antagonist (AH7614) were used. The cells, previously co-transfected with miRFP703-Ikβα reporter or HSP probe, were incubated overnight with 0.1% (v/v) DMSO (agonist and antagonist solvent) and 0.2% (v/v) absolute ethanol (fatty acids vehicle) in DMEM + GlutaMAX^{TM}-I culture medium supplemented with 10% FBS and 100U/mL penicillin and 100 µg/mL, as mentioned. This overnight incubation is intended to decrease the DMSO and ethanol influence on microglial cells' response. Before microscope analyses, a 30 min pre-incubation with the mentioned fatty acids and TUG-891 or AH7614 was

performed as described in **Figures 2.3 and 2.4**, respectively. Thus, the overnight solution was replaced by the HBSS testing solution. An ethanol and DMSO control were used as the untreated control. In summary, the cells were recorded for 5 min (baseline reading) in the presence of the testing solutions or ethanol. Then all cell groups were recorded in the presence of the stimulus solution (PA+Fructose) or ethanol and DMSO (negative control) for 15 min (stimulation period). During the assay, cells were kept under 37 ºC in HBSS with $CaCl_2$ and $MgCl_2$ (Thermo Fisher Scientific, Massachusetts, USA).

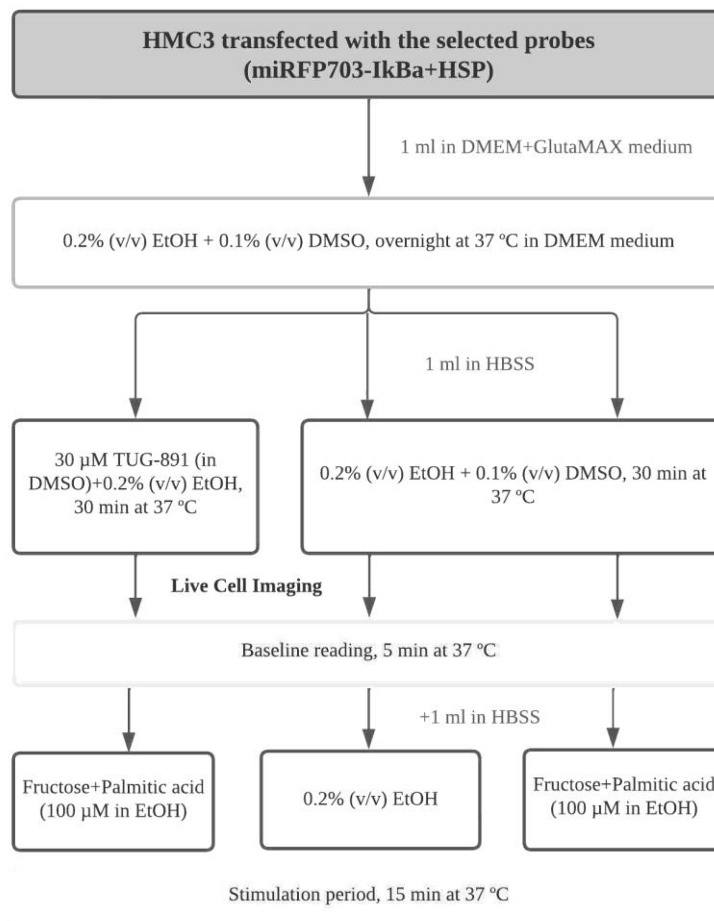

Figure 2.3- Schematic representation of the experimental protocol for the evaluation of GPR120/FFA4 activation, by using TUG-891 GPR120/FFA4 agonist, in microglia activation.

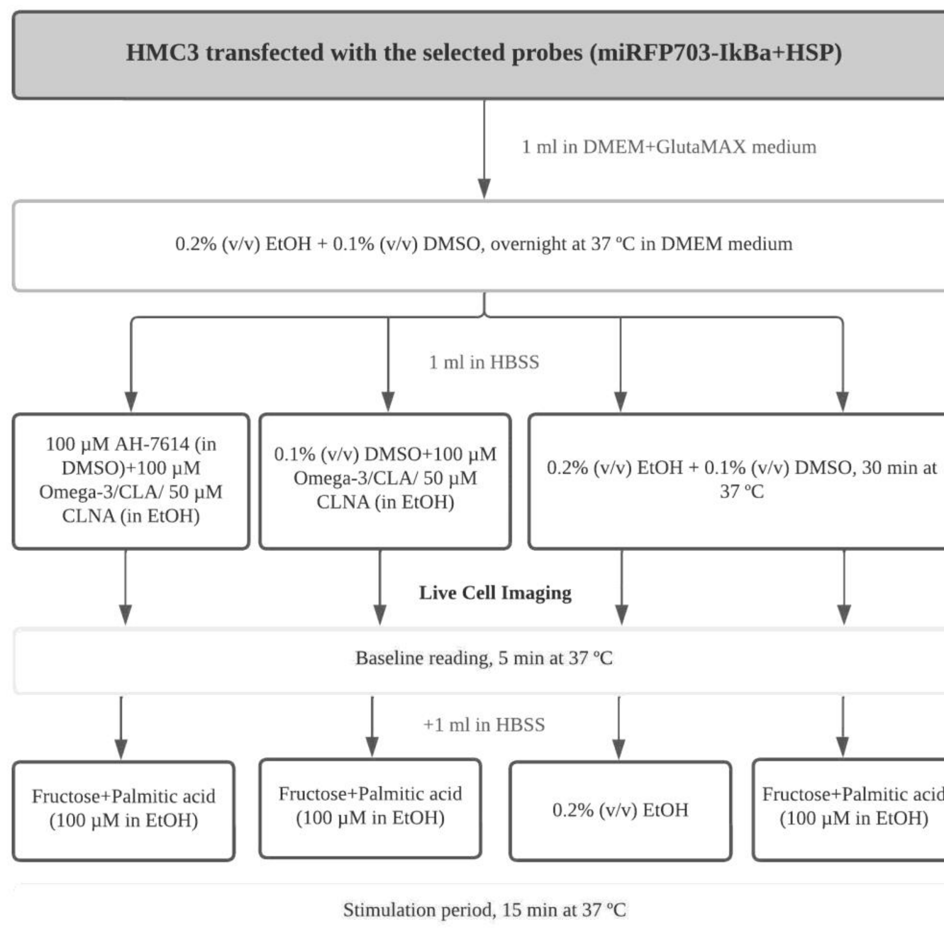

Figure 2.4- Schematic representation of the experimental protocol for the evaluation of omega-3, CLA, and CLNA action in GPR120/FFA4 chemical inhibition, by using AH7614 GPR120/FFA4 antagonist, in microglia activation.

2.11. Statistical Analysis

Experimental units in individual biological replicates were evaluated *a priori* for Gaussian distribution using the D'Agostino & Pearson omnibus normality test. For live-cell imaging experiments when comparing two or more groups with two independent variables (fatty acid treatment and time), a two-way ANOVA with Bonferroni's multiple comparison test was used to evaluate the statistical significance of all groups. In the specific case of the GFP-p65 experiments, four groups were compared considering only one factor: fatty acid treatment at the end of the experimental time. Thus, in this specific case, an ordinary One-way ANOVA followed by Bonferroni's multiple comparison test was used for data with normal distribution. All quantifications were performed using Graph Pad Prism 6.0 (Graphpad® software, San Diego, California, USA). A 95% confidence interval was used and $p<0.05$ was considered as a statistically significant difference between the analyzed groups. Experimental groups were randomly assigned, and all quantifications were performed blindly. More details on statistical analysis are indicated in figure legends.

3. Results

3.1. Cytotoxicity

Exposure of HMC3 cells to 100 µM of PA+Frut and 100 µM of omega-3 and CLA did not affect the cell viability after 5 and 24 h exposure. In the case of the CLNA isomer, exposure to 50 µM of a PUA solution did not affect the cell viability after 5 h exposure (**Figure 2.5**). Nevertheless, 24 h exposure to this PUFA seems to induce cytotoxicity in the experimental cell line. Since our experiment for FRET analysis involved a 45 min exposure to this fatty acid and we demonstrated that there was no cytotoxicity observed after a 5 h exposure, we decided to use the 50 µM concentration since, as discussed, it was described as a bioactive concentration. The results are reported as the percentage of cell viability as compared with the control (cells without treatment).

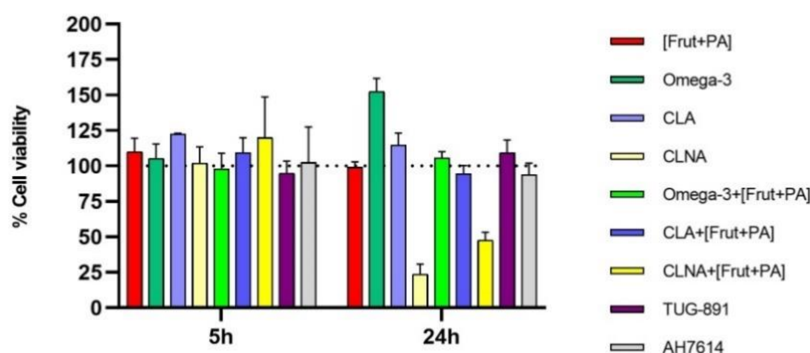

Figure 2.5 – **Cytotoxicity of HMC3 microglia cell** after 5 and 24 h exposure to the study PUFAs. Bars represent cell viability, measured by the PrestoBlue assay in percentage (%) after treatment.

3.2. Omega-3, CLA, and CLNA prevent NF-κB pathway activation by palmitic acid and fructose in microglia

Studies show that PA activates microglia and triggers a pro-inflammatory response, partially through the NF-κB pathway (31,60). In addition, short-term fructose ingestion affects the brain without significant involvement of peripheral tissues (26). However, the effect of the combination of fructose and PA, the two major constituents of a WD, on microglia activation and inflammation is entirely elusive. Glucose transporter 5 (GLUT5) is a hexose transporter involved in fructose transport which is primarily expressed in microglia within the CNS. Such transporter shows greater affinity to fructose than to glucose. Fructose is known to activate the NF-κB pathway, similarly to PA (25). The expression of GLUT5 was confirmed by Western Blot **(Figure S1A supplementary material)**. The presence of GLUT5 in the Western Blot was confirmed by the presence of a band with 100 kDa. Two other bands were detected with ≈75 kDa and ≈45 kDa. Previous works have reported that GLUT5 protein presents a 45-70 kDa molecular weight (80–84).

To evaluate the role of PA and fructose in microglia inflammation, we assessed the activation of the canonical NF-κB pathway (the primary pro-inflammatory driver in myeloid cells,

including microglia). We visualized NF-κB activity using a biosensor-based approach coupled to live-cell imaging in cultured microglia expressing the miRFP703-Ikβα nanosensor (to detect the amounts of the canonical NF-κB inhibitor), and the GFP-p65 probe (to detect the nuclear accumulation of the catalytic p65 subunit of the NF-κB complex). Exposure to a WD-mimicking solution (PA+Fructose; 2:1; 100 μM) caused a fast and sustained decrease in the signal of the miRFP703-Ikβα reporter in living microglia (**Figure 2.6A**), suggesting increased Ikβα degradation and consequent NF-κB pathway regulation, and increased the nuclear accumulation of GFP-tagged p65 NF-κB catalytic subunit (**Figure 2.6B**). We concluded that exposure to a WD-mimicking solution leads to the activation of NF-κB pathway in microglia.

Although SFAs induce microglia activation, other FAs, such as PUFAs, can normalize the inflammatory effects caused by SFAs (61,85). Because omega-3 FA, EPA, and DHA, mostly through fish oil supplementation studies, were shown to have a significant positive impact in reversing HFD-induced inflammation in the hypothalamus, we hypothesized that other PUFAs could display similar effects. Thus, we explored the modulatory effect of omega-3 and CFAs, CLA (specifically RA and C18:2 *t*10*c*12 CLA isomer), and CLNA (PUA) in cultured microglia. In this study, we intended to approach these PUFAs' action on microglia as a potential preventive strategy to the negative PA+Fructose-induced effects. Thus, we used them as a pre-treatment. Interestingly, the omega-3 (EPA and DHA; 100 μM) combination prevented the PA+Fructose-induced nuclear accumulation of GFP-p65 NF-κB catalytic subunit **(Figure 2.6B)** but did not prevent the PA+Fructose -induced Ikβα degradation (**Figure 2.6A**). Thus, the omega-3 prevention of NF-κB pathway activation was observed with the GFP-p65 probe but not with the miRFP703-Ikβα nanosensor. Such results might be related to the short pre-incubation time used in the assays, suggesting that a more prolonged pre-incubation period might be required to detect omega-3 effects in NF-κB pathway through Ikβα degradation with the miRFP703-Ikβα reporter. Moreover, we found that pre-incubation of microglia with either CLA (100 μM) or CLNA (50 μM), significantly prevented the PA+Fructose-induced canonical NF-κB pathway activation (using the miRFP703-Ikβα nanosensor) by restoring Ikβα levels and the nuclear accumulation of the catalytic p65 subunit of the NF-κB complex (using the GFP-p65 probe) (**Figure 2.6A and B**). The inhibitory effect was more pronounced in microglia pre-incubated with the CLNA isomer, PUA, than in microglia exposed to CLA (**Figure 2.6A and B**).

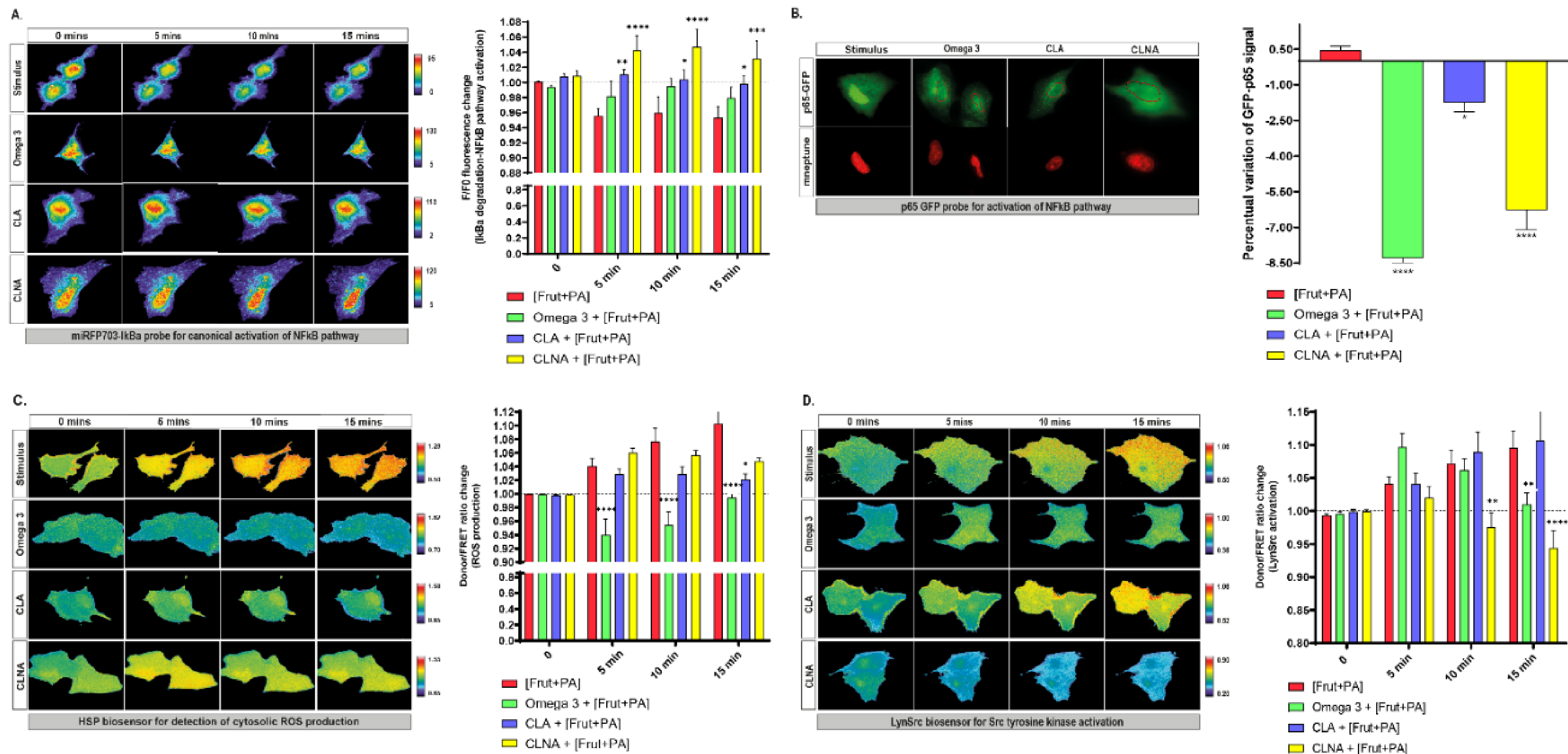

Figure 2.6- Omega-3, CLNA, and CLA fatty acids can revert Fructose and Palmitic acid-induced microglia inflammatory imbalance elicited by NF-κB pathway activation, ROS production, and Src Tyrosine activation. (A) Fluorescence imaging and results of the quantification of human microglia cell line (HMC3) expressing the miRFP703-Ikβα sensor in cells subjected to the stimulus solution (Palmitic acid+Fructose) and in cells pre-incubated with the studied fatty acids (omega-3, CLA and CLNA) for the selected time points (0, 5, 10 and 15 min). A decreased signal means a bigger Ikβα degradation and higher NF-κB pathway activation. The error bar represents the SEM calculated from n>10 cells from two independent cultures. Two-way ANOVA in relation to Palmitic acid+Fructose (PA+Frut), where no pre-incubation with the selected fatty acids was performed. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. (B) Fluorescence imaging and results of the quantification of human microglia cell line (HMC3) expressing the GFP-p65 and mneptune biosensor in cells subjected to the stimulus solution (Palmitic acid+Fructose) and in cells pre-incubated with the studied fatty acids (omega-3, CLA and CLNA). The mnpetune probe allows nucleus staining. The results correspond to n of the sensor signal of human microglia expressing the GFP-p65 sensor after the 15 min incubation time. This variation is in relation to the baseline measured for each experiment. A negative signal means a decreased signal in relation to the baseline, meaning that the catalytic p65 subunit of the NF-κB complex migration to the nucleus decreased in cells exposed to a pre-incubation with the studied fatty acids. In cells only subjected to the stimulus solution the signal increased after baseline measure, indicating catalytic p65 subunit translocation to the nucleus. Error bar represents the SEM calculated from n>10 cells from two independent cultures. One-way ANOVA in relation to Palmitic acid+Fructose (PA+Frut), where no pre-incubation with the selected fatty acids was performed. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. (C) Fluorescence imaging and results of the quantification of human microglia cell line (HMC3) expressing the ROS FRET sensor HSP in cells subjected to the stimulus solution (Palmitic acid+Fructose) and in cells pre-incubated with the studied fatty acids (omega-3, CLA and CLNA) for the selected time points (0, 5, 10 and 15 min). Error bar represents the SEM calculated from n>15 cells from two independent cultures. Two-way ANOVA in relation to Palmitic acid+Fructose (PA+Frut), where no pre-incubation with the selected fatty acids was performed. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. (D) Fluorescence imaging and quantification of human microglia cell line (HMC3) expressing the LynSrc FRET sensor in cells subjected to the stimulus solution (Palmitic acid+Fructose) and in cells pre-incubated with the studied fatty acids (omega-3, CLA and CLNA) for the selected time points (0, 5, 10 and 15 min). Error bar represents the SEM calculated from n>15 cells from two independent cultures. Two-way ANOVA in relation to Palmitic acid+Fructose (PA+Frut), where no pre-incubation with the selected fatty acids was performed. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.3. Omega-3 and CLA display antioxidant capacity

Microglia response to tissue damage and infection is characterized by the production and release of ROS to the surrounding CNS milieu (86,87), which directly changes cell metabolism, transcription, and the secretion of bioactive molecules such as cytokines, chemokines, and neurotransmitters. Studies in BV-2 microglia-like cells and primary microglia showed that besides increasing the microglial secretion of pro-inflammatory cytokines, SFAs promote ROS production (58). Thus, we assessed the PA+Fructose impact on ROS production, as well as omega-3 (EPA and DHA), CLA (RA and C18:2 *t*10*c*12 CLA isomer), and CLNA (PUA) antioxidant potential in HMC3 human microglia. We found that PA+Fructose stimulation caused a time-dependent increase in ROS production in living microglia (**Figure 2.6C**). However, pre-incubation of microglia with 100 µM omega-3 abrogated the PA+Fructose-induced ROS generation in living microglia, suggesting that, under these experimental conditions, omega-3 displays an antioxidant potential. Pre-incubation of microglia with 100 µM CLA also significantly prevented the ROS-generating effect of PA+Fructose (**Figure 2.6C**). Although CLNA treatment promoted a noticeable tendency to suppress ROS generation triggered by PA+Fructose, the inhibitory effect of CLNA on ROS production, under the tested experimental conditions, did not reach statistical significance (**Figure 2.6C**). These results indicate that omega-3 and CLA attenuate the oxidative stress generated by PA+Fructose and are potential regulators of the cytosolic redox balance in microglia.

3.4. CLNA and omega-3 prevent Src tyrosine kinase activation by fructose and palmitic acid

Activation of the cytosolic tyrosine kinase Src is essential to produce inflammatory mediators by microglia (55,77). Thus, we tested whether PA+Fructose could also modulate Src in microglia. To answer this question, we used the LynSrc FRET sensor, which reports specifically the activity of c-Src, but not other Src family kinases (SFKs), at the plasma membrane of living cells. We observed that PA+Fructose leads to a time-dependent increase of Src activity in living microglia (**Figure 2.6D**). Interestingly, whereas the pre-incubation of microglia with CLNA or omega-3 significantly suppressed the activation of Src triggered by PA+Fructose, no inhibitory effect was observed by pre-incubating the cells with CLA (**Figure 2.6D**).

3.5. Chemical activation of the GPR120/FFA4 receptor does not suppress NF-κB pathway activation but inhibits fructose and palmitic acid-induced ROS production

Some PUFAs, such as the omega-3 fatty acids, ALA, DHA, and EPA, are known to activate the GPR120 receptor (38). Moreover, activation of GPR120 is considered a significant mediator of omega-3 anti-inflammatory actions (88). The *ortho*-biphenyl ligand 4-{[4-fluoro-4′-methyl(1,1′-biphenyl)-2-yl]methoxy}-benzenepropanoic acid (TUG-891) is the first selective

agonist of GPR120 (89), with reported biological effects in the range of 10 µM (68–70). This is considered a significant selective agonist ligand for GPR120, showing good potency at both human and mouse GPR120 (89–91).

The expression of GPR120 in our cell line was confirmed through Western Blot by the detection of a band of approximately 70 kDa (**Figure S1B supplementary material**), which agrees with previous studies reporting molecular weights bands ranging from 42 kDa to 90 kDa (92). Indeed, the detection of higher molecular weight receptor dimers and oligomers are features commonly found after SDS-PAGE in G-Protein coupled receptors in general (93). Since GPR120 was confirmed to be expressed in the HMC3 cell line, TUG-891 was assayed to activate GRP120 in microglia following exposure to PA+Fructose. The results suggest that TUG-891-modulation of GPR120 did not prevent the PA+Fructose -mediated activation of the canonical NF-κB pathway, during the assayed time (**Figure 2.7A**) but completely suppressed the generation of ROS elicited by PA+Fructose (**Figure 2.7B**). These results may suggest that activation of GRP120 alone may modulate the redox balance without affecting the classical inflammatory status of microglia exposed to a WD-mimicking solution.

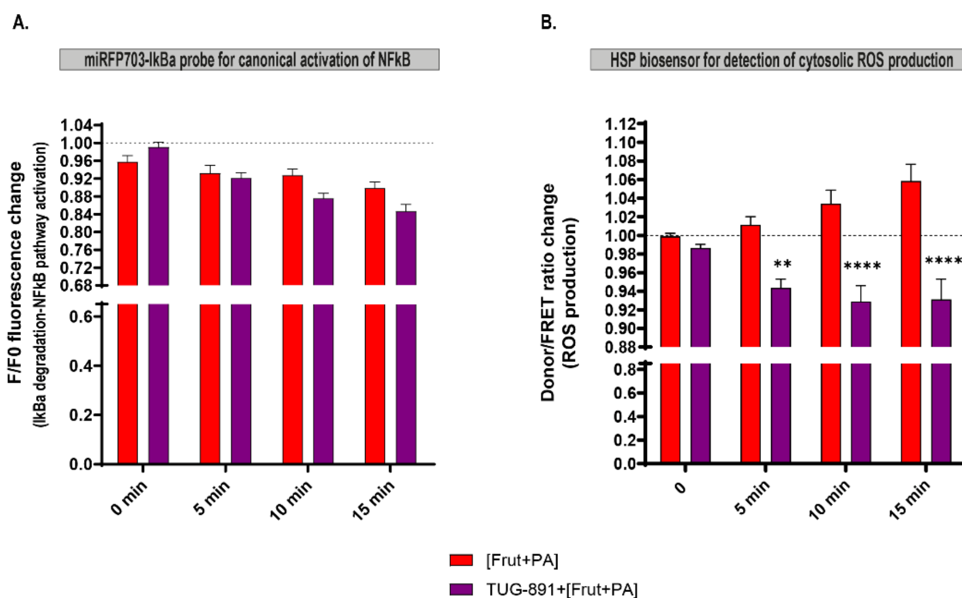

Figure 2.7- Chemical activation of GPR120 by TUG-891 enhances NF-κB pathway activation and inhibits ROS production by Fructose and Palmitic acid. (A) Results of the quantification of human microglia expressing the miRFP703- Ikβα sensor for the selected time points (0, 5, 10, and 15 min). A decreased signal means a bigger Ikβα degradation and higher NF-κB pathway activation. Error bar represents the SEM calculated from n>12 cells from two independent cultures. (B) Results of the quantification of human microglia expressing the HSP-FRET sensor for the selected time points (0, 5, 10, and 15 min). Error bar represents the SEM calculated from n>10 cells from two independent cultures. (A) and (B) Two-way ANOVA in relation to Palmitic acid+Fructose (PA+Frut). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.6. Omega-3, CLA, and CLNA anti-inflammatory, but not antioxidant action, occurs through the GPR120 receptor in microglia

Considering that some studies have demonstrated that omega-3 can activate GPR120 (94,95), we hypothesized whether CLA (RA and C18:2 *t*10*c*12 CLA isomer) and CLNA (PUA) may

modulate NF-κB pathway and ROS production through GPR120, in microglia. Thus, we tested AH7614, a non-competitive antagonist of the GPR120 receptor (71), in microglia exposed to PA+Fructose. We found that the pharmacological blockade of GPR120 by 100 μM AH7614 abolished the inhibitory effect on the NF-κB pathway triggered by omega-3 **(Figure 2.8A1)**, CLA **(Figure 2.8A2)**, and CLNA **(Figure 2.8A3)** following exposure to PA+Fructose. Interestingly and opposing to the results obtained with TUG-891 (GPR120 agonist), treatment with AH7614 did not block the effect of omega-3 **(Figure 2.8B1)** or CLA **(Figure 2.8B2)** on ROS production upon PA+Fructose exposure. Thus, these data strongly suggest that distinct pathways control the anti-inflammatory and antioxidant potential of omega-3 and CFAs (CLA and CLNA) in microglia exposed to a WD-mimicking solution.

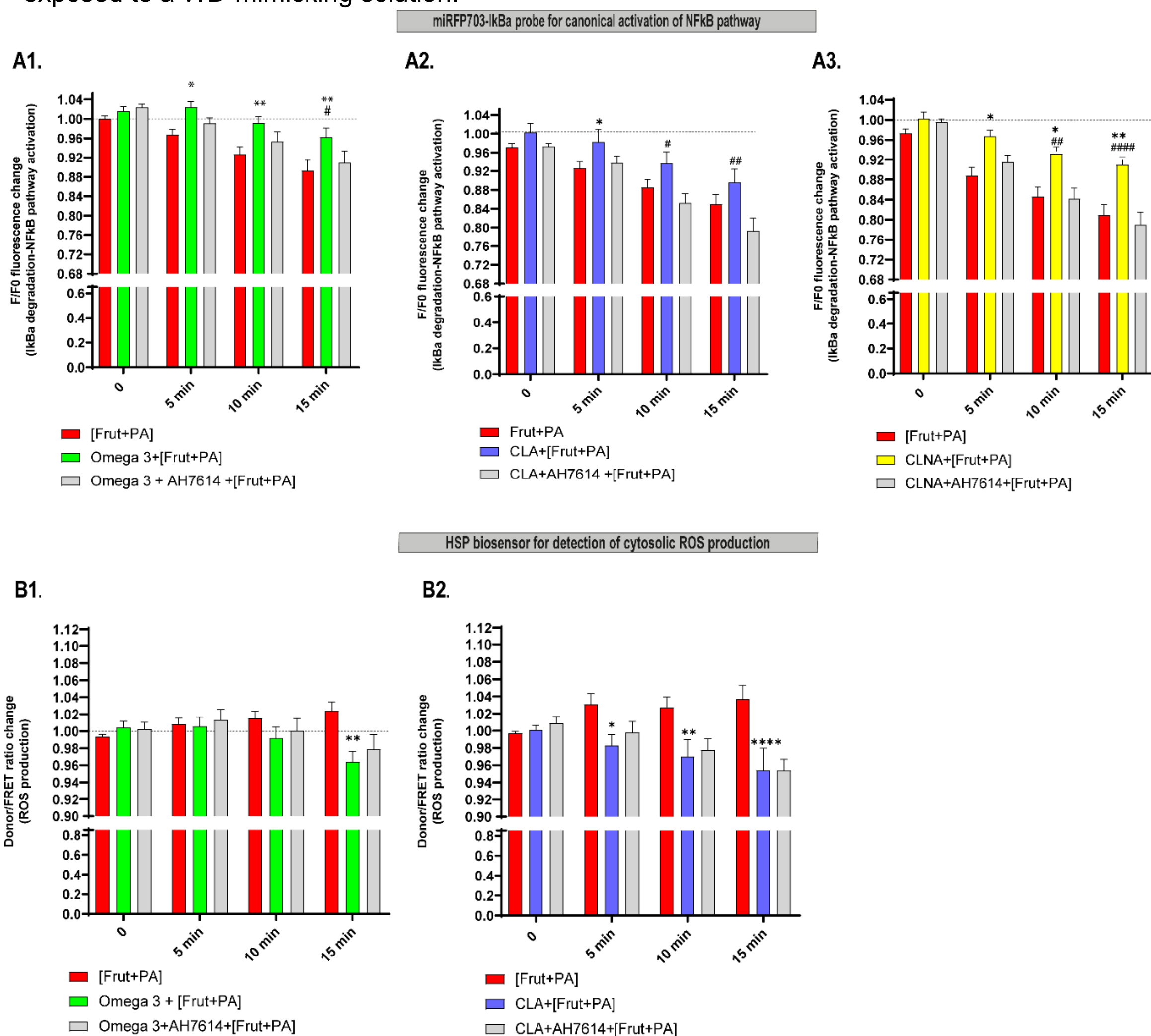

Figure 2.8- Chemical inhibition of GPR120 by AH7614 prevents omega-3, CLA, and CLNA anti-inflammatory action but is not involved in omega-3 and CLA antioxidant action. (A) Results of the quantification of human microglia expressing the miRFP703-Ikβα sensor for the selected time points (0, 5, 10, and 15 min). A decreased signal means a bigger Ikβα degradation and higher NF-κB pathway activation. Error bar represents the SEM calculated from n>20 cells from two independent cultures. AH7614 inhibition of GPR120/FFA4 in cells exposed to (A1) omega-3, (A2) CLA (rumenic acid and C18:2 t10c12 CLA isomers), and (A3) CLNA (PUA isomer). Two-way ANOVA in relation to Palmitic acid+Fructose (PA+Frut), where no pre-incubation with the selected fatty acids was performed: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and in relation to cells which were pre-incubated with omega-3/CLA/CLNA and AH7614, a GPR120 inhibitor: #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001. (B) Results of the quantification of human microglia expressing the HSP-FRET sensor for the selected time points (0, 5, 10, and 15 min). Error bar represents the SEM calculated from n>12 cells from two independent cultures. AH7614 inhibition of GPR120/FFA4 in cells exposed to (B1) omega-3 and (B2) CLA (rumenic acid and C18:2 t10c12 CLA isomers). Two-way ANOVA in relation to Palmitic acid+Fructose (PA+Frut) (*), where no pre-incubation with the selected fatty acids was performed: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and in relation to cells which were pre-incubated with omega-3/ CLA and AH7614 (#), a GPR120 inhibitor: #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001.

4. Discussion

PA is an important SFA, indeed its average dietary intake is around 20-30g/day and it is found in many dietary sources, with levels of 20-30% in animal lipids and 10-45% in vegetable oils (96). Significantly, as reported by Nadjar et al. (32) dietary fatty acids can cross the BBB and modulate CNS biochemistry. Different lipids, mainly SFAs, and PUFAs, have been proven to induce microglia activity. Indeed, chronic or acute exposure of microglia in culture to SFAs activates the NF-κB pathway (31) and classical downstream inflammatory cascades (57). Although the exact mechanisms through which FA exerts their action are elusive, microglia express a wide range of lipid-sensitive receptors (32) that could potentially initiate microglia inflammation. Therefore, SFAs' entry into the CNS could be a potential nutritional trigger for hypothalamic inflammation in the context of diet-induced obesity (31). Importantly, high-fructose consumption has also been associated with metabolic syndrome development and obesity development, in part related to inflammatory pathways activation (including NF-κB) (23,97). Fructose consumption has been increasing and has been highly associated with obesity prevalence. In fact, since the 1970s fructose intake has increased to a mean of around 7.5% of total energy intake, in the USA (98). Interestingly, high fructose consumption has been associated with cognitive dysfunction in rats. Moreover, such effects may be amplified by diets low in omega-3 FAs, suggesting the importance of these FAs in fructose-induced effects. In addition, combining fructose with SFAs (coconut oil) causes worse memory defects than when combined with fats rich in omega-6 PUFAs (99). Recently, it was suggested that neuroinflammatory and neurodegenerative diseases, such as Alzheimer’s development may be related to the activation of the fructose survival pathway by eating excess sugar and fructose (99). Suggesting a mechanism of endogenous fructose production in the brain.

Mammalian NF-κB functions as a dimer composed of p50 (NF-κB1) and p65 (RelA). Under normal conditions, NF-κB remains inactive in the cytoplasm by binding to a set of proteins from the IκB inhibitory protein family. In the canonical pathway, following cellular stimulation, activation of IKKβ induces IκB phosphorylation by IKKβ, which results in proteasomal degradation, NF-κB nuclear translocation, and transcription of its target genes, including TNF-α, IL-1β, and cyclooxygenase-2 (COX-2) (100,101). PA induces the phosphorylation and nuclear translocation of the p65 subunit of NF-κB in BV-2 microglia, resulting in proinflammatory activation (58). Recently, it was demonstrated that PA-induced time- and dose-dependent lipotoxicity in BV-2 microglia cells, since it decreases cell viability and increases cell death (102). Our data suggest that the combined action of PA+Fructose induces Ikβα degradation and p65 subunit nuclear translocation in HCM3 human microglia. PA+Fructose exposure also leads to ROS production and Src activation (critical regulators of microglia inflammation), suggesting that a WD may trigger neuroinflammatory and neurotoxic effects in the CNS.

In the brain, PUFAs are esterified to phospholipids and largely located in the membranes of neurons, glial cells, and endothelial cells (32). PUFAs account for 35% of total lipids in the adult

brain (103). AA and DHA, which make up 50% and 40% of brain PUFAs, are essential to brain development and function. Although SFAs act deleteriously on microglia (*i.e.,* inflammation triggers), PUFAs can normalize these harmful effects. The anti-inflammatory effect of the omega-3 FAs EPA and DHA is thought to involve the inhibition of the phosphorylation of the inhibitory subunit of NF-κB – Ikβ (104). In this work, exposure to omega-3 (EPA and DHA) blocked the NF-κB pathway activation and the production of ROS triggered by PA+Fructose. These results agree with others reported elsewhere showing a reduction in ROS production in BV-2 microglia supplemented with PUFAs (105). Omega-3 also prevented the activation of Src in microglia. Indeed, Src tyrosine kinase is recognized as a potential therapeutic target for neuroinflammation-related diseases, including Parkinson’s (106) and Alzheimer’s disease (107). Besides EPA and DHA, CLA (RA and C18:2 *t*10*c*12) inhibited NF-κB pathway activation and ROS production by PA+Fructose. Exposure to CLNA (PUA) also inhibited the NF-κB pathway and Src activation. This is the first demonstration of a potential modulatory role for CFAs in microglia to the best of our knowledge. Interestingly, the inhibitory effect on the NF-κB pathway was more pronounced in microglia pre-incubated with the CLNA isomer, PUA, than in microglia exposed to the CLA isomers. Importantly, we have reported that microglia activation requires c-Src and that its activation *per se* is sufficient to trigger a classical proinflammatory signature following acute LPS or hypoxia challenging (55,56). In addition, we have also demonstrated that overexpression of a constitutively active c-Src was sufficient to increase the nuclear accumulation of p65 suggesting that c-Src promotes NF-κB activation during microglia activation (108). These observations may explain the difference in NF-κB pathway regulation capacity when comparing CLA and CLNA isomers. The CLNA (PUA) effect on Src activation, which is not observed with the CLA isomers, may contribute, in part, to CLNA's capacity to strongly regulate the NF-κB pathway.

SFAs activate TLR4 and this one regulates the Myd88, TAK1/TAB1 modulation, and NF-κB activation. On the other hand, omega-3 fatty acids, such as EPA and DHA, activate GPR120. This interaction recruits β-arrestin 2, leading to the internalization of the GPR120-β-arrestin 2 complex. Such complex interacts with TAB1, explicitly inhibiting TAK1 phosphorylation and activation (39,109). Indeed, GPR120 is known to bind some omega-3 fatty acids, namely DHA and EPA (38) and ALA (37). GPR120 is thought to be expressed predominantly in microglia cells, whereas GPR40/FFA1 is in POMC and NPY neurons (110). GPR120 activation by the synthetic agonist TUG-891 failed to block NF-κB pathway activation by PA+Fructose. Indeed, contradictory results have been reported: in mouse intestinal epithelial endocrine cell line, STC-1, exposure to TUG-891 for 30 min did not induce GPR120 internalization and presented no effects on NF-κB, while the opposite was reported in Caco-2 cells (111). On the other hand, in our study, inhibiting GPR120 with AH7614 abolished the effect of omega-3 (EPA and DHA) on the NF-κB pathway, confirming that omega-3 anti-inflammatory action occurs via GPR120 activation as reported by previous studies (88,110). There is a lack of information about the interaction of CFAs with GPR120, especially in microglia. The CLA isomer RA, but not the C18:2 *t*10*c*12 isomer, was found to enhance GPR120 expression (112). Here, CLA (RA and C18:2 *t*10*c*12) and CLNA (PUA)

actions on the NF-κB pathway inhibition were mediated through GPR120 signaling in HMC3 microglia. CLA and CLNA may have a mechanism of action similar to that of omega-3 on microglia, probably via GPR120 activation and modulation of NF-κB-associated inflammatory pathways. Moreover, our results, using TUG-891 agonist and AH7614 antagonist may suggest that although omega-3, CLA, and CLNA fatty acids action on the NF-κB pathway is mediated by GPR120 other mechanisms may be linked and are necessary for such action. Indeed, TUG-891, a selective agonist for GPR120, failed to suppress NF-κB pathway activation in our study. In a different study, it was suggested that the ability of GPR120 to couple to Gαq or β-arrestin-2 may explain the divergence observed in different cell types since GPR120 stimulation could induce distinct signaling pathways (111). In addition, some studies have reported that attenuation of microglial activation by lauric acid (a medium-chain saturated fatty acid) may occur via the GPR40-dependent pathway (113). Moreover, another study demonstrated that exposure of murine primary hepatocytes to DHA for 12 h increased both GPR40 and GPR120 mRNA levels (114). Thus, the combined role of GPR40 and GPR120 on NF-κB pathway regulation cannot be disregarded. Thus, further studies should focus on describing the ligand-receptor interaction to fully understand if the response is dependent on the FAs. Regarding ROS production, interestingly, GRP120 activation by TUG-891 prevented the oxidative stress generated by PA and fructose. However, omega-3 and CLA antioxidant capacity was not mediated by GPR120. These results indicate that the anti-inflammatory action and antioxidant potential of omega-3, CLA, and CLNA occur through different signaling mechanisms, as previously reported elsewhere for omega-3 DHA in macrophages (115). This is highly relevant since ROS production has been suggested to be intrinsically connected to NF-κB pathway activation in microglia (101). Indeed, the redox state is widely accepted to control NF-κB nuclear levels (87), and inhibition of NF-κB associates antioxidant and anti-neuroinflammatory effects of specific bioactive molecules on microglia (116). Other antioxidants, such as ascorbate (55) and piperlongumine (117) also exert their effects by suppressing NF-κB activity in microglia. Our data suggest that the antioxidant potential of omega-3 and CLA isomers was not related to NF-κB pathway inhibition. For instance, it has been reported that CLA can directly scavenge ROS in the human neuroblastoma cell line SH-SY5Y (117) and a modest scavenge potential was demonstrated for omega-3 DHA (115). Nevertheless, this is a controverse theme considering that some concerns are present due to their potential pro-oxidant effect, having detrimental effects in several tissues, especially in the CNS. On the other hand, the action of oxidized omega-3 fatty acids is known to be directed against kelch-like ECH-associated protein 1 (keap1). This protein is the negative regulator of the nuclear factor erythroid 2-related factor 2 (Nrf2) (118). Interestingly, tiliroside, a natural dietary glycosidic flavonoid, protects BV-2 microglia from LPS/IFN-γ-induced neuroinflammation via Nrf2 antioxidant mechanisms (119). An Nrf2-dependent antioxidant role for omega-3 fatty acids EPA and DHA may also occur in rat primary astrocytes (120). Thus Nrf2-dependent antioxidant mechanism for omega-3, and eventually for CLA and CLNA, cannot be excluded. The biological mechanisms proposed in this work are illustrated in **Figure 2.9.**

Despite their relevance and novelty, these results must be carefully considered since a cell line was used. In fact, cell lines are incredibly advantageous due to their ease, especially considering the performed assays using FRET technology. Nevertheless, it must be pointed out that a major disadvantage is their susceptibility to be dedifferentiated and the possibility that the immortalization process may alter the microglial phenotype. Indeed, recent studies have pointed out that microglia cell lines differ genetically and functionally from primary microglia and *ex vivo* microglia. Despite such limitations, microglia cell lines are still suitable and a relevant tool for biochemical and molecular approaches as well as for high-throughput screening studies due to high cell number requirements such as in this experiment. Moreover, although primary cells are often used, they present several limitations regarding transfection processes, for instance. Moreover, rodent primary microglia face a major limitation due to their evolutionary divergence from humans. On the other hand, human primary microglia, besides ethical constraints, present limited availability of (healthy) human brain tissue, limited control over the *ante mortem* conditions, and *post-mortem* delay, which affect the microglia phenotype (121,122). Despite the limitations of using a cell line, HMC3 cells present the advantage of being authenticated by ATCC in terms of morphology evaluation, karyotyping, and PCR-based approaches to confirm their identity and rule out contaminations. Significantly, recent studies have also shown that HMC3 (ATCC CRL-3304) cells retain most of the original antigenic properties (121).

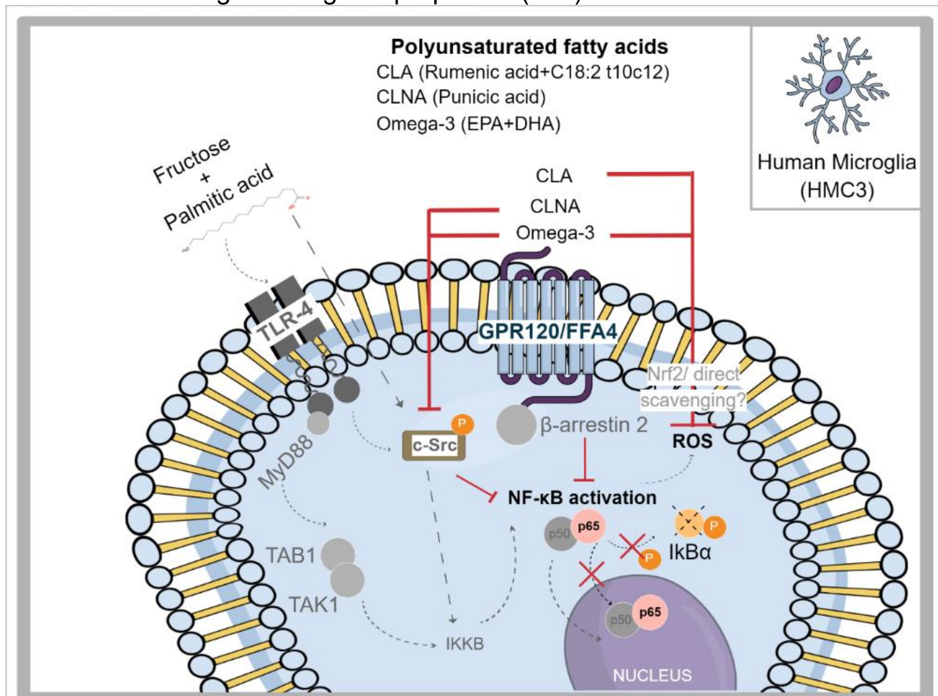

Figure 2.9 – Proposed molecular mechanisms involved in omega-3, CLA, and CLNA isomers' inhibitory action on microglia activation induced by Fructose and Palmitic acid. In our proposed mechanism Fructose and Palmitic acid combined action activate NF-κB through TLR4 binding. The activation of myeloid differentiation factor (MyD88) leads to TAK1 activation and consequent interaction with TAB1. Such effects result in IKKβ activation which ultimately leads to Ikβ phosphorylation and NF-κB nuclear translocation. Fructose and Palmitic acid are also able to induce oxidative stress through reactive oxygen species production (ROS) and Src tyrosine kinase activation. Omega-3 (EPA and DHA), CLA isomers (Rumenic acid and C18:2 t10c12), and CLNA isomer (Punicic acid) stimulate GPR120/FFA4 receptor which recruits β-arrestin 2. Their internalization inhibits NF-κB nuclear translocation and consequently its activation. Moreover, CLA and omega-3 show antioxidant potential that might be a result of direct scavenging or be mediated by Nrf2 antioxidant mechanisms. CLNA and omega-3 inhibit c-Src activation, which may also affect NF-κB pathway activation.

5. Conclusion

By a combination of live cell imaging and FRET technology, we were able to describe some of the mechanisms involved in PA+Fructose microglia activation. We have demonstrated that Fructose and PA combined action induces an immediate pro-inflammatory response in the human microglia cell line (HMC3). In fact, exposure to the WD-mimicking solution caused increased IkBα degradation and nuclear accumulation of GFP-tagged p65 NF-κB catalytic subunit, strong indicators of NF-κB pathway activation. Moreover, this stimulus caused a time-dependent increase in ROS production. For the first time, we have demonstrated that this stimulus increased Src activity in living microglia. Importantly, both ROS production and Src activation are critical regulators of microglia inflammation. Such results suggest a neuroinflammatory and neurotoxic effect of WD in the brain. In contrast, we have demonstrated that omega-3 and for the first time, CLA and CLNA isomers, prevented the induced-NF-κB pathway activation. However, only omega-3 and CLA displayed antioxidant potential by inhibiting ROS production, protecting the cells against the generated oxidative stress. Furthermore, only omega-3 and CLNA isomer PUA demonstrated an effect on Src activation. The effect of CLNA on Src tyrosine kinase activation may contribute to its strong regulatory action on NF-κB pathway regulation when compared to CLA. Interestingly, using a GPR120 antagonist (AH7614) it was observed that although a role between ROS production and NF-κB pathway activation is widely accepted, under these experimental conditions, while omega-3, CLA, and CLNA regulatory action on the NF-κB pathway is mediated by GPR120, the antioxidant potential of omega-3 and CLA isomers is mediated by different cellular mechanisms. Moreover, we have demonstrated that in microglia cells and under our experimental conditions, the anti-inflammatory action of CFAs was mediated by GPR120 and not by the widely reported PPARs.

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CHAPTER 3

Omega-3 and conjugated fatty acids bioaccessibility

This chapter intends to describe the bioaccessibility and bioavailability prediction of omega-3 and conjugated fatty acids from different matrixes.

This chapter was published as follows and is presented in this thesis with slight modifications:

Salsinha, A.S., Cunha, S., Machado, M., Rodriguez-Alcalá, L.M., Relvas, J.B., Pintado, M. (2023). Assessment of the bioaccessibility and bioavailability prediction of omega-3 and conjugated fatty acids by in vitro standardized digestion model (INFOGEST) and cell model. Food Bioscience, 53, 102635. https://doi.org/10.1016/j.fbio.2023.102635

Chapter 3

Chapter 3

Preface

What is the impact of the GIT on the bioaccessibility and bioavailability of omega-3 and CFAs?

To achieve therapeutical doses for omega-3 and CFA it is necessary to consume high amounts of these FAs. Therefore, the use of enriched oils has been used as an alternative, and oral supplementation is a preferable way of delivering such compounds since it allows an easy, straightforward, and regular delivery. This thesis aims to explore the anti-obesity potential of omega-3 and CFAs through the oral delivery of enriched oils. It is important to consider that several factors influence the bioavailability of a given FA, namely the type and composition of the matrix, the molecular form in which they are delivered, and the presence of certain antinutritional constituents. In addition, the GIT is an important player in the release of bioactive compounds, and it must not be neglected. Therefore, the study of bioaccessibility is of foremost importance to understand the full nutraceutical potential of these bioactive lipids considering the relevant modulatory effect of GIT digestion in these bioactive lipids. Moreover, a bioavailability prediction is essential to understand the potential of blood-stream absorption through oral delivery. This is important when studying a possible systemic therapy targeting the brain, as explored in **Chapter 2**.

Therefore, in this chapter, the bioaccessibility of different bioactive PUFAs, in different commercially available matrixes was determined using a standardized static GIT *in vitro* protocol (INFOGEST). The bioactive lipid content of the matrixes was assessed throughout the GIT by calculating the recovery index (RI) as well as the antioxidant potential. To simulate the intestinal permeability in the small intestine two different methods were used: the use of dialysis membranes and a Caco-2/HT-29-MTX coculture to predict the bioavailability of the different bioactive FAs. These studies allow the prediction of the content of two different fractions of the bioactive FAs after GIT digestion: serum-available and colon-available fractions. While the serum-available fraction is important for the modulation of different organs namely adipose and ultimately the brain. The colon-available fraction is valuable for gut microbiota modulation. So, these are important steps for the next studies specifically assessing the potential of these fatty acids in gut microbiota modulation.

Chapter 3

Assessment of the bioaccessibility and bioavailability prediction of omega-3 and conjugated fatty acids by *in vitro* standardized digestion model (INFOGEST) and cell model.

Ana Sofia Salsinha[a,b], Sara A. Cunha[a], Manuela Machado[a], Luís Miguel Rodríguez-Alcalá[a], João B. Relvas[b,c] and Manuela Pintado[a]*

a. Universidade Católica Portuguesa, CBQF - Centro de Biotecnologia e Química Fina –Laboratório Associado, Escola Superior de Biotecnologia, Rua de Diogo Botelho, 1327, 4169-005, Porto, Portugal

b. Instituto de Investigação e Inovação em Saúde and Instituto de Biologia Molecular e Celular (IBMC), Universidade Do Porto – Rua Alfredo Allen, 208, 4200-135, Porto, Portugal

c. Departmento de Biomedicina, Faculdade de Medicina da Universidade do Porto (FMUP), 4200-319 Porto, Portugal

Abstract

Omega-3 EPA and DHA are polyunsaturated fatty acids with relevant health benefits. CLA and CLNA are known for their anti-carcinogenic effect, anti-inflammatory properties, and body weight reduction. To achieve therapeutical doses, high amounts of these fatty acids' food sources must be consumed. Thus, the intake of enriched oils with a high concentration of these fatty acids is often used. However, several factors influence their bioavailability. Here, by using the INFOGEST static *in vitro* protocol of gastrointestinal tract digestion we studied the bioaccessibility of these fatty acids in different matrixes: Pomegranate and Fish oil and Omega-3, CLA, and CLNA soft-gel enriched capsules content. After digestion, the Recovery Index for the major bioactive PUFAs is very low: Pomegranate oil is 2%, Fish oil 11-13%, CLNA 17%, CLA 6%, and Omega-3 capsules 3%. Higher initial concentrations of these PUFAs seem to be related to higher degrees of oxidation. In Pomegranate oil, CLNA, and Omega-3 capsules, the digestion process negatively influenced the antioxidant potential. The opposite was verified for the Fish oil and CLA capsules. Importantly, bioaccessibility studies of similar matrixes are very scarce, and intestinal permeability is absent in most of the studies. Intestinal permeability studies were performed using a Caco-2/HT29-MTX co-culture: there is a significative incorporation of the bioactive fatty acids into the intestinal cells, which may affect their permeability performance. Interestingly, most fatty acids remain in the non-bioaccessible fraction which may be relevant when designing oral routes of administration and in gut microbiota modulation.

Keywords: Fish oil; Pomegranate oil; INFOGEST digestion method; Bioaccessibility; Intestinal Permeability; Bioactive fatty acids

Graphical Abstract

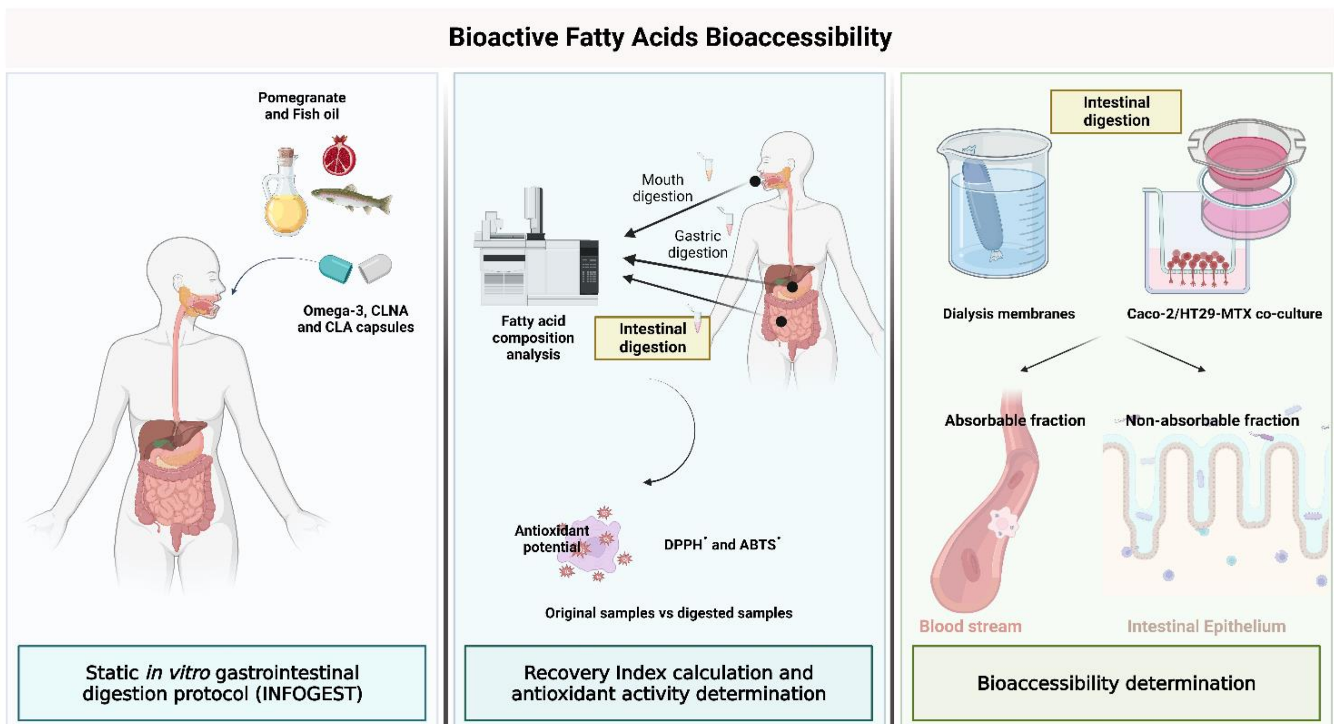


Figure 3.1 – Graphical abstract. By using the INFOGEST static in vitro protocol of GIT digestion it was studied the bioaccessibility of bioactive FAs – omega-3 EPA and DHA, CLA isomers RA and C18:2 t10c12 and CLNA isomer PUA - in different matrixes: Pomegranate and Fish oil and Omega-3, CLA and CLNA soft-gel enriched capsules. The RI (%) of those bioactive FAs was determined throughout the different stages of the GIT and the antioxidant potential was determined. Intestinal permeability studies were performed using a Caco-2/HT29-MTX co-culture model and 3.5 kDa dialysis membranes.

1. Introduction

The importance of lipids in our health is now well-established. Among the main important lipid molecules are TGs) and their metabolites (mono- and diglycerides and FAs) (1). Regarding FAs, PUFAs are the ones most widely recognized and studied due to their proven health benefits. The omega-3 PUFAs EPA (C20:5 *c*5*c*8*c*11*c*14*c*17) and DHA (C22:6 *c*4*c*7*c*10*c*13*c*16*c*19) are considered essential nutrients found in fatty fish such as mackerel, herring, and salmon (2). They have been associated with the reduction of TGs and cholesterol levels, normalization of blood pressure, and the consequent promotion of cardiovascular health (3). Some studies have also reported potential antioxidant properties, which may be relevant in the treatment of Sickle cell disease (4) or T2DM (5). Other PUFAs have been gaining attention due to their role in health promotion. For instance, CLA isomers have been shown as an agonist of several PPAR isoforms, being thus, responsible for the reduction of inflammatory responses (6). Additionally, CLNA isomers, specifically PUA (C18:3 *c*9*t*11*c*13), the most studied CLNA isomer, have been demonstrated to have a positive on body weight (7–9) and its effects on peripheral tissues, are widely recognized (10).

EFSA has established that 250 to 500 mg/day of EPA and DHA are the dietary recommended daily dose for European adults, based on cardiovascular risk considerations (11). Regarding CFAs, namely CLA – specifically RA and C18:2 *t*10*c*12 - and CLNA – specifically PUA – the effective doses have been described as 3 g/day (12) and 2-3 g/day (13), respectively. Consequently, one of the biggest concerns is that considerably high amounts of Fish, Pomegranate seed oil, CLA, or CLNA food products would have to be consumed to achieve an adequate intake of their respective bioactive FAs to attain their therapeutic benefits. Thus, the adopted strategy has been raising PUFA's plasma concentration by supplementation of enriched oils containing high concentrations of the mentioned bioactive FAs in the form of soft gel capsules or emulsions (14). Indeed, oral supplementation has been a preferable way of delivering such compounds. Nevertheless, several factors such as the type and processing of food or even the presence of a certain antinutritional constituent, influence the bioavailability of a given FA (15). This justifies the interest in FA composition and FA bioaccessibility and the relevance of this study: it aims to assess the bioaccessibility of bioactive FAs, omega-3 EPA, and DHA, CLNA isomer PUA, and CLA isomers RA and C18:2 *t*10*c*12, using a standardized static *in vitro* protocol (INFOGEST 2.0) mimicking the GIT digestion. Importantly, the GIT has been described as an important player in the release of bioactive compounds, being responsible for both positive and negative effects on bioactive compounds' bioaccessibility. This perception justifies the importance of GIT simulation, to fully comprehend the potential of bioactive ingredients present in a certain matrix after oral consumption. Considering that *in vivo* studies are very time-demanding, require large resources, and present some analytical and ethical constraints (16), *in vitro* models have been used for many decades to simulate the digestion of food. But as far as we are concerned there are only a few studies on lipid bioaccessibility in general and even fewer using similar

matrixes as the ones studied here. 7 studies were found applying *in vitro* GIT models to access FAs or lipid bioaccessibility, 2 studies (14,17) used a dynamic GIT model (TIM system) and 5 studies (15,18–20) used a static *in vitro* model, only 3 (19–21) used INFOGEST protocol. INFOGEST is a recent standardized protocol developed in 2014 and optimized to an improved digestion method (INFOGEST 2.0) in 2019 (22). Such protocol was created since *in vitro* simulations have used a wide range of different conditions that often have very little physiological relevance, and this prevents the meaningful comparison of results. It has been reported considerable discrepancies between different digestion models, which were attributed to variations in the experimental parameters, *e.g.,* chyme transit and enzyme concentration (23). Thus, the importance of using a standardized method to compare the bioaccessibility results obtained is of foremost importance. In addition, in most experiments aiming to study the bioaccessibility of FAs, these studies do not ultimately assess intestinal permeability lacking an adequate comprehension of bioavailability. Since there are very few studies addressing the bioaccessibility of FAs on bioactive oil matrixes and even fewer were performed using INFOGEST protocol, we performed a deep study that intends to validate the most relevant PUFAs' bioaccessibility. So, this study intends to assess the impact of *in vitro* simulation of GIT on FAs composition and their bioactive properties, specifically on antioxidant activity. Moreover, to study the bioavailability of these FAs, we evaluated the absorption and intestinal permeability after digestion.

2. Materials and Methods

2.1. Chemicals and reagents

For the *in vitro* GIT tract digestion α-amylase from human saliva (A1031-5KU), Bile salts (bile extract porcine – B8631), and Pancreatin from porcine pancreas (P7545) were purchased from Sigma-Aldrich (Missouri, USA). Rabbit gastric extract (RGE 15) was obtained from Lipolytech (Marseille, France). The Dialysis 3.5 kDa tubing membranes (Pre-wetted RC Tubbing, Spectra/Por®6 Dialysis Membrane; 734-0652) were purchased from VWR Chemicals (Pennsylvania, USA).

For the FAs profile analysis, Hexane, Methanol, Dimethylformamide (DMF), and Acetonitrile were HPLC grade and purchased from VWR Chemicals. Sulphuric acid was obtained from Honeywell (North Carolina, USA). Sodium methoxide was from Acros Organics (Geel, Belgium). Tritridecanoin (33-1300-13) internal standard was from Larodan Research Grade Lipids (Solna, Sweden).

Regarding the antioxidant activity assessment, the DPPH reagent (2,2-diphenyl-1-picrylhydrazyl free radical, 44150) was obtained from Alfa Aesar (Kandel, Germany). Methyl-β-cyclodextrin (332615), Fluorescein (F6377), 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, A1888), (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, 238813) and 2,2'-Azobis(2-methyl-propionamidine) dihydrochloride (AAPH, 440914) were purchased from Sigma-Aldrich. Ethyl acetate (E/0906/17) was HPLC grade from Thermo Fisher Scientific (Massachusetts, USA). Ethanol absolute (4146052) was from Carlo Erba reagents (Val de Reuil, France) and Acetone from Honeywell.

2.2. FAs Sources

Fish oil from Menhaden (F8020) was purchased from Sigma-Aldrich. Pomegranate Kernel Oil cold pressed, as a PUA source, was supplied by All Organic Treasure (Germany). CLA, CLNA, and Omega-3 capsules were purchased from commercially available sources from two relevant Portuguese sports nutrition brands. The use of the capsules is intended to assess the relevance of different matrixes in the bioactive FAs' bioaccessibility. Indeed, using both oils and capsules the two main relevant sources of commercially available options for bioactive FAs oral supplementation, were covered. CLA capsules are MEGACLA A95 from Gold Nutrition, which presents as the main ingredient CLARINOL® A-95 Amber capsules with a 50:50 ratio of C18:2 *c*9*t*11 and *t*10*c*12 isomers. Omega-3 Fully concentrated EPA&DHA capsules (here defined as Omega-3 capsules) were purchased from Prozis and are described to be comprised of 360 mg of EPA and 240 mg of DHA. As a CLNA isomer source, specifically PUA, Xanthigen® capsules (here simply defined as CLNA capsules) purchased in Cellulase were used, with 100 mg of Pomegranate oil extract/capsule. The capsule content was extracted and further analyzed. The objective of this study was not to assess the gel capsules as a vehicle to deliver omega-3 FAs or

conjugated FAs, but instead to determine how these FAs behave throughout the GIT tract in different formulations and concentrations.

2.3. *In vitro* GIT tract digestion

For the simulation of the GIT tract digestion, the standardized static digestion model INFOGEST 2.0 protocol (22) was followed. The experimental procedure is divided into the oral, gastric, and intestinal phases with the corresponding fluids to better simulate the *in vivo* conditions. Shortly, mouth digestion includes the dilution of food 1:1 (w/w) with simulated salivary fluid complemented with salivary α-amylase from human saliva. The mastication of the food is simulated using an Orbital Shaker MaxQ 6000 at 200 rpm and 37 °C for 2 min. As described in the INFOGEST protocol, the oral bolus is then diluted 1:1 (v/v) with simulated gastric fluid and gastric enzymes – pepsin and lipase from rabbit gastric extract – and agitated in the mentioned orbital shaker at 130 rpm and 37 °C, at pH 3.0 for 2 h. The resultant gastric chyme is subsequently diluted 1:1 (v/v) with simulated intestinal fluid, bile salts, and pancreatic enzymes (pancreatin from the porcine pancreas) and incubated at pH 7.0 for 2 h, at 45 rpm and 37 °C in the orbital shaker. For each sample (3 g/sample) two replicas were performed and a negative control without a sample, using distilled water, was used. The initial mass was set at 3 g/ sample considering that the effective dose for CLA and CLNA (PUA) is described to be 2-3 g/day. Although the recommended dose for EPA and DHA is 250-500 mg/day, supplemental intakes of EPA and DHA combined at doses up to 5 g/day do not raise safety concerns for adults (11). Thus, for uniformization reasons and to cover all the effective doses for the different bioactive FAs the assay value was set at 3 g. To screen the action of GIT tract digestion conditions in the lipid profile of the samples, aliquots were collected at the different stages of digestion (oral, gastric, and intestinal phases).

2.4. Bioaccessibility assessment using dialysis membranes

After intestinal digestion a segment of dialysis tubing (3.5 kDa molecular weight cut-off) was filled with the digested samples and placed inside a recipient filled with distilled water and further incubated overnight in the orbital shaker, at 37 °C and 50 rpm, mimicking peristaltic movements. The dialysis membrane size was selected according to the recommendations of the INFOGEST protocol (3-10 kDa) (22) and previous studies (24–26). The dialysis process had the goal of simulating the passage of the digested samples by the small intestine (specifically the duodenum and jejunum, at least) (27). At the end of the process, the solution left outside (OUT) represented the sample that was available for absorption (serum-available), and the solution that was left inside (IN) the dialysis membrane represented the non-absorbable sample (colon-available) (26,28).

After, the dialysis submitted samples, both retentate (IN) and permeate (OUT) were lyophilized and stored for analysis of the bioactive lipids.

2.5. Stability and bioaccessibility of compounds through *in vitro* GIT tract

2.5.1. FAs profile

2.5.1.1. Sample preparation

The FAs profile of the samples and the action of the *in vitro* GIT tract in the different stages of the digestion was assessed through GC-FID and compared with the original samples which were not subjected to the GIT tract conditions. 250 µL of digested samples and 50-100 mg of lyophilized dialyzed solutions were prepared according to previous studies (29,30). Briefly, for quantification purposes, 200 µL of tritridecanoin acid (1.5 mg/mL) was added before the derivatization process as an internal standard. Afterward, 2.26 mL of methanol, 1 mL of hexane, and 240 µL of sodium methoxide (5 M) were added. Samples were vortexed and incubated at 80 °C for 10 min. After cooling in ice, 1.25 mL of DMF and 1.25 mL of sulphuric acid (3M) were added. Again, samples were vortexed and then incubated at 60 °C for 30 min. After cooling, 800 µL of hexane was added. The samples were vortexed and centrifuged (1250xg, 18- °C for 5 min). The upper layer containing methyl esters (FAME) was collected for gas chromatography analysis.

2.5.1.2. FAs content analysis with gas chromatography

FAME extracts were analyzed using a gas chromatograph HP6890A (Hewlett-Packard, Avondale, PA, USA) equipped with a flame-ionization detector (GC-FID) and a BPX70 capillary column (SGE Europe Ltd, Coutaboeuf, France). As previously described (30), the injector temperature was 250 °C, split 25:1, the injection volume was 1 µL, and the detector (FID) temperature was 275 °C. Moreover, hydrogen was the carrier gas at 20.5 psi. The oven temperature program was as follows: starting at 60 °C (held for 5 min), then raised at 15 °C/min to 165 °C (held for 1 min), and finally at 2 °C/min to 225 °C (held 2 min). GLC-Nestlé36 was assayed for calculation of response factors and detection and quantification limits (LOD: 0.79ng FA/mL and LOQ:2.64ng FA/mL).

2.5.2. Nutritional quality indexes

To evaluate the nutritional quality values and assess the FA composition of the various samples analyzed, the atherogenicity index (AI), thrombogenicity index (TI), and hypocholesterolemic/hypercholesterolemic ratio (HH) were calculated according to previous studies (31,32) and are described in Equations 3.1, 3.2 and 3.3, respectively.

$$AI = \frac{C12{:}0 + (4 \times C14{:}0) + C16{:}0}{\sum MUFA + \sum PUFA} \quad \textbf{(Equation 3.1)}$$

$$TI = \frac{C14{:}0 + C16{:}0 + C18{:}0}{0.5 \times \sum MUFA + 0.5 \times \sum PUFA\ omega{-}6 + 3 \times \sum PUFA\ omega{-}3 + \frac{\sum PUFA\ omega{-}3}{\sum PUFA\ omega{-}6}} \quad \textbf{(Equation 3.2)}$$

$$HH = \frac{\mathrm{C18{:}1n{-}9 + C18{:}2n{-}6 + C18{:}3n{-}3 + C20{:}4n{-}6 + C20{:}5n{-}3}}{C14{:}0 + C16{:}0} \quad \textbf{(Equation 3.3)}$$

2.5.3. Recovery and bioaccessibility index

The RI (%, **Equation 3.4**) and bioaccessibility index (BI %, **Equation 3.5**) were calculated to determine the GIT digestion effect on the different matrixes based on previous studies (28,33,34). The values of the bioactive FAs in the original samples before digestion were assumed as 100%. Accordingly, the RI calculation allows the determination of the amount of a given main component - bioactive FAs, specifically -, in the tested matrix after digestion (in the oral, gastric, and intestinal phases). So, RI is calculated as follows:

$$\mathrm{RI}\ (\%) = \frac{\text{Bioactive content in the digested sample } (\mathrm{BC_{DF}})}{\text{Bioactive content quantified in the test matrix } (\mathrm{BC_{TF}})} \times 100 \quad \textbf{(Equation 3.4)}$$

Bioaccessibility refers to the amount of a given compound that is released from its matrix in the digestive tract and could potentially become available for bloodstream absorption. The bioaccessibility index is defined as the percentage of the bioactive compound that is available for absorption after intestinal dialysis. Such concept, here determined by using the *in vitro* GIT tract simulation and intestinal dialysis, is important for determining the bioavailability of a given compound, which corresponds to the fraction of an ingested bioactive compound or nutrient that reaches the systemic circulation and performs its bioactive functions (26). Consequently, bioavailability includes the term bioaccessibility. Thus, the BI corresponds to the percentage of the bioactive compound that passes the dialysis membrane and consequently, this index defines the proportion of the bioactive compound that could, potentially, become available for absorption in the systemic circulation. The BI index is calculated as:

$$\mathrm{BI}\ (\%) = \frac{\text{Bioactive content in the digested sample after the dialysis step } (\mathrm{BC_{S}})}{\text{Total bioactive content in the digested sample after the dialysis step } (\mathrm{BC_{DF}})} \times 100 \quad \textbf{(Equation 3.5)}$$

Where BC_S corresponds to the OUT fraction of the dialysis step, the absorbable fraction. And BC_{DF} to the total bioactive content, IN (retentate/ non-absorbable) + OUT (permeate/ absorbable) fractions.

Importantly, most studies refer to RI as BI, without taking into consideration that bioaccessibility is a term that refers not only to the effect of GIT in a given bioactive molecule of a food matrix but also to the quantity of the bioactive compound that can be absorbed and potentially reach the systemic circulation.

2.5.4. Effect of *in vitro* GIT digestion on antioxidant activity

The antioxidant activity of the fully digested and lyophilized samples was determined using ABTS and DPPH methods as described in the following sections and using a multi-detection plate reader (Synergy H1, Vermont, USA). The activity of the lyophilized digested samples was compared to the non-digested original samples. All analyses were performed in triplicate and expressed in µmol of Trolox equivalent (TE)/g of sample. Both ABTS and DPPH assays were performed in 96 wells of UV Flat Bottom Microtiter® Plates (8404, Thermo Fisher Scientific).

2.5.4.1. DPPH

The DPPH method followed was based on the method from (35) and (36), with slight modifications: the DPPH stock solution is dissolved in ethyl acetate. After, the DPPH stock solution is diluted with ethyl acetate to obtain a solution with an absorbance of 0.600±0.100 at 515 nm. This working solution was prepared daily. A Trolox standard curve was prepared in ethyl acetate. Samples were prepared at concentrations of 60 and 100 mg/mL in ethyl acetate and further diluted in the same solvent. Ethyl acetate was successfully used as a solvent for lipidic samples in a previous study (37). We the solvent chosen since no other tested solvent – ethanol, methanol, hexane, and acetone - was suitable for the total dissolution of the samples (results not shown).

2.5.4.2. ABTS

The ABTS method followed was based on the method from (38) and (39) with slight modifications: the ABTS and the potassium persulfate were dissolved in ultrapure water and stirred overnight. The ABTS stock solution was then filtered with a 0.45 µm syringe filter and diluted with ethanol to an absorbance of 0.70 (±0.02) at 734 nm. The radical working solution was freshly prepared. A Trolox standard curve was prepared in ethyl acetate. Samples were prepared at concentrations of 60, 100, and 200 mg/mL in ethyl acetate and further diluted in the same solvent.

2.6. Assessment of intestinal permeability effect on digested samples: human intestinal epithelium Caco-2/HT29-MTX cell lines co-culture

2.6.1. Cell preparation

The caco-2 (Caucasian colon adenocarcinoma) cell line is established for the study of intestinal permeation of bioactive molecules. Nevertheless, a monoculture of Caco-2 does not represent accurately duodenum epithelia due to the presence of tight junctions typical of the colon

but not of the small intestine, leading to limited absorption of hydrophilic molecules (40). Moreover, Caco-2 monoculture is exclusively composed of enterocytes and overexpress efflux transporters, which characterizes an excretory rather than absorptive epithelia. Thus, the HT29-MTX (Caucasian colon adenocarcinoma grade II) cell line is used in co-culture with Caco-2 cells. Importantly, HT29-MTX possesses mucus-producing ability, mimicking what happens in the duodenum mucosa.

HT29-MTX E12 (ECACC 12040401) and Caco-2 (ECACC 86010202) cell lines were grown separately in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) L-glutamine, 1% (v/v) penicillin and streptomycin and 1% (v/v) of non-essential amino acids. Cells were maintained at 37 °C under a 5% CO_2 water-saturated atmosphere. Upon 70-80% confluence, cells were collected using trypsin. Co-culture seeding in Transwells (Milicell hanging cell culture insert, PET 0.4 µm, 12-well, 48/pk, Merck) was performed in a 9:1 ratio of Caco-2 (3×10^5 cells/well) and HT29 (3×10^5 cells/well) cells, respectively (40).

2.6.2. Cell layer integrity

Transepithelial electrical resistance (TEER) was used to assess the cell layer integrity of the Caco-2/HT29 co-culture. TEER (**Supplementary Material Table S1**) was determined at different time points during the permeability assay, to assess the cell growth rate and cell viability after contact with tested samples. For TEER measurement, it was used a Millicell® ERS-2 Voltohmmeter (Merck, Germany) (41). During permeability experiments, TEER values were always above 250 $\Omega\cdot cm^2$, indicating that the cells were viable along the assay and the cell layer integrity was not compromised (42).

2.6.3. FAs transepithelial diffusion across intestinal (Caco-2/HT29-MTX) cell layers

Permeability assay was assessed in the mentioned Transwell inserts, using 12-well plates. Caco-2/HT29-MTX co-culture was seeded into the inserts to mimic the absorptive epithelia of the human intestine. For culture medium replacement, the medium was removed from the wells and 0.5 and 1.5 mL of fresh culture medium were added to the apical and basolateral sides, respectively. The incubation was performed for 21 days. On the day of the study, the culture medium was removed. Medium in the basolateral side (receptor part) was replaced with 1.5 mL of fresh medium. The medium on the apical side (donor part) was replaced with the samples. Briefly, 100 µL of digested samples (already diluted in 1:10) was added to 900 µL of the medium, fully dissolved by vortexing, and further diluted at 1:100 in the culture medium. This concentration was determined based on previous tests on cell cytotoxicity. 500 µL of each sample solution was added in duplicate to the plate. DMSO (30% v/v) was used as the negative control and culture medium as the positive control. 500 µL of samples were withdrawn from the basolateral side at 0,

1, 2, 3, and 6 h. After 6 h the apical content was completely removed, and cells were collected using 500 μL of NaOH 0.1M solution.

2.6.3.1. FAs content analysis with gas chromatography

To calculate the apparent permeability (Papp, **Equation 3.6**), the analysis of FAs content in both apical and basolateral sides, and cells was assessed through gas-chromatography. As described in **Section 2.5.1.1** the upper layer containing FAs methyl esters was collected and further analyzed in a gas chromatograph Agilent 8860 (Agilent, USA), equipped with a flame ionization detector and a BPX70 capillary column (60 m x 0.25 mm x 0.25 μm; SGE Europe Ltd, Courtaboeuf, France). Analysis conditions were as follows: injector (split 25:1; injection volume 1 μL), injector, and detector temperatures were 250 ˚C and 275 ˚C, respectively; hydrogen was used as a carrier gas at a flow rate of 1 mL/min. The oven temperature was initially at 60 ˚C and then increased to a final temperature of 225 ˚C. Supelco 37 was used for the identification of FAs.

The Papp is calculated as follows (43):

$$Papp\left(\frac{cm}{s}\right) = \frac{dQ}{(dt(A \times C0))} \quad \textbf{(Equation 3.6)}$$

Where dQ is the total amount of permeated fatty acid (μg/mL), A is the diffusion area (cm^2**)** C_0 is the initial concentration of fatty acids (μg of fatty acid /μL of the sample), and dt is the time of the experiment (s). The coefficient dQ/dt represents the flux of FAs across the monolayer.

2.7. Statistical analysis

Results are reported as mean values ± standard deviation. Analyses were performed using IBM SPSS Statistics 28 (SPSS Inc., IBM Corporation, NY, USA). First, data were analyzed for normal distribution using the Shapiro-Wilk test (n<50). Levene's test was applied to verify the homogeneity of the variances. When comparing the means of two groups a t-student test was applied and one-way ANOVA was for three or more groups. The Bonferroni post hoc test was used to determine differences among groups. When data didn't follow a normal distribution, it was transformed using the log base 10 function. When the transformation failed to employ normality, Kruskal-Wallis' test was used as a non-parametric test to compare the means of the groups. The level of significance was set at 0.05.

3. Results and Discussion

3.1. Stability and bioaccessibility of bioactive lipids through in vitro GIT tract

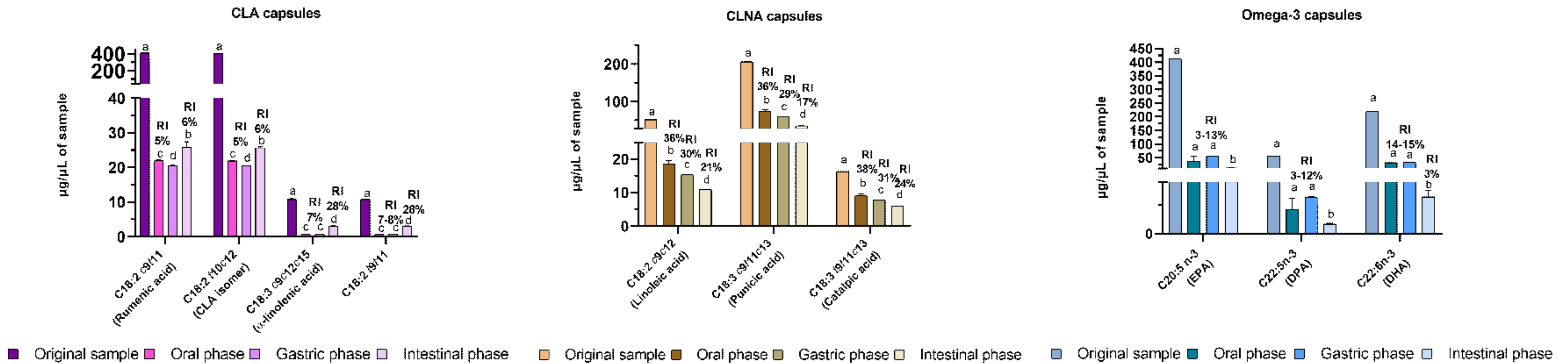
3.1.1. FAs profile and recovery index calculation

Four classes of lipids are usually found in vegetable oils: TAGs, diacylglycerols, polar lipids, and FFAs (44). Pomegranate seed oil, here simply mentioned as Pomegranate oil, is reported to yield oil contents ranging from 12.20 to 24.69% (45,46). The most prevalent forms of lipids were glycolipids (23.90%) and phospholipids (24.35%) (46). High contents of PUFAs have been reported ranging from 46.44-89.00% (45,46). TAGs are conveyed as important constituents of Pomegranate oil being the punicic acid-punicic acid-punicic acid, the punicic acid–punicic acid–catalpic acid (47), and the Stearic-Punicic-Punicic acid, some of the most reported TAGs (48). Regarding Fish oil, the primary chemical constituent has been reported to be TAGs, it also contains variable amounts of phospholipids and glycerol ethers (49,50). These oils are rich in omega-3 PUFAs, typically containing between 20 and 30%, and DHA and EPA account for more than 80% of these total omega-3 PUFAs (51). The omega-3 capsules contain Fish oil in their composition as a source of omega-3 FAs, so similarly to Fish oil, the primary chemical constituent is TAGs. The CLA capsules used in this study present as the main ingredient CLARINOL® A-95 and as described by the manufacturer (Stepan Lipid Nutrition, USA) it is an FFA mixture of CLA isomers. The CLNA capsules are Xanthigen® capsules which are described by the manufacturer as a source of PUA (≥ 35% (w/w)) derived from Pomegranate seed oil and fucoxanthin (≥ 0.43% (w/w)) from brown seaweed.

The effect of INFOGEST GIT digestion on the total FA profile is described in **Figure 3.2** where the most relevant PUFAs for each sample are emphasized (and a general FA composition is presented in **Tables 3.1, 3.2, and 3.3**). In the different GIT tract phases (oral, gastric, and intestinal) significant variations in FAs profile were observed ($p < 0.05$). As previously reported, fat digestion occurs mainly in the intestine, where 80% of the lipolysis reaction occurs (33). Here, for the most relevant PUFAs, we observed that indeed in Fish and Pomegranate oil and CLNA capsules' samples (**Figure 3.2**), the major variation is observed in the intestinal phase where the RI values are lower: between 1.90 and 14.35% in Pomegranate oil, 11.10 and 12.59% in Fish oil and 17.03-24.45% in CLNA capsules. Nevertheless, oral, and gastric digestion presents an important action in the facilitation of lipid intestinal digestion (33,52). Other studies have been reporting the negative effects of mastication in the mouth and the acidic pH of the stomach in fat-rich foods, resulting in lipid peroxidation (53) concerning the lipid profile. RI between 20 and 40% in the oral and gastric phases have been reported in a previous study with fat-rich matrixes (olive pomace) (33). These RI values indicated that a strong degradation effect on lipid fraction is occurring. In this study for Pomegranate oil, the RI is slightly higher than the reported values for olive pomace, with values between 31.86-67.96%. Nevertheless, the RI values for Fish oil and CLNA capsules agreed with the reported values: 27.69-40.31% and 29.78-37.82%, respectively

(33). Regarding CLA (**Figure 3.2 and Table 3.2**) and Omega-3 capsules (**Figure 3.2 and Table 3.3**), the observations are slightly different: there is a high degradation of general FAs content in the oral phase and a slight increase in the intestinal phase. The RI is extremely low in both the oral and gastric phases, in CLA capsules it is situated at 5.02-5.42%, and in omega-3 capsules at 7.79-14.92%. The RI values increase slightly in the intestine to 6.34-6.36% and 2.94-3.11% in CLA and Omega-3 capsules, respectively. A factor that was recently stated as a potential explanation for the extensive loss of FAs, was the presence of oxygen during all the steps of INFOGEST GIT tract digestion. Thus, it has been suggested and reinforced in this study, that the use of N_2 gas at the start of each digestion step of fat-rich foods should be applied to reduce fat oxidation (33,54). Considering this, such a step should be mentioned or included in further INFOGEST-based protocols. Oxidation of omega-3 food supplements, such as capsules as used here, is an important issue that has been reported in early studies (55). Nonetheless, it is worth mentioning that the GIT negative effect was applied to the same degree for all FAs. It is important to mention again that in this study the capsules were not used in the GIT digestion, instead their content was removed and used directly for the digestion procedures. Although FA oxidation can be a plausible explanation for the degradation observed it is important to mention that all the capsules, for instance, present antioxidants in their composition, namely tocopherols. Importantly, besides oxidation due to the presence of oxygen in all the steps of the digestion, after consumption FAs are prone to oxidation. Indeed, they interact with the pro-oxidative environment of the digestive tract, often leading to limited bioavailability. Such processes highly depend on the physicochemical properties of the initial food, as well as the characteristics of the consumer (20). Recently, a study aiming to assess the bioaccessibility of omega-3 FAs in different matrixes (including soft capsules with concentrated Fish oil and antioxidants) and the oxidation effects throughout GIT, showed that regardless of raw material used there was a high degree of oxidation by the end of the digestion processes which ultimately resulted in the decreased bioaccessibility of omega-3 FAs. The authors described that primary oxidation takes place in the gastric phase and secondary oxidation mainly takes place in the intestinal phase. Interestingly, the authors described that antioxidants presence, either natural or added, was not sufficient to protect PUFAs from the pro-oxidative conditions in the GIT (20); the same was possibly observed here in the CLA and Omega-3 capsules.

Moreover, the differences in RI observed between the Pomegranate and Fish oil and CLA and Omega-3 capsules seem to not be related to the initial concentration of PUFAs in these matrixes. Some studies have reported that the rate and degree of oxidation, for instance, were found to be strongly correlated with the initial concentration of the bioactive FAs (20). Here, considering in general the MUFA/ PUFA content we couldn’t verify any correlation between the initial concentration and the RI calculated after the digestion process.



$$\text{RI}\,(\%) = \frac{\text{Bioactive content in the digested sample}}{\text{Bioactive content quantified in the test matrix}} \times 100$$

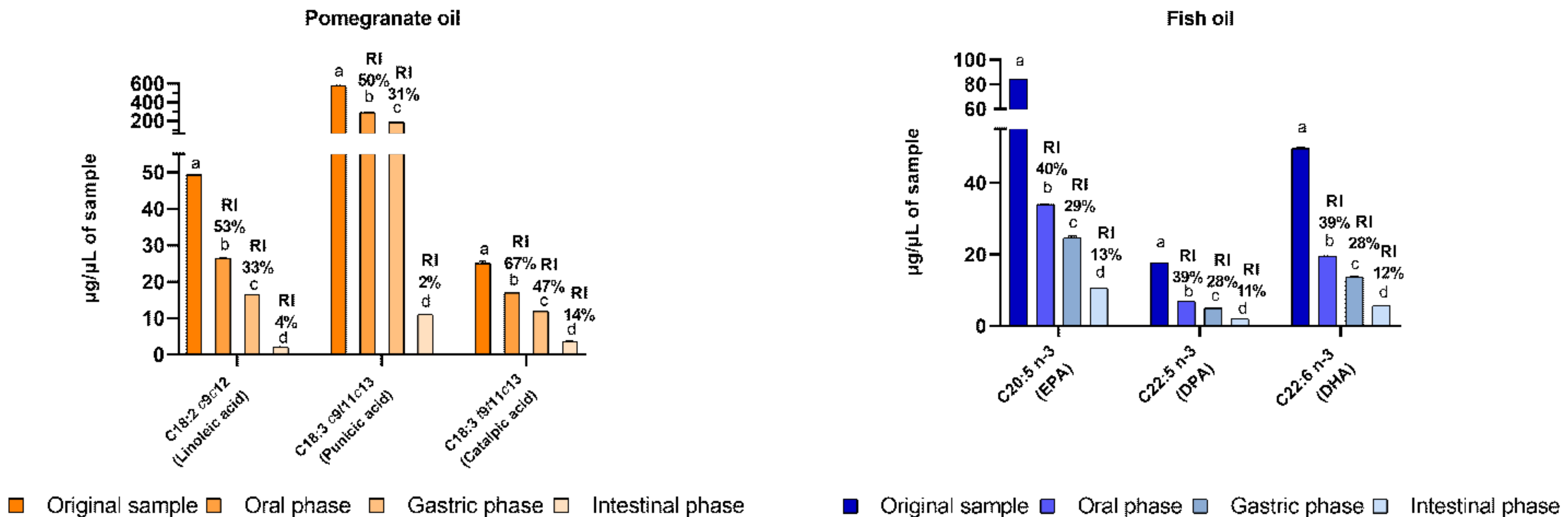


Figure 3.2 - Major FAs content of each sample in the different steps (oral, gastric and intestinal phases) of GIT tract digestion. The RI (%) was calculated for each phase using the equation presented in the figure. Values are expressed as mean±standard deviation of µg of FAs/ µL of the sample and are the result of two biological and two analytical replicates. [a,b,c,d]Different superscript letters for significant differences ($p < 0.05$) between the same FAs.

Interestingly, MUFAs and PUFAs presented a higher RI when compared to SFAs in the studied oils (Pomegranate and Fish oil), but not in CLNA capsules, where the values are identical. Regarding the bioactive FAs in these matrixes, in Pomegranate oil, PUA presented an RI of 50.25% in the oral phase, and 31.86% in the gastric phase and it was highly degraded in the intestinal phase since the RI lowered to 1.90%. Interestingly, smaller RIs were observed for both the oral (36.07%) and gastric phase (29.38%), but a higher RI (17.03%) was observed for PUA in CLNA capsules in the intestinal phase. The initial concentration of PUA was higher in Pomegranate oil (577.33 µg/µL of the sample) when compared to the CLNA capsules (205.84 µg/µL of the sample). Regarding Fish oil, the RI of the main bioactive components was observed at the same degree for all of them. EPA presented an RI of 40.31% in the oral phase, 29.24% in the gastric phase, and 12.59% in the intestinal phase; DPA showed an RI of 39.03%, 28.07%, and 11.10% in the oral, gastric, and intestinal phases, respectively. Lastly, DHA presented an RI of 39.24% in oral, 27.69% in gastric, and 11.46% in intestinal phases. These values were slightly higher than the ones presented for the omega-3 capsules: EPA – 12.29%, 13.36%, and 3.04%; DPA – 7.79%, 11.66%, and 3.11%; DHA – 14.11%, 14.92% and 2.94%, in oral, gastric and intestinal phases, respectively. The initial concentrations of the mentioned FAs were higher in the Omega-3 capsules (**Table 3.3**). In agreement with what was discussed for PUA, higher concentrations of the bioactive FAs result in lower RIs after digestion. Interestingly, in Fish oil as previously reported (15), the RI of EPA (12.59%) and DHA (11.46%) was lower than that of oleic acid (14.13%) and total MUFA (15.49%). Moreover, an early study assessing salmon FA bioaccessibility using an *in vitro* GIT model (not INFOGEST-based), demonstrated that higher levels of unsaturation decreased RI (described in the study as bioaccessibility, BI%). The authors reported higher RI values (higher than 50%) for FFAs. These different results are explained by the different matrixes: salmon slices in the mentioned study here compared to the Fish oil. The differences in RI values can be explained by the gradual release of FAs from the matrix and by the intrinsic antioxidant mechanisms present in the muscle tissue of fish, which may protect the FAs from such effects (20). Interestingly, in the same study, the authors described that RI values are enhanced by chain length but reduced with higher levels of FA unsaturation. Nevertheless, a study assessing the *in vitro* bioaccessibility of milk FAs and the C18:2 *c*9*t*11 CLA isomer using a TIM GIT *in vitro* model, reported that the degree of absorption of saturated FA decreased with chain length (17). Here, we observed that the RI shows a positive correlation (r=0.98) regarding chain length in SFAs in Pomegranate oil. However, a negative correlation (r=-0.93) was verified with Fish oil (**Figure 3.3**). In this study, no statistically significant correlation was observed for the degree of unsaturation. The differences in the studies may be related to the GIT model since different models were used in all the mentioned studies. Indeed, in a work using two different GIT models – TIM-1 and an *in vitro* method adapted from the INFOGEST standardized static model - , discrepancies in the RI of individual FAs were observed. The authors stated that this can be attributed to variations in the experimental parameters, such as chyme transit and enzyme concentrations (23). In the mentioned study using enriched CLA isomers milk, the reported RIs (described in the study as bioaccessibility BI%) for both C18:2 *c*9*t*11 and C18:2 *t*10*c*12 CLA

isomers were much higher than the ones reported here for the CLA capsules: ≈86.00% (17). In our study, we obtained similar RIs for both C18:2 *c*9*t*11 and C18:2 *t*10*c*12 CLA isomers (≈6.00%). Such differences between our results and the ones reported by the mentioned studies may be explained by the naturally present antioxidants in milk and their possible release during GIT digestion. Nevertheless, besides the antioxidant content of milk, other important aspects must be considered such as the chemical composition of the capsules: as mentioned the CLA content in the capsules is presented as FFAs while in milk FAs can be found esterified to the different compounds in fat majorly as TAGs (56). Although FAs can be found in milk as FFAs, as well, their major form is as TAGs and such difference may explain their distinct stability during GIT.

Interestingly, regarding the initial concentration for the mentioned bioactive FAs, we observed that, possibly, higher concentrations in the original matrix, before digestion, seem to be related to lower RI. In fact, regarding omega-3 FAs, we observed that the initial concentration of EPA in Omega-3 capsules was higher (411.95±0.03 μg of FA/μL of the sample) than in Fish oil (84.07±0.11 μg of FA/μL of the sample), resulting in a lower RI (3.04%) for Omega-3 capsules when comparing to Fish oil (12.59%). The same was observed for DHA: Omega-3 presented a higher initial concentration (220.13±0.38 μg of FA/μL of the sample), resulting in lower RI (2.94%) than Fish oil (49.54±0.22 μg of FA/μL of the sample and RI=11.46%). Similar observations were detected for PUA in Pomegranate oil and CLNA capsules. The Pomegranate oil presented a

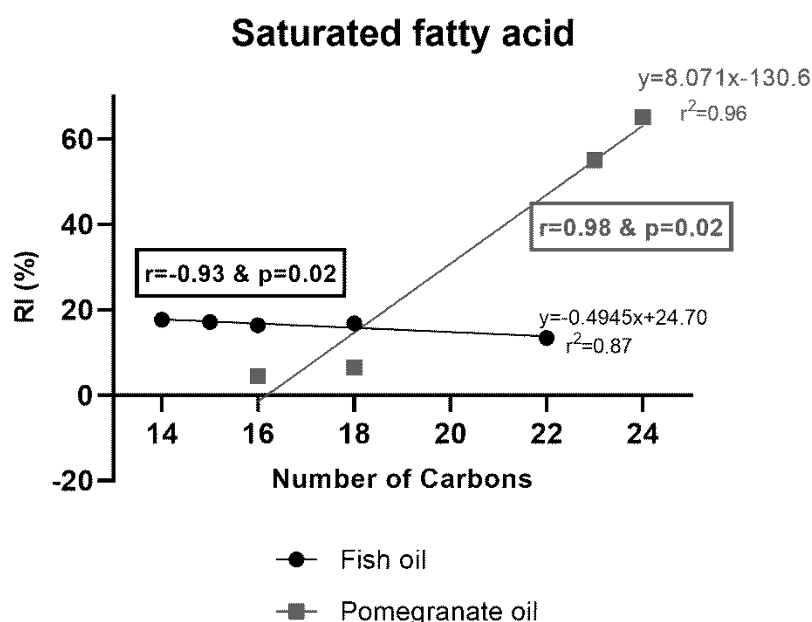


Figure 3.3 - Correlation between the number of carbons (chain length) and RI (%) for pomegranate and Fish oil. The p value concerns the slope parameter.

higher initial concentration (577.33±2.17 μg of FA/μL of the sample) when compared to CLNA capsules (205.84±0.38 μg of FA/μL of the sample), which resulted in lower RI: 1.90% vs 17.03%, respectively. Importantly, a recent study reported relevant results: a strong correlation between the initial concentration and oxidation rate. In fact, they observed that the higher the initial concentration of a specific PUFA was, the higher the degree of oxidation was. Furthermore, this

correlation was observed regardless of the length of the chain and degree of unsaturation (20). Such observations may explain the mentioned results obtained in this study.

Moreover, the nutritional indices for the different samples were calculated to assess dietary fat quality according to the AI, TI, and HH as described in **Section 2.5.1.3**. These indices are demonstrated for Fish and Pomegranate oil in **Table 3.1**, CLNA and CLA capsules in **Table 3.2**, and Omega-3 in **Table 3.3**. AI characterizes the atherogenic potential of an FA. Thus, this index is a correlation between specific SFAs (C12:0, C14:0, and C16:0) and unsaturated FAs (57). The mentioned SFAs are considered pro-atherogenic, meaning that they favor the adhesion of lipids to cells of the circulatory and immunological systems. The unsaturated FAs are considered anti-atherogenic since they can inhibit the accumulation of plaque and reduce the levels of phospholipids, cholesterol, and esterified FAs. Thus, the consumption of food or products with a low AI is associated with a reduction in the levels of total cholesterol and low-density lipoprotein cholesterol in human blood plasma (32). The AI in all the samples was very low (<1) which agrees with the high unsaturated FA content of all the samples. The AI was higher in Fish oil, which is consistent with high SFA contents, especially PA. In this study, we observed an increase in AI indices throughout GIT which may be explained by the effect of GIT on the general content of UFA. Nevertheless, the values remained very low. In addition, the TI indicates the thrombogenic potential of FAs, predicting the potential of blood clot formation. It considers the relationship between the pro-thrombogenic FAs (C12:0, C14:0, and C16:0) and the anti-thrombogenic FAs (MUFAs and the PUFAs, specifically the ones from the omega-3 and omega-6 families). The consumption of foods and/or products with low TI is beneficial for cardiovascular health (32). Like what was observed for AI the TI values were low in all the samples (<1), especially in the Omega-3 sample due to their high omega-3 and low SFA content. In addition, HH characterizes the relationship between hypocholesterolemic FA (C18:1 c9 and PUFAs) and hypercholesterolemic FA. This index more accurately reflects the effect of FA composition on CVD. High HH indices were reported for all the samples. Fish oil presented the lowest HH ratio (0.74-0.96) which is due to its high PA (C16:0) and myristic acid (C14:0) contents, despite the high omega-3 content. On the other hand, the omega-3 sample presented the highest HH ratio due to high omega-3 content and low PA (C16:0) and myristic acid (C14:0) content. Nevertheless, the values for Fish oil agree with values previously reported for fish which range from 0.87 to 4.83 (32). In general, there was no significant variation of these indices along the GIT and the values reflect the beneficial effects of these samples in cholesterol and overall cardiovascular health.

Table 3.1 - FAs profile of original tested samples (Pomegranate and Fish oil) and samples after *in vitro* INFOGEST GIT tract digestion in the different phases of the process (oral, gastric, and intestinal). The RI (%) was calculated for each phase. Values are expressed as mean±standard deviation of µg of FAs/ µL of the sample. [a,b,c,d] Different superscript letters within a row for significant differences ($p < 0.05$) between the same sample (Pomegranate oil or Fish oil).

µg of FAs/ µL of sample		Pomegranate oil							Fish oil						
		Original	Oral phase	RI (%)	Gastric phase	RI (%)	Intestinal	RI (%)	Original	Oral phase	RI (%)	Gastric phase	RI (%)	Intestinal	RI (%)
		Saturated FAs (SFAs)													
C14	Myristic acid	n.d.	n.d.		n.d.		n.d.		53.19±0.39^{a}	21.00±0.02^{b}	39.47	15.36±0.18^{c}	28.88	9.41±0.03^{d}	17.69
C15		n.d.	n.d.		n.d.		n.d.		3.35±0.03^{a}	1.29±0.01^{b}	38.56	0.94±0.00^{c}	28.13	0.57±0.01^{d}	17.13
C16	Palmitic acid	24.29±0.05^{a}	12.94±0.03^{b}	53.28	7.85±0.02^{c}	32.32	1.10±0.11^{d}	4.51	115.93±0.80^{a}	44.48±0.13^{b}	38.37	32.57±0.45^{c}	28.09	19.01±0.03^{d}	16.40
C18	Stearic acid	19.61±0.08^{a}	10.82±0.12^{b}	55.21	6.60±0.00^{c}	33.67	1.30±0.05^{d}	6.62	21.76±0.17^{a}	8.17±0.05^{b}	37.56	6.01±0.09^{c}	27.64	3.67±0.01^{d}	16.85
C22	Behenic acid	n.d.	n.d.		n.d.		n.d.		1.63±0.01^{a}	0.61±0.00^{b}	37.21	0.46±0.01^{c}	28.11	0.22±0.00^{d}	13.42
C23		1.80±0.03^{a}	1.27±0.08^{a,b}	70.55	1.23±0.00^{a,b}	67.58	0.99±0.11^{b}	55.00	n.d.	n.d.		n.d.		n.d.	
C24	Lignoceric acid	1.32±0.00^{a}	1.11±0.07^{a}	84.70	1.01±0.02^{a}	77.07	0.86±0.07^{a}	65.06	n.d.	n.d.		n.d.		n.d.	
TOTAL (SFAs)		**45.22**	**24.87**	**55.00**	**15.46**	**34.2**	**3.26**	**7.21**	**357.23**	**75.55**	**21.15**	**55.34**	**15.49**	**32.88**	**9.20**
		Monounsaturated FAs (MUFAs)													
C16:1c9	Palmitoleic acid	n.d.	n.d.		n.d.		n.d.		69.82±0.41^{a}	27.43±0.09^{b}	39.28	20.29±0.27^{c}	29.06	10.96±0.04^{d}	15.70
C18:1 *t9*	Elaidic acid	n.d.	n.d.		n.d.		n.d.		0.84±0.00^{a}	0.31±0.01^{b}	36.88	0.22±0.02^{c}	26.29	0.12±0.01^{d}	14.48
C18:1 *t11*	Vaccenic acid	1.26±0.11^{a}	1.31±0.14^{a}	103.54	1.03±0.04^{a}	82.04	0.33±0.02^{b}	26.46	0.84±0.01^{a}	0.35±0.01^{b}	41.59	0.25±0.01^{c}	30.23	0.20±0.01^{d}	23.76
C18:1 *t12*		1.15±0.08^{a}	1.45±0.12^{a}	126.29	1.23±0.00^{a}	107.08	0.43±0.02^{b}	37.68							
C18:1 *c9*	Oleic acid	48.8±0.14^{a}	26.28±0.14^{b}	53.85	15.98±0.05^{c}	32.75	2.18±0.08^{d}	4.47	51.25±0.28^{a}	19.66±0.13^{b}	38.36	14.49±0.21^{c}	28.27	7.24±0.05^{d}	14.13
C18:1 *c11*	cis-vaccenic acid	3.79±0.01^{a}	2.19±0.04^{b}	57.84	1.43±0.02^{c}	37.83	0.43±0.00^{d}	11.23	22.18±0.15^{a}	8.51±0.07^{b}	38.35	6.29±0.11^{c}	28.37	3.38±0.04^{d}	15.23
C18:1 *c4/ t6*		2.76±0.09^{a}	1.9±0.1^{b}	68.76	1.30±0.00^{c}	47.31	0.26±0.01^{d}	9.57	0.76±0.01^{a}	0.28±0.00^{b}	37.70	0.21±0.01^{c}	28.17	0.12±0.01^{d}	15.89
C20:1 *c11*	Gondoic acid	n.d.	n.d.		n.d.		n.d.		4.17±0.01^{a}	1.67±0.02^{b}	39.97	1.23±0.02^{c}	29.49	0.61±0.01^{d}	14.68
C24:1 *c15*	Nervonic acid	0.6±0.01^{a}	0.33±0.01^{b}	55.26	0.19±0.00^{c}	31.19	n.d.		n.d.	n.d.		n.d.		n.d.	
TOTAL (MUFAs)		**58.36**	**33.46**	**57.33**	**21.16**	**36.26**	**3.63**	**6.22**	**146.10**	**58.21**	**39.84**	**42.98**	**29.42**	**22.63**	**15.49**
[MUFA/SFA]		**0.77**	**1.34**		**1.37**		**1.11**		**0.41**	**0.77**		**0.78**		**0.69**	
		Polyunsaturated FAs (PUFAs)													
C18:2 *c9c12*	Linoleic acid	49.34±0.12^{a}	26.48±0.12^{b}	53.67	16.49±0.03^{c}	33.41	2.07±0.08^{d}	4.19	7.94±0.05^{a}	3.10±0.03^{b}	39.01	2.3±0.04^{c}	28.91	0.90±0.02^{d}	11.39
C18:3 *c6c9c13*	γ-linolenic acid	0.4±0.00^{a}	0.21±0.00^{b}	53.87	0.15±0.00^{c}	38.71	n.d.		4.92±0.02^{a}	1.97±0.01^{b}	39.97	1.45±0.01^{c}	29.47	0.73±0.00^{d}	14.88
C18:2 *c9t11*	Rumenic acid	1.51±0.13^{a}	1.57±0.12^{a}	104.53	1.27±0.00^{a}	84.1	0.35±0.01^{a}	23.21	0.36±0.01^{a}	0.14±0.00^{b}	37.82	0.11±0.01^{c}	30.35	n.d.	n.d.
C18:2 *t10c12*	CLA isomer	1.65±0.07^{a}	1.77±0.02^{a}	107.39	1.32±0.00^{a}	79.83	0.27±0.02^{a}	16.12	13.36±0.01^{a}	5.41±0.05^{b}	40.45	3.94±0.06^{c}	29.47	1.78±0^{d}	13.31
C18:3 *c9c12c15*	α-linolenic acid	6.59±0.05^{a}	0.23±0.02^{b}	3.55	0.22±0.00^{b}	3.35	n.d.		7.76±0.01^{a}	2.88±0.04^{b}	37.16	2.14±0.03^{c}	27.54	1.08±0.01^{d}	13.92
C18:2 t9t11		3.05±0.31^{a}	3.39±0.25^{a}	111.32	2.67±0.03^{a}	87.47	0.65±0.03^{b}	21.38	1.67±0.00^{a}	0.69±0.02^{b}	41.14	0.49±0.00^{c}	29.41	0.23±0.00^{d}	13.84
C20:3 *c8c11c14*	Dihomo-γ-linolenic acid	n.d.	n.d.		n.d.		n.d.		5.46±0.01^{a}	2.13±0.03^{b}	39.05	1.59±0.01^{c}	29.03	0.95±0.04^{d}	17.30
C20:3 *c11c14c17*		0.96±0.01^{a}	0.52±0.00^{a}	54.69	0.32±0.01^{b}	33.19	n.d.		1.50±0.03^{a}	0.65±0.05^{b}	43.68	0.58±0.04^{b}	39.05	0.40±0.02^{d}	26.90
C20:4 n-6	Arachidonic acid	n.d.	n.d.		n.d.		n.d.		8.05±0.01^{a}	3.26±0.02^{b}	40.51	2.39±0.04^{c}	29.67	1.10±0.01^{d}	13.65
C20:5 n-3	EPA	n.d.	n.d.		n.d.		n.d.		84.07±0.11^{a}	33.89±0.22^{b}	40.31	24.58±0.48^{c}	29.24	10.59±0.02^{d}	12.59

C18:3 *c*9*t*11*c*13	Punicic acid	577.33±2.17[a]	290.12±2.73[b]	50.25	183.96±1.27[c]	31.86	10.97±0.13[d]	1.90	n.d.	n.d.		n.d.		n.d.	
C18:3 *c*9*t*11*t*13	α-eleostearic acid	8.62±0.43[a]	5.76±0.28[b]	66.91	4.41±0.21[b]	51.14	1.17±0.14[c]	13.57	n.d.	n.d.		n.d.		n.d.	
C18:3 *t*9*t*11*c*13	catalpic acid	24.99±0.64[a]	16.99±0.16[b]	67.96	11.83±0.11[c]	47.33	3.59±0.25[d]	14.35	n.d.	n.d.		n.d.		n.d.	
C18:3 *t*9*t*11*t*13	β-eleostearic acid	5.09±0.3[a]	4.76±0.09[a]	93.62	3.38±0.11[b]	66.42	2.34±0.08[c]	46.06	n.d.	n.d.		n.d.		n.d.	
C22:5n-3	DPA	n.d.	n.d.		n.d.		n.d.		17.71±0.00[a]	6.91±0.03[b]	39.03	4.97±0.03[c]	28.07	1.97±0.02[d]	11.10
C22:6n-3	DHA	0.29±0.00[a]	0.14±0.00[b]	49.01	0.08±0.00[c]	25.80			49.54±0.22[a]	19.44±0.01[b]	39.24	13.72±0.21[c]	27.69	5.68±0.05[d]	11.46
TOTAL (PUFAs)		**679.79**	**351.94**	**51.77**	**226.10**	**33.26**	**21.41**	**3.15**	**202.34**	**80.47**	**39.77**	**58.26**	**27.31**	**25.39**	**12.55**
[PUFA/SFA]		**15.03**	**14.15**		**14.62**		**6.57**		**0.57**	**1.06**		**1.05**		**0.77**	
Nutritional Quality Indexes															
AI		7.00x10^{-4}	1.40x10^{-3}	-	2.10x10^{-3}	-	2.29x10^{-2}	-	7.33x10^{-2}	0.17	-	0.22	-	0.5	-
TI		0.59	0.76	-	0.73	-	0.84	-	0.34	0.32	-	0.32	-	0.42	-
HH		4.31	4.09	-	4.16	-	3.86	-	0.94	0.96	-	0.96	-	0.74	-

Table 3.2 - FAs profile of original tested samples (CLA capsules and CLNA capsules) and samples after *in vitro* INFOGEST GIT tract digestion in the different phases of the process (oral, gastric, and intestinal). The RI (%) was calculated for each phase. Values are expressed as mean±standard deviation of μg of FAs/ μL of the sample (n=2). ^{a,b,c,d}Different superscript letters within a row for significant differences ($p < 0.05$) between the same sample (CLA capsules or CLNA capsules).

μg of FAs/ μL of sample		CLA capsules							CLNA capsules						
		Original	**Oral phase**	**RI (%)**	**Gastric phase**	**RI (%)**	**Intestinal**	**RI (%)**	**Original**	**Oral phase**	**RI (%)**	**Gastric phase**	**RI (%)**	**Intestinal**	**RI (%)**
Saturated FAs (SFAs)															
C14	Myristic acid	0.11±0.00	n.d.		n.d.		n.d.		0.10±0.00	n.d.		n.d.		n.d.	
C15		0.02±0.00	n.d.		n.d.		n.d.		0.09±0.00^{a}	0.03±0.00^{b}	38.69	n.d.		n.d.	
C16	Palmitic acid	4.80±0.00^{a}	0.27±0.00^{b}	5.59	0.27±0.00^{b}	5.61	n.d.		65.92±0.14^{a}	22.82±1.13^{b}	34.61	18.81±0.07^{c}	28.53	14.00±0.04^{d}	21.24
C18	Stearic acid	2.76±0.04^{a}	0.34±0.00^{b}	12.15	0.42±0.01^{b}	15.39	n.d.		23.65±0.08^{a}	8.02±0.37^{b}	33.91	6.63±0.02^{c}	28.04	4.81±0.02^{d}	20.36
C24	Lignoceric acid	0.56±0.00	n.d.		n.d.		n.d.		0.66±0.00^{a}	0.21±0.00^{b}	31.60	0.17±0.00^{c}	26.45	n.d.	
TOTAL (SFAs)		**8.77**	**0.64**	**7.30**	**0.69**	**7.86**			**90.42**	**31.08**	**34.37**	**25.61**	**28.32**	**18.81**	**20.80**
Monounsaturated FAs (MUFAs)															
C16:1*c*9	Palmitoleic acid	0.52±0.00^{a}	0.03±0.00^{b}	5.60	n.d.		0.08±0.00^{c}	15.87	0.04±0.00	n.d.		n.d.		n.d.	
C18:1 *t*9	Elaidic acid	n.d.	n.d.		n.d.		n.d.		n.d.	n.d.		n.d.		n.d.	
C18:1 *t*11	Vaccenic acid	0.63±0.05^{a}	0.22±0.01^{c}	34.90	0.27±0.00^{c}	43.33	0.35±0.22^{b}	55.66	n.d.	n.d.		n.d.		n.d.	
C18:1 *t*12		6.48±0.25^{a}	0.77±0.04^{c}	11.93	0.92±0.02^{c}	14.22	1.9±0.74^{b}	29.37	n.d.	n.d.		n.d.		n.d.	
C18:1 *c*9	Oleic acid	35.94±0.04^{a}	2.14±0.01^{c}	5.96	2.12±0.01^{c}	5.91	4.58±0.06^{b}	12.76	357.69±1.14^{a}	123.35±6.03^{b}	34.49	100.97±0.02^{c}	28.23	66.79±0.20^{d}	18.68
C18:1 *c*11	cis-vaccenic acid	2.7±0.02^{a}	0.24±0.00^{b}	9.02	0.28±0.01^{b}	10.40	0.55±0.07^{b}	20.26	9.21±0.01^{a}	4.20±0.20^{b}	45.65	3.48±0.04^{c}	37.82	2.42±0.00^{d}	26.27
C18:1 *c*4/*t*6		2.45±0.01^{a}	0.13±0.00^{c}	5.25	0.13±0.00^{c}	5.33	0.35±0.01^{b}	14.37	1.03±0.01^{a}	0.40±0.01^{b}	38.67	0.37±0.00^{b}	35.74	0.29±0.00^{c}	28.62
C24:1 *c*15	Nervonic acid	n.d.	n.d.		n.d.		n.d.		0.07±0.00	n.d.		n.d.		n.d.	
TOTAL (MUFAs)		**48.20**	**3.50**	**7.26**	**3.72**	**7.72**	**7.73**	**16.04**	**368.04**	**127.95**	**34.76**	**104.82**	**28.71**	**69.50**	**18.88**
[MUFA/SFA]		**5.50**	**5.47**		**5.39**				**4.07**	**4.11**		**3.70**		**3.69**	
Polyunsaturated FAs (PUFAs)															
C18:2 *c*9*c*12	Linoleic acid	6.31±0.01^{a}	0.35±0.00^{b}	5.59	0.34±0.00^{b}	5.42	n.d.		51.71±0.16^{a}	18.63±0.97^{b}	36.03	15.39±0.01^{c}	29.76	11.00±0.02^{d}	21.27
C18:3 *c*6*c*9*c*13	γ-linolenic acid	n.d.	n.d.		n.d.		n.d.		4.28±0.03^{a}	1.40±0.06^{b}	32.78	1.17±0.00^{c}	27.24	0.68±0.03^{d}	15.85
C18:2 *c*9*t*11	Rumenic acid	407.99±0.12^{a}	21.97±0.17^{c}	5.39	20.49±0.11^{d}	5.02	25.85±1.56^{b}	6.34	0.12±0.02^{bc}	0.10±0.00^{c}	80.92	0.13±0.00^{bc}	107.56	0.18±0.00^{ab}	141.54
C18:2 *t*10*c*12	CLA isomer	403.54±0.39^{a}	21.88±0.18^{c}	5.42	20.43±0.11^{d}	5.06	25.68±0.42^{b}	6.36	0.26±0.00^{a}	0.11±0.00^{d}	44.05	0.12±0.00^{c}	48.18	0.13±0.00^{b}	51.28
C18:3 *c*9*c*12*c*15	α-linolenic acid	10.78±0.25^{a}	0.71±0.00^{c}	6.55	0.75±0.02^{c}	6.91	3.04±0.21^{b}	28.17	4.15±0.001^{a}	1.38±0.06^{b}	33.26	1.14±3.2x10^{-4} ^{c}	27.43	0.72±0.01^{d}	17.30
C18:2 t9t11		10.80±0.00^{a}	0.72±0.05^{c}	6.65	0.83±0.01^{c}	7.71	3.06±0.08^{b}	28.3	0.07±0.00^{a}	0.03±0.00^{b}	50.93	n.d.		n.d.	
C18:3 *c*9*t*11*c*13	Punicic acid	0.46±0.01	n.d.		n.d.		n.d.		205.84±0.38^{a}	74.24±4.33^{b}	36.07	60.48±0.02^{c}	29.38	35.05±0.07^{d}	17.03
C18:3 *c*9*t*11*t*13	α-eleostearic acid	0.17±0.01	n.d.		n.d.		n.d.		16.33±0.00^{a}	9.15±0.40^{b}	56.01	7.72±0.008^{c}	47.28	5.99±0.03^{d}	36.68
C18:3 *t*9*t*11*c*13	catalpic acid	0.44±0.00	n.d.		n.d.		n.d.		23.80±0.06^{a}	9.00±0.46^{b}	37.82	7.39±0.04^{c}	31.08	5.82±0.12^{d}	24.45

C18:3 *t*9*t*11*t*13	β-eleostearic acid	0.51±0.00	n.d.		n.d.		n.d.		5.04±0.00[a]	2.04±0.09[b]	40.53	1.82±0.00[b]	36.23	2.48±0.05[c]	49.21
C22:5n-3	DPA	n.d.	n.d.		n.d.		n.d.		0.22±0.00[a]	0.08±0.00[c]	36.11	0.09±0.00[b]	39.91	n.d.	
C22:6n-3	DHA	n.d.	n.d.		n.d.		n.d.		0.17±0.02	n.d.		n.d.		n.d.	
TOTAL (PUFAs)		**841.00**	**45.63**	**5.42**	**42.84**	**5.09**	**57.63**	**6.85**	**311.19**	**116.16**	**37.32**	**95.62**	**30.72**	**50.19**	**16.13**
[PUFA/SFA]		**95.90**	**71.29**		**62.09**				**3.44**	**3.74**		**3.73**		**2.67**	
Nutritional Quality Index															
AI		1.0×10^{-3}	1.39×10^{-2}	-	1.48×10^{-2}	-		-	4.00×10^{-2}	0.01	-	0.01	-	0.02	-
TI		0.12	0.10	-	0.11	-		-	0.40	0.40	-	0.40	-	0.44	-
HH		10.80	11.85	-	11.89	-		-	6.26	6.28	-	6.25	-	5.61	-

Table 3.3 - FAs profile of original tested samples (Omega-3 capsules) and samples after *in vitro* INFOGEST GIT tract digestion in the different phases of the process (oral. gastric and intestinal). The RI (%) was calculated for each phase. Values are expressed as mean±standard deviation of μg of FAs/ μL of the sample. ^{a,b,c,d}Different superscript letters within a row for significant differences ($p < 0.05$).

μg of FAs/ μL of sample		Omega-3 capsules						
		Original	**Oral phase**	**RI (%)**	**Gastric phase**	**RI (%)**	**Intestinal**	**RI (%)**
		Saturated FAs (SFAs)						
C14	Myristic acid	0.73±0.01^{a}	0.07±0.02^{b}	9.58	0.1±0.01^{b}	13.69	0.11±0.02^{b}	15.06
C16	Palmitic acid	3.86±0.01^{a}	0.31±0.07^{b}	8.03	0.45±0.01^{b}	11.65	2.61±0.34^{a}	67.62
C18	Stearic acid	5.26±0.01^{a}	0.39±0.04^{b}	7.41	0.55±0.01^{b}	10.45	2.20±0.35^{b}	41.82
C22	Behenic acid	1.02±0.02^{a}	0.14±0.01^{b}	13.72	0.14±0.00^{b}	13.72	n.d.	
TOTAL (SFAs)		**10.87**	**0.91**	**8.37**	**1.24**	**11.41**	**4.92**	**45.26**
		Monounsaturated FAs (MUFAs)						
C16:1c9	Palmitoleic acid	1.96±0.01^{a}	0.17±0.06^{b}	8.80	0.24±0.00^{b}	12.24	0.23±0.05^{b}	11.73
C18:1 *c9*	Oleic acid	10.75±0.00^{a}	0.58±0.10^{c}	5.39	1.22±0.01^{b}	11.35	1.71±0.26^{b}	15.90
C18:1 *c11*	cis-vaccenic acid	3.76±0.01^{a}	0.29±0.08^{b}	7.71	0.41±0.01^{b}	10.90	0.33±0.06^{b}	10.67
TOTAL (MUFAs)		**16.47**	**1.04**	**6.33**	**1.87**	**11.35**	**2.27**	**13.78**
[MUFA/SFA]		**1.51**	**1.14**		**1.51**		**0.46**	
		Polyunsaturated FAs (PUFAs)						
C18:2 *c9c12*	Linoleic acid	1.50±0.00^{a}	0.12±0.04^{c}	8.00	0.19±0.00^{c}	12.70	0.91±0.13^{b}	40.67
C18:3 *c6c9c13*	γ-linolenic acid	1.89±0.00^{a}	0.16±0.07^{b}	8.47	0.24±0.00^{b}	12.70	n.d.	
C18:2 *c9t11*	Rumenic acid	0.40±0.00^{a}	0.07±0.01^{b}	17.5	0.07±0.01^{b}	17.50	n.d.	
C18:2 *t10c12*	CLA isomer	20.66±0.03^{a}	2.72±0.02^{b}	13.36	2.92±0.02^{b}	14.13	1.15±0.52^{b}	5.57
C18:3 *c9c12c15*	α-linolenic acid	3.50±0.00^{a}	0.23±0.03^{b}	6.57	0.34±0.00^{b}	9.71	0.21±0.06^{b}	6.00
C18:2 t9t11		1.94±0.00^{a}	0.18±0.07^{b}	9.27	0.25±0.00^{b}	12.88	n.d.	
C20:3 *c8c11c14*	Dihomo-γ-linolenic acid	12.87±0.00^{a}	1.13±0.56^{b}	8.78	1.73±0.02^{b}	13.44	0.74±0.35^{b}	5.75
C20:3 *c11c14c17*		0.83±0.02^{a}	0.96±0.08^{b}	115.67	0.85±0.01^{b}	102.81	0.18±0.06^{b}	21.69
C20:4 n-6	Arachidonic acid	18.17±0.01^{a}	2.01±0.11^{b}	11.08	2.34±0.08^{b}	12.88	0.90±0.4^{c}	4.95
C20:5 n-3	EPA	411.95±0.03^{a}	50.64±0.47^{a}	12.29	55.05±0.51^{a}	13.36	12.53±1.43^{b}	3.04
C22:5n-3	DPA	55.03±0.06^{a}	4.29±1.93^{a}	7.79	6.42±0.06^{a}	11.66	1.71±0.14^{b}	3.11
C22:6n-3	DHA	220.13±0.38^{a}	31.06±0.39^{a}	14.11	32.84±0.19^{a}	14.92	6.48±1.08^{b}	2.94
TOTAL (PUFAs)		**748.87**	**93.54**	**12.49**	**103.24**	**13.79**	**24.81**	**3.31**
[PUFA/SFA]		**68.89**	**102.79**		**83.26**		**5.04**	
		Nutritional Quality Index						
AI		3.00x10^{-4}	1.90x10^{-3}	-	1.90x10^{-3}	-	0.05	-
TI		5.00x10^{-3}	3.00x10^{-3}	-	4.00x10^{-3}	-	0.07	-
HH		97.14	141.00	-	107.53	-	5.98	-

3.1.2. Bioaccessibility assessment using dialysis membranes

Bioavailability is a term that can be referred to as the speed of absorption and the quantity of the substance (molecule) absorbed. Traditionally, bioavailability can also be considered the amount of a substance that reaches systemic circulation. *In vivo*, the absorption of a bioactive molecule occurs in the GIT only to a certain extent, and such a process depends on many factors (58). Considering the potential health benefits of these bioactive FAs, bioavailability determination is extremely important to understand if the studied matrixes can have an impact on human health through oral supplementation. As mentioned, bioavailability includes the term bioaccessibility. So, the calculation of the BI, of a certain bioactive compound, is relevant for the determination of its bioavailability.

Regarding the BI, shown in **Table 3.4**, it was possible to observe that most FAs were not detected in the permeate (OUT) phase – serum-available - but instead in the retentate (IN) – colon available - after the dialysis process. The BI for most of the FAs was thus, null, or very low 0.02-0.91%. Only the identified smaller chain FAs, such as myristic (C14:0), PA (C16:0), and palmitoleic (C16:1 *c*9) acids presented higher BI values. Similar values for BI were also observed in a recent study using the same 3.5 kDa dialysis membranes for many of the same FAs (33). The bioavailability of omega-3 FAs, for instance, varies depending on the type of chemical binding (lipid structure) (59). Moreover, bioaccessibility is also limited by the moderate to poor solubility of the FAs (43). Interestingly, the capsule samples (CLA, CLNA, and omega-3) presented, in general, a higher BI compared to the oil samples (Pomegranate and Fish oil). In addition, chain length has been considered to have an impact on FAs' bioavailability. In a study aiming to determine the intestinal absorption of FAs from milk, especially CLA, short-to-medium chain saturated FAs (up to C12:0) were described to be absorbed more efficiently than long-chain saturated FAs C14:0, C16:0, and stearic acid C18:0 (60). Here, we observed that in the capsules, lower chain-length FAs (C14:0 and C16:0) presented considerably higher BIs than longer chain-length FAs. These results may suggest that the FAs concentration and matrix composition may affect the specific FAs response to the GIT conditions and consequently the BI after the dialysis process.

Importantly, bioavailability is a complex term and when referring to nutrients the definition is still poorly specified and needs to be better standardized (61). As mentioned in this study, it is important to define that the calculation of the BI for each of the FAs present in the studied matrixes is relevant for the prediction of their bioavailability, but it must be carefully considered since it is only a prediction of the same measure. It has been reported that the chemical form of omega-3 PUFAs affects their bioavailability, but there is still a lack of strong and consistent evidence to quantify the extent of the differences. However, it has been reported that the bioavailability of EPA and DHA in ethyl ester form was significantly lower than that in TAG form. In addition, the FFA form seems to be the more bioavailable structure. Indeed, different factors could affect the *in vivo*

bioavailability of different forms of omega-3 PUFAs, including lymphatic recovery of FAs, the position of FAs in the glycerol backbone of TG, the position of double bond from carboxyl-end, the molecular weight of omega-3 PUFA formulations, and the actual matrix in which the omega-3 PUFA were provided. Another important aspect to consider is the pancreatic lipase activity, which again justifies the importance of using GIT standardized *in vitro* methods (61). Here only CLA capsules presented a different formulation of the bioactive PUFAS (as FFA) compared to the other matrixes (FAs mainly as TAGs), but there were no observable differences considering their BI.

There are very few studies aiming to determine the BI of a certain fatty acid where permeability assays, such as the ones presented here, are mentioned. However, we believe that such studies are a relevant prediction tool to consider in this kind of static *in vitro* test and must be used as screening tools.

Since these results indicated that most FAs were retained in the non-bioaccessible fraction (retentate, IN), this could be interesting for further studies regarding their effects on gut health modulation.

Table 3.4 - Major FAs of samples submitted to the dialysis, with 3.5kDa membranes, process after in vitro INFOGEST GIT tract digestion. The IN corresponds to the retentate and the OUT samples to the permeate. The bioaccessibility index (BI%) was calculated for each sample. Values are expressed as mean±standard deviation of μg of FAs/ μL of the sample.

μg of FAs/ mg of sample		**CLA capsules**			**CLNA capsules (Xanthigen)**			**Omega-3**			**Pomegranate oil**			**Fish oil**		
		Retentate (IN)	**Permeate (OUT)**	**BI (%)**	**Retentate (IN)**	**Permeate (OUT)**	**BI (%)**	**Retentate (IN)**	**Permeate (OUT)**	**BI (%)**	**Retentate (IN)**	**Permeate (OUT)**	**BI (%)**	**Retentate (IN)**	**Permeate (OUT)**	**BI (%)**
C14	Myristic acid	0.17±0.01	0.17±0.00	49.78	0.24±0.00	0.21±0.00	47.42	0.29±0.01	0.31±0.00	51.94	n.d.	n.d.		66.55±0.04	0.98±0.00	1.46
C16	Palmitic acid	7.40±0.02	0.86±0.01	10.44	89.61±0.27	1.27±0.04	1.40	3.64±0.02	1.88±0.01	34.07	29.59±0.03	2.04±0.03	6.44	176.14±0.21	1.83±0.00	1.03
C16:1*c*9	Palmitoleic acid	0.60±0.00	0.28±0.01	32.02	4.86±0.01	0.76±0.03	13.52	1.1±0.01	0.91±0.01	45.32	n.d.	n.d.		86.76±0.19	n.d.	
C18	Stearic acid	5.21±0.06	0.14±0.01	2.69	37.38±0.20			4.66±0.02	0.14±0.00	3.00	25.39±0.03	0.22±0.01	0.86	38.29±0.04	0.17±0.01	0.45
C18:1 *c*9	Oleic acid	42.06±0.06	0.42±0.03	0.99	460.79±1.60	0.36±0.02	0.08	7.88±0.03	0.04±0.00	0.50	56.57±0.17	1.23±0.03	2.13	70.21±0.08	0.03±0.00	0.05
C18:1 *c*11	cis-vaccenic acid	3.93±0.07	n.d.		16.34±0.11	n.d.		2.59±0.01	1.19±0.03	31.49	4.49±0.04	n.d.		30.98±0.05	1.32±0.00	4.09
C18:2 *c*9*c*12	Linoleic acid	7.52±0.01	n.d.		63.19±0.11	n.d.		1.80±0.01	0.05±0.00	2.86	51.13±0.04	0.1±0.01	0.20	11.0±0.02	n.d.	
C18:2 *c*9*t*11	Rumenic acid	186.27±1.56	n.d.		1.10±0.10	n.d.		0.20±0.01	n.d.		0.92±0.11	n.d.		0.36±0.00	n.d.	
C18:2 *t*10*c*12	CLA isomer	185.05±0.42	0.08±0.00	0.04	1.67±0.15	n.d.		10.25±0.05	n.d.		1.28±0.08	n.d.		13.12±0.07	n.d.	
C18:3 *c*9*c*12*c*15	α-linolenic acid	21.87±0.21	n.d.		6.19±0.07	n.d.		2.45±0.01	n.d.		7.92±0.06	n.d.		11.7±0.03	0.11±0.01	0.91
C18:2 t9t11		22.01±0.08	n.d.		5.94±0.04	n.d.		±	n.d.		7.84±1.39	n.d.		1.66±0.03	n.d.	
C20:3 *c*8*c*11*c*14	Dihomo-γ-linolenic acid	n.d.	n.d.			n.d.		7.72±0.03	n.d.		n.d.	n.d.		6.26±0.02	n.d.	
C20:4 n-6	Arachidonic acid	n.d.	n.d.			n.d.		10.83±0.13	n.d.		n.d.	n.d.		8.93±0.08	n.d.	
C20:5 n-3	EPA	n.d.	n.d.			n.d.		214.34±0.56	0.05±0.00	0.02	n.d.	n.d.		80.68±0.29	0.1±0.00	0.12
C18:3 *c*9*t*11*c*13	Punicic acid	n.d.	n.d.		109.09±0.83	n.d.		n.d.	n.d.		180.58±1.37	0.04±0.00	0.02	n.d.	n.d.	
C18:3 *c*9*t*11*t*13	α-eleostearic acid	n.d.	n.d.		5.58±0.00	n.d.		n.d.	n.d.		14.4±0.06	0.07±0.00	0.47	n.d.	n.d.	
C18:3 *t*9*t*11*c*13	catalpic acid	n.d.	n.d.		59.22±0.38	n.d.		0.30±0.02	n.d.		115.82±1.03	n.d.		n.d.	n.d.	
C18:3 *t*9*t*11*t*13	β-eleostearic acid	0.36±0.01	n.d.		70.32±0.13	n.d.		n.d.	n.d.		222.57±1.78	0.12±0.00	0.05	n.d.	n.d.	
C22:5n-3	DPA	n.d.	n.d.		n.d.	n.d.		32.21±0.03	n.d.		n.d.	n.d.		18.94±0.06	n.d.	
C22:6 n-3	DHA	n.d.	n.d.		n.d.	n.d.		118.29±0.31	n.d.		0.35±0.01	n.d.		47.32±0.25	n.d.	

3.1.3. Antioxidant potential

Human studies have shown that PUFAs in general improve the metabolic and anti-inflammatory/antioxidant capacity of several organs. In the heart, it was demonstrated that PPARγ activation is a mechanism by which Fish oil n-3 PUFAs enhance mitochondrial FA oxidation and antioxidant capacity in the human atrial myocardium (62). Besides, they have been mentioned as enhancer factors in antioxidant defense against ROS (63). These results have also been demonstrated in *in vivo* studies with rats for ovarian ischemia/reperfusion. Interestingly, a low-dose Pomegranate seed oil extract showed anti-inflammatory potential by reducing TNF-α levels and significantly increased antioxidant activity (measured by superoxide dismutase activity and glutathione levels) (64). The scavenging potential of these FAs and FAs' sources was also demonstrated *in vitro*, as it is going to be discussed in this section.

The antioxidant activity of the studied samples – CLA, CLNA, and Omega-3 capsules and Fish and Pomegranate oil – before and after digestion was determined by two different methods: DPPH and ABTS (**Figure 3.4** and **Supplementary Material Table S2**). DPPH and ABTS are examples of electron transfer methods (33). In this study, the ABTS method showed higher antioxidant values compared to DPPH. Such a result was not expected since DPPH has been described as more efficient in measuring the antioxidant activity of less polar compounds, due to its solubilization only in organic solvents. Nevertheless, both DPPH and ABTS assays confirmed the presence of free radical scavenging activity in the studied samples.

The antioxidant effect of the samples was concordant in both ABTS and DPPH methods: CLA capsules presented a higher antioxidant potential after the digestion process compared to the sample before GIT digestion in both methods (ABTS, DPPH). Fish oil presented the same result as CLA capsules in ABTS, while no statistically significant difference for the samples before and after digestion was observed for DPPH. The antioxidant potential of Omega-3 and CLNA capsules and Pomegranate oil was negatively influenced by GIT tract digestion since they present lower antioxidant activity after the digestion process. Pomegranate juices are known to possess significant antioxidant activity due to their phenolic content (65). Indeed, a significant correlation was found between the antioxidant activity of Pomegranate seed oil and the total content of tocopherols, suggesting the contribution of tocopherols to the antioxidant properties of this oil (66). Moreover, CLNA isomers such as PUA (67) and even, PUFAs (68) in general, are known for their antioxidant capacity *in vivo*. Although other compounds in Pomegranate oil have important antioxidant potential, we expected that since a reduction of these FAs is observed after GIT digestion the overall antioxidant potential was also affected. Importantly, similar values for the antioxidant activity of Pomegranate seed oil were reported elsewhere: 171.44 mg TE/100 g of sample (≈6.85 µmol TE/g sample) (69). CLNA capsules showed lower antioxidant activity in DPPH (0.07±0.00 µmol TE/g sample) when compared to Pomegranate oil, but higher antioxidant activity with ABTS (1.83±0.09 µmol TE/g sample). Such results may be related to the fact that DPPH, due to its lipophilic nature, is better suited for samples with high lipidic contents such as oils. In

addition, a study has reported that high-pigmented and hydrophilic antioxidants are better reflected by ABTS than DPPH (70). Besides, it is also important to mention that ABTS allows the measure of antioxidant activity of both hydrophilic and lipophilic compounds, whereas DPPH, by its low solubility in aqueous compounds is appropriate for analysis of lipophilic compounds, which is an important limitation to consider when interpreting the role of hydrophilic antioxidants (33). CLNA capsules (Xanthigen®) present pigments and have a lower percentage of Pomegranate oil in their composition. In addition, it is relevant to mention that antioxidants are incorporated in the CLNA capsules. In addition, there is also the presence of polyphenols naturally present in the Pomegranate seed oil, as discussed. Thus, the antioxidant activity measured is affected by all these compounds and not only by their FA content.

We were only able to identify one *in vitro* study that specifically assessed the Fish oil antioxidant potential. In this study Fish oil was described as presenting a good free scavenging activity due to the presence of omega-3 FAs: DPPH• inhibition values were around 97% (in

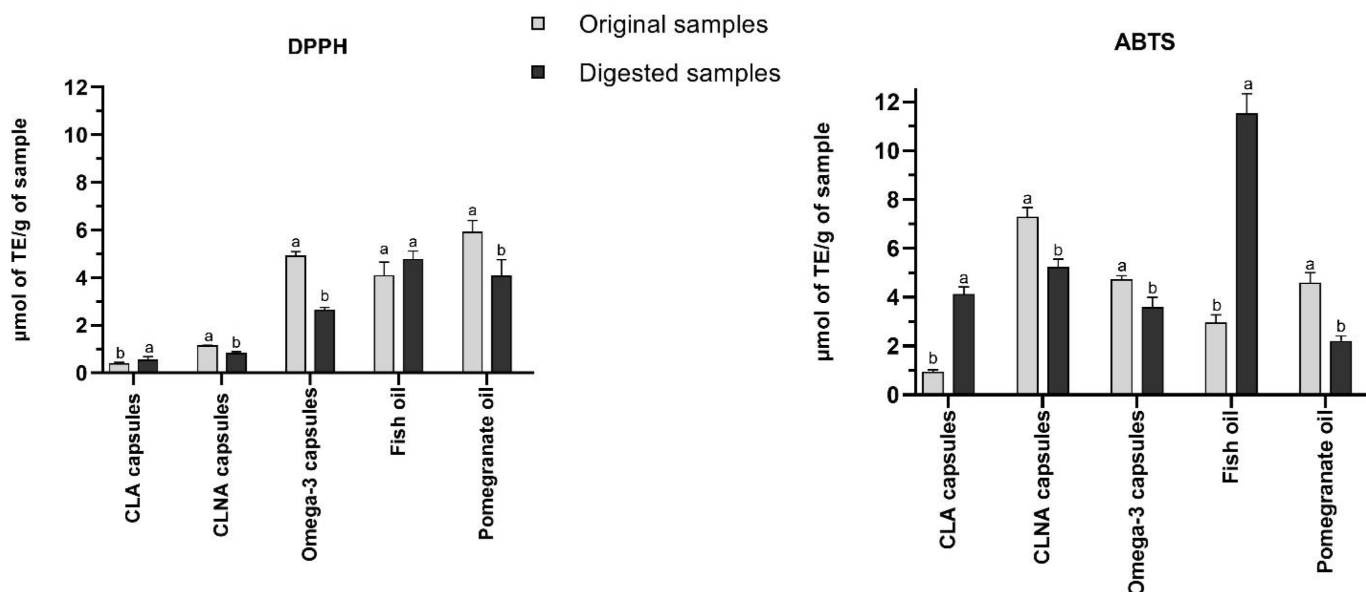


Figure 3.4 - Antioxidant potential assessment through DPPH and ABTS methods before and after GIT tract digestion of CLA, CLNA and Omega-3 capsules and Fish and Pomegranate oil. Values are expressed as mean±standard deviation of µmol of Trolox Equivalents/ g of sample (n=3). a,b Different superscript letters for significant differences ($p < 0.05$) between original and digested samples.

smaller concentrations of 4.00 mg/mL of Fish oil) and 90% for ABTS• (4.00 mg/mL of Fish oil) (71). Some natural and synthetic antioxidants have been reported to be added to Fish oil to prevent lipid oxidation processes (72) and this must be considered for its antioxidant activity potential. Nevertheless, omega-3 EPA and DHA have been reported to have antioxidant activity (4) or promote antioxidant cellular responses (73) in some *in vitro* studies using cell lines. Here, before GIT digestion, we obtained significative values (4.11±0.55 µmol TE/g sample), comparable to the Pomegranate oil (5.93±0.47 µmol TE/g sample). Lower values were obtained for the ABTS method, 2.97±0.31 µmol TE/g sample for Fish oil and 4.60±0.40 µmol TE/g sample in Pomegranate oil, which can be explained by the high lipidic content of the sample, as already discussed. Slightly higher results were obtained for Omega-3 capsules – 0.16±0.03 and

0.23±0.03 µmol TE/g sample, for DPPH and ABTS, respectively -, which can be explained by the added tocopherol content (10 mg α-TE) in the capsules.

The antioxidant activity of CLA isomers has been reported as a possible mechanism behind its anticarcinogenic and antiatherogenic effects. Nevertheless, pro-oxidant properties have been attributed to them (74,75), and thus, controversial reports have been made regarding their antioxidant potential. It has been reported that C18:2 *c*9*t*11 and C18:2 *t*10*c*12 CLA isomers reacted and quenched DPPH• at several concentrations (75,76). It is important to mention, again that an important antioxidant activity is due to the added tocopherol content (3.00 mg/g). Here, it is shown that indeed CLA capsules present some antioxidant activity, although the lowest among the tested samples in both DPPH (1.23±0.04 µmol TE/g sample) and ABTS (1.19±0.04 µmol TE/g sample) methods.

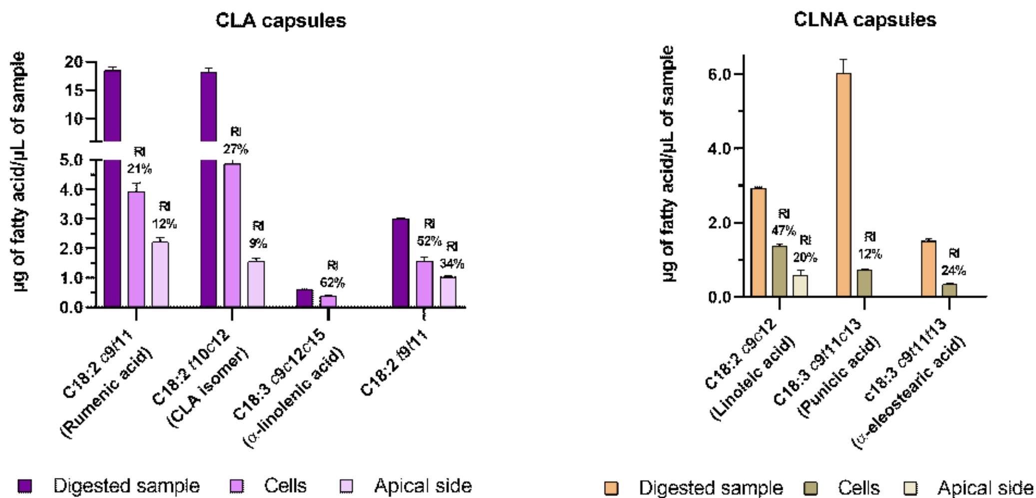
3.2. Assessment of intestinal permeability effect on digested samples: mimicking the human intestinal epithelium with a Caco-2/HT29-MTX co-culture

To predict the bioavailability of the studied samples after GIT digestion, specifically of their main bioactive FAs, besides the use of dialysis membranes (as discussed in **Section 3.1.2** from this chapter) we performed a permeability assay using a co-culture of Caco-2/HT29-MTX cell lines. It was not possible to quantify any FAs on the basolateral side (receptor side) meaning that permeability was affected. Thus, the Papp was not possible to be calculated. To determine if the FAs were incorporated in the cells, the cells after the experimental time were collected. **Figure 3.5** depicts the FAs profile of the original samples, cells, and apical side (donor side), after normalization with the negative control to eliminate the FFAs naturally present in the culture medium and cells. The RI was calculated concerning the concentration of the original sample added to the apical side (donor side). We observed that in CLNA capsules and Pomegranate oil, 12.23 and 10.25%, respectively, of PUA is incorporated into the cells as well as 16.50-23.65% of other CLNA isomers, such as α-eleostearic acid (C18:3 *c*9*t*11*t*13), catalpic acid (C18:3 *t*9*t*11*c*13) and β-eleostearic acid (C18:3 *t*9*t*11*t*13). An early study aiming to investigate the uptake of CLNA isomers (α-eleostearic acid (C18:3 *c*9*t*11*t*13), catalpic acid (C18:3 *t*9*t*11*c*13), and β-eleostearic acid (C18:3 *t*9*t*11*t*13)) and their metabolism into CLA in Caco-2 cells, showed that a 24 h incubation with these isomers showed to induce an accumulation of these FAs, while the control cells were free of these FAs. Interestingly, the authors also demonstrated that these FAs can be converted into CLA (77,78).

Regarding CLA capsules, C18:2 *c*9*t*11 and C18:2 *t*10*c*12 isomers were detected in cells with an RI of 21.29 and 26.71%, respectively. Nevertheless, a considerable RI was calculated on the apical side: 12.01 and 8.58%, for the same isomers, respectively. The C18:2 *c*9*t*11 and C18:2 *t*10*c*12 were found to alter the distribution of tight junction proteins occluding and ZO-1 in a Caco-2 cell model increasing the paracellular permeability. Such observations were associated with the incorporation of CLA isomers in the cellular membrane (79,80). Thus, these results may explain

the low permeability that was observed in our study since a considerable amount of CLA isomers were retained on the apical side.

Regarding Fish oil, 11.03% of EPA was detected in cells and 12.59% in the sample collected from the apical side after the experimental time. In addition, 8.44% of DHA was detected in the cells and 8.53% on the apical side. Indeed, one mechanism that is linked to the several regulatory properties attributed to omega-3 PUFAs is precisely the alteration and modulation of membrane FA composition and this has been demonstrated in several cell types including intestinal cells. As reviewed by Durkin et al. (81) it has been demonstrated that EPA supplementation of Caco-2 cells increased their membrane content of EPA, and long incubations (96 h) with EPA and DHA increased their respective amounts in these cells. Interestingly, it has been reported that EPA accumulates mostly in the phospholipid fraction and DHA and ALA in the



$$\text{RI}\ (\%) = \frac{\textbf{Bioactive content in the apical side/cell}}{\textbf{Bioactive content quantified in the test sample (digested)}} \times \textbf{100}$$

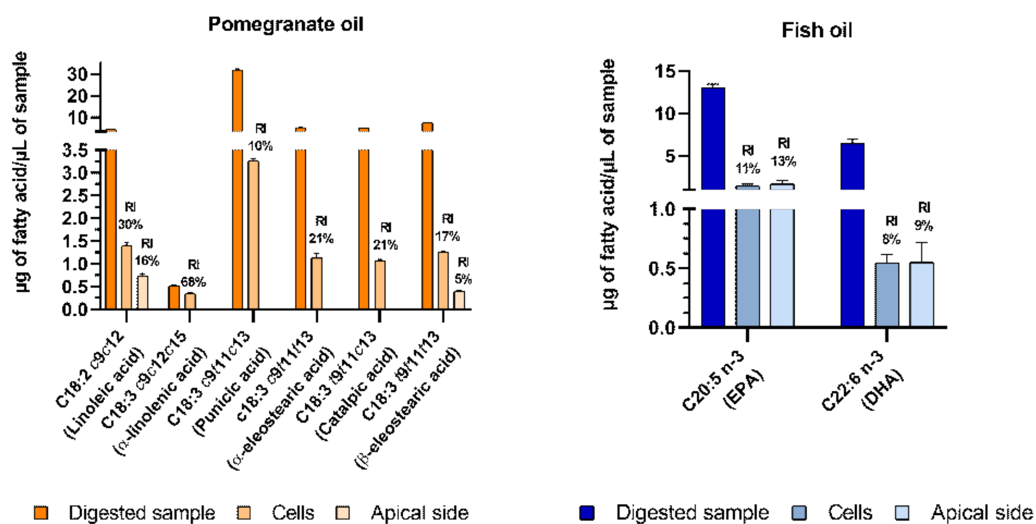


Figure 3.5 - Major FAs in the digested samples and their quantification in apical side and cell content after permeability assays using a Caco-2/HT29-MTX cell lines co-culture. The RI (%) was calculated using the equation presented in the image. Values are expressed as mean±standard deviation of µg of FAs/ µL of the sample (n=4).

neutral lipid pool. Moreover, bioconversion of exogenous omega-3 PUFAs can also occur in epithelial cells. One explanation for the decreased permeability observed here and the considerable content of such FAs still found in the apical side, besides FAs incorporation in cells, may be related to the fact that EPA and DHA can improve barrier integrity in *in vitro* studies in caco-2 cells. In those studies, 24 h EPA supplementation reduced the permeability of horseradish peroxidase (82) and incubation with EPA for 96 h was able to attenuate increased permeability in heat stress-impaired caco-2 monolayers (83).

The incorporation of omega-3 FAs and CFAs in intestinal cells presents great interest since these FAs may develop important functions in gut health. A recent study reviewed the positive effects of omega-3 FAs, specifically EPA, DHA, and ALA. Omega-3 is known to influence the gut microbiota community since these FAs were shown to exert beneficial effects by decreasing the growth of *Enterobacteria* and increasing the growth of *Bifidobacteria*, inhibiting the inflammatory response associated with endotoxemia. Moreover, omega-3 may also reduce gut inflammation by increasing Treg differentiation and decreasing IL-17 production. Interestingly, these FAs can also reduce intestinal epithelial cell damage caused by inflammatory factors, which may be highly relevant in some intestinal inflammatory diseases (84). Moreover, as discussed CLNA isomers, PUA, and eleostearic acid are known to activate PPARγ to suppress inflammatory responses. PUA and eleostearic acid have been demonstrated to modulate mucosal immune responses by a PPARγ-dependent mechanism, ameliorating colitis (85). Considering the ability of CLA isomers to activate PPARs, it is not surprising that they have been described for their anti-inflammatory potential, as well. Indeed, in mouse models of inflammatory bowel disease, CLA isomers are shown to have anti-inflammatory properties and other studies showed that CLA possesses anti-carcinogenic activity in the rat colon (86).

It was also possible to observe that there is high incorporation and possible synthesis (RI>100%) of PA (C16:0), stearic acid (C18:0), and oleic acid (C18:1 *c*9) in the cells after 6 h. PA was found to be incorporated in cellular phospholipids in a greater proportion than oleic acid (87). The same results were observed here for all the samples studied; PA presented higher RI values than oleic acid (**Table S3 Supplementary Material**). Another study has reported that only 7% of PA was able to cross the Caco-2 cells monolayer, and 90% of it was incorporated into the cells (88). Importantly, once again some FAs appear to alter intestinal permeability: PA was demonstrated to affect intestinal permeability without inducing cytotoxicity or oxidative stress; it seems to cause a functional change in tight junctions and adherens junction barrier (89).

The same experience was not possible for Omega-3 capsules since the TEER values obtained after 6h of incubation indicated that the membrane integrity and cell viability were compromised.

4. Conclusion

This work assesses the bioaccessibility of bioactive PUFAs, mostly PUA, present in Pomegranate oil and CLNA capsules (Xanthigen®), EPA, DHA, and DPA from Fish oil and Omega-3 capsules and RA and C18:2 *t*10*c*12 CLA isomer from CLA capsules. By using an *in vitro* static protocol, the effect of the GIT digestion was assessed, and it was demonstrated that significant variations in FAs profile occur in the different GIT phases: for the Pomegranate and Fish oil and CLNA capsules a major variation is observed in the intestine since lower RI (%) values were obtained here. In CLA and Omega-3 capsules, it was observed a high degradation of general FA content right after the oral phase. Indeed, this observation may be explained by the oxidative process of PUFAs after digestion, which is demonstrated not to be protected by the presence of both natural and added antioxidants. After the intestinal digestion process, the RI value for PUA in Pomegranate oil is 1.90% and 17.03% for CLNA capsules. In Fish oil, the RI values for EPA, DPA, and DHA are between 11.10-12.59%, and in Omega-3 capsules around 2.94-3.11%. In CLA capsules the RI for RA and C18:2 *t*10*c*12 CLA isomer is 6.34-6.36%. In addition to the low RI obtained in our study, higher initial concentrations seem to be correlated with higher oxidation rates of PUFAs. All these observations – composition of matrixes, PUFA concentration in the matrix, oxidation processes, and the low RI -, supported from previous studies, are important considerations when aiming to design an oral route of administration of the bioactive PUFAs studied here.

Moreover, using 3.5 kDa dialysis membranes it was detected that the BIs (%) were very low or null for most of the major bioactive FAs of all the samples. These results indicated that most FAs were retained in the non-bioaccessible fraction. In addition, intestinal permeability was assessed using a Caco-2/HT29-MTX co-culture. Reinforcing the results obtained with the dialysis membrane, it was observed a significant incorporation of the bioactive FAs into the intestinal cells. This membrane incorporation negatively affects intestinal cells' permeability capacity. Indeed, one mechanism that is linked to the several regulatory properties attributed to omega-3 PUFAs is precisely the alteration and modulation of membrane FA composition and this has been demonstrated in several cell types including intestinal cells. Such evidence increases the importance of our study: first, it is important to consider the low bioavailability potential of these PUFAs when aiming to understand their potential health benefits by providing them as oral supplements. Thus, such results are relevant for future considerations of bioavailability studies of these FAs reinforcing the need to look for further mechanisms of target-delivering. Secondly, considering their low permeability it is highly relevant to study and understand their potential in microbiota modulation and their role in gut dysbiosis, specifically, considering their anti-inflammatory potential, in inflammatory diseases such as Inflammatory bowel disease, for example.

In conclusion, this work provides a full bioaccessibility assessment of different bioactive FAs using distinct matrixes, with diverse formulations (commercial soft-gel capsules and enriched

oils), compositions, and bioactive PUFA concentrations. The major strengths of this work rely on it being a complete study using a standardized *in vitro* static protocol (INFOGEST 2.0 protocol) and providing some insights on intestinal permeability and absorption. The use of a standardized protocol is an important point in this study since it allows the accurate comparison of inter-laboratory results. Nevertheless, some limitations in this study are important to identify. It is important to mention, that although *in vitro* protocols are a valuable and easily accessible tool, when considering the simulation of GIT, this type of protocol only provides a predictive potential. Indeed, static digestion models are described as simplifications of a dynamic physiological process (90). Thus, further studies using semi-dynamic or dynamic models, which include the physicochemical and mechanical processes as well as the gradual changes that occur in the GIT digestion *in vivo* and ultimately *in vivo* studies, are required to fully characterize the bioaccessibility and bioavailability potential of these PUFAs. Another important aspect that needs to be considered regarding the bioavailability prediction made in this study is the lack of concordance with *in vivo* data. Several *in vivo* (human and animal studies) have reported higher bioavailability levels when considering, specifically, omega-3 FAs, EPA and DHA. Nevertheless, important considerations need to be made regarding this observation. First, as mentioned the reader must always consider that *in vitro* protocols present only a predictive potential and, in this study, the bioavailability is just a prediction from the determined BIs (%). Secondly, as reviewed in a study by Ghasemifard and collaborators (61) there are still several limitations presented in these *in vivo* studies which difficult a comparison between them and a correct prediction: the lack of definition and proper characterization of the “bioavailability” term, the lack of standardization of analytical methodology, differences in the methodology of bioavailability measure, failure to provide equal amounts of omega-3 FAs, failure to measure fatty acid control and lack of control of fat intake. Indeed, it is important to point out that in this *in vitro* study the quantity of bioactive FAs provided is equivalent to just one intake, with a single dose of 3 g. This dose is lower than the ones used in most of the *in vivo* studies presented in the mentioned review article. It is also important to consider that in most *in vivo* studies the FAs intake was provided more than once during different studies duration. Additionally, the omega-3 FAs were incorporated with high-fat meals, which will impact the bioavailability measured. Besides, in most studies, there was no information or control of the dietary fat intake. In addition, it is relevant to mention that in *in vivo* studies, these supplementations are part of a normal daily diet which will, as expected, have an impact on the bioavailability. This kind of methodology is not possible to be completely translated in an *in vitro* “isolated” study as the one presented here. Importantly, in one example of the importance of fat intake in bioavailability measures, the authors pointed out that a high-fat breakfast, for instance, containing omega-3 PUFAs, followed by a high-fat lunch (lacking omega-3 PUFAs) can lead to a second peak in the appearance of omega-3 PUFAs in plasma lipids. In fact, it was suggested that some of the PUFAs from the breakfast were stored in the enterocytes and released after the high-fat lunch. All these aspects may explain the differences observed between this *in vitro* study and the *in vivo* reports regarding PUFAs' bioavailability.

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CHAPTER 4

The effect of omega-3 and conjugated fatty acids in the modulation of a healthy microbiota.

This chapter intends to describe the effects and the therapeutical potential of omega-3 and conjugated fatty acids in the modulation of healthy human microbiota.

This chapter is awaiting submission and is going to be presented as:

Salsinha, A.S., Araújo-Rodrigues, H., Dias, C., Cima, A., Rodríguez-Alcalá, Relvas, J.B., Pintado, M. Omega-3 and conjugated fatty acids impact on human microbiota modulation using an *in vitro* fecal fermentation model.

Chapter 4

Chapter 4

Preface

What is the impact of omega-3 and CFAs on the human gut microbiota?

Is there any preventive potential for obesity development?

In the last years, a causal and direct relationship between obesity development and microbiota dysbiosis has been unraveled. Indeed, studies using omega-3 have demonstrated that besides dietary fibers, bioactive FAs may also present a prebiotic effect and can modulate gut microbiota. Moreover, gut-brain axis communication has been described and its importance in several neurodegenerative and neuroinflammatory diseases such as Alzheimer's disease and Multiple sclerosis has been unraveled. So, the possibility of a gut-brain axis role in obesity development must be equally considered.

Considering the results obtained in **Chapter 3**, regarding the bioaccessibility of these FAs, there is a possible modulatory impact of these FAs in gut microbiota. Indeed, as described in **Chapter 3** the oral delivery of the described enriched oils results in a very significant amount of these FAs being retained in the non-absorbable fraction. This means that they are available for gut microbiota modulation. Besides, it seems that these FAs are incorporated in the epithelial cells using the Caco-2/HT-29 MTX model described, which will have an impact on gut permeability.

Taking into consideration all these observations, the possible effect of these bioactive FAs in gut microbiota cannot be overlooked, and first, the effect of these FAs in human gut microbiota and its modulation as a possible obesity preventive strategy was assessed. For that, *in vitro* fecal fermentations were performed using fecal samples from human donors, and the effects of these FAs were assessed on gut microbial community modulation and metabolic products and consequently a possible beneficial role in gut-brain axis regulation was proposed.

Chapter 4

Omega-3 and conjugated fatty acids impact on human microbiota modulation using an *in vitro* fecal fermentation model

Ana Sofia Salsinha[a,b], Helena Araújo-Rodrigues[a,b], Cindy Dias[a], André Cima[a], Luís Miguel Rodriguez-Alcalá[a], João B. Relvas[b] and Manuela Pintado[a*]

a. Universidade Católica Portuguesa, CBQF - Centro de Biotecnologia e Química Fina –Laboratório Associado, Escola Superior de Biotecnologia, Rua de Diogo Botelho, 1327, 4169-005 Porto, Portugal

b. Instituto de Investigação e Inovação em Saúde and Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto – Rua Alfredo Allen, 208, 4200-135 Porto, Portugal

Abstract

Gut microbiota has been gaining more and more attention and its important role in the maintenance of a general good health condition is already established. The potential of microbiota modulation through diet is an important research focus to be considered. Lipids in general, including dietary lipids, are essential in gut permeability and exert either pro- or anti-inflammatory actions, and have demonstrated a positive role in the modulation of the gut microbiome. Omega-3 fatty acids, specifically, are well known for their beneficial role on organs and corresponding diseases. However, their impact on gut microbiota is still poorly defined and studies on the role of other polyunsaturated fatty acids, such as CLA and CLNA are even scarcer. Thus, by using an *in vitro* human fermentation model, we assessed the effect of omega-3, CLA isomers, and punicic acid on microbiota modulation. Fish oil, Omega-3, and CLA samples presented a positive impact on *Akkermansia* spp. and *Bifidobacterium* spp. growth. Moreover, all the samples supported *Roseburia* spp. growth after 24 h of fermentation and, importantly, they were able to maintain the Firmicutes: Bacteroidetes ratio near 1. All the bioactive fatty acids samples, except Pomegranate oil, were able to significantly increase butyrate levels compared to those found in the positive control (FOS) sample. Moreover, Fish oil and Omega-3 capsules were able to increase the concentration of GABA, alanine, tyrosine, phenylalanine, isoleucine, and leucine between 12 and 24 h of fermentation, which may be relevant in gut-brain axis modulation.

Keywords: Omega-3; Conjugated linoleic acid; Conjugated linolenic acid; Microbiota modulation; GABA; *in vitro* fecal fermentation.

Graphical abstract

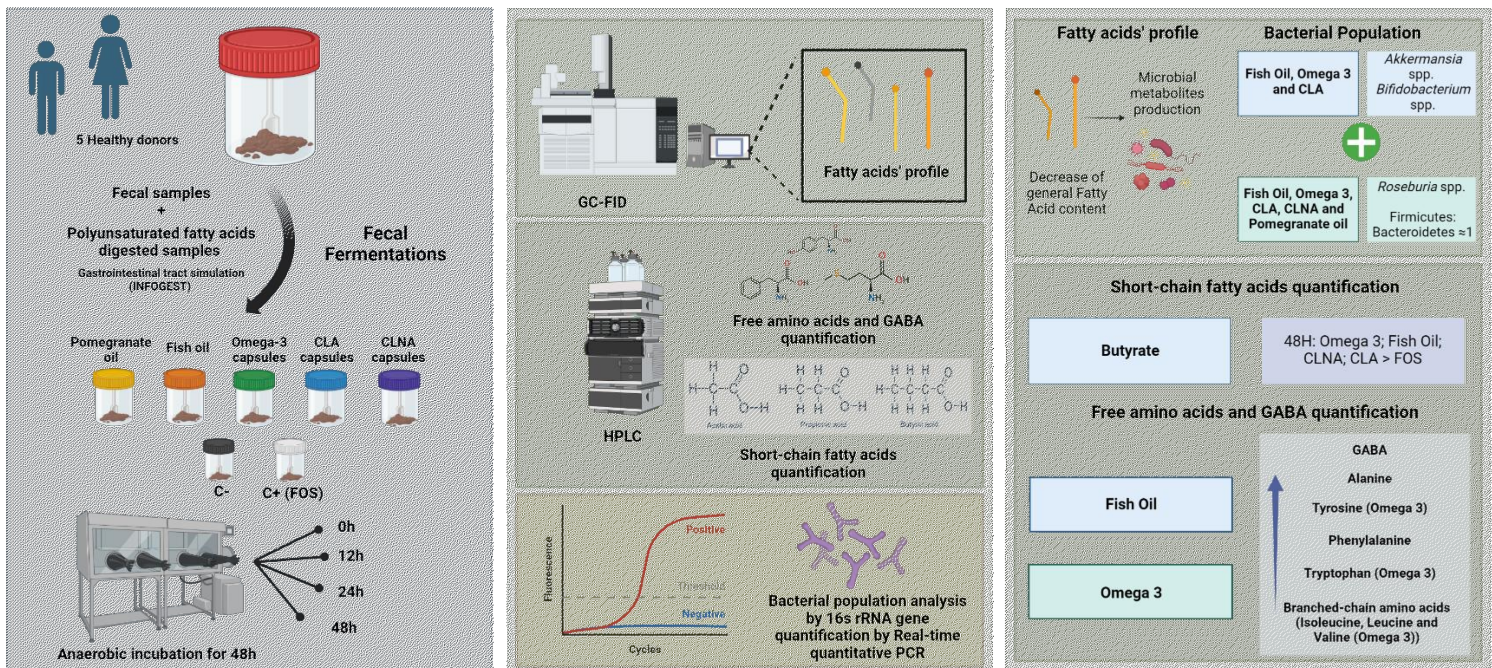

Figure 4.1 – Graphical abstract. Use of an in vitro human fermentation model to assess the effect of omega-3, CLA isomers, and punicic acid (CLNA isomer) on microbiota modulation. Fecal samples from 5 healthy donors were used. The FAs' profile was analyzed through GC-FID, the Free amino acids and GABA and SCFA quantification was assessed by HPLC. The bacterial population was quantified by 16s rRNA gene by Real-time quantitative PCR. It was observed a decrease of general FA content which was due to their use for microbial metabolite production. Fish oil, Omega-3, and CLA samples presented a positive impact on Akkermansia spp. and Bifidobacterium spp. growth. Moreover, all the samples supported Roseburia spp. growth after 24 h of fermentation. All the bioactive FAs samples, except Pomegranate oil, were able to significantly increase butyrate levels compared to those found in the positive control (FOS) sample. Moreover, Fish oil and Omega-3 capsules were able to increase the concentration of GABA, alanine, tyrosine, phenylalanine, isoleucine and leucine between 12 and 24 h of fermentation.

1. Introduction

The evolution of *Homo sapiens* has been intrinsically connected to a mutualistic association between bacteria that cohabit at different sites of the human body. One of the most relevant sites is the GIT. Gut microbiota includes archaea, viruses, and fungi but more than 99% of the microbial genes detected in the gut are bacterial genes (1,2). More than 90% of the bacterial species comprising the human microbiome belong to 4 major phyla: Firmicutes (65%), Bacteroidetes (16%), Actinobacteria (9%), and Proteobacteria (5%) (3). In adults, most bacteria belong to the genera *Bacteroides* (Bacteroidetes phylum), *Parabacteroides* (Bacteroidetes phylum), and *Clostridium* (Firmicutes phylum). Nevertheless, it is important to consider that each GIT site presents a distinctive microbiota (3). Importantly, the diversification and composition of the microbiota are influenced by several factors, such as perinatal features (mode of delivery: caesarian section or vaginal delivery), nutrition, and weaning, among others. Moreover, environmental, and host-specific factors, such as genotype, age, and gender, as well as habitat and most importantly diet, are determinants to define the host's microbiome (1–3).

Gut microbiota is an evolving scientific field and over the past few years, the important role of microbiota in the host's homeostasis has been increasingly recognized. Three major functions are attributed to gut microbiota: *i*) protection against pathogen colonization by nutrient competition and production of antimicrobial agents; *ii*) stimulation of innate immunity and *iii*) promotion of nutrient absorption through indigestible dietary fibers by tri/tetrasaccharides metabolization to monosaccharides ultimately producing B-group vitamins (4). Moreover, gut microbiota also interacts with enteroendocrine cells, and produces vitamins, steroid hormones, and neurotransmitters such as GABA and serotonin (5). Thus, considering the fundamental role that gut microbiota presents in health conditions and the effect of food on microbiota, the potential of microbiota modulation through diet has been gaining increasing attention.

Importantly, lipids, including dietary lipids, are the major cell membrane constituents and, thus, essential elements in gut permeability. Additionally, they are important in the modulation of the gut microbiome (6). Besides their structural role, lipids regulate multiple cell functions through intercellular and intracellular signaling mediators present both in the brain and the enteric system (7). These lipids are known as bioactive lipids. Importantly, multiple bioactive lipids exert either pro- or anti-inflammatory actions on the gut microbiome, influencing several important processes such as immune regulation, inflammation, and homeostasis (8). One of the most relevant lipids, when considering gut microbiota are SCFAs. SCFAs are the main metabolites produced in the proximal colon by bacterial fermentation of dietary fibers and resistant starch. SCFAs are important regulators of several gastrointestinal system mechanisms, namely in colon energy supply, in the regulation of Treg cells (9), and in maintaining the integrity of the epithelial barrier by regulating mucus production and tight junction expression (5). Besides, they also are known to possess immunomodulatory effects since they can promote anti-inflammatory properties. In recent years, there has been growing evidence supporting the possibility of SCFAs exerting

physiological effects on several organs, including the brain (9). SCFAs can interact with the BBB, by upregulating tight junction proteins and consequently modulating BBB permeability and through T-cell differentiation towards regulatory subtypes (Treg) (5). Besides, SCFAs can cross BBB via monocarboxylate transporters in endothelial cells. In the CNS, SCFAs can also influence neuroinflammation by interacting with glial cells, and consequently modulate brain function (9).

Nevertheless, knowledge of the role of other lipids in gut microbiota is still very scarce. The known beneficial roles of PUFAs in organs both in health and disease are well documented, specifically for omega-3 FAs. However, the impact of this bioactive FA on the gut microbiota is poorly defined and studies on the role of other PUFAs, such as CFAs are even scarcer. Omega-3 FAs have shown promising results in adults' microbiota modulation. In fact, omega-3 supplementation has been demonstrated to induce common changes in gut microbiota, which are characterized by a decrease in *Faecalibacterium* associated with an increase in Bacteroidetes and butyrate-producing bacteria (10). Moreover, in several diseases, omega-3's potential action in gut microbiota is related to restoring the dysbiosis that is characteristic of such pathologies. For instance, Firmicutes: Bacteroidetes ratio dysbiosis is often observed in weight gain and obesity, insulin resistance, HFD consumption, gut permeability, inflammatory bowel disease, and depression. Interestingly, omega-3 can restore the Firmicutes: Bacteroidetes ratio and increase Lachnospiraceae bacteria, increasing the production of butyrate, a relevant anti-inflammatory SCFA. In addition, omega-3 PUFAs increase LPS-suppressing bacteria and bifidobacteria and decrease LPS-producing bacteria such as enterobacteria, suppressing endotoxemia responsible for low-grade systemic inflammation (10). Recently in a study aiming to evaluate, among other parameters, the effect of CLA on the gut microbiome and intestinal barrier integrity of mice, a 1% CLA (commercial mixture) supplementation showed an increase in the abundance of beneficial bacteria (e.g., *Lachnoclostridium*, *Roseburia*, *Dubosiella*, *Oscillibacter*, and *Anaerostipes*) and a lower abundance of pro-inflammatory bacteria (e.g., *Tyzzerella* and *Alistipes*) (11).

Since there is still a lack of knowledge regarding this theme and considering that CFAs are also known for their anti-inflammatory potential in some organs, for instance in adipose tissue (12), by using an *in vitro* human fermentation model, we aim to investigate the effect of different PUFAs, including omega-3, CLA isomers and PUA, a CLNA isomer, in microbiota modulation and corresponding metabolites production. We expect that different FAs will present distinct impacts on the microbiota of healthy donors and that the studied FAs will improve the growth of anti-inflammatory bacteria and consequently butyrate production.

2. Material and Methods

2.1. Chemicals and reagents

For the GIT digestion α-amylase from human saliva (A1031-5KU), Bile salts (bile extract porcine – B8631), and Pancreatin from porcine pancreas (P7545) were purchased from Sigma-Aldrich (Missouri, USA). Rabbit gastric extract (RGE 15) was obtained from Lipolytech (Marseille, France).

Regarding the fecal *in vitro* fermentations, the Cysteine hydrochloride monohydrate (1.02839.0100), Tryptone Soya Broth without dextrose (T3938), Ammonium chloride (101145) was from Merck (Darmstadt, Germany). The Sodium chloride (746398), Calcium chloride dihydrate (C7902), Potassium phosphate dibasic trihydrate (P5504), and Resazurin sodium salt (199303) were obtained from Sigma-Aldrich (Missouri, USA). The Bactopeptone (peptone bacteriological, RM001) was from Himedia Labs (Einhausen, Germany). The Yeast nitrogen base (DF0392-15-9239210) was purchased from Fisher Scientific (Massachusets, USA). The Magnesium chloride hexahydrate (459337) was purchased from Carlo Erba Diagnostics (Milano, Italy). The Trace mineral supplement solution was from ATCC (Virginia, USA). Fructooligosaccharides (FOS, P-FOS28) were from Megazyme (Wicklow, Ireland). Concerning the DNA extraction and real-time quantitative polymerase chain reaction, the Tris-EDTA buffer (TE, 10x concentrate, PPB010) and Lysozyme (P00698) were from Sigma-Aldrich (Missouri, USA). The iQ SYBR Green Supermix (1708882) was acquired from Bio-rad (California, USA). The NZY Tissue gDNA for DNA extraction (MB13502) was purchased from NZYTech (Lisbon, Portugal).

For the FAs profile analysis, Hexane, Methanol, Dimethylformamide (DMF), and Acetonitrile were HPLC grade and purchased from VWR Chemicals (Pennsylvania, USA). Sulphuric acid was obtained from Honeywell (North Carolina, USA). Sodium methoxide was from Acros Organics (Geel, Belgium). Tritridecanoin (33-1300-13) internal standard was from Larodan Research Grade Lipids (Solna, Sweden).

Regarding the SCFAs analysis, the standards were Butyric acid (B10,350-0) and Propionic acid (P1386) from Sigma-Aldrich. Acetic acid (20104.323) and Lactic acid (20366.293) were purchased from VWR.

For the free amino acids analysis, Perchloric acid (244252), Homoserine and Norvaline standards, and 2-mercaptoethanol (M6250) were from Sigma-Aldrich. The analytical standards used for the amino acids identification and calibration curve development were: γ-amino-n-butyric acid (GABA, A-5835), L-lysine (L-5626), L-leucine (L-5652), L-valine (V-0500), L-tryptophan (T-0254); L-isoleucine (I2752), L-phenylalanine (P2125), L-arginine (A-8094), L-methionine (M-9625), L-tyrosine (T-3754), L-alanine (A7627), L-glutamine (49419), L-cysteine (168149), L-threonine (T8625), L-histidine (H-8000), L-serine (54311), L-glutamic acid (G-1251), L-asparagine (A0884), L-aspartic acid (A9256), all from Sigma-Aldrich. Glycine (33226H) was from Honeywell.

2.2. FAs Sources

The samples used in this study are thoroughly described in **Chapter 3 in Section 2.2**.

2.3. *In vitro* GIT tract digestion

The GIT digestion was performed as described in **Chapter 2 Section 2.3.** Briefly, before submitting the FAs' sources to the *in vitro* fecal fermentations each sample was submitted to GIT to mimic as closely as possible *in vivo* conditions. To simulate GIT the standardized static digestion model INFOGEST 2.0 protocol (13) was followed. The protocol is divided into oral, gastric, and intestinal phases and the correspondent fluid is used to better simulate the *in vivo* conditions.

To simulate the passage of the digested samples by duodenum and jejunum, the samples were incubated overnight at 37 ºC and 50 rpm inside a dialysis tubing (3.5 kDa molecular weight cut off). At the end of the process, the solution that remains in the interior of the membrane represents the non-absorbable fraction (colon-available) which is accessible to the gut microbiota (14–16).

2.4. Gut microbiota simulation: fecal fermentations

2.4.1. Fecal samples collection

Human fecal samples were obtained fresh (on the morning of the assay day) at the Center for Biotechnology and Fine Chemistry from the Catholica University of Porto. The samples collected were donated voluntarily from five healthy adult donors – 4 women and 1 man aged 24 to 31. The donors were informed about the study design and a consent agreement was signed. All the volunteers met the study inclusion criteria: normal omnivorous diets, had not ingested any antibiotics or other medicines known to affect the microbiota for at least 6 months before the study, and were not regular consumers of probiotics or prebiotics. The fecal samples were maintained under anaerobic conditions for a maximum of 2 h before being used.

It was performed a 100 g/L dilution of the fecal samples in Reduced Physiological Salt solution (RPS) (comprised of 0.5 g/L cysteine hydrochloride (Merck, Darmstadt, Germany) and 8.5 g/L NaCl (LabChem, Zelienople, USA) with a final pH value of 6.8. The mixture was prepared and homogenized using a stomacher (Serward, Worthing, UK) for 2 min at 460 paddle beats per min. This produced a fecal slurry to be used as the inoculum (17,18).

2.4.2. *In vitro* human fecal fermentations

The *in vitro* fecal fermentations were performed based on a method previously developed (14) with slight modifications. The fermentation medium used for the fermentations consisted of

5.0 g/L of trypticase soy broth (TSB) without dextrose, 5.0 g/L of bactopeptone, 5.0 g/L of a yeast nitrogen base, 0.5 g/L cysteine hydrochloride, supplemented with a 1% (v/v) salt solution A (100 g/L of Ammonium chloride (NH_4Cl), 10 g/L of magnesium chloride hexahydrate ($MgCl_2 \cdot 6H2O$) and 10 g/L of $CaCl_2 \cdot 2H_2O$), 0.2% (v/v) of a salt solution B (200 g/L of Potassium phosphate dibasic trihydrate ($K_2HPO_4 \cdot 3H_2O$), 0.2% (v/v) of 0.5 g/L resazurin solution and a 10 mL/L of a trace minerals solution. The final pH was adjusted to 6.8 and before sterilization, the medium was placed under a nitrogen stream.

Shortly, for the fecal fermentations the positive control (FOS) and the digested samples (Fish oil, Pomegranate oil, Omega-3, CLA, and CLNA capsules) were added to sterile urine flasks at 2% (w/v) and the fecal inoculum was added to the flasks at a concentration of 2% (v/v). Importantly, in the digested samples the pH was adjusted to 8, before fermentation assays, to ensure that the gastric lipase and pepsin were irreversibly inactivated (19–21). In the negative control, no sample was added, just 2% (v/v) of fecal inoculum. The total volume of each sample was then divided into 13 mL sealed sterile tubes. For each sample (FOS, Fish oil, Pomegranate oil, Omega-3, CLA, and CLNA capsules and negative control), it was used the fecal inoculum from each of the five donors (separately) and aliquots were obtained at 0, 12, 24, and 48 h of incubation. pH values were measured at each of the mentioned time points using a MicropH 2002 pH meter (Crison, Barcelona, Spain), equipped with a 52-07 pH electrode (Crison, Barcelona, Spain). All the procedures were performed in an anaerobic workstation (Don Whitley Scientific, West Yorkshire, UK).

2.5. Bacterial population analysis

2.5.1. DNA extraction and real-time quantitative polymerase chain reaction

For the DNA extraction, 4 mL of the fecal fermentation sample was centrifuged at 4000 xg for 10 min at 4 ºC. The resultant pellet was dissolved in 1 mL of TE buffer. The solution was centrifuged at 4000 xg for 10 min at 4 ºC and 180 µL of a lysozyme solution (10 mg/ml in TE buffer) was added to the pellet. The solution was incubated at 37 ºC for 2 h. The solution was further centrifuged at 4000 xg for 10 min at 4 ºC and the resulting pellet was used for DNA extraction using the NZYTissue gDNA isolation kit according to the manufacturer's instructions.

Afterward, the 16S rRNA gene from the Firmicutes, *Lactobacillus* spp., *Clostridium leptum*, Bacteroidetes, *Bifidobacterium* spp., *Akkermansia* spp. and *Roseburia* spp. groups were quantified using specific primers obtained from STABvida (Lisbon, Portugal), according to a real-time polymerase chain reaction (RT-PCR) using iQ SYBR Green Supermix and a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, USA), under conditions adapted and optimized from previous studies as described in **Table 4.1** (17,22).

Table 4.1- Primer sequence information and Genomic DNA standard used for gut microbiota analysis.

Target group	Primers' sequence (5'-3'; F- forward, R- reverse)	Annealing temperature (ºC)	Genomic DNA standard	
Firmicutes Total	**F:** ATG TGG TTT AAT TCG AAG CA **R:** AGC TGA CGA CAA CCA TGC AC	55	*Lactobacillus gasseri* (ATCC 33323)	DSM20243
Bacteroidetes	**F:** CAT GTG GTT TAA TTC GAT GAT **R:** AGC TGA CGA CAA CCA TGC AG	55	*Bacteroides vulgatus* (ATCC 8482)	DSM 1447
Lactobacillus spp.	**F:** CAC CGC TAC ACA TGG AG **R:** AGC AGT AGG GAA TCT TCC A	59	*Lactobacillus gasseri* (ATCC 33323)	DSMZ 20243
Roseburia spp.	**F:** TAC TGC ATT GGA AAC TGT CG **R:** CGG CAC CGA AGA GCA AT	60	*Roseburia hominis*	DSM 16839
Bifidobacterium spp.	**F:** CGC GTC YGG TGT GAA AG **R:** CCC CAC ATC CAG CAT CCA	60	*Bifidobacterium longum* subsp. infantis	DSM 20088
Clostridium leptum subgroup	**F:** GCA CAA GCA GTG GAG T **R:** CTT CCT CCG TTT TGT CAA	58	*Clostridium leptum*	DSM 753
Akkermansia spp.	**F:** CAG CAC GTG AAG GTG GGG AC **R:** CCT TGC GGT TGG CTT CAG AT	62	*Akkermansia muciniphila*	DSM 22959

2.5.2. Fatty acid composition analysis by GC-FID

2.5.2.1. Sample preparation

The FAs profile of the samples after GIT digestion and at the different time points (0, 12, 24, and 48 h) of the *in vitro* fecal fermentations was assessed through GC-FID. 250 µL of digested samples and 500 µL of fecal fermentation samples were prepared according to previous studies (23,24) as described in **Chapter 3 Section 2.5.1.1**. For quantification purposes, 200 µL of tritridecanoin acid (1.5 mg/mL) was added before the derivatization process as an internal standard. After derivatization, FAMEs were collected for gas chromatography analysis.

2.5.2.2. GC-FID analysis

The FA composition of the FAME extracts was determined and quantified using a gas chromatograph Agilent 8860 (Agilent, USA) equipped with a flame ionization detector and a BPX70 capillary column (60 m x 0.25 mm x 0.25 µm; SGE Europe Ltd, Courtaboeuf, France). The analysis conditions were described in **Chapter 3 Section 2.6.3.1**.

2.5.3. Short-chain Fatty acids and Lactic acid quantification

SCFA analysis was determined by collecting the fecal fermentation samples supernatant (14,17). The analyses were performed using a Beckman Coulter System Gold HPLC (Knauer, Berlin, Germany) coupled to IR and UV detector using Aminex HPX-87H column (Bio-rad, Berkeley, USA) at 55 ºC and 5 mM H_2SO_4 as mobile phase (flow rate: 0.6 mL/min). The identification was achieved by comparison of the relative retention times of sample peaks with

adequate standards and the quantification by using a calibration curve in the range of concentrations of 0.2–20 mg/mL.

2.5.4. Free Amino acids and GABA detection and quantification

For free amino acids detection, 1 mL of fecal fermentation samples were dissolved in 2 mL of 0.6 M perchloric acid. Each mixture was placed in a roller mixer for 1 h and further centrifuged at 4100 xg, for 15 min at 4 ºC. The supernatant was collected and passed through a 0.22 µm filter (25).

The samples were subsequently analyzed by HPLC (26). The chromatographic analysis was performed using two eluents, A and B. Eluent A is a solution comprised of 10 g/L sodium phosphate dibasic dihydrate, 7.4 g/L propionic acid, 20 mL/L dimethyl sulfoxide (DMSO), 65 mL/L acetonitrile and ultrapure water, with the final pH value being adjusted to 6.65. Eluent B was obtained by a mixture of 330 mL/L methanol, 70 mL/L DMSO, 400 mL/L acetonitrile, and ultrapure water. The reagent A consisted of 120 mL/L of a previously prepared internal standard solution (20 mg/L of homoserine and norvaline in 0.1 M HCl), 4.8 mL/L mercaptoethanol, 20 g/L of sodium tetraphenylborate and the volume adjusted with borate buffer (6.2 g/L H_3BO_3, pH 9.5). Reagent B was comprised of 35 g/L of iodoacetic acid and the volume was completed with borate buffer (6.2 g/L H_3BO_3, pH 9.5) adjusted to a pH value of 9.5. Reagent C was prepared by mixing 4.5 g/L of OPA (Phthaldialdehyde), 100 mL/L of methanol, and completed with borate buffer (6.2 g/L H_3BO_3, pH 9.5). Then, 10 mL/L of mercaptoethanol was added and the solution was bubbled with N_2. For the characterization and quantification of the fermentation samples' free amino acid content it was used a liquid chromatography apparatus (HPLC Gold 128 solvent module, Beckman Coulter, Brea, USA) with a High-Resolution Fluorescence Detector (λexcitation 356 nm; λemission 445 nm; Waters 474, Milford, USA) and an autosampler (model 410 Varian prostar, Agilent Technologies, Santa Clara, USA). The system was connected to a Chromolith® Performance RP18 (4.6 × 100 mm) (Merck, Darmstadt, Germany), operating at a flow rate of 0.8 mL/min. 100 µL of the filtered samples were mixed with 250 µL of reagent A, and 250 µL of reagent B. After 3 min, 250 µL of reagent C was added and 10 µL of the mixture was injected into the HPLC system. The identification of the individual free amino acids in each sample was achieved by comparison of the relative retention times of sample peaks with adequate standards and the quantification by using a calibration curve in the range of concentrations of 0.1–10 mg/L.

2.6. Statistical Analysis

Results are reported in both tables and figures as mean values ± standard deviation. For the statistical analysis, IBM SPSS Statistics 28 (SPSS Inc., IBM Corporation, NY, USA), was used. The first step consisted of analyzing data for normal distribution, through Shapiro-Wilk's test.

When data didn't follow a normal distribution, it was transformed using the log base 10 functions. To verify the homogeneity of the variances Levene's test was used. One-way ANOVA was employed to compare the means of three or more groups. Tukey's post hoc test was used to determine differences among groups. When normality was not achieved Kruskal-Wallis' test was applied. The level of significance was set at 0.05.

A principal component analysis (PCA) using IBM SPSS 28 was applied to identify the PUFA samples (Fish oil, Omega-3, Pomegranate oil, CLNA, and CLA) that produced a differentiating effect on modulating a healthy human microbiota and which are the parameters (pH, FAs, amino acids, and SCFAs) that better explained such effect. To reduce the factors to identify the high and low correlations among variables, the Varimax method was used to produce orthogonal transformations. Loadings are a representation of the importance of the variable in explaining the data variability in the respective component. Consequently, a loading of ≥ 0.7 (absolute value) is used to determine the dominant variables in the respective component. The scores represent the distribution of the data in the rotated system composed of the principal components (27).

3. Results and discussion

3.1. Fatty acid composition analysis of the fermentation samples

First, to determine the effect of the *in vitro* fermentation and, specifically, of gut microbiota on the major bioactive FAs of the different samples, a total FA analysis was performed for each sample at different time points (12, 24, and 48 h) and the results are displayed in **Figure 4.2**. The FA profile, for the major bioactive FAs, of the fermented samples was compared to the original sample used for the fermentation assays. This sample was previously subjected to GIT digestion, as explained in **Section 2.3** of this chapter, and only the non-absorbed fraction was used to be tested in the microbiota assay. It was observed that in general there was a decrease in the concentration of most FAs after 12 h fermentation. A recent study performing an *in vitro* fermentation assay using human fecal samples also reported similar results: it was demonstrated that the concentration of several FAs decreased during fermentation. The authors hypothesized that such reduction may suggest a substantial conversion of FAs in microbial metabolites, which may also be a plausible explanation for what was observed here. Additionally, in this study, it was also reported the presence of LA metabolites in the fermentation pellets. This may suggest that besides metabolic conversion, the incorporation of microbial FA metabolites into bacterial cells may be an explanation for the decrease in the FA concentration during fermentation (30). To better assess this decrease, the RI (%) was calculated **(Equation 4.1)** for the major FAs detected in each sample **(Supplementary Material Table S4)**.

$$\text{RI (\%)} = \frac{\text{Fatty acid content quantified after fermentation}}{\text{Fatty acid content quantified in the digested sample before fermentation}} \times 100 \qquad \textbf{(Equation 4.1)}$$

Regarding the most prevalent SFAs, it was observed that for myristic (C14:0), PA (C16:0), and stearic (C18:0) acids the RI was higher in Fish oil (23.39-27.30% for C14:0 and C16:0 and 29.01-32.04% for C18:0) than in Omega-3 samples (0, 13.17-16.57% and 20.84-27.03% for C14:0, C16:0 and C18:0, respectively). This observation may be explained by the higher initial concentration of these SFAs in Fish oil (11.36±3.52 for C14:0, 23.17±4.61 for C16:0 and 3.92±0.08 µg of FA/µL of sample for C18:0) than in Omega-3 (0.075±0.004, 0.59±0.04 and 0.47±0.04 µg of FA/µL of sample for C14:0, C16:0 and C18:0, respectively). The same was observed for the Pomegranate oil and CLNA samples: the C16:0 showed a slightly higher RI in the CLNA samples (23.51-29.59%) when compared to Pomegranate oil (17.59-19.28%) since the concentration of this FA was higher in the digested CLNA samples (7.98±2.91 µg of FA/µL of sample) than in Pomegranate oil (3.90±1.75 µg of FA/µL of sample). When considering C18:0 both Pomegranate oil and CLNA samples present similar initial concentrations – 2.76±1.02 and 2.65±0.67 µg of FA/µL of the sample, respectively – and this is translated in similar RIs (26.26-34.65%). Intriguingly, although CLA samples presented lower concentrations of C16:0 (0.64±0.01

µg of FA/µL of the sample) and C18:0 (0.31±0.007 µg of FA/µL of the sample) the RIs were higher by 24.34-53.90% for C16:0 and 94.17% for C18:0 at the end of the fermentation comparing to the other samples. Interestingly, at 12 and 24 h of fermentation, the C18:0 presents a RI>100%. Such results may suggest that there is a production of C18:0 in this sample along with a consumption of LA (RI≈27.58-34.15%). Indeed, the production of C18:0 from LA has been previously described (28). Considering the most prevalent MUFA, the oleic acid (C18:1 *c*9), similar results were obtained: the higher RI in CLNA (≈19.43%) and Fish oil (≈18.63%) was correlated to higher initial concentrations of this FA (43.65±17.33 and 10.02±2.20 µg of FA/µL of sample, respectively) when comparing to Pomegranate oil (RI≈13.90%, 7.50±3.52 µg of FA/µL of sample) and Omega-3 (RI≈5.95%, 1.46±0.10 µg of FA/µL of sample), correspondingly. Together, these results suggest that the initial concentration of a determined FA may be a relevant parameter to consider when assessing its effect on microbiota modulation.

Moreover, the microbial digestion of dietary lipids, as those observed here, is considered a detoxifying mechanism used by several bacteria such as *Lactobacillus*, *Roseburia,* and *Bifidobacterium*, to transform growth-inhibiting PUFAs into less toxic FAs. One of these important mechanisms is the process through which LA is converted into CLA isomers. Indeed, it has been reported that *Bifidobacterium* can transform LA into different CLA isomers, including C18:2 *c*9*t*11 and its precursor C18:1 *t*11 (Vaccenic acid) (29). Interestingly in this study, it was observed in Pomegranate oil, CLNA and CLA capsules a statistically significant increase (RI>100%, p>0.05) in C18:2 *t*9*t*11 and C18:1 *t*11 FAs concentration. In the CLNA sample, the same was observed for the C18:3 *t*9*t*11*t*13 (β-eleostearic acid) (**Figure 1**). Since the values were significantly higher than the original samples (RI>100%) this indicates a production of these CLA and CLNA isomers. This was accompanied by a significative decrease, right after 12 h, of LA, which is consistent with the microbial conversion of this FA to a less toxic form. Similar results were reported in a recent study but in that study, an increase in RA, another CLA isomer, was also reported (30). In the present study it was observed an increase in its precursor - C18:1 *t*11 (Vaccenic acid) – but differently, here it was observed a significative decrease of RA in the CLA sample (RI≈1.02%). Nevertheless, the authors from the mentioned study demonstrated that microbial metabolism of LA is different depending on the form in which LA is provided (30), which may explain the different results obtained here.

Omega-3 FAs, similarly to what was already previously discussed, seemed to be metabolized by the gut microbiota bacterial community. In fact, omega-3 PUFAs are known to be partially metabolized by anaerobic bacteria, such as *Bifidobacterium* and *Lactobacillus*, in the distal intestine (31). Regarding EPA and DHA, the RIs were higher for Fish oil (≈11.11% and ≈7.51%) than for omega-3 samples (≈2.49% and ≈1.61%). Contrarily to the observations made for the previously discussed FAs, EPA and DHA presented lower initial concentrations in Fish oil (18.91±7.77 and 11.10 ±0.44 µg of FA/µL of sample) compared to omega-3 (39.91±3.11 and 27.71±1.96 µg of FA/µL of sample).

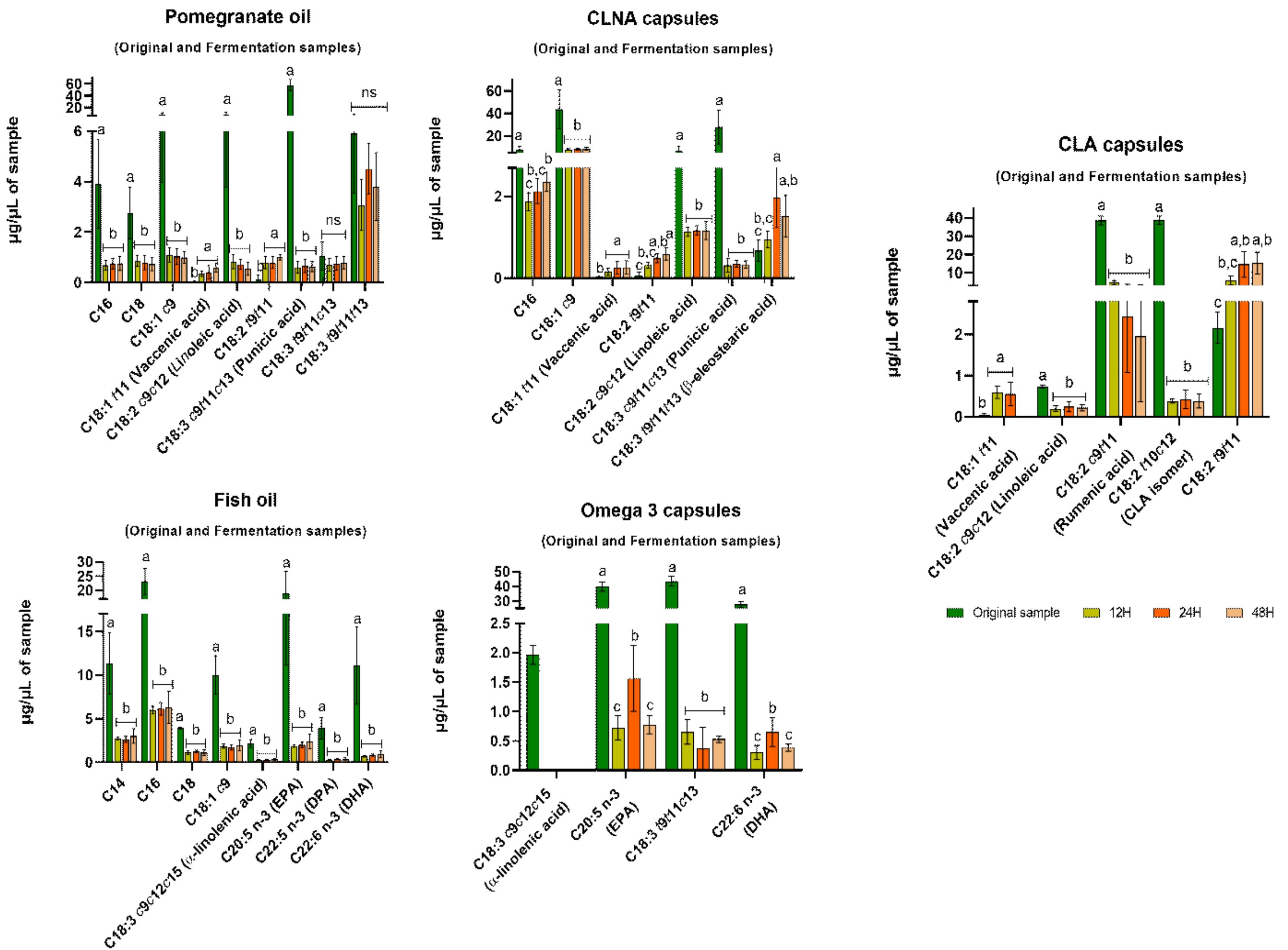

Figure 4.2- Total fatty acid analysis of the original samples (after gastrointestinal tract digestion) and the corresponding samples after *in vitro* fermentation after 12, 24 and 48 h. Results are the means of five donors and the error bars represent the standard deviation. Different letters indicate statistically significant differences ($p < 0.05$).

3.2. Fecal microbial communities' analysis

To understand the effect of the bioactive FAs in the gut microbiota of the human donors, the 16S rRNA gene from the Firmicutes, *Lactobacillus* spp., *Clostridium leptum*, Bacteroidetes, *Bifidobacterium* spp., *Akkermansia,* and *Roseburia* groups was quantified. As mentioned, Firmicutes and Bacteroidetes are the two major phyla of bacterial species in the human microbiome. *Clostridium* is one of the major bacteria found in the gut microbiota of human adults (3). Moreover, besides the enormous interest that *Lactobacillus* and *Bifidobacterium* strains have been receiving as probiotics (32), they are also known to possess an effect in metabolizing omega-3 FAs (29). Indeed, a positive effect of omega-3 on *Bifidobacterium* growth has been previously reported (33). In addition, *Roseburia* and *Akkermansia* have been associated with positive effects in several inflammatory diseases, such as inflammatory bowel disease (34) and obesity (35), where PUFAs, such as CLA and omega-3, are known to possess a positive impact. Considering this, during fermentation, samples were taken at 0, 12, 24 and 48 h. FOS was used as the positive control since it is a compound with a known prebiotic effect (36). Each donor's fecal microbiota composition was determined using a negative control, where no sample was added. With this assay three of the four dominant phyla in the human gut were evaluated, *i.e.,* Firmicutes (total, *C. leptum*, *Roseburia* spp.), Bacteroidetes (total) and Actinobacteria (*Bifidobacterium* spp.). *Lactobacillus* spp. was also evaluated, as stated, but, in this study, the 16S rRNA gene quantification was under the quantification limit for all the study groups. This agrees with previous studies that have been reporting lower numbers of these bacteria in normal gut microbiota (1.90-2.90 log 16S rRNA gene copies/ng of DNA) (17,22,37).

In **Figure 4.3** and **Supplementary Material Table S5** the log 16S rRNA gene copies/ng of DNA are illustrated for the different groups of bacteria analyzed in this work. Moreover, in **Figure 4.4** the relative differences (RD (%)) in reference to the negative control were calculated as demonstrated in **Equation 4.2**.

$$RD\ (\%) = \frac{\mathrm{SMC}-\mathrm{CMC}}{CMC} \times 100 \qquad \textbf{(Equation 4.2)}$$

SMC represents the mean of the log 16S rRNA gene copies/ng of DNA of a determined sample at a certain time (12, 24, and 48 h) and CMC is the mean of the log 16S rRNA gene copies/ng of DNA of the negative control sample at the correspondent conditions. Positive (%) values represent an increase in the number of log 16S rRNA gene copies/ng of DNA copies relative to the negative control. By analyzing the RD presented in **Figure 4.4** it was observed a tendency of the Positive control, FOS, to induce a positive effect (an increased abundance) in the growth of most of the studied bacterial groups (Firmicutes total after 24 h, *C.leptum*, *Roseburia* spp., *Bifidobacterium* spp.). A negative effect was observed in general for the Bacteroidetes phylum. Nevertheless, it is important to mention that when comparing the values of the log 16S rRNA gene copies/ng of DNA (**Figure 4.3**) for Bacteroidetes there are no statistically significant differences between all the studied groups and the controls (FOS and the negative control). It is noteworthy that these observations are due to high standard deviations that are intrinsically

connected with the high variability related to the use of different human donors, from different sexes and with different dietary consumptions. Regarding the samples used, Pomegranate oil seems to induce a negative effect (decreased abundance) in most of the mentioned groups, which is translated by negative RD values (**Figure 4.4**). Such observation may be related to the already discussed toxic effect of LA in bacterial growth. As observed, higher amounts of LA may inhibit bacterial growth and accelerate the transformation of LA into less toxic FAs such as C18:2 *t*9*t*11 CLA isomer (30). It has been reported that the bacterial lag phase was dependent on LA concentrations and was proportional to the FA concentration (29). Indeed, along with CLNA (7.17±3.48 µg/µL of the sample), Pomegranate oil is the sample with higher LA concentrations (8.21±4.42 µg/µL of the sample), which is consistent with the negative RD observed. Thus, considering the other source of PUA used in this study, CLNA capsules, similar results were obtained. When considering the log 16S rRNA gene copies/ng of DNA values Pomegranate oil presents a statistically significant <0.05) negative effect for *C.leptum* in all the studied times (12, 24, and 48 h). CLNA capsules also induced a negative effect ($p<0.05$) on *C.leptum* at 24 and 48 h of fermentation and on Bacteroidetes after 48 h of fermentation ($p<0.05$). Indeed, several species of the *Clostridium* genera have been described to be able to hydrogenate LA to other FAs (28,38), which may explain the negative effect of these FAs on *C.leptum* growth. Nevertheless, PUA supplementation in HFD obesity-induced mice has been demonstrated to be able to restore the decreased levels of Bacteroidetes and *Roseburia* groups that were induced by the HFD (39). Here, at 24 h Pomegranate oil and CLNA showed a positive RD index for *Roseburia* spp. group (**Figure 4.4**). Nevertheless, a negative RD was observed for these samples in the Bacteroidetes group. Indeed, CLNA capsules presented at 48 h a lower statistically significative log 16S rRNA gene copies/ng of DNA value (for Bacteroidetes) when compared to the negative control (**Figure 4.3**). Similar results were observed for CLA capsules, which presented a lower concentration of LA (0.731±0.04 µg/µL of sample): it was observed a statistically significant negative effect ($p<0.05$) on *C. leptum* bacteria (at 12, 24, and 48 h) and Bacteroidetes group after 24 and 48 h fermentation. CLA treatment in obesity-induced mice has been shown to decrease Bacteroidetes, which was consistent with the observations made in this study. On the other hand, in the mentioned study CLA was shown to increase *Roseburia* bacteria (11). In fact, in the present study when considering the *Roseburia* group, although log 16S rRNA gene copies/ng of DNA presented no statistically relevant difference among the different groups, considering just the RD values there is a small positive impact of CLA on *Roseburia* growth at 24 and 48 h, translated by a positive RD (**Figure 4.4**). Importantly, *Roseburia* has been described to metabolize LA by the same pathway found in ruminal bacteria (40), which may explain the negative effect of Pomegranate oil, CLA, and CLNA on its growth at 12 h.

Omega-3 PUFAs, such as EPA and DHA, have been demonstrated to exert a relevant positive impact on several diseases known to cause dysbiosis in gut microbiota. Omega-3 effects are described to be mediated in three ways: (*i*) by modulating the type and abundance of gut microbiota, (*ii*) by altering the levels of proinflammatory mediators (*e.g.* endotoxins and IL-17),

and (*iii*) by regulating the levels of SCFAs or SCFAs salts (31). Besides, human and animal studies have demonstrated the ability of omega-3 PUFAs to modulate the gut-brain axis by acting on gut microbiota composition. Although these promising benefits, there are few studies on the impact of dietary fats such as omega-3 on the gut microbiota (10). As already discussed, it has been demonstrated that, in general, omega-3 PUFAs can restore eubiosis in gut microbiota by restoring Firmicutes: Bacteroidetes ratio, increasing Bacteroidetes, butyrate-producing bacteria belonging to the Lachnospiraceae family (*e.g*. *Roseburia*) and LPS-suppressing bacteria, such as *Bifidobacterium* (10). Here, in the Fish oil and the Omega-3 capsules, the sources of EPA and DHA, it was not observed any statistically relevant negative effect on all the bacteria groups analyzed in this work. Through the analysis of the RD, alone, it seems that both omega-3 and Fish oil may have a positive effect on *Bifidobacterium* spp growth after 24 h fermentation, but further studies are required to validate this result. Although a recent study assessing the effect of omega-3 α-linolenic acid demonstrated that oral supplementation with blended omega-3-rich oils, highly increased the relative abundance of *C. leptum* in volunteers with borderline hypercholesterolemia (41), here we observed that both Fish oil and omega-3 presented negative RD. This may be explained by the different omega-3 FAs used. Considering the Bacteroidetes group, after 48 h there was an inhibition of this group by CLA and CLNA samples, but not by omega-3 and Fish oil.

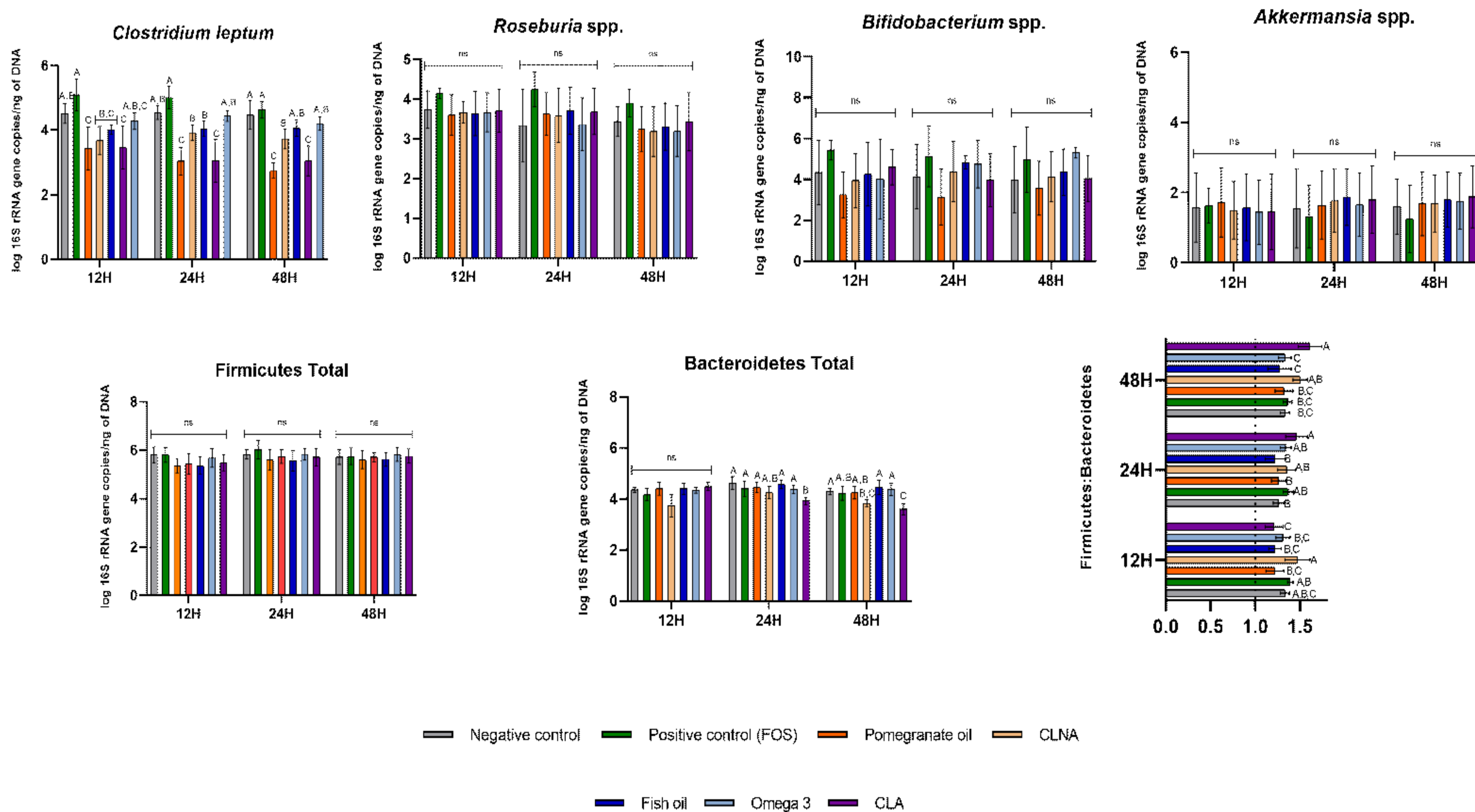

Figure 4.3 - Values of log 16S rRNA gene copies/ng of DNA of gut bacterial population after 12, 24 and 48 h of *in vitro* fecal fermentation of different bioactive lipids sources (Fish and Pomegranate oil, CLA, CLNA and Omega-3 capsules. Results are the means of five donors and the bars represent standard deviation. Different letters indicate significant differences ($p < 0.05$). The capital letters indicate the differences among samples Negative control, Positive control (FOS), and bioactive lipids sources (Fish and Pomegranate oil and Omega-3, CLA and CLNA capsules) for the population of same microbial genus at the same time.

This study also evaluated the effect of the assessed bioactive lipids sources on *Akkermansia* spp. *Akkermansia* has been associated with a positive impact on obesity by modulating glucose metabolism and low-grade inflammation (42,43). Importantly, a recent study has demonstrated that a high relative abundance of *Akkermansia* is associated with a low risk of obesity and this association declines with aging (35). It seems that when analyzing the RD (**Figure 4.4**) of the studied samples and comparing them with FOS, there is a positive effect of all the samples on *Akkermansia spp.* bacteria, translated by a positive RD, especially after 24 h for Pomegranate and Fish oil, CLNA, and Omega-3 capsules and after 48 h for CLA.

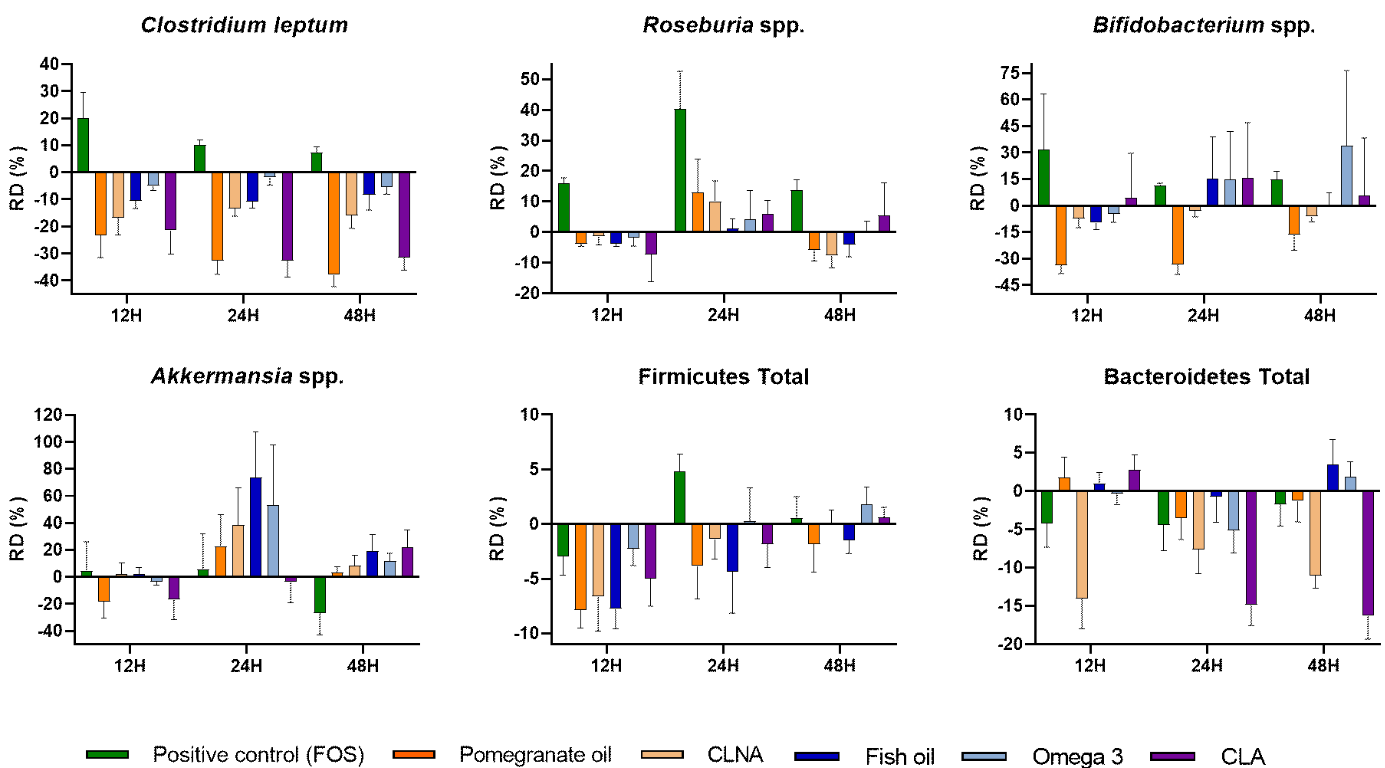

Figure 4.4 – **Relative difference (RD;%) values of gut bacterial population after 12, 24 and 48 h of *in vitro* fecal fermentation of different bioactive lipids sources** (Fish and Pomegranate oil, CLA, CLNA and Omega-3 capsules). Results are the means of five donors and the bars represent standard error mean.

As mentioned, the Firmicutes/Bacteroidetes ratio is a highly relevant parameter to consider when assessing a possible prebiotic effect. It has been described that healthy people display ratios of Firmicutes to Bacteroidetes near 1:1 and an alteration in this ratio is associated with several diseases, particularly obesity. Omega-3, CLA, and CLNA (specifically PUA) FAs have been demonstrated to be able to attenuate the increase of the Firmicutes: Bacteroidetes ratio associated with obesity (10,11,31,39,44). Relevantly, in this study, the samples tested have not altered the Firmicutes: Bacteroidetes (**Figure 4.3**) ratio and no statistically different effect was observed when compared to a positive control (FOS), except for CLA at 48 h ($p<0.05$).

3.3. Short-chain Fatty acids and Lactic acid production

SCFAs are metabolites produced by gut microbiota in the colon by fermentation of food components that are unabsorbed/undigested, such as dietary fibers and resistant starch (45). Acetate, propionate, and butyrate are the main SCFAs produced. Although anaerobic fermentation of these dietary products is the largest source of these SCFAs, they can also be produced from amino acid metabolism, however, less than 1% of gut microbiota uses these metabolic pathways to produce SCFAs (9,45).

Considering this, the production of lactic acid and the major SCFAs acetate, propionate, and butyrate was assessed as displayed in **Figure 4.5**. It was observed that lactic acid was the major metabolite produced during the entire fermentation of FOS (**Figure 4.5**), which agrees with the increase of lactate-producing bacteria such as *Bifidobacterium* spp*.,* demonstrated by a positive RD (**Figure 4.4**). This rise in lactic acid production was translated by a decrease in pH (**Figure 4.6**). It was observed an accumulation of lactic acid in FOS samples, which may indicate that the first was not used as a substrate during fermentation. Such results agree with previous studies using similar *in vitro* fermentation models and using a human fecal slurry as inoculum (17,22). Since this study was not conducted using a pH control, the significative decrease of pH in the FOS samples after 12 h may have not allowed the growth of lactate-utilizing bacteria (*e.g., Eubacterium hallii*, *Anaerostipes caccae, Propionibacterium avidum, and Veillonella ratti*), leading to an accumulation of lactic acid during the fermentation (46,47). Inhibition of these bacteria growth has been reported previously in pH values lower than 5.2, as detected here (46). For all the other samples – Pomegranate and Fish oil, Omega-3, CLA, and CLNA capsules-, it was detected a decrease of lactic acid throughout the fermentation time which is consistent with the higher pH value in these samples compared to FOS (**Figure 4.6**). Although Fish oil and Omega-3 also presented a positive effect on *Bifidobacterium* spp*.* growth (positive RD) this was not translated into higher lactic acid production. It is important to consider that several bacteria genera are known producers of lactic acid, such as *Leuconostoc*, *Lactococcus*, *Lactobacillus*, *Pediococcus*, *Enterococcus*, *Streptococcus*, *Vagococcus, Aerococcus, Carnobacterium, Tetragenococcus, Oenococcus and Weissella*. In this study, it was only possible to characterize the effect of the samples in *Bifidobacterium.* Thus, the possible differentiating effect of FOS, Fish oil, and Omega-3 capsules on different lactic acid bacteria (LAB) may explain the differences observed here between these samples regarding lactic acid production. Besides, lactic acid is an organic compound that is produced via fermentation using different carbohydrate sources. Here, the only carbohydrate source used was FOS (48). Besides, in Pomegranate and Fish oil, CLNA, CLA, and Omega-3 capsules, the maintenance of the pH ≈ 6 allowed the use of LAB by the lactate-utilizing bacteria. In addition, the PUFA samples used in this study did not present any relevant carbohydrate source. All these aspects may explain the mentioned difference between FOS and the remaining samples.

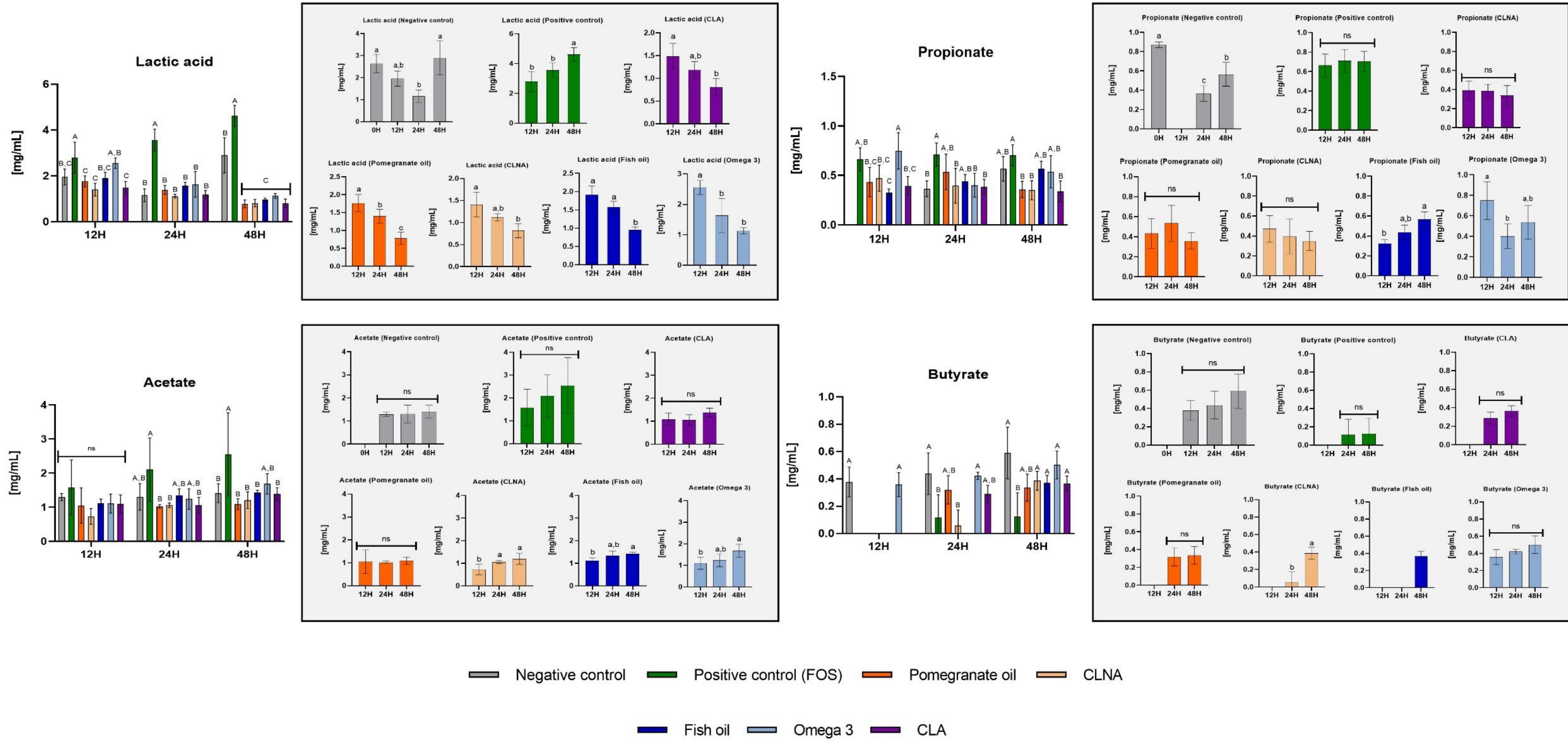

Figure 4.5 – Concentrations of short-chain fatty acids (mg/mL) – Acetate, Propionate and Butyrate - and Lactic acid after 12, 24 and 48 h of the *in vitro* fecal fermentation of different bioactive lipids sources (Fish and Pomegranate oil, CLA, CLNA and Omega-3 capsules). The values presented are the means of five donors and the bars represent the standard deviation. Different letters indicate significative differences ($p < 0.05$). The samples are presented individually at different time points in the light grey square. The small letters (a,b,c) indicate the differences for the same sample over time for the same short-chain fatty acid. In the left graphs, the different samples are compared. The capital letters (A,B,C) indicate the differences among the different samples - Negative control, Positive control (FOS), and bioactive lipids sources - for the same short-chain fatty acid at the same time.

Regarding SCFAs, acetate is produced by several anaerobic bacteria in the human gut. Importantly, it binds to co-enzyme A being involved in fat and carbohydrate metabolic pathways in intestinal cells. Compared to other SCFAs, higher concentrations of acetate have been found in blood circulation and colon lumen (49). In this study, FOS, Fish oil, Omega-3, and CLNA presented an increase in this SCFA throughout the fermentation time. But importantly, the values were not statistically different from the negative control, during the studied times, except for FOS fermentation at 48 h. Similar observations were previously described in a similar study using Brewer's spent grain (22). The increasing concentration of acetate along with the decreasing concentration of lactic acid, in Fish oil, Omega-3, and CLNA may be due to a cross-feeding effect among intestinal bacteria, using lactic acid as a substrate to produce acetate as the final metabolite (50,51). This observation was not evident during FOS fermentation, where it was observed an accumulation of lactic acid, as already discussed. At 48 h the concentration of acetate for Pomegranate and Fish oil, CLNA, and CLA samples was significantly lower than the FOS sample. Importantly, *Clostridium* bacteria are known producers of acetate (49). Since there is a statistically significant reduction, as discussed in **Section 3.2** of this chapter, of *C. leptum* bacteria, in Pomegranate oil, CLA, and CLNA samples compared to FOS, especially at 48 h, this may explain the lower acetate levels between these samples. Additionally, more and more evidence has been showing that PUFAs, namely omega-3 and CFAs, can attenuate the metabolic syndrome that is closely associated with obesity (12). Interestingly, it has been reported that butyrate can mitigate insulin resistance in mice and attenuate inflammation in 3t3-L1 adipocytes, while acetate was demonstrated to promote metabolic syndrome in HFD-fed mice via parasympathetic activation (52,53). So, the ability of all these FAs sources to decrease acetate production, compared to FOS, while increasing butyrate may be highly relevant and have a positive impact on these types of diseases.

On the other hand, propionate is a highly relevant SCFA since it can decrease hepatic cholesterol synthesis and improve fat metabolism. It also presents anti-inflammatory and antibacterial properties. *Akkermansia muciniphila* is recognized as a highly relevant bacteria for propionate production (49). Besides, some Bacteroidetes bacteria and *Roseburia* species have also been described as propionate producers (54). The concentration of this SCFA was constant throughout time in all the samples except for Fish oil where it was observed an increasing tendency throughout time (**Figure 4.5**). Compared to FOS, Omega-3, and Fish oil samples present a comparable production of propionate. On the other hand, CLNA, Pomegranate oil, and CLA presented a lower concentration ($p<0.05$) of propionate production compared to this positive control (FOS) at 48 h.

Butyrate has been widely documented, especially in obesity studies, due to its effects on the immune system. This SCFA can induce Treg differentiation and thus, control inflammation. Besides it also acts as an energy source for colonocytes, regulates multiple gut functions, cell differentiation, and gene expression, and reduces oxidative stress, among other relevant functions (49). Here, butyrate production after 48 h of fermentation is higher ($p>0.05$) in Omega-

3, Fish oil, CLNA, and CLA samples when compared to the FOS sample. A similar, but not statistically significant effect was observed for the Pomegranate oil sample. One explanation may be the cross-feeding effect that uses acetate to synthesize butyrate via the butyryl-CoA: acetate CoA-transferase route (49). This explains the lower acetate, compared to FOS, discussed above, and the higher butyrate concentrations. Most known butyrate producers-bacteria belong to the *Clostridium* cluster of the Firmicutes phylum, such as *Faecalibacterium*, *Roseburia*, *Eubacterium*, *Anaerostipes*, *Coprococcus*, *Anaerobutyricum* and *Blautia*. They can metabolize carbohydrates via the butyryl-CoA: acetate CoA-transferase pathway, as mentioned, and use butyrate kinase terminal enzymes to produce butyrate (31,55). Here we only analyzed *Roseburia* spp. and FOS was the sample with the most relevant impact on its growth, which was not translated into more butyrate production. So, there may be other mechanisms involved in the increased production of butyrate. Although in low levels, amino acids can also be used to generate butyrate via glutamate and lysine pathways (55).

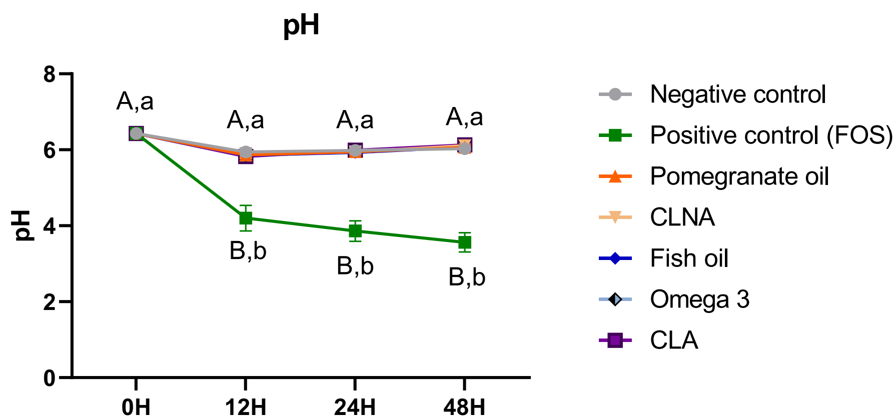

Figure 4.6 -pH determination at different fermentation times (0, 12, 24, and 48 h) for the different bioactive lipids' sources (Fish and Pomegranate oil, CLA, CLNA, and Omega-3 capsules) and for the Negative and Positive control (FOS). The results are the means of five different donors. The bars represent the standard deviation.

3.4. Free Amino acids and GABA detection

Amino acids also play an important role in gut microbiota homeostasis, in fact upon uptake by bacteria, they can be directly incorporated into bacterial cells and used as protein building blocks or become catabolized (56). Importantly, as mentioned, propionate and butyrate are formed as products, although at low levels, from peptide and amino acid fermentations (54). In fact, protein fermentation usually takes place in the distal large intestine where carbohydrates are already depleted (9). It is estimated that the colon receives approximately 13 g of protein and peptides per day and peptides seem to be preferred over free amino acids by gut microbiota (54,56). To fully understand the effect of the studied bioactive FAs in gut microbiota metabolism, free amino acids, and GABA detection and quantification analysis were performed. The major amino acids detected are demonstrated in **Figure 4.7** and **Supplementary Material Table S6**.

Regarding the large intestine, it is observed that amino acids are not significantly absorbed by the colonic mucosa; instead, they undergo intensive metabolism by the large intestinal microbiota (56). This heightened bacterial protein fermentation is associated with the elevated pH and reduced carbohydrate availability in the large intestine (57). Colonic bacteria exhibit a preference for certain amino acid substrates, such as lysine, arginine, glycine, valine, and isoleucine (56). This metabolism results in the production of a diverse array of metabolic end products, including ammonia, short-chain fatty acids (acetate, propionate, and butyrate), and branched-chain fatty acids (valerate, isobutyrate, and isovalerate). The increased rate of protein fermentation in the large intestine has been linked to the alkaline pH and limited carbohydrate availability (56). On the other hand, low gut pH and the presence of carbohydrates reduce peptide and amino acid fermentation *in vitro* (57). Such observation may explain the reason why for most of the studied amino acids there are no differences in concentration throughout time (12 and 24 h) in FOS samples.

The preferred amino acid substrates of colonic bacteria include lysine, arginine, glycine, and the branched-chain amino acids leucine, valine, and isoleucine. For instance, threonine and glutamate have been described to be able to be metabolized to acetate (56). It was observed a decrease in threonine for the Fish and Pomegranate oil, CLNA, and Omega-3, and a decrease in glutamic acid for the pomegranate oil, CLNA, and CLA samples. An increase in acetate, as discussed in **Section 3.3**, was described for CLNA, Omega-3, and Fish oil, which may suggest that these two amino acids (threonine and glutamic acid) may be used as precursors for acetate synthesis in these samples. In addition, *in vitro* fermentations using fecal inoculums have shown that propionate can be produced from aspartate, alanine, threonine, and methionine and Bacteroidetes were described as having important roles in propionate production from peptides (54). Here we demonstrated that alanine concentrations decrease from 12 to 24 h ($p<0.05$) in FOS samples, but there is no increase in propionate concentration at the mentioned times. This suggests that perhaps due to low pH inhibition, the decrease in concentration observed for the alanine is not related to propionate production. Interestingly, threonine has been reported as the

major precursor of propionate (56). Regarding this amino acid, there is a decrease in its concentration from 12 to 24 h for the Pomegranate and Fish oil, CLNA, and Omega-3 samples. There was an increasing concentration of propionate (**Figure 4.5**) in Fish oil samples. Importantly, the decrease of threonine concentration along with the positive effect of Fish oil in Bacteroidetes may suggest a production mechanism of propionate from threonine in the Fish oil sample. Considering methionine, a reduction from 12 to 24 h in this amino acid concentration is only observed in the Pomegranate oil sample, which is not translated into an increase in propionate levels, suggesting that here the decrease in methionine concentration is not related to propionate synthesis.

Several pathways of glutamate degradation into butyrate, have been described in *Clostridium* species (54). The glutamate degradation pathways meet the main butyrate pathway either via pyruvate (3-methylasparate pathway) or crotonyl-CoA (4-aminobutyrate pathway) and 2-hydroxyglutarate pathway (reviewed in more detail by Louis and Flint (54)). This last pathway is also found in different Firmicutes species, such as *Acidaminococcus fermentans*, *Clostridium sporosphaeroides*, *Clostridium symbiosum*, *Fusobacterium* spp., and *Peptostreptococcus asaccharolyticus*. As discussed, there is an increase in butyrate production from 12 to 24 h in Pomegranate and Fish oil, CLNA, CLA, and FOS samples. This is accompanied by a decrease in glutamic acid concentration in CLNA, CLA, and Pomegranate oil samples in the same time frame. Interestingly, although there is a negative effect of these samples in *C. leptum* species, there is no significative negative effect of these samples on Firmicutes (total), which may suggest that there is a production of butyrate from glutamic acid by Firmicutes bacteria in CLA, CLNA, and CLA samples. Glutamate was also reported to be degraded to GABA under acidic stress to maintain intracellular pH homeostasis in several gut bacteria (58). Considering this, it was expected an increase of GABA production in FOS samples, since there is a pH shift after 12 h. However, no effect either in glutamic acid or GABA concentration was observed in these samples. Besides, alanine, serine, and cysteine can be broken down into pyruvate. And, for example, in *Clostridium propionicum*, alanine fermentation leads to the production of propionate via the pyruvate pathway (54). There was a decrease in alanine concentration in the FOS sample from 12 to 24 h. The possible synthesis of propionate from alanine may contribute to the maintenance of propionate levels.

By serving as precursors for SCFA synthesis, amino acids influence microbial activity by regulating SCFA homeostasis. Besides, mice studies have revealed that the distribution of free amino acids in the GIT can be altered by gut microbiota. Thus, gut microbiota can affect the bioavailability of amino acids in the host. It is also relevant to point out that microbiota in the large intestine is enriched with genes involved in essential amino acid biosynthesis, but the specific microbiota contribution to whole-body amino acid metabolism in humans is still uncertain (56). Interestingly, here it was observed that both Fish oil and Omega-3 capsules promote an increase in the concentration of several amino acids from 12 to 24 h, namely GABA, alanine, tyrosine, phenylalanine, isoleucine, and leucine (**Figure 4.7**). More relevantly, in alanine, tyrosine,

phenylalanine, and isoleucine (in the case of Omega-3 capsules) the rise in concentration was significative ($p<0.05$) higher than the FOS sample. These results are highly relevant considering that D-Alanine, for instance, was shown to be a potent co-agonist of the glycine-binding site on the N-methyl-D-aspartate receptors, acting as a neurotransmitter and neuromodulator in the mammalian brain (59). Moreover, gut microbiota production of tyrosine, a precursor for dopamine, has been positively correlated with cognitive function in Schizophrenia patients (60). More importantly, certain neuroactive compounds have similar roles to those of neurochemicals, reducing inflammation and facilitating gut-brain axis communication. These centrally-acting compounds include the discussed SCFAs and neurotransmitters such as GABA and serotonin (33). In this study, as mentioned, it was observed an increase in GABA production in both Fish oil and Omega-3 capsules from 12 to 24 h, which was statistically equivalent to FOS. Interestingly, although diet can be a direct source of GABA, it has also been demonstrated that acetate that passes the BBB can alter the levels of GABA in the hypothalamus. Here, along with an increase in GABA concentration it was observed, an increase in acetate concentration from 12 to 24 h (**Figure 4.5**) in the same samples. On the other hand, the essential amino acid L-tryptophan is the precursor of the neurotransmitter serotonin. Serotonin biosynthesis is another humoral gut-brain communication pathway that seems to be affected by SCFAs, such as propionate, as well as dietary intake (61,62). Indeed, several studies have demonstrated that elevated levels of dietary tryptophan can present a suppressive effect on aggressive behavior and post-stress plasma cortisol concentrations (61). Relevantly, in this study, the Omega-3 sample showed a significant ability to increase tryptophan levels from 12 to 24 h. Omega-3 and Fish oil samples were also able to increase both phenylalanine and isoleucine concentrations from 12 to 24 h. Some *in vivo* studies have reported a significant increase in these amino acids in the feces and blood of Alzheimer's disease mouse models. Moreover, it has been demonstrated that both phenylalanine and isoleucine are associated with the promotion of both differentiation and proliferation of peripheral inflammatory Th1 cells (63). Although these are highly relevant observations that need to be considered, it is important to keep in mind the anti-inflammatory potential that omega-3 and CFAs present. Indeed, omega-3 FAs and probiotics were shown to significantly reduce inflammatory biomarkers and are described as important influencers of gut-brain axis modulation (33). Additionally, a previous *in vitro* study from our group, using a human microglia cell model, showed an anti-inflammatory potential in the central nervous system of both omega-3 (EPA and DHA) and CLA and CLNA (PUA) isomers by inhibiting NF-κB pathway activation through GPR120 receptor (64).

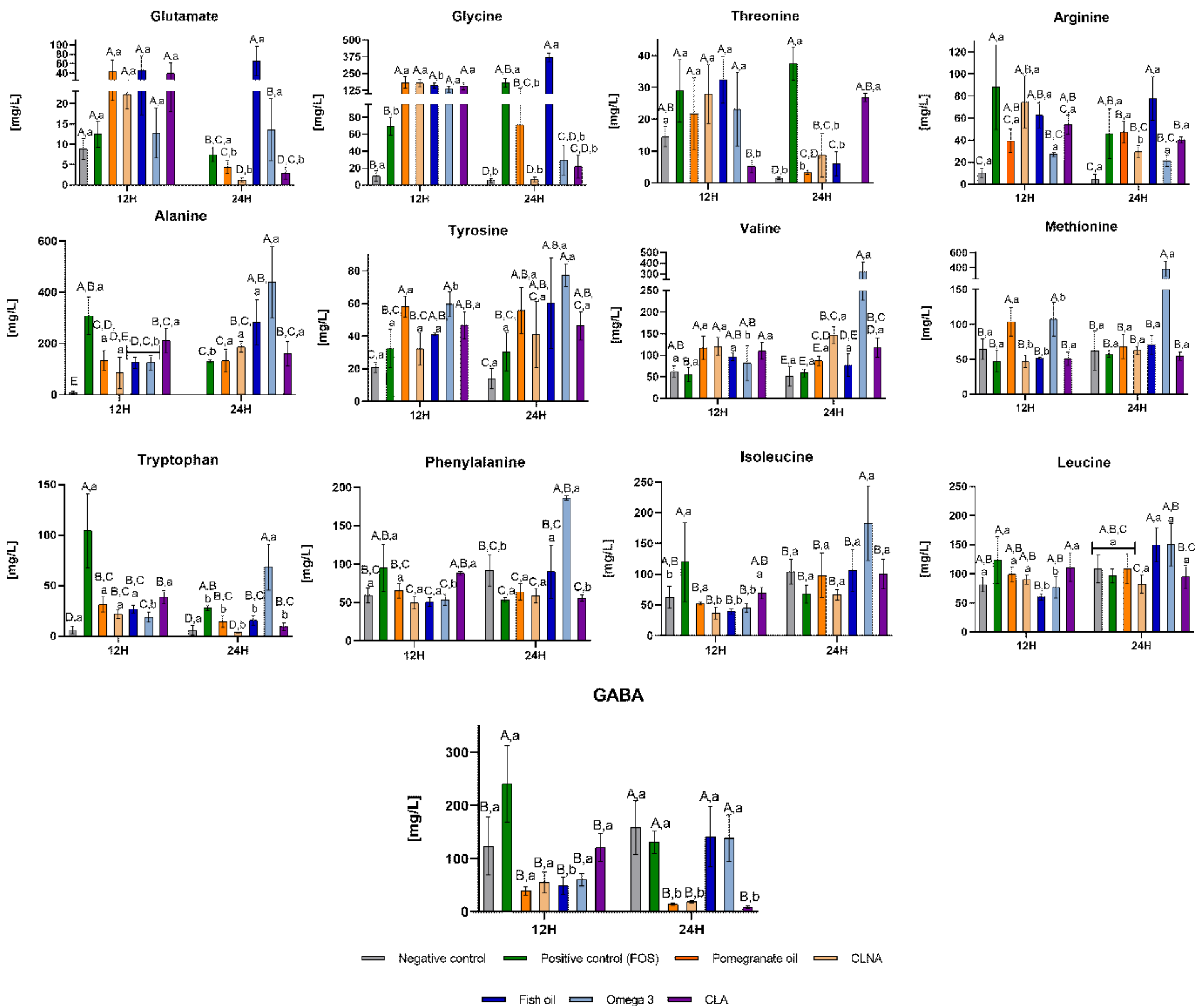

Figure 4.7- Concentrations of amino acids (mg/L) after 12 and 24 h of the *in vitro* fecal fermentation of different bioactive lipids sources (Fish and Pomegranate oil, CLA, CLNA, and Omega-3 capsules). The values presented are the means of five donors and the bars represent the standard deviation. Different letters indicate significant differences ($p < 0.05$). The capital letters (A, B, C, D, E) indicate the differences among samples - Negative control, Positive control (FOS), and bioactive lipids sources - for the same amino acid at the same time. The small letters (a and b) indicate the differences for the same sample over time for the same amino acid.

3.5. Assessment of the effect of PUFA samples in healthy human microbiota modulation by PCA analysis

The PCA was performed to evaluate which samples produced a differentiating effect on gut microbiota modulation and which parameters (pH, FAs, amino acids, and SCFAs) can better explain such effect after 12 and 24 h of fermentation. The PCA performed explains approximately 60% of the data variation using three Principal Components (PC1=23.45%, PC2=19.78%, and PC3=16.69%). **Figure 4.8** represents the PC1 vs PC2 and PC1 vs PC3 biplots and the

correspondent scores and loading plots, considering the different samples and parameters analyzed. Through the analysis of the score plot (**Figure 4.8 (A2)**), there is a clear differentiation of three groups of samples (marked with circles in the graph): Fish oil, Pomegranate oil, and CLNA groups, and a fourth corresponding to the Negative control. Analyzing the loading plot (**Figure 4.8 (A1)**), it is possible to understand that along PC1 the dominant parameters (*i.e.,* with a component weight ≥ 0.7) that are responsible for the isolation of the Fish oil sample from the other groups are the FAs Palmitic (0.963) and Myristic acids (0.968), as well as DHA (0.813), EPA (0.817) and DPA (0.974). In **Section 3.4** it was demonstrated that the Fish oil promotes an increase in the concentration of GABA, alanine, tyrosine, phenylalanine, isoleucine, and leucine from 12 to 24 h. Through this PCA analysis, it was observable that glycine (0.733) and glutamic acid (0.789) are the major amino acids explaining Fish oil differentiation from the remaining samples at both 12 and 24 h of fermentation. So, these two amino acids are also important metabolites to be considered in the fermentation of Fish oil. To the best of our knowledge, a possible relation between glycine and omega-3 FAs was only observed in another study aiming to assess the effect of dietary supplementation with *Lactobacillus* in piglets. In this study, it was found that serum PUFAs C18:2*c*9*c*12 (LA), C18:3*c*9*c*12*c*15 (ALA), C20:4 n-6 (AA) and relevantly C22:6 n-3 (DHA) were elevated along with serum free amino acids glycine, alanine, valine, isoleucine, asparagine, aspartate, glutamine, methionine, phenylalanine and leucine in the supplemented group compared with the control group (65). Moreover, as mentioned, omega-3 FAs, specifically EPA and DHA are greatly known due to their anti-inflammatory potential. Likewise, intake of diets with high content of glycine has been associated with decreased accumulation of fat mass in rodent studies and glycine was previously associated with anti-inflammatory effects (66). Considering this, the higher content of both glycine along with DHA, EPA, and DPA observed in this study in Fish oil after 24 h of fermentation, may be relevant in an anti-inflammatory perspective specifically in diseases where an inflammatory imbalance is observed.

On the other hand, along PC2 the dominant parameters are the FAs PUA, Catalpic acid, and β-eleostearic. The high concentration of these two FAs explains the differentiation of both Pomegranate oil and CLNA samples. In PC3, combining the analyses of the score and loading plots **(Figure 4.8 (B1 and B2))** it is observable the separation of the Omega-3 capsules (24 h) from the remaining samples mainly due to the amino acids alanine (0.909), tyrosine (0.686), valine (0.788), methionine (0.847), phenylalanine (0.912), isoleucine (0.854) and leucine (0.794). By analyzing this score plot is also observable an increase from 12 to 24 h, which is consistent with what was previously discussed in **Section 3.4**. Nevertheless, with this PCA analysis, besides the already discussed amino acids (alanine, tyrosine, phenylalanine, isoleucine, and leucine), valine seems to be an important amino acid that needs to be considered in Omega-3 capsules. Importantly, a recent study demonstrated that gut microbiota could synthesize essential amino acids and that the biosynthesis of branched-chain amino acids including leucine, isoleucine, and valine was positively correlated with circulating levels of these amino acids in a healthy population (25). Again, careful considerations must be made here considering that some studies have

associated elevated levels of branched-chain amino acids with the promotion of an inflammatory response (67). Nevertheless, branched-chain amino acids are also described as playing an important role in the brain, such as promoting the synthesis of neurotransmitters, participating in intracellular transduction, regulating the levels of inflammation, and influencing mitochondrial function. Moreover, in a recent study using a Parkinson's disease mouse model, it was observed that during disease progression the related alterations of gut microbiota composition led to the peripheral decrease of the branched-chain amino acids. Interestingly, the supplementation with these amino acids was shown to attenuate the inflammatory levels observed in Parkinson's disease mice model and reverse motor and non-motor dysfunctions and dopaminergic neuron impairment (68). So, it is highly relevant the capacity of Omega-3 capsules, observed in this study, to promote the biosynthesis of these amino acids and this sample may be a relevant therapeutical option. Additionally, considering the impact on all the mentioned amino acids, it is also relevant to consider the potential of these samples, especially Fish oil and Omega-3 capsules in gut-brain axis modulation.

Moreover, reflecting on the distinction observed between Fish oil and Omega-3 capsules in the PCA, and specifically considering EPA and DHA, it is also important to point out that this omega-3 FA concentration, as previously discussed, may be important in microbiota modulation and must be considered when designing studies where the impact of similar samples in gut microbiota modulation are determined.

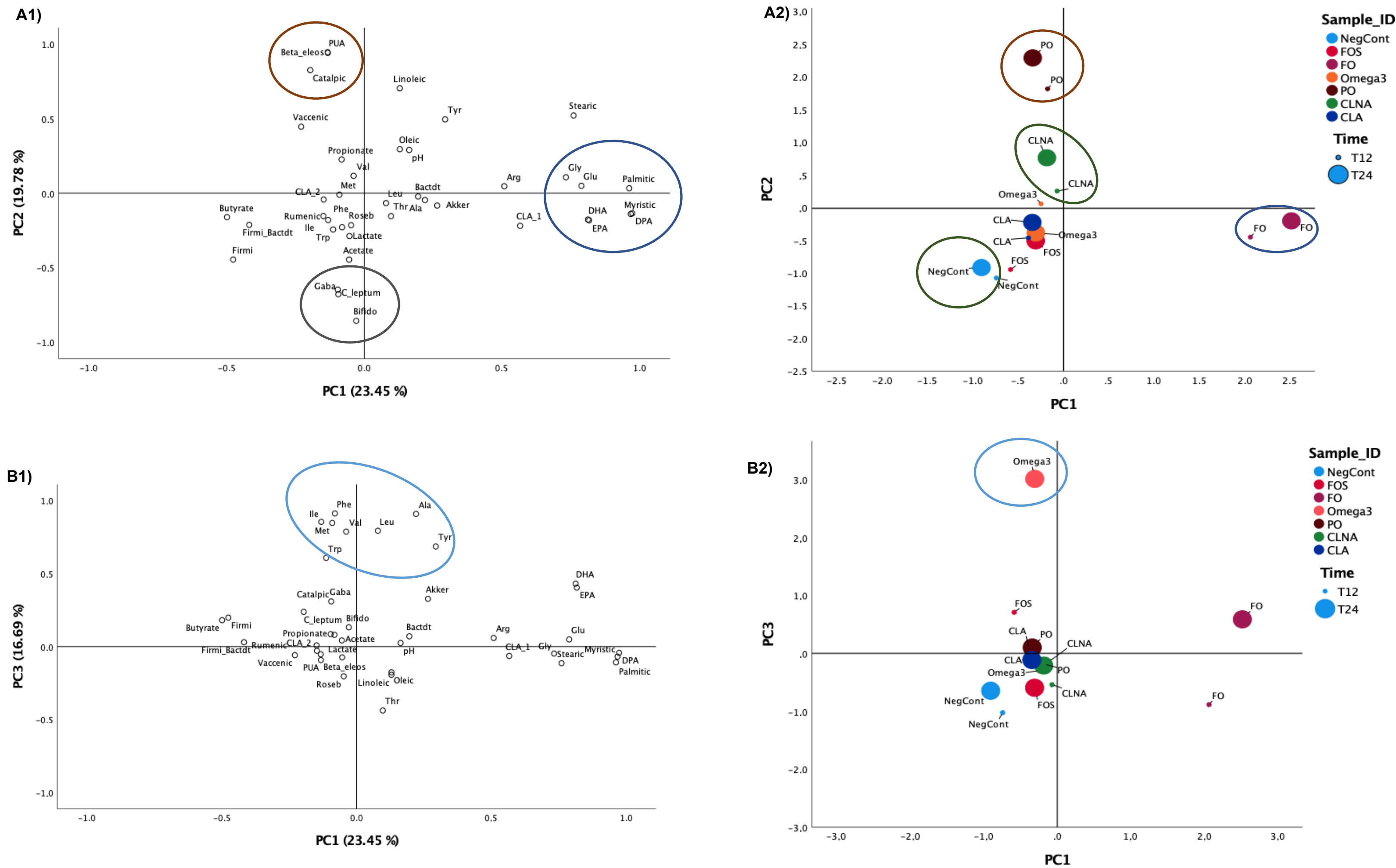

Figure 4.8 – A. **Principal component analysis** biplot PC1 vs PC2: (A1) Variables loadings distribution along each component and (A2) data scores along the components, for 12 and 24 h. B. Principal component analysis biplot PC1 vs PC3: (B1) variables loadings distribution along each component and (B2) data scores along the components, for 12 and 24 h. The samples are represented as: NegCont – Negative control; FOS – Positive control (FOS); FO- Fish oil; Omega3- Omega-3 capsules; PO- Pomegranate oil; CLNA – CLNA capsules and CLA- CLA capsules. All the variables analyzed were considered for the Principal component analysis. pH and Fatty acids: Myristic – C14; Palmitic – C16; Stearic – C18; Oleic – C18:1 c9; Vaccenic – C18:1 t11; Linoleic – C18:2 c9c12; Rumenic – C18:2 c9t11; CLA_1 – C18:2 t10c12; CLA_2 – C18:2 t9t11; EPA – C20:5 n-3; PUA – C18:3 c9t11c13; Catalpic – C18:3 t9t11c13; beta_eleos – C18:3 t9t11t13; DPA – C22:5 n-3; DHA – C22:6 n-3. Short-chain fatty acids - Acetate, Propionate and Butyrate - and Lactate. Amino acids: Glu – Glutamic acid; Gly – Glycine; Thr – Threonine; Arg – Arginine; Ala – Alanine; GABA; Tyr – Tyrosine; Phe – Phenylalanine; Iso – Isoleucine; Leu- Leucine. Bacteria: Roseb – Roseburia spp.; Bifido- Bifidobacterium spp.; Bactdt – Bacteroidetes; C_leptum – Clostridium leptum; Firmi – Firmicutes total; Akker – Akkermansia spp. and Firmi_Bact – Firmicutes:Bacteroidetes ratio.

4. Conclusion

With this work the impact of omega-3 and CFAs on the gut microbiota of feces from healthy donors was assessed by using Fish oil and Omega-3 capsules as omega-3 FAs sources (EPA and DHA), CLA capsules as the source of CLA isomers (C18:2 *c*9*t*11 – RA- and C18:2 *t*10*c*12) and Pomegranate oil and CLNA (Xanthigen® capsules) as PUA source. To the best of our knowledge, this is the first study aiming to evaluate both the impact of omega-3 and other relevant PUFAs, such as CFAs, on the gut microbiota of healthy human donors using *in vitro* fermentation assays. This assay allowed us to study not only the impact on the gut microbial population but also consider the metabolic impact of these samples, by evaluating SCFAs and amino acids production. Although in some of the assessed bacterial groups, no statistical significance was reached when compared to negative control, it is important to consider two important aspects: first, these observations are related to the natural microbial variability (evident as high standard deviations) that are closely connected with the use of human donors, from different sexes and dietary intakes (although standard requirements were applied to each donor). Secondly, most studies using omega-3 and a few using CFAs were focused on the impact of these FAs on obesity effects. So, these were studies where there is a dysbiosis of gut microbiota already installed. In this assay, feces from healthy donors with no expected significative alteration of gut microbiota were used. Nevertheless, all the bioactive FAs samples in general presented a positive impact on *Akkermansia* spp. and *Bifidobacterium* spp. (except for Pomegranate oil and CLNA), translated by a positive RD in reference to the negative control. These are bacteria that have been widely reported to have a positive impact on obesity. Importantly, all the samples were also able to maintain the Firmicutes: Bacteroidetes ratio near 1. These are all important parameters to consider not only in cases of an already established dysbiosis, such as obesity but also in maintaining homeostasis (10,11,31,35,39,44). Besides, the observation at 48 h of an increased production of butyrate higher than FOS, which was accompanied by a decrease in acetate production is highly relevant in obesity since butyrate has been associated with both an anti-inflammatory potential and a beneficial effect on insulin resistance. On the other hand, acetate is known to promote metabolic syndrome (52,53). Besides, it was demonstrated that Fish oil and Omega-3 capsules were able to increase the concentration of several amino acids from 12 to 24 h fermentation, namely GABA (a highly relevant neurotransmitter), alanine, tyrosine, phenylalanine, isoleucine, and leucine. For instance, alanine has been associated with a therapeutical potential in diseases such as schizophrenia, Alzheimer's disease, and renal disease (59). In addition, the cognitive function of patients with schizophrenia is associated with the potential for gut microbiota to biosynthesize tyrosine, a precursor for dopamine (60). Despite all these interesting and promising results, especially in obesity therapy and neuroinflammatory diseases by gut-brain axis modulation, it is important to point out, however, that although fecal fermentations represent a valid approach, several sources of bias have to be considered: intestinal transit and permeability as well as metabolite transportation (9). Therefore, although this

study highlights relevant evidence, it is important to complement these studies with *in vivo* assays and clinical trials to confirm such conclusions.

5. References

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CHAPTER 5

The effect of omega-3 and conjugated fatty acids in restoring the Western diet induced-alterations in gut microbiota.

This chapter intends to describe the effects and the therapeutical potential of omega-3 and punicic acid in restoring the WD-induced alterations.

This chapter was submitted to Food & Function as:

Salsinha, A.S., Cima, A., Araújo-Rodrigues, H., Viana, S., Reis, F., Coscueta, E.R., Rodríguez-Alcalá, L.M., Relvas, J.B., Pintado, M. The use of an *in vitro* fecal fermentation model to uncover the beneficial role of omega-3 and punicic acid in gut microbiota alterations induced by a Western diet. Submitted on February 3rd 2024 to *Food & Function* journal.

Chapter 5

Chapter 5

Preface

Can a mixture of Fish oil and Pomegranate oil present a synergistic effect in restoring the gut microbiota alterations induced by Western-type diet consumption?

Considering the important role of bioactive FAs, specifically the ones present in Fish oil and Pomegranate oil, in gut microbiota modulation and following the promising results obtained in **Chapter 4** the next step of this work was focused on understanding the role of these bioactive molecules in restoring the alterations that are often associated with WD consumption. Indeed, WD consumption-induced alterations in gut microbiota have been increasingly associated with the development of several diseases, including obesity. So, the role of gut microbiota in obesity onset, development, and treatment cannot be overlooked and must be an important parameter to take into consideration. So, in this perspective, this chapter intended to understand if these enriched bioactive FAs matrixes can restore or improve the alterations in gut microbiome bacterial composition, as well as describe the potential in modulating their metabolic activity. For that, *in vitro* fecal fermentations were performed using cecal samples collected from rats subjected to two types of diet: a control diet (CD) and a Western-type diet (WD) comprised of high-sugar and high-fat. Besides, a screening of a possible beneficial role in gut-brain axis regulation was performed.

Chapter 5

The use of an *in vitro* fecal fermentation model to uncover the beneficial role of omega-3 and punicic acid in gut microbiota alterations induced by a Western diet

Ana Sofia Salsinha[a,b], André Cima[a], Helena Araújo-Rodrigues[a,b], Sofia Viana[d,e], Flávio Reis[d], Ezequiel R. Coscueta[a], Luis Miguel Rodríguez-Alcalá[a], João B. Relvas[b,c] and Manuela Pintado[a]*

a. Universidade Católica Portuguesa, CBQF - Centro de Biotecnologia e Química Fina –Laboratório Associado, Escola Superior de Biotecnologia, Rua de Diogo Botelho, 1327, 4169-005 Porto, Portugal

b. Instituto de Investigação e Inovação em Saúde and Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto – Rua Alfredo Allen, 208, 4200-135 Porto, Portugal

c. Departmento de Biomedicina, Faculdade de Medicina da Universidade do Porto (FMUP), 4200-319 Porto, Portugal

d. Coimbra Institute of Clinical and Biomedical Research (iCBR), Faculty of Medicine and Center for Innovative Biomedicine and Biotechnology (CIBB), University of Coimbra, Coimbra, Portugal; Clinical Academic Center of Coimbra, Coimbra, Portugal

e. Instituto Politécnico de Coimbra, Escola Superior de Tecnologia da Saúde de Coimbra, Rua 5 de Outubro – S. Martinho Bispo, Apartado 7006, 3046-854 Coimbra, Portugal

Abstract

The influence of gut microbiota in the onset and development of several metabolic diseases has gained increased attention over the last few years. Diet plays an essential role in gut microbiota modulation. Western diet, characterized by high-sugar and high-fat consumption, alters gut microbiome composition, diversity index, microbial relative levels, and functional pathways. Despite the promising health effects demonstrated by polyunsaturated fatty acids, their impact on gut microbiota is still overlooked. The effect of Fish (omega-3 source) and Pomegranate oil (punicic acid source), and a mixture of both oils in gut microbiota modulation were determined by subjecting the oil samples to *in vitro* fecal fermentations. Cecal samples from rats from two different dietary groups: control diet (CD) and high-fat high-sugar diet (WD), were used as fecal inoculum. 16S amplicon metagenomics sequencing showed that Fish oil+Pomegranate oil from the WD group increased α-diversity. This sample also increased the relative abundance of the Firmicutes and Bacteroidetes phylum as well as *Akkermansia* and *Blautia*, which were affected by the WD consumption. All samples were able to increase butyrate and acetate concentration in the WD group. Moreover, tyrosine concentrations, a precursor for dopamine and norepinephrine, increased in the Fish oil+Pomegranate oil WD sample. GABA, an important neurotransmitter, was also enhanced in WD samples. These results suggest a positive impact of these oils' mixture on gut-brain axis modulation. It was demonstrated, for the first time, the great potential of using a mixture of both Fish and Pomegranate oil to restore the gut microbiota changes associated with WD consumption.

Keywords: Omega-3, Punicic acid, Western diet, gut microbiota, *in vitro* fecal fermentation

Graphical Abstract

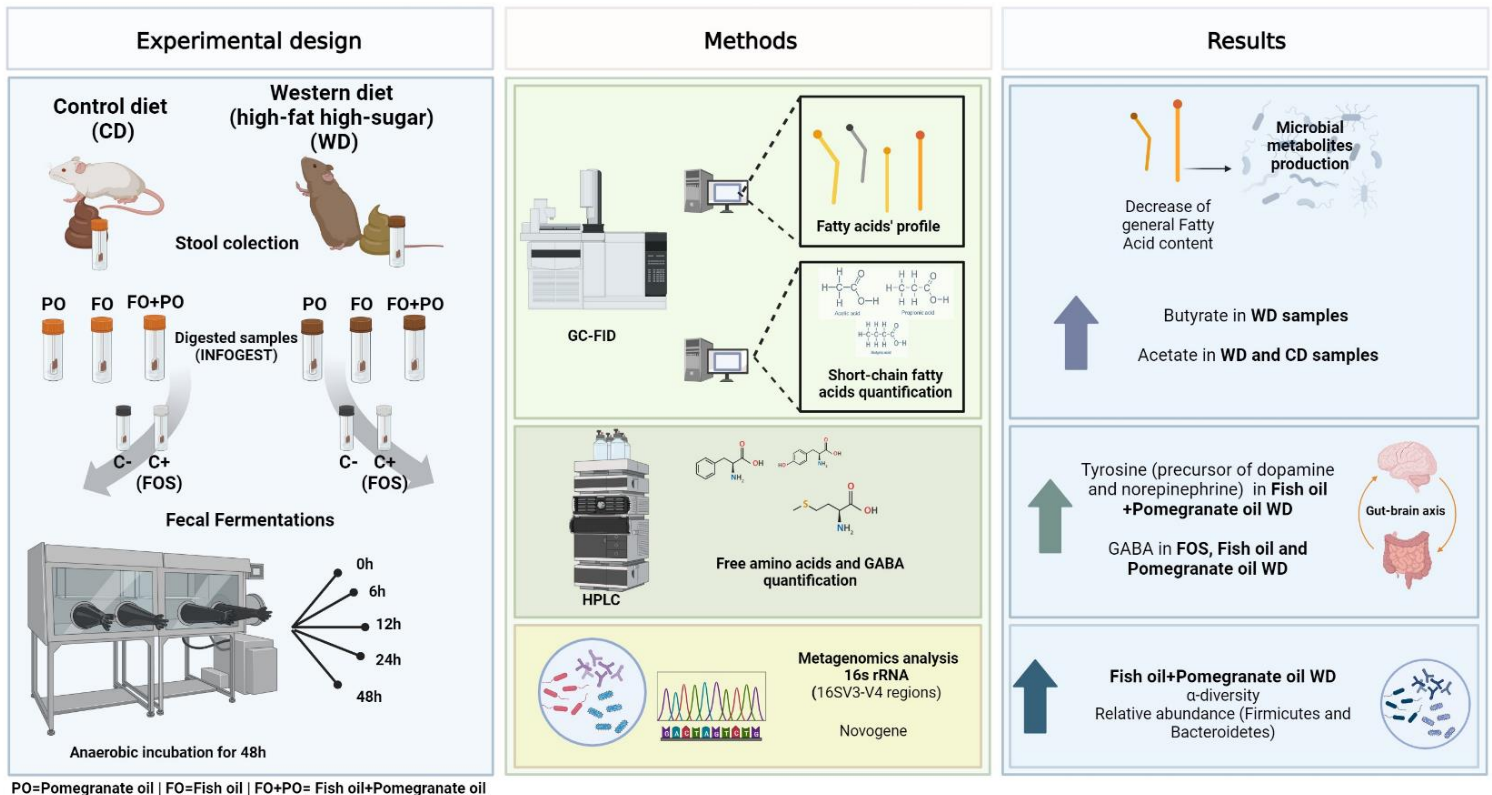


Figure 5.1 – Graphical abstract. Assessment of the modulatory effects of both omega-3 EPA and DHA present in Fish oil and PUA in Pomegranate oil in restoring the gut microbiota alterations induced by a WD consumption in a rat model. Cecal samples from rats subjected to two different diets - CD or WD - were subjected to *in vitro* fermentations using FOS as a positive control and Fish oil, Pomegranate oil and a mix of both oils. The total FAs profile and the SCFAs quantification were performed by GC-FID, free amino acids and GABA quantification were achieved by HPLC and the bacterial population was analysed by 16s rRNA metagenomics analysis.

1. Introduction

The so-called "Western diseases", namely obesity, T2DM, IBD, and food allergies, are rising in countries where significant industrial advances and shifts toward urbanized living have been observed. Indeed, the Industrial Revolution changed how humans interact with their physical environment. This social change promoted considerable shifts in eating behaviors, sleeping patterns, housing conditions, and medical advances. These transformations have been linked with considerable alterations in the human body's bacterial communities which have been associated with the development of several intestinal and systemic metabolic and inflammatory diseases (1).

According to the Human Microbiome Project Consortium, a "normal" human gut microbiome is often characterized mainly by Firmicutes, *Bacteroides*, *Proteus*, Actinomycetes, Fusobacteria, and Verrucomicrobia. Its core functions include the biodegradation of polysaccharides, the production of SCFAs, the enrichment of specific LPS, and the production of vitamins and essential amino acids (2). Moreover, a "healthy" gut microbiota is generally highly diverse, and conversely, the relative lack of diversity in the gut microbiota leads to the mentioned diseases (2–5).

Diet is a key factor influencing gut microbiota composition (6). So, the dietary alterations that are associated with all the technological and lifestyle changes, namely the rise of the WD, which is in part characterized by high-fat and high-sugar consumption, have been pointed out as relevant players in the induced changes observed in the human gut microbiota. Indeed, rodent studies have demonstrated that the Western-type HFD strongly influences gut microbiota's genetic composition and metabolic activity. Moreover, a reduced gut microbiota diversity is also observed when comparing humans subjected to an HFD and high-fat high-sugar diets (here defined as WD) compared to diets with higher proportions of plant polysaccharides (7–11). WD is also known to influence several microbial mechanisms, such as BA expression levels, LPS and SCFAs production, and trimethylamine N-oxide levels in the brain (12). SCFA synthesis, for instance, was shown to be reduced in HFD and WD models due to a reduced abundance of SCFA-producing bacteria (as reviewed by Chen et al. (13)). SCFAs, essentially acetate, propionate, and butyrate, are essential regulators since they can inhibit inflammatory reactions through NF-κB inhibition, maintain the anaerobic environment, and regulate appetite (through glucagon-like peptide-1 and peptide YY inhibition), control energy metabolism, glucose, and lipid metabolism (14–17). This SCFA reduction is shown to result in energy metabolism disorder and excessive production of the pathogenic facultative anaerobic bacteria *Escherichia coli* and the production of pro-inflammatory factors (18,19). WD-induced changes in gut microbiota are seen as a trigger for metabolic impairments associated with obesity (20).

Moreover, although the causal relationship between gut microbiota and T2DM and its underlying mechanisms are still not fully elucidated, it has been demonstrated that the gut microbiota imbalance associated with excessive fat intake can lead to changes in the ratio of

Bacteroidetes and Firmicutes, a decrease in butyrate-producing bacteria and an increase in the abundance of pathogens. This imbalance is thought to disturb host metabolism, resulting in decreased amounts of SCFA and immunoglobulin A, ultimately leading to T2DM (21). So, these WD-induced alterations in gut microbiota have been associated with increased susceptibility to diseases not only to obesity, diabetes, and gastrointestinal intestinal diseases, as we already mentioned, but also to cancer, namely colorectal cancer, neurodegenerative, and CVD (22,23). So, considering the effect of WD in gut microbiota modulation and consequently, in the development of several diseases, the research about interventions with probiotics, prebiotics, and symbiotics to correct these associated microbiota alterations is increasing.

Prebiotics are essential functional ingredients with positive health effects that are naturally present in some plant and animal foods (24). Indeed, they are known to modulate the intestinal microbiota, increasing the production of SCFAs, and reducing the risk of several diseases, including obesity and T2DM. For a long time, prebiotic action was mainly attributed to polysaccharides (resistant starch, pectin, and dextrin) and oligosaccharides such as FOS, galactooligosaccharide (GOS), xylooligosaccharides (XOS), isomaltooligosaccharides (IMO), mannan oligosaccharides (MOS), raffinose oligosaccharides (RFOs), arabinoxylan oligosaccharides (AXOS), lactulose and inulin (25). Besides polysaccharides, other functional molecules have been demonstrated to potentially modulate gut microbiota. Accordingly, the term prebiotic has been recently revised to include other ingredients that allow changes to the composition and the activity of the gastrointestinal microbiome and may confer benefits upon host well-being and health (24). Recently, in a 6-week dietary intervention study using omega-3 versus a well-characterized prebiotic (inulin), it was demonstrated that dietary omega-3 alters gut microbiome composition. Some of the beneficial cardiovascular effects that have been attributed to it appear to be mediated by its effect on gut microbial fermentation products (26). So, omega-3 has been suggested as a prebiotic nutrient. Previous studies have mostly reported increases in *Clostridiaceae* (Firmicutes), *Enterobacteriaceae* (Proteobacteria), *Akkermansiaceae* (Verrumicrobia), and *Desulfovibrionaceae* (Proteobacteria) families, as well as *Bifidobacterium* (Actinobacteria), *Lachnospira* (Firmicutes) and *Roseburia* (Firmicutes) genera (27,28), following omega-3 PUFA supplementation. Importantly, butyrate is generally linked to anti-inflammatory properties, and an increase in butyrate-producing bacterial genera is observed after omega-3 supplementation (27). Very few studies have addressed the impact of other PUFAs, specifically CFAs, on microbiota modulation. Nevertheless, some promising results have emerged for PUA: PUA supplementation in HFD-fed mice reverses the Bacteroidetes reduction and restores the Firmicutes/Bacteroidetes ratio affected by the HFD. Besides, PUA was able to decrease, at a class level, the relative abundance of Desulfovibrionales (Thermodesulfobacteriota) and Deltaproteobacteria (Proteobacteria). In addition, at the family level, PUA was able to restore the upregulation of *Desulfovibrionaceae* (Thermodesulfobacteriota) as well as the downregulation of *Muribaculaceae* (Bacteroidota) and *Bacteroidaceae* (Bacteroidota) caused by the HFD.

Moreover, PUA supplementation was able to increase the relative abundance of *Lactobacillus* (Firmicutes), *Roseburia* (Firmicutes), and *Oscillibacter* (Firmicutes) (29).

Since there is still a lack of knowledge regarding this theme and considering that SCFAs can attenuate the HFD-induced inflammation effects and insulin resistance and present anti-obesity effects (30,31), by using an *in vitro* fermentation model we aim to investigate the effect of omega-3 and PUA, a CLNA isomer, on microbiota modulation and corresponding metabolites production. Our objective is to elucidate the influence of these PUFAs, both individually and synergistically, on gut microbiota modulation using cecal samples from rats administered either a Control diet (CD) or a WD. We posit that these PUFAs may exert varying effects on the cecal microbiota depending on the diet—CD or WD. Furthermore, considering the documented positive impacts of both omega-3 and PUA, we propose that these bioactive FAs possess the potential to ameliorate or rectify certain gut microbiota disturbances instigated by the WD and restore these alterations.

2. Material and Methods

2.1. Chemicals and reagents

In this study, the Fish oil, Pomegranate oil, and the GIT INFOGEST reagents are described in **Chapter 3 Section 2.1 and 2.2.**

Regarding the fecal *in vitro* fermentations, Soya peptone (84616.0500) and Tween 80 (28830,291) were purchased from VWR Chemicals (Pennsylvania, USA). Yeast extract (A1202) and Tryptone (A1401 HA) were from Biokar Diagnostics (Cedex, France). Sodium chloride (31434) was from Honeywell (North Carolina, USA), and Potassium dihydrogen phosphate (1.04871.1000), Sodium bicarbonate (1.06329.1000), L-cysteine hydrochloride (1.02839.0100) and Calcium chloride (102378) were from Merck (New Jersey, USA). Heme chloride (A11165) was from Alfa Aesar (Massachusetts, USA). Magnesium sulfate hexahydrate (459337) was from Carlo Erba (Emmendingen, Deutschland). D-(+)-Maltose monohydrate (63419), D-(+)-Glucose (G8270), Resazurin sodium salt (199303), Bile salts (48305) and Vitamin K1 (V3501) were all acquired from Sigma-Aldrich (Missouri, USA).

For the DNA extraction, it was used Tris-EDTA buffer (TE, 10x concentrate, PPB010) and Lysozyme (P00698) from Sigma-Aldrich (Missouri, USA). The NZY Tissue gDNA kit for DNA extraction (MB13502) was purchased from NZYTech (Lisbon, Portugal).

Hexane, Methanol, DMFFA, and Acetonitrile (HPLC grade) from VWR Chemicals (Pennsylvania, USA), Sulphuric acid from Honeywell (North Carolina, USA), and Sodium methoxide from Acros Organics (Geel, Belgium) were used for the FAs profile analysis. Tritridecanoin (33-1300-13) from Larodan Research Grade Lipids (Solna, Sweden) was used in this assay as the internal standard.

Regarding the SCFAs analysis, Diethyl ether (HPLC grade, 99% stabilized with ethanol, 1009212500) from Sigma was used for the extraction process. For the quantification, the standards were Acetic acid (20104.323) from VWR Chemicals (Pennsylvania, USA), Butyric acid (B10350-0), Propionic acid (P1386), Isobutyric (58360) and Isovaleric acid (59850) from Sigma-Aldrich (Missouri, USA). Valeric acid (94530) from Sigma-Aldrich was used as an internal standard.

For the free amino acids analysis, the following standards were used for quantification and identification purposes: γ-amino-n-butyric acid (GABA, A-5835), L-lysine (L-5626), L-leucine (L-5652), L-valine (V-0500), L-tryptophan (T-0254); L-isoleucine (I2752), L-phenylalanine (P2125), L-arginine (A-8094), L-methionine (M-9625), L-tyrosine (T-3754), L-alanine (A7627), L-glutamine (49419), L-cysteine (168149), L-threonine (T8625), L-histidine (H-8000), L-serine (54311), L-glutamic acid (G-1251), L-asparagine (A0884), L-aspartic acid (A9256), all from Sigma-Aldrich. Glycine (33226H) was from Honeywell. Moreover, Perchloric acid (244252), Homoserine and Norvaline internal standards, and 2-mercaptoethanol (M6250) were from Sigma-Aldrich.

2.2. FAs Sources

The samples used in this study as the sources of the main bioactive FAs (omega-3 - EPA and DHA - and PUA, a CLNA isomer) for the *in vitro* fermentation assays are described in **Chapter 3 Section 2.2**. As the source of omega-3 EPA and DHA, Fish oil from Menhaden was used. In addition, as the source of CLNA isomer PUA, it was used Pomegranate Kernel Oil cold pressed.

2.3. *In vitro* GIT tract digestion

The GIT digestion simulation was performed using the INFOGEST 2.0 static *in vitro* protocol as described in **Chapter 3 Section 2.3**. The samples, Fish oil, Pomegranate oil, and a Fish oil/Pomegranate oil blend mixed at the assayed proportion of 1:1 (w/w), were submitted to GIT digestion following the mentioned standardized INFOGEST protocol.

After the GIT digestion, a dialysis tubing (3.5 kDa molecular weight cut off) was used to simulate the passage of the digested samples by the small intestine (namely duodenum and jejunum, at least). The samples were incubated overnight at 37 ºC and 50 rpm inside the dialysis membrane. At the end of the dialysis process, the retentate, *i.e.,* the solution that remains inside the membrane, represents the non-absorbable fraction (colon-available), which is accessible to the gut microbiota (32,33).

2.4. Gut microbiota simulation: Fecal fermentations

2.4.1. Rats cecal sample collection

Procedures involving animals and their care were conducted according to the National and European Communities Council Directives of Animal Care, ARRIVE guidelines and received approval (17/2021) by the local (iCBR) Animal Welfare Body (ORBEA). Experiments were conducted on twelve-week-old male Wistar rats (Charles River Laboratories, Barcelona, Spain) weighing about 400 g. Following one week of acclimatization, rats were pair-housed in ventilated cages and kept under stable environmental conditions, temperature (22±1 ºC) and humidity (50-60%), in a room with a 12/12 h reversed light/dark cycle and ad libitum access to tap water and two different types of diet, for sixteen weeks: standard isocaloric chow (CD, Mucedola 4RF21: 3.0% of energy as fat, 53.5% as carbohydrates and 18.5% as protein, with a total of 3.15 kcal/g; n=10) and a Western-type diet (WD, Teklad TD.08811: 44.6% of energy as fat, 40.7% as carbohydrates and 14.7% as protein, with a total of 4.7 kcal/g; n=9). Body weight, food/beverage consumption, and metabolic status were monitored throughout the experimental study. At the end of the study, animals were terminally anesthetized in a saturated chamber with isoflurane (IsoFlo®, Abbott). Individual intraluminal cecal contents were immediately collected into a sterile tube, snap-frozen on dry-ice, and stored at -80 ºC.

2.4.2. *In vitro* fecal fermentation assays

The *in vitro* fecal fermentation assay protocol using rodent cecal samples was based on a recent work aiming to assess *Fortunella margarita's* effect on mice's fecal microbiota (34). First, two different preculture inocula were prepared by mixing 1 g of equal amounts of fecal material from 10 and 9 rats from the two different dietary groups (CD and WD, respectively) with phosphate-buffered saline (PBS, 0.01M, pH 7.4) in a final concentration of 1:10 (m/V). The mixed fecal samples were then precultured (16% (v/v)) in a preculture medium (10 g/L tryptone, 5 g/L yeast powder, 10 g/L sodium chloride, 5 g/L glucose, 6 g/L maltose) in an anaerobic chamber for 18 h. The two independent preculture inocula (CD and WD) were then filtered through sterile gauze sponges to remove large particles (35). For the fermentation assays, a carbon-free medium was prepared using 2 g/L soybean peptone, 2 g/L yeast powder, 0.1 g/L sodium chloride, 0.04 g/L potassium dihydrogen phosphate, 0.01 g/L magnesium sulfate heptahydrate, 0.01 g/L calcium chloride hexahydrate, 2 g/L sodium bicarbonate, 2 mL/L Tween 80, 0.05 g/L heme chloride, 10µL/L vitamin K1, 0.5 g/L L-cysteine hydrochloride, 0.5 g/L bile salt, and 4 mL/L resazurin (0.05 % (m/V)). The pH was adjusted to 6.83, and the medium was sterilized by autoclaving (34). Based on previous studies, the fecal fermentations were conducted by adding the positive control (FOS) and the digested samples (Fish oil, Pomegranate oil, Fish oil+Pomegranate oil) to sterile urine flasks at a final concentration of 2% (w/v) in the described carbon-free medium (36–39). The two preculture inoculum were added separately to the corresponding flasks at a concentration of 5% (v/v) (34). A negative control was prepared to identify the basal microbiota for the two preculture groups. No sample was added in this control, just 5% (v/v) of the respective preculture inoculum (CD or WD). The resulting volume of each sample was then divided into 13 mL sealed sterile tubes. Each sample (FOS, Fish oil, Pomegranate oil, Fish oil+Pomegranate oil, and Negative control) was cultured in triplicate for each group (CD and WD), and aliquots were obtained for the different time points at 0, 6, 12, 24 and 48 h of incubation. pH values were measured at each mentioned time point using a MicropH 2002 pH meter (Crison, Barcelona, Spain), equipped with a 52-07 pH electrode (Crison, Barcelona, Spain). All the procedures were performed in an anaerobic workstation (Don Whitley Scientific, West Yorkshire, UK).

2.4.3. Bacterial DNA extraction and Metagenomics analysis

To perform a metagenomics analysis of the fermented samples, first, 4 mL of the fecal fermentation sample was centrifuged at 4000 x*g* for 10 min at 4 ºC. The resultant pellet was dissolved in 1 mL of TE buffer, and subsequent centrifugation at 4000 x*g* for 10 min (4 ºC) was performed. After, 180 µL of a lysozyme solution (10 mg/ml in TE buffer) was added to the pellet. The solution was incubated at 37 ºC for 2 h and subsequently centrifuged at 4000 xg for 10 min at 4 ºC. According to the manufacturer's instructions, the resulting pellet was used for DNA extraction using the NZYTissue gDNA isolation kit.

The 16S amplicon metagenomics sequencing analysis was performed by Novogene (Beijing, China). 16S rRNA genes of the 16SV3-V4 regions were amplified using specific primers connecting with barcodes (5'-CCTAYGGRBGCASCAG-3' and 5'-GGACTACNNGGGTATCTAAT-3'). The PCR reactions were carried out using 15 µL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs). 0.2 µM of forward and reverse primers and 10 ng of template DNA were used. The PCR conditions were as follows: initial denaturation at 98 ºC for 1 min, followed by 30 cycles of denaturation at 98 ºC for 10 s, annealing at 50 ºC for 30 s and elongation at 72 ºC for 30 s and 72 ºC for 5 min. The resulting PCR products were detected by electrophoresis using a 2% agarose gel, and the PCR products were subsequently purified using a Universal DNA Purification kit (DP214, TianGen, China). Sequencing libraries were generated using the NEB Next® Ultra™ II FS DNA PCR-free library Prep Kit (E7430L, New England Biolabs, USA), and index codes were added. The library was then checked with Qubit and real-time PCR for quantification and bioanalyzer for size distribution detection. The quantified libraries were pooled and sequenced on Illumina platforms. Amplicon sequence variants (ASVs), species annotation, and phylogenetic relationship construction were performed using QIIME2 software (version QIIME2-202006). α-diversity was calculated to analyze the communities' diversity, richness, and uniformity. For that, it was used 7 indices in QIIME2 software: Observed_otus, Chao1, Shannon, Simpson, Dominance, Good's coverage, and Pielou_e. To evaluate the complexity of the community composition and to compare the differences between groups of samples, β-diversity was calculated based on weight and unweight unifrac distances in QIIME2.

2.4.4. Fatty acid composition analysis by GC-FID

2.4.4.1. Sample preparation

To obtain the FAs profile of each sample, before and after *in vitro* fermentations at the different time points (0, 6, 12, 24, 48 h), 250 and 500 µL of samples, respectively, were prepared according to previous works (40,41). The sample preparation for the derivatization process is described in **Chapter 3 Section 2.5.1.1**.

2.4.4.2. GC-FID analysis

The FA composition of the FAME extracts was determined and quantified using a gas chromatograph Agilent 8860 (Agilent, USA) equipped with a flame ionization detector and a BPX70 capillary column (60 m x 0.25 mm x 0.25 µm; SGE Europe Ltd, Courtaboeuf, France). The analysis conditions were described in **Chapter 3 Section 2.6.3.1**.

2.5. Short-chain Fatty acids quantification

The SCFAs quantification analysis was also performed by GC-FID. The extraction of the SCFAs from the fermentation fluids samples was performed according to the method described by Scortichini and collaborators (42). Briefly, 250 mg of each sample was acidified with 200 µL of sulphuric acid (50% (w/v)) and mixed by vortex for 1 min. 10 µL of internal standard solution

(Valeric acid, C5) was added to the acidic solution to achieve the sample's final concentration of 450 μM. The SCFAs were extracted by adding 800 μL of diethyl ether and centrifuge for 5 min at 2800 x*g*. The organic phase was transferred to a new collection tube, and the extraction process was repeated three times, collecting a total of 2400 μL of the organic phase. The identification of the SCFA in each sample was achieved by comparison of the relative retention times of sample peaks with the adequate standards and the quantification by using a calibration curve in the range of concentrations of 0.86-7000 μM for the most prevalent SCFAs in the sample – acetic, propionic and butyric acids.

2.6. Free Amino acids and GABA detection and quantification

For the free amino acids and GABA detection and quantification analysis, 1 mL of fecal fermentation samples were precipitated with 2 mL of 0.6 M perchloric acid. The mixture was agitated for 1 h and centrifuged at 4100 x*g*, for 15 min at 4 ºC (43). The supernatant was collected, filtered, and analyzed by HPLC (44) as thoroughly explained in **Chapter 4 Section 2.5.4.**

2.7. Statistical analysis

Table and figure results are reported as mean values of three biological replicates ± standard deviation. The statistical analysis was performed using IBM SPSS Statistics 28 (SPSS Inc., IBM Corporation, NY, USA). First, data was analyzed for normal distribution using Shapiro-Wilk’s test. The homogeneity of variances was verified using Levene’s test. To compare the means of three or more groups, was used One-way ANOVA. Tukey’s post-hoc test was used to determine differences among groups. When only two samples were analyzed, a T-student test was applied. The level of significance (*p*) was set at 0.05.

3. Results and discussion

3.1. Fatty acid composition analysis of the fermented samples

While the microbial metabolism of carbohydrates and proteins in the gut is well studied, the interaction between gut microbiota and dietary fats is still overlooked (45). Considering this, an FA analysis was performed for each sample at the different fermentation time points (6, 12, 24, and 48 h). The major bioactive FAs in the fermented samples were compared to the original digested sample used for the fermentation assays, and the results are displayed in **Figure 5.2**. As discussed, this sample was previously subjected to GIT digestion, and only the non-absorbed fraction was used to be tested for microbiota modulation. Furthermore, a RI (%) was calculated, according to **Equation 5.1**, for the major bioactive FAs detected in each fermented sample (**Table 5.1**) concerning the sample before fermentation.

$$\text{RI (\%)} = \frac{\text{FA content quantified after fermentation}}{\text{FA content quantified in the digested sample before fermentation}} \times 100 \qquad \textbf{(Equation 5.1)}$$

By analyzing both **Figure 5.2** and the calculated RI (**Table 5.1**) it was observed that, in general, for most samples, there is a decrease in the concentration of most FAs after 6 h of fermentation, which is observable by a RI<100%. Similar results have been reported in recent studies: in an *in vitro* fermentation assay using human fecal inoculums, several FAs were reduced during the fermentation time. This reduction is justified by the conversion of FAs into microbial metabolites. Indeed, it has been recognized that besides dietary fiber and proteins, the gut microbiota is also able to metabolize lipids and produce bioactive metabolites such as LA and CLA isomers (45). Moreover, in this study, the authors also observed the presence of LA metabolites in the fermentation sediments (45). Such results suggest that there is also the incorporation of microbial FA metabolites into bacterial cells. These observations are also powerful possibilities to explain what was observed in our study.

Regarding the major SFAs identified in the fermented samples – C14 (myristic acid), C16 (palmitic acid), and C18 (stearic acid) - it was observed that the RI of these FAs in Fish oil and Pomegranate oil samples was lower than for the Fish oil+Pomegranate oil in samples from both models (CD and WD). For the myristic acid, the RI for the Fish oil sample was ca. 52.4-62.6% and 43.5-60.4% for CD and WD samples, respectively, compared to 79.47%-102.6% and 85.90-92.81% in Fish oil+Pomegranate oil. Interestingly, the initial concentration of the Fish oil for this SFA is higher (11.36±3.52 µg of FA/µL of the sample) compared to 1.66 ±0.62 µg of FA/µL of the sample obtained for the Fish oil+Pomegranate oil sample. Similar observations were made for palmitic acid: in Fish oil, the RI was between 49.5-58.7% for CD and 48.9-54.4% for WD, corresponding to an initial concentration of 23.17±4.61 µg of FA/µL of the sample of the sample. These RI values were lower compared to Fish oil+Pomegranate oil with an RI of 59.9-79.95% for CD and 66.48-76.10% for WD, with an initial concentration of 4.37±1.41 µg of FA/µL of the sample. The same was observed for Pomegranate oil: 37.26-49.88% for CD and 40.9-44.02% for

WD, with an initial concentration of 5.75 µg of FA/µL of the sample, comparing to the mentioned values of Fish oil+Pomegranate oil. Similar observations were made for stearic acid, but for this FA regarding Fish oil+Pomegranate oil samples in both models, the RI is higher than 100%. This observation indicates that there is bacterial production of this FA. Interestingly, the bacterial production of stearic acid from LA has been previously described by Wallace et al. (46). This may be a plausible explanation for the results observed in our study, considering that along with the increase observed for C18, there was a reduction of LA in these samples (RI=53.1-84.5%). Nevertheless, by analyzing **Figure 5.2**, it seems that the difference between the concentration of stearic acid in the Fish oil+Pomegranate oil fermented samples compared to the original sample is not statistically different.

The most relevant MUFA in all the fermented samples is oleic acid (C18:1 *c*9). As discussed for the SFAs, oleic acid RIs were higher in Fish oil+Pomegranate oil samples (40.9-139.12%) than in Fish oil (28.9-67.4%) and Pomegranate oil (16.4-58.4%). Again, although oleic acid RI was higher for Fish oil+Pomegranate oil samples, the initial concentrations were lower in these samples (2.68±0.94 µg of FA/µL of the sample) compared to Fish oil (10.02±2.21 µg of FA/µL of the sample) and Pomegranate oil (11.32±1.89 µg of FA/µL of the sample). Regarding PUFAs, similar observations were made for LA and ALA. Regarding the major omega-3 FAs, namely EPA, DHA, and DPA in Fish oil and Fish oil+Pomegranate oil, the same observation was made: higher RIs in the latter, although lower initial concentrations in the digested samples (2.12±0.98, 1.51±0.66 and 0.55±0.22 µg of FA/µL of sample in Fish oil+Pomegranate oil, vs 18.91±7.77,11.10±4.43 and 3.94±1.22 µg of FA/µL of sample in Fish oil, for EPA, DHA and DPA, respectively). Regarding PUA, one of the major bioactive FAs in Pomegranate oil and Fish oil+Pomegranate oil, although the initial concentration in Pomegranate oil (139.87±22.65 µg of FA/µL of sample) was much higher than in Fish oil+Pomegranate oil (10.58±4.92 µg of FA/µL of sample) the RIs are similar in both samples (≈2.45% and 4.55%, respectively). Together, these results suggest that the initial concentration of a determined FA may be a relevant parameter to consider when assessing its effect on microbiota modulation.

Intestinal bacteria can interact with FAs through hydration and biohydrogenation of the unsaturated bonds of MUFAs and PUFAs (47). In Pomegranate oil samples, the C18:2 *t*9*t*11 RI is considerably higher than 100%. **Figure 5.2** shows that compared to the original sample, the concentration of this FA in the fermented samples is significantly higher. The same is observed for β-eleostearic acid (C18:3 *t*9*t*11*t*13) in the Pomegranate oil sample for both groups and in Fish oil+Pomegranate oil for the CD group. These results indicate a production of these FAs in the mentioned samples. Notably, the microbial digestion of specific dietary lipids is considered a detoxifying mechanism. Several bacteria, including *Lactobacillus*, *Roseburia,* and *Bifidobacterium* use this mechanism to transform specific growth-inhibiting PUFAs into less toxic FAs.

An example is the bacterial mechanism through which LA is converted into CLA isomers. In fact, through a biohydrogenation process, LA is converted to rumenic acid (C18:2 *c*9*t*11), which is subsequently hydrogenated to vaccenic acid (C18:1 *t*11) and <a hydration step in which LA is

converted to a hydroxy-C18:1 FA, which is also a precursor of CLA isomers (47). *Bifidobacterium*, for instance, can transform LA into C18:2 *c*9*t*11 and its precursor C18:1 *t*11 (Vaccenic acid) (48). Similar to what we observed here, recent work has reported an increase in the C18:2 *t*9*t*11 CLA isomer in *in vitro* human fecal fermentations (45), and the same was observed in the subcutaneous adipose and colon tissue of germ-free mice fed a low-fat diet (49). Some *Bifidobacterium* strains, such as *Bifidobacterium breve* 2258, were previously identified as C18:2 *t*9*t*11 producers (50). Moreover, an early study identified *Roseburia* and *Bifidobacterium fibrisolvens* as vaccenic acid producers, *Propionibacterium freudenreichii subsp. Shermani* as the C18:2 *c*9*t*11, C18:2 *t*9*t*11, and C18:2 *t*10*c*12 and *Bifidobacterium breve* as C18:2 *c*9*t*11 and C18:2 *t*9*t*11 producers (51). Here, from these FAs, the only one that showed increased concentration was the mentioned CLA isomer C18:2 *t*9*t*11. Nevertheless, it has been demonstrated that LA's microbial metabolism differs depending on the form in which LA is provided (52) which may explain the different results obtained here.

Regarding β-eleostearic acid (C18:3 *t*9*t*11*t*13), an increase in concentration ($p<0.05$) was observed in the Pomegranate oil and Fish oil+Pomegranate oil samples. As far as we are concerned, there are no reports of gut bacterial production of this FA. Although the identification was positively made in all the samples by comparing the retention times with the retention time of the reference standards, it is important to mention that other CLNA isomers with similar retention times may co-elute. For instance, it has been shown that several *Bifidobacterium* isolates and *Lactobacillus plantarum* ZS2058 can convert free linolenic acid into C18:3 *c*9*t*11*c*15 and C18:3 *t*9*t*11*c*15 CLNA isomers at different levels (53,54), which may explain the increased concentration observed here. We were able to verify that C18:3 *c*9*t*11*c*15 isomer and β-eleostearic acid present different retention times and can be separated under GC-FID analysis conditions. The same was not verified for the C18:3 *t*9*t*11*c*15 isomer, which may present a similar retention time as β-eleostearic acid. So, careful considerations need to be drawn here considering these CLNA isomers, and further studies are required for a more accurate identification.

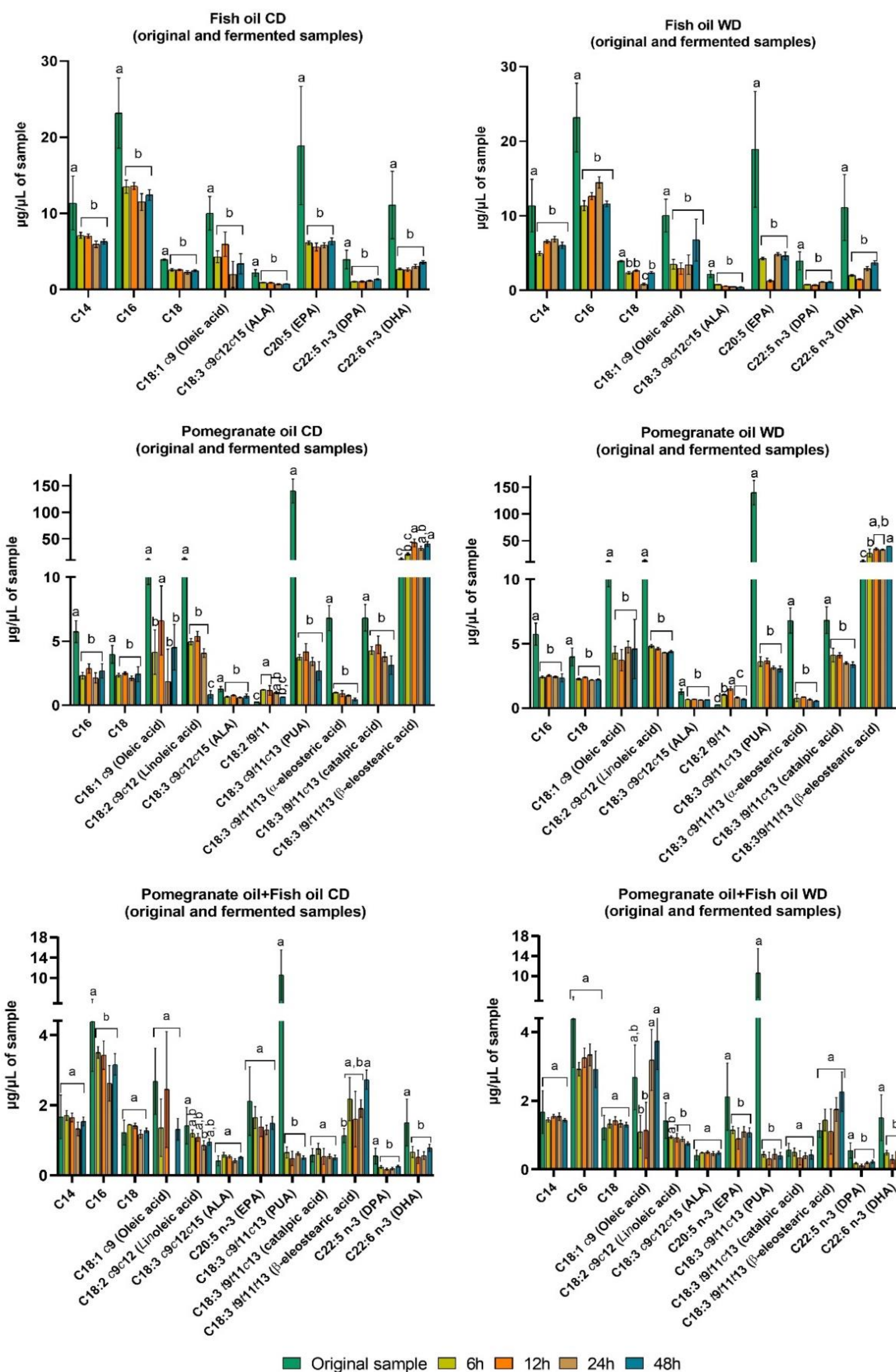


Figure 5.2 – **Major FAs concentration (µg/µL of the sample) of the different samples at the different fermentation times (0, 6, 12, 24, and 48 h).** CD-Control Diet and WD-Western Diet models. The values presented are the means of three biological and two analytical replicates the bars represent the standard deviation. Different letters indicate significant differences ($p < 0.05$). The capital letters (A,B,C) indicate the differences among samples - Negative control, Positive control (FOS), and bioactive lipids sources (Fish oil, Pomegranate oil, and a mixture of both) - at the same time. The small letters (a,b,c) indicate the differences for the same sample over time.

Table 5.1 - RI for the major FAs for the different fermented samples at different times (6, 12, 24, and 48 h) in relation to the sample before fermentation. CD-Control Diet and WD-Western Diet models. n.d.- not detected

Fatty acids		RI (%)																							
		Fish oil								Pomegranate oil								Fish oi+Pomegranate oil							
		CD				WD				CD				WD				CD				WD			
		6h	12h	24h	48h	6h	12h	24h	48h	6h	12h	24h	48h	6h	12h	24h	48h	6h	12h	24h	48h	6h	12h	24h	48h
Myristic acid	C14	62.63	61.88	52.42	55.42	43.50	57.47	60.46	53.03	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	102.61	98.90	79.47	92.20	86.57	92.81	92.22	85.90
Palmitic acid	C16	58.27	58.73	49.57	53.58	48.96	54.56	62.39	50.07	40.18	49.88	37.26	46.63	42.05	44.02	42.26	40.90	79.95	78.27	59.90	72.25	66.60	74.14	76.10	66.48
Stearic acid	C18	66.09	66.02	57.26	62.98	60.29	67.17	21.07	61.09	59.10	62.78	53.02	61.10	56.59	60.41	54.92	55.56	118.79	115.83	96.60	104.92	108.83	116.40	109.16	106.68
Oleic acid	C18:1 *c*9	42.90	59.37	28.93	33.76	34.82	29.15	34.35	67.40	36.69	58.48	16.40	39.98	37.94	32.94	41.91	40.48	50.62	91.34	n.d.	49.03	40.92	42.49	118.78	139.12
cis-vaccenic acid	C18:1 *c*11	61.38	61.29	53.07	54.09	66.55	60.59	46.75	55.02	78.85	79.24	56.61	67.10	70.79	78.96	68.49	73.38	106.11	123.86	100.22	108.97	114.19	125.09	114.66	113.96
Linoleic acid	C18:2 *c*9*c*12	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	38.72	41.99	31.74	6.65	37.54	36.01	33.60	34.14	84.54	76.56	60.38	67.29	66.50	65.11	62.04	53.14
α-linolenic acid	C18:3 *c*9*c*12*c*15	42.18	40.87	31.90	33.35	35.54	26.40	22.00	19.47	53.63	61.07	48.83	57.32	52.84	53.72	50.12	50.73	141.73	129.21	100.57	123.81	118.41	121.12	112.50	117.30
CLA isomer	C18:2 *t*9*t*11	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	485.66	467.85	399.31	256.22	417.01	608.58	335.83	275.83	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Eicosapentaenoic acid (EPA)	C20:5 n-3	32.40	29.52	30.76	33.53	22.50	6.74	25.63	24.44	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	77.82	65.36	61.17	70.35	54.23	42.27	51.69	50.81
Punicic acid	C18:3 *c*9*t*11*c*13	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.68	2.98	2.44	1.92	2.58	2.63	2.25	2.18	6.16	4.56	5.85	4.75	4.12	2.96	4.22	3.79
α-eleostearic acid	C18:3 *c*9*t*11*t*13	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	14.58	13.35	10.99	6.58	11.40	12.58	9.83	8.06	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
catalpic acid	C18:3 *t*9*t*11*c*13	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	108.66	120.07	95.93	79.71	104.75	104.48	89.08	85.75	132.40	94.16	95.00	88.66	88.60	59.42	70.70	75.04
β-eleostearic acid	C18:3 *t*9*t*11*t*13	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	172.81	347.85	266.32	329.45	221.71	280.17	273.44	325.18	191.72	141.43	167.96	240.39	126.69	97.42	153.92	198.88
Docosapentaenoic acid (DPA)	C22:5 n-3	26.27	26.76	29.01	33.87	19.81	17.36	28.74	28.47	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	43.52	31.62	34.54	47.67	32.88	20.85	35.19	39.77
Docosahexaeoic acid (DHA)	C22:6 n-3	24.20	23.50	27.57	32.19	17.98	13.37	26.15	33.33	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	44.13	34.53	37.28	51.96	32.17	19.46	34.70	34.13

3.2. Metagenomics analysis

Several diseases mainly affecting Westernized countries are usually associated with gut microbiota dysbiosis and loss of microbial diversity. Interestingly, loss of microbial diversity has been pointed out as one of the most constant findings of intestinal dysbiosis. Indeed, this phenomenon is common in diseases such as Chron's disease, irritable bowel syndrome, and colorectal cancer (55). Considering the importance of the bacterial population diversity, the 16S amplicon metagenomics sequencing analysis performed by Novogene and described in detail in **Section 2.4.3** allowed the taxonomic annotation of the top 10 and 30 taxa of each sample or group at different taxonomic ranks. **Figure 5.3** displays the distribution histogram of the relative abundance for each sample and groups of samples for both Phylum **(Figure 5.3 A1 and B1)** and Species and Genus **(Figure 5.3 A2 and B2)**. Such analysis allowed the observation of the taxa with a higher relative abundance and their proportion in these two different classification levels. In our study, analyzing the relative abundance at the mentioned taxonomical levels, it is possible to observe by comparing the Negative control samples from both groups (CD and WD) at time 0 h, which represents the basal microbiota before fermentation, that the control group (CD) appears to present a higher bacterial diversity than the WD group.

Nevertheless, regarding the Firmicutes group, the CD group presented a higher relative abundance than the WD group. However, at the genus level, following what was previously reported for HFD (56), there seems to be a higher prevalence in the WD group of *Escherichia-Shigella*. HFD has been associated with a reduced abundance of *Lactobacillus, Bifidobacterium, Akkermansia, Faecalibacterium,* and *Blautia* (57)*,* also observed in this study. WD has been shown to increase *Clostridium*, *Eubacterium*, *Anaerotruncus,* and *Holdemania* and decrease *Candidatus arthromitus* (58), which was not observed here. In this study, the authors stated that low gut microbial community changes were observed in the WD (42.5% kcal from fat and 40.5% from carbohydrates, 30 g of sucrose/ 100 g) compared to CD (59).

Nevertheless, another study with a different WD composition (53.9% kcal from fat and 20 g of sucrose/ 100 g) reported drastic changes in gut microbial composition, including a lower abundance of *Lactobacillus* (60). These differences may be related to the higher fat percentage in the last study. Indeed, the different nutritional composition reported in several studies is responsible for different gut microbiota responses and is a critical aspect to consider when interpreting the gut microbiota data.

3.3. Bacterial population diversity richness

3.3.1. α-diversity assessment

To analyze the communities' diversity, richness, and uniformity, α-diversity was calculated. **Figure 5.4A** shows the flower diagrams according to the analysis results of the feature sequences (corresponding to the OTU representative sequence). These diagrams display the

common and unique information for different groups of samples and each sample through time. In addition, the α-diversity indexes were determined, and the results are demonstrated in **Figure 5.4B** using Chao1 (OTU richness) and Shannon (OTU evenness) diversity indexes. By analyzing **Figures 5.3 and 5.4**, although CD presents a higher diversity than WD considering the Shannon index, there is no statistical significance between the CD and WD groups **(Figure 5.4B)**. Some studies have reported a higher Shannon index for HFD than control groups (61,62). Moreover, a recent study reported lower Shannon indexes in mice fed an HFD after 4 and 12 weeks, but no significant change was found at 8 weeks (63). If considering specifically a WD, some studies reported no statistically significant difference between the α-diversity Chao1 index of the control group and the WD group (59), which was also observed here. Furthermore, another study using a WD with a higher fat content reported similar observations: the Shannon and Chao1 indexes were lower in the WD group compared to the control group, but no statistical difference was achieved (60).

By analyzing **Figures 5.3 and 5.4**, the sample Fish oil+Pomegranate oil, especially in the WD group, presents higher diversity than the remaining samples, most relevantly after 12 h of fermentation. This sample presented higher α-diversity Shannon and Chao1 indexes than the remaining samples. Relevantly, the Shannon and Chao1 indexes were significantly higher ($p<0.05$) than all the samples of the same group (WD) and the negative control, FOS, and Fish oil samples from the CD group. In a recent systematic review and meta-analysis study, the authors observed that a significantly lower α-diversity (Shannon index) was described in obese versus non-obese adults in nine studies (56). In addition, similar results have been reported in T2DM: a decline in bacterial diversity (based on the Shannon index) among T2DM human subjects (64,65).

Consequently, different results have been reported regarding α-diversity in an HFD or WD context. Nevertheless, there is usually a loss of microbial diversity observed in several diseases often associated with WD consumption. Thus, the capacity of the Fish oil+Pomegranate oil sample to significantly increase the α-diversity indexes in the WD group to higher values than the remaining samples, especially to higher values than the FOS sample (a known prebiotic) of the control group (CD group), may be highly relevant in a therapeutical setting.

3.3.2. β-diversity assessment (PCoA analysis)

The β-diversity analysis comprises an assessment of microbial community structure differences among groups of samples. Such a difference in community structure between two samples can be visualized by the unifrac distance between two samples and be visualized in two dimensions through multivariate statistical methods such as Principal coordinates Analysis (PCoA). From the 16S information analysis process, the value matrix composed of the OTU abundance of two different samples is used for the PCoA analysis. Thus, unweight Unifrac was calculated and then PCoA is able to pick up the main variables from complex multidimensional variable data, investigating also the ecological distance. The results are demonstrated in **Figure 5.5**. Although no statistical significance was reached (PERMANOVA analysis; unweighted-

UniFrac) the PCoA biplot showed a clear tendency to differentiate the two diet types (CD and WD) through along PCoA1 (46.76%). Confirming what was already discussed along PcoA2 (10.09%) there is a visible tendency of differentiation between Fish oil+Pomegranate oil sample in the WD (at 12 and 24 h) from the remaining WD samples.

3.3.3. Bacterial relative abundance analysis

By 16S rRNA gene sequencing, HFD was described to increase Desulfovibrionaceae, Rikenellaceae RC9 gut group, and *Mucispirillum* and reduce the abundance of *Lactobacillus*, *Bifidobacterium*, *Akkermansia*, *Faecalibaculum* and *Blautia.* Nevertheless, no significative change in Firmicutes abundance was observed (62). Similarly, a WD (40.6% kcal from fat, 40.7% kcal from carbohydrates, and 18.2% (w/v) sucrose) intervention study in mice demonstrated a decrease in *Bifidobacterium*, Bacteroidetes, and *Akkermansia.* Contradictory results regarding Firmicutes phylum were reported*:* increased relative abundance of Firmicutes (*Lactobacillus* and *Oscillospira*) (66).

Interestingly, Fish oil+Pomegranate WD seems to increase the relative abundance of the Firmicutes phylum to similar levels as the CD group control (the negative control time 0 h). The contradictory results reported in different studies regarding the effect of WD in Firmicutes' relative abundance are probably related to different nutritional compositions of the diets and different species of rodent models used. Since it was observed in this study a reduction of Firmicutes relative abundance in the WD group, the capacity of the Fish oil+Pomegranate oil WD sample to restore those levels may be highly relevant from a therapeutical perspective. In fact, previous studies have reported that omega-3 can increase Firmicutes abundance (28,67). Interestingly, this capacity to increase Firmicutes abundance was only observed in the fermentation with both oils (Fish oil and Pomegranate oil), *i.e.,* the same was not observed for Fish oil alone. It can be hypothesized that this increase may be a result of the synergistic action of both oils since PUA (present in Pomegranate oil) has also been described to increase *Lactobacillus*, *Roseburia,* and *Oscillibacter* bacteria (31), all belonging to the Firmicutes phylum. In addition, the Fish oil+Pomegranate oil sample can also increase the Bacteroidetes' relative abundance **(Figure 5.3)**. So, the Fish oil+Pomegranate oil sample can decrease the elevated Firmicutes/Bacteroidetes ratio of the WD group to levels like the ones observed in control mice (subjected to a CD) at time 0 h.

Moreover, looking at the relative abundance, it seems that Fish oil+Pomegranate oil of the WD group can increase *Akkermansia* and *Blautia* relative abundance, which were affected by the WD compared to the CD group. A *t*-test **(Figure 5.6)** was performed to determine bacteria with significant variation between Fish oil, Pomegranate oil, and Fish oil+Pomegranate oil for the corresponding CD vs. WD groups ($p< 0.05$) at different taxon ranks. In agreement with what was reported before, Fish oil and Pomegranate oil **(Figure 5.6)** presented statistically significative higher mean values of the abundance of *Escherichia-Shigella, Klebsiella,* and *Weisella* in the WD group compared to the corresponding control (CD). Bacterial strains belonging to

Enterobacteriaceae, like *Escherichia*, *Klebsiella*, and *Shigella* were also dominant in HFD treatments in male C57BL/6J mice (68). On the opposite, in Fish oil+Pomegranate oil, the mean value of abundance of *Escherichia-Shigella, Klebsiella,* and *Weissela* in WD is similar to the abundance values observed for the CD group. These results indicate that compared to Fish oil and Pomegranate oil alone, combining both oils (Fish oil+Pomegranate oil sample) can decrease the relative abundance of these bacteria that were affected by the WD. The observations regarding the *Klebsiella* genus are highly relevant if we consider the negative effect of these bacteria on diseases like obesity. A higher abundance of *Klebsiella* has been demonstrated in overweight and obese subjects (69,70). In addition, *Klebsiella pneumoniae* colonization has been associated with higher weight gain in rats. Importantly, when the bacterium was eradicated, the mean weight decreased and became almost like the control rats. It has been hypothesized that *K. pneumoniae* may lead to more energy harvesting from the food resulting in obesity (71).

On the other hand, *Weissela* is a recently identified human-derived LAB. *Weissella cibaria* is the most prevalent species in kimchi and has been reported to have antioxidant and anti-inflammatory effects. A recent study has suggested that *W.cibaria* MG5285 may be a potential anti-obesity LAB since it showed a significantly reduced expression of lipogenic proteins, *e.g.,* peroxisome proliferator-activated receptor γ, CCAAT/enhancer-binding protein α, FA synthase, and adipocyte-protein 2 (72). Although its increase may be beneficial in an obesity context, it is essential to mention that relatively few reports on its anti-obesity and hepato-protective roles are available, and contradictory results have been reported for other WD-related conditions, such as T2DM. Recent studies have demonstrated that *Weissella confusa* relative abundance was higher in mice with T2DM (73), and other studies demonstrated that *Weissella* decreased after treatment in animal models of T2DM (74–76). These contradictory results may be explained by the effect of these bacteria being species-specific or even dependent on the disease. Importantly, considering the negative effect of the mentioned bacteria, the capacity of Fish oil+Pomegranate oil to maintain or even decrease the *Escherichia-Shigella, Klebsiella,* and *Weissella* levels of the WD to similar values as the CD group may be highly relevant. Nevertheless, further studies are required.

Although presenting small relative abundance levels and no statistical significance was reached in the t-test, it was observed a higher relative abundance of *Akkermansia* in FOS, Fish oil, Pomegranate oil, and Fish oil+Pomegranate oil WD samples than in the fermented samples of the CD group. This may be a relevant observation since therapeutic intervention with *A. muciniphila* in intestinal microbiota is nowadays considered a promising strategy for preventing and treating obesity and metabolic disorders such as T2DM. Supplementation with this bacteria decreased body weight and improved liver dysfunction and inflammation in human trials (77). Additionally, some beneficial *Clostridium* bacteria, such as *Clostridium leptum,* are decreased in T2DM (78), but some HFD and WD studies have been reporting an increase in these bacteria (60,79). In our study, we observed that Fish oil+Pomegranate oil of the WD group also showed a higher relative abundance of Clostridia_UCG-014 than most of the remaining samples, including samples from the CD group. Notably, the decreased prevalence of Clostridia_UCG-014 has been

associated with impaired gut barrier function in Chron’s disease (80). Again, these results show the potential of a mixture of Fish oil and Pomegranate oil in restoring the community alterations that a WD often induces.

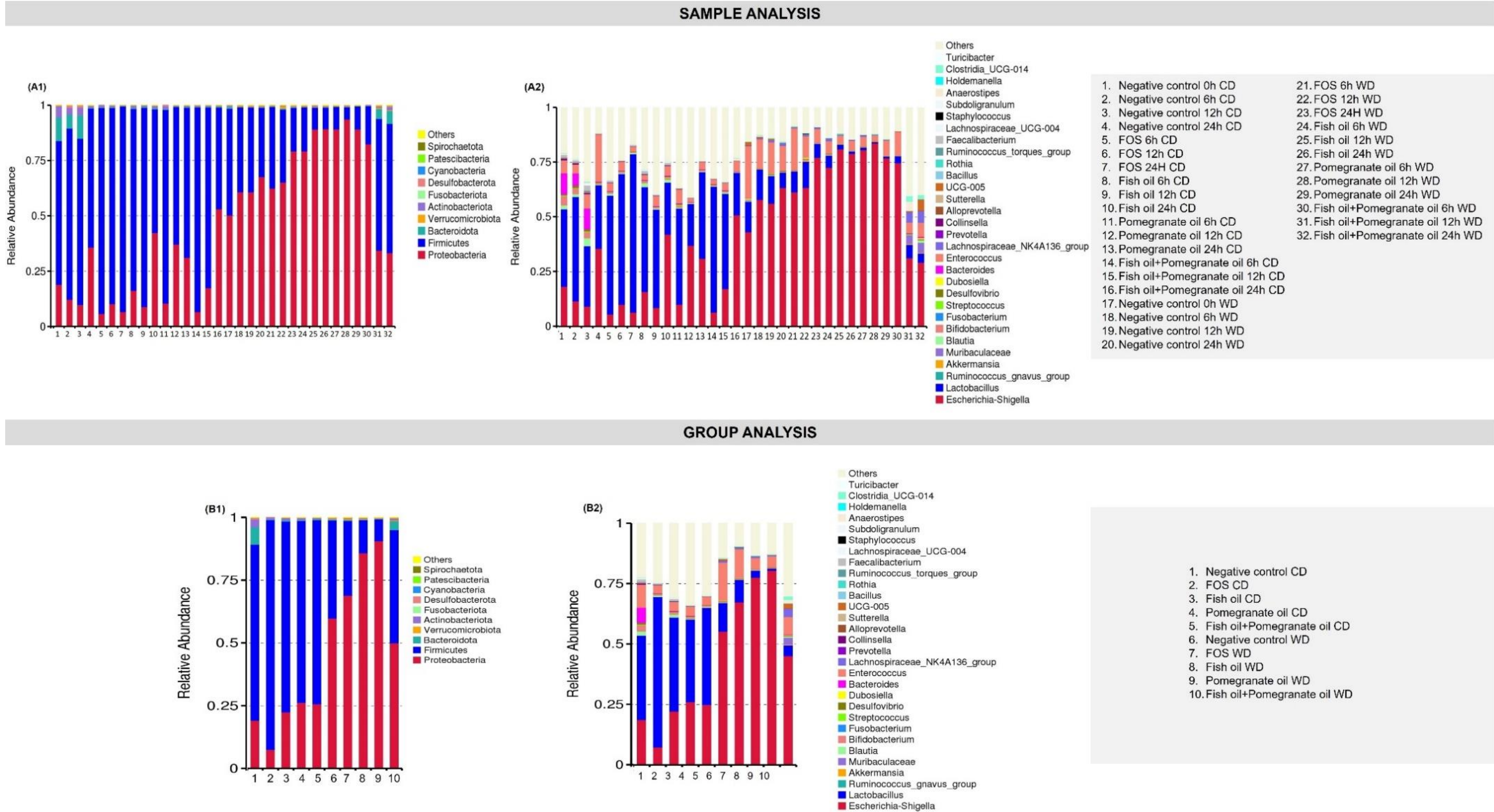
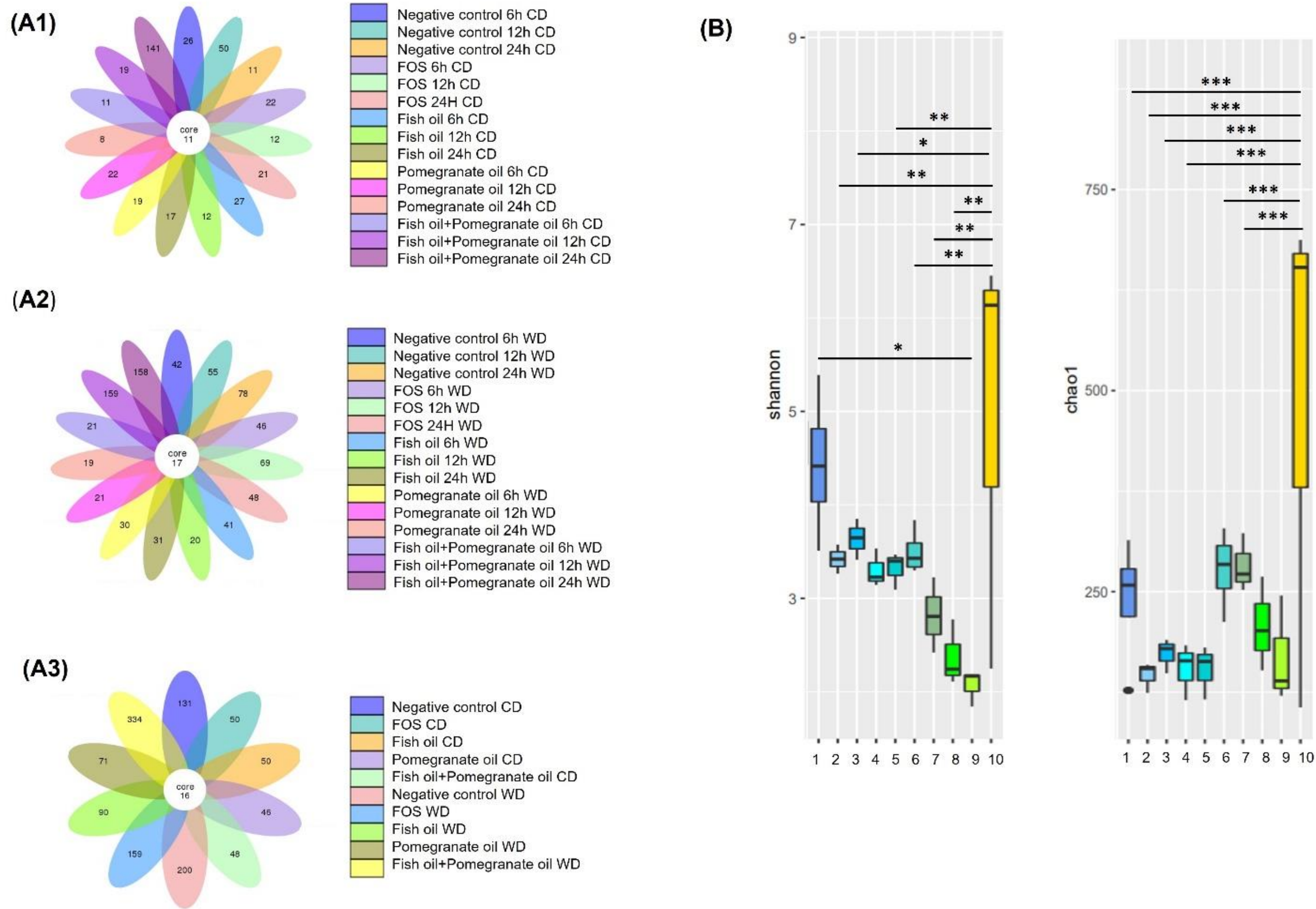


Figure 5.3 – **Relative abundance analysis of the different samples (Fish oil and Pomegranate oil and the mixture of both oils) and the negative and positive controls (FOS) for the two different groups CD and WD.** (A) Barplot representation of the relative abundance analysis for all the samples at the different fermentation time points (0, 6, 12 and 24 h) (A1) top 10 taxa of each sample at the phylum level and the (A2) top 30 taxa of each sample at genera and species level. (B) Barplot representation of the relative abundance analysis for all the samples for the different sample groups (B1) top 10 taxa of each group at the phylum level and the (B2) top 30 taxa of each group at the genus and species level.



1. Negative control CD
2. FOS CD
3. Fish oil CD
4. Pomegranate oil CD
5. Fish oil+Pomegranate oil CD
6. Negative control WD
7. FOS WD
8. Fish oil WD
9. Pomegranate oil WD
10. Fish oil+Pomegranate oil WD

Figure 5.4 - **(A) Flower diagram** of (A1) CD samples and (A2) WD group at the different fermentation time points (0, 6, 12 and 24 h) and (A3) grouped analysis of all the samples of both the common and unique information for different samples based on the OTUs analysis. **(B) α-diversity analysis using Chao1 and Shannon diversity index.** Kruskal-Wallis test was used to determine statistical differences between samples, where **p*<0.05, ***p*<0.01 and ****p*<0.001.

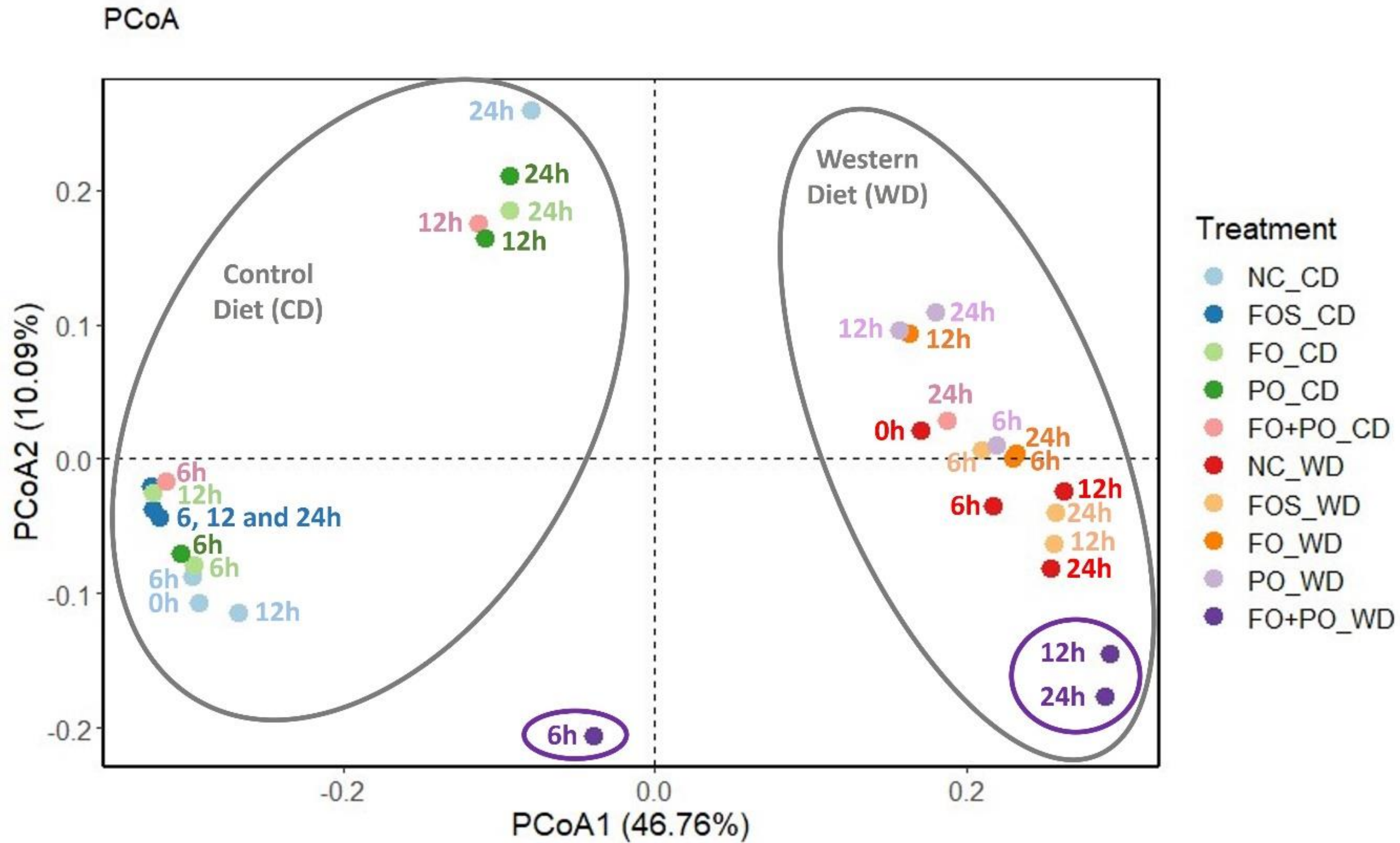


Figure 5.5 – Principal coordinate analysis (PCoA) considering Unweighted Unifrac distances. The 2D PCoA diagram is presented in the first and second principal coordinates.Each point represents a sample from a different time point colored by group, plotted by a principal component on the X-axis and another principal component on the Y-axis, which was colored by group. The percentage on each axis indicates the contribution value to discrepancy among samples.

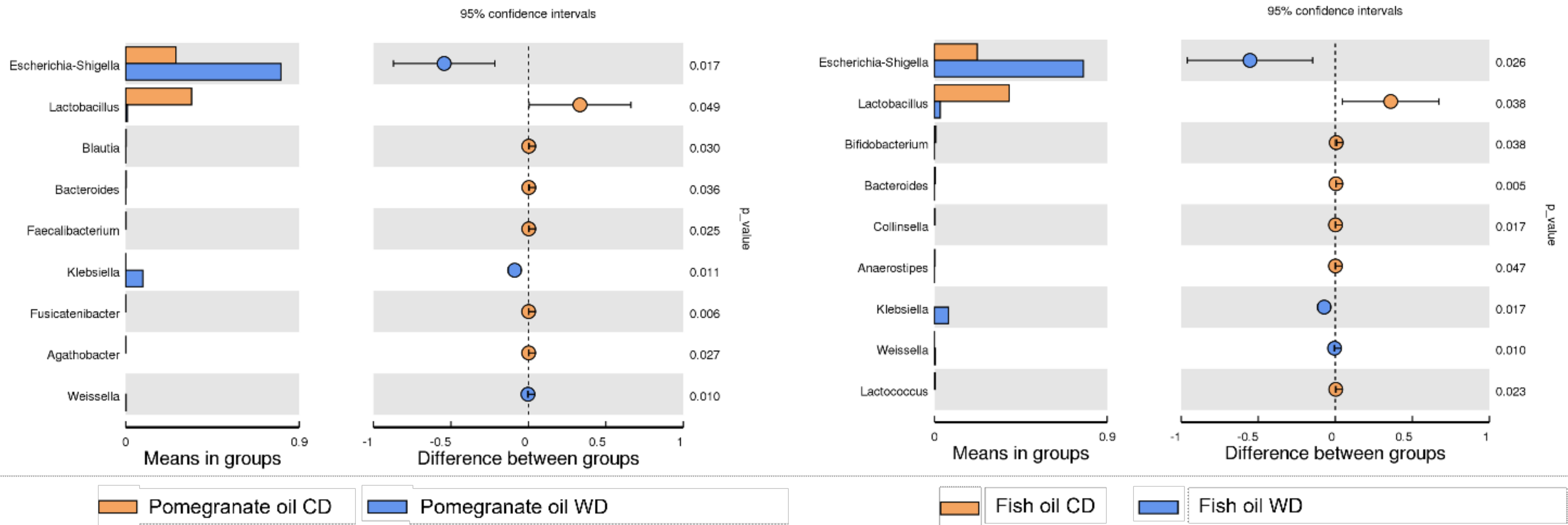


Figure 5.6 – Between-group T-test analysis for the species showing significant differences between groups. Each bar represents the mean value of the abundance in each group of the species showing significant differences between groups. The right panel is the confidential interval of between-group variations. The left-most part of each circle stands for the lower limit of 95% confidential interval, while the right-most part is the upper limit. The center of the circle stands for the difference in the mean value. The color of the circle agrees with the group whose mean value is higher. The right-most value is the p-value of the significance test of between-group variation.

3.4. Short-chain fatty acids concentration analysis

The production by gut microbiota of metabolic products and signaling molecules promotes beneficial or detrimental effects on human health. Among those metabolites, SCFAs, FAs with less than 6 carbons in their aliphatic chain, are gaining attention due to their potential in modulating several diseases. Acetate, propionate, and butyrate are the major SCFAs accounting for 80% of all SCFAs, and are produced by anaerobic gut bacterial saccharolytic fermentation of complex resistant carbohydrates, such as resistant starch, simple sugars, polysaccharides, and FOS. These molecules escape digestion and absorption in the small intestine and can be used to produce SCFAs. Afterward, the produced SCFAs play regulatory functions in the lipids' cholesterol and glucose metabolism, anti-inflammatory and immune responses, and gut barrier integrity (81). Considering this, it is easily understood that SCFAs play a crucial role in restoring the adverse effects of WD consumption. Indeed, several tissues express SCFA receptors and can respond to the beneficial effects that these molecules may present (82).

Moreover, the type of diet can significantly modulate SCFA production and/or abundance of SCFA-producing bacteria. Besides the mentioned dietary fibers that are greatly responsible for SCFA production, other bioactive molecules, namely omega-3, correlate with higher levels of SCFAs and SCFA-producing bacteria (81). Taking this into consideration, we evaluated the major SCFA concentrations (acetate, propionate, and butyrate) in the different samples (Pomegranate and Fish oil and the mixture of both) and compared them to the positive control (FOS sample), where a known prebiotic (FOS) was used, as well as to the negative control, where no active sample was added. In the colon and the stools, butyrate, propionate, and acetate are found in an approximate molar ratio of 20:20:60. But these values are dependent on the microbiota composition, SCFA substrates, and gut transit (81). Indeed, here the major SCFA observed in the negative control, which represents the basal gut microbiota (at time 0 h), was acetate. This SCFA was observed in this sample at a concentration of 30.48±4.62 μmol/g of fermented sample in the CD model and 7.68±2.03 μmol/g of fermented sample in the WD model. Indeed, lower SCFAs levels have been reported in an HFD (22) and WD (83) context. Interestingly, in a WD model the expression of GPR43, which is a SCFA receptor, was found to be reduced in WD-fed mice similar to what was observed in Chron's disease patients (84). Regarding propionate and butyrate concentrations, at time 0 h the values were under the quantification limit.

3.4.1. Acetate

After the fecal fermentations **(Figure 5.7)** using the bioactive lipids samples, it was observed that there was an increase in acetate production in all the samples during the fermentation time. Pomegranate oil showed a statistically significant ($p<0.05$) lower acetate concentration (0.52±0.07 and 0.85±0.03 μmol/g of CD fermented sample and 0.85±0.12 and 3.06±0.73 μmol/g of WD fermented sample, at 24 and 48 h respectively) compared to the remaining samples. At the same time, the Fish oil sample in the WD model (48.10±29.77 μmol/g of WD fermented sample) showed similar concentration values at 48 h when compared to FOS

(48.03±9.27 μmol/g of WD fermented sample) and Negative control (54.51±2.75 μmol/g of WD fermented sample). In the CD model, Fish oil presented lower levels of acetate (10.55±1.18 μmol/g of CD fermented sample) when compared to the WD samples (Negative control, FOS, and Fish oil). However, these values were not statistically different from the Negative control (26.28±3.03 μmol/g of CD fermented sample, 48 h) and FOS CD samples (23.14±1.89 μmol/g of CD fermented sample, 48 h). Interestingly, comparable observations were made for the Fish oil+Pomegranate oil mixture (36.43±4.21 μmol/g of CD fermented sample, 48 h). Previous studies have reported consistent results: statistically equivalent values were observed between the Negative control and the fermentation of Brewer's spent grain (85). This study was a result of human *in vitro* fecal fermentation. Some studies have demonstrated that acetate promotes metabolic syndrome in HFD-fed mice via parasympathetic activation (86,87).

Moreover, it was reported that acetate can increase the production of pro-inflammatory cytokines (IL-6, CXCL1, and CXCL2) (88). In this context, the decrease in acetate production by Pomegranate oil may be relevant, especially in the WD model. In contrast, acetate was demonstrated to protect adipose and hepatic metabolic perturbations (89). Long-term acetate depletion is also considered a risk factor for cognitive decline (90). Considering this, the capacity of Fish oil and Fish oil+Pomegranate oil to differentially increase the acetate concentration in the WD model may be highly relevant. These conflicting results are speculated to be related to the local concentration of SCFAs and the activated receptors (88). However, the capacity of this sample to increase acetate concentration is especially relevant, considering that this SCFA is often found to be decreased in a WD and HFD context. Importantly, this reduction often results in energy metabolism disorder and excessive reproduction of the pathogenic *Escherichia coli* (22).

3.4.2. Propionate

Furthermore, acetate, propionate, and butyrate treatment were demonstrated to regulate the body weight and diabetic parameters that HFD induced. Supplementation with these SCFAs was also able to reverse T cell polarization and cytokine profile (91). In our study, despite the ability of most of the fermented samples to increase acetate concentration, the same was not observed for propionate **(Figure 5.7)**. Indeed, at 48 h the samples of Fish oil WD, Pomegranate oil, and Fish oil+Pomegranate oil for both models showed lower propionate levels ($p<0.05$) when compared to Negative control and FOS for the WD model. Nevertheless, in all the samples, propionate production was observed from 6 to 48 h.

3.4.3. Butyrate

Butyrate is produced considerably at 48 h for all the samples for the WD model. HFD triggers low-grade inflammation, and propionate and butyrate have been shown to suppress pro-inflammatory cytokines and play an essential role in reversing these effects. Interestingly, butyrate was found to be decreased in rats fed an HFD (92). Moreover, in the case of WD-fed mice, a co-administration of inulin and butyrate was shown to attenuate liver steatosis (93), revealing the critical role of butyrate in restoring the effects of WD consumption. Importantly, although not

statistically significant, it seems that both Fish oil (2.49±0.08 μmol/g of the fermented sample) and Fish oil+Pomegranate (2.37±0.67 μmol/g of the fermented sample) WD samples tend to increase butyrate concentration when compared to Negative control WD (1.39±0.50 μmol/g of the fermented sample). This capacity was also higher when comparing these two samples with the Pomegranate oil sample (1.19±0.17 μmol/g of WD fermented sample, $p<0.05$).

3.4.4. SCFA synthesis pathways

To better understand how the different samples and the concentration of different bacteria may also indirectly and directly influence the levels of the identified SCFAs, it is also essential to consider these SCFA synthesis pathways. Acetate is known to be produced from pyruvate via 2 different pathways: acetyl-CoA is produced through the decarboxylation of pyruvate, and then acetyl-CoA is hydrolyzed. Bacteria such as *Bifidobacterium* and *Lactobacillus,* as well as *Prevotella* spp., *Ruminococcus* spp., *Bacteroides* spp., *Clostridium* spp., *Streptococcus* spp., *Akkermansia muciniphila*, and *B. hydrogenotrophica* use this pathway to produce most of acetate. Acetogenic bacteria produce one-third of the colonic acetate via the Wood–Ljungdahl pathway. In this pathway, the reduction of carbon dioxide generates carbon monoxide, which reacts with coenzyme A to produce acetyl-CoA. Acetyl-CoA is the substrate to produce acetate (81,82). Although there is a higher relative abundance of *Lactobacillus* in the CD group compared to WD groups, this was not translated into an increase in acetate levels in these samples. Interestingly, even though during the fermentation time, Fish oil presented significantly lower ($p<0.05$) acetate values in the CD group than in the WD group, the concentration of *Bifidobacterium*, *Bacteroides,* and *Streptococcus* was significantly higher in the CD group **(Figure 5.6)**. These results suggest that the reduction in the relative abundance of the mentioned bacteria does not affect acetate production, which, considering the positive, although controversial, effects of acetate may be highly relevant.

On the other hand, propionate is produced by colonic bacteria using the succinate, acrylate, and propanediol pathway. The succinate pathway has a four-carbon pathway comprising malate, fumarate, succinate, and methylmalonyl-CoA as the intermediates. Bacteroidetes and several Firmicutes belonging to the Negativicutes class are known to use this pathway. In the end, 2 molecules of propionate and 1 molecule of acetate are generated. In the acrylate pathway, lactate is used as the precursor. This pathway is only present in a very reduced number of gut bacteria, including *Coprococcus catus*. In the propanediol pathway, propionate is synthesized by converting deoxy-sugars, fucose, and rhamnose. Only a few bacteria genera can form propionate, and these pathways are more conserved and substrate-specific. *Salmonella enterica* and *Roseburia inulinivorans* are bacteria known to use this pathway. As well as *A. muciniphila*, the major propionate producer (81,82). The small relative abundance of Bacteroidetes and the decreased relative abundance of Negativicutes (results not shown) may explain the lower propionate concentrations observed in this study.

Lastly, butyrate is mainly produced by glycolysis from carbohydrates and formed via 2 different pathways: butyrate kinase pathway, where it is formed from acetoacetyl-CoA by phosphotransbutyrylase and butyrate kinase using two acetyl-CoA molecules. *Ruminococcus* spp. is the primary producer of butyrate using this pathway. Acetate can be converted to butyrate via the butyryl-CoA: acetate CoA-transferase route, mainly used by butyrate-producing bacteria (*Anaerostipes* spp., *Coprococcus catus*, *Eubacterium rectale*, *Eubacterium hallii*, *Faecalibacterium prausnitzii* and *Roseburia* spp.). In the case of lactate-utilizing bacteria, acetyl-CoA can be produced from lactate (81,82). In this study, there is no observation of a decreased concentration of acetate that may undoubtedly justify its use for the synthesis of butyrate. Notably, butyrate can also be formed from amino acids such as lysine, glutamate, and 4-aminobutyrate pathways. These pathways are found in Firmicutes bacteria such as *Fusobacterium* spp., *Peptostreptococcus asaccharolyticus*, *Clostridium sporosphaeroides*, *Acidaminococcus fermentans*, and *Clostridium symbiosum* (82). Considering the high relative abundance of Firmicutes found in all the samples **(Figure 5.3)**, the production of butyrate from these pathways

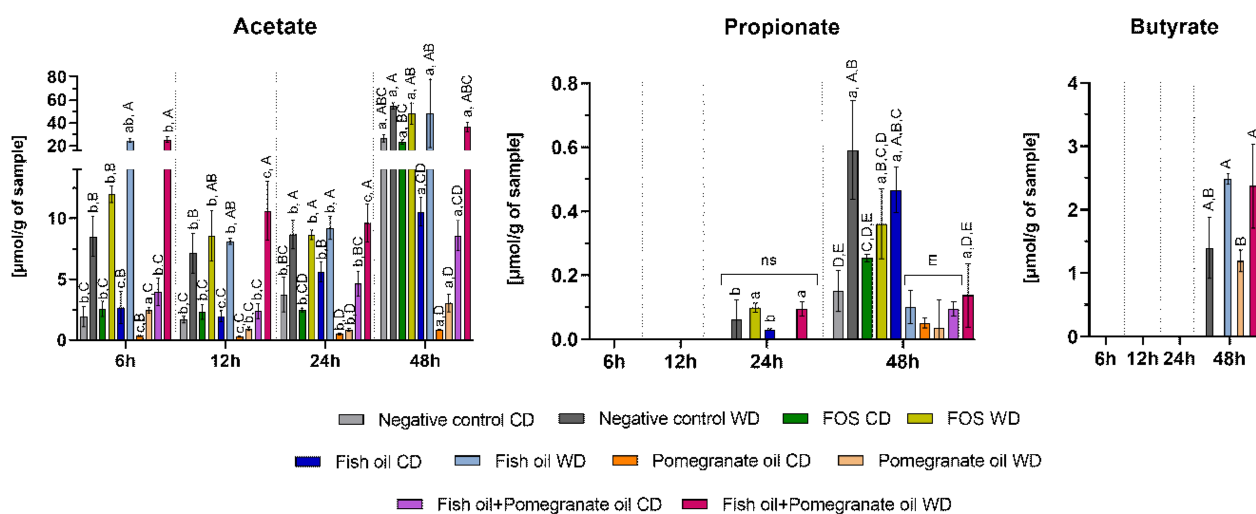


Figure 5.7 - Major SCFAs (Acetate, Propionate and Butyrate) concentration (µmol/g of the sample) of the different samples at the different fermentation times (0, 6, 12, 24 and 48 h). CD-Control Diet and WD- Western Diet models. The values presented are the means of three biological and two analytical replicates the bars represent the standard deviation. Different letters indicate significant differences ($p < 0.05$). The capital letters (A,B,C) indicate the differences among samples - Negative control, Positive control (FOS) and bioactive lipids sources (Fish oil, Pomegranate oil and a mixture of both) - at the same time. The small letters (a,b,c) indicate the differences for the same sample over time.

may be a possible explanation for the butyrate production in WD samples, and this possibility is explored in the next section.

3.4.5. pH variations throughout the fermentation time

In addition to SCFA quantification, changes in pH are known to vary depending on the SCFA concentration. Here, a decrease in pH for all the samples from 0 to 6 h was observed, which may be consistent in some samples with the increased production of some SCFA (**Figure 5.8 and Supplementary Table S7)**. Nevertheless, FOS samples presented lower pHs ($p<0.05$) than the remaining samples. The production of different metabolites, such as lactic acid, may explain this. Although, this study did not measure lactic acid concentrations, previous works using

similar *in vitro* fermentation models but using human fecal slurry as inoculum reported lactic acid as the major metabolite produced during the entire fermentation of FOS, and such increment was associated with a decrease in pH (36,38). Moreover, the difference in the pH for the FOS WD and CD samples may also be associated with lower production of lactic acid in the FOS WD samples due to the lower relative abundance of *Lactobacillus* bacteria in this group **(Figure 5.3).**

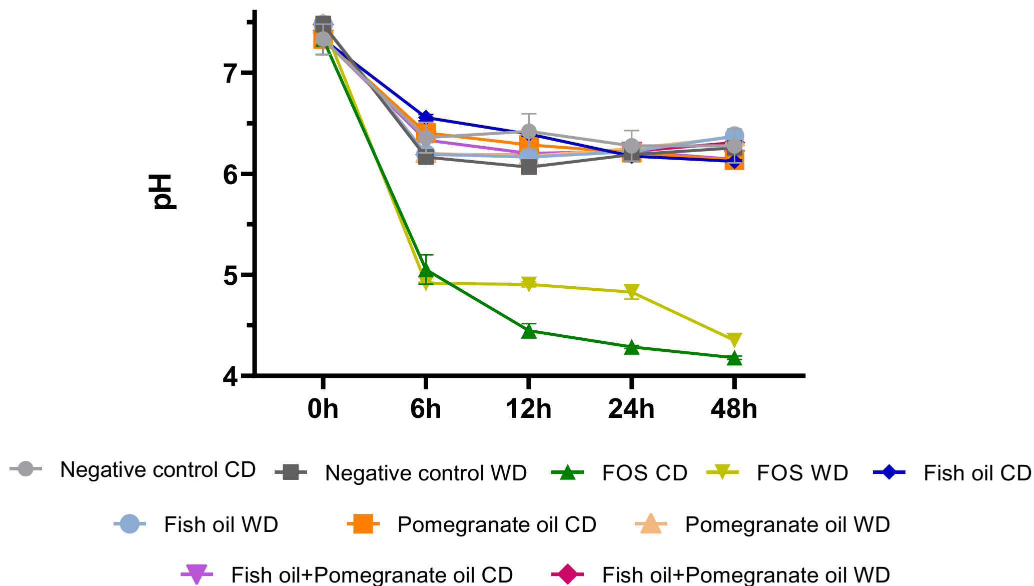


Figure 5.8- pH determination at different fermentation times (0, 6, 12, 24, and 48 h) for the different bioactive lipids' sources (Fish and Pomegranate oil and a mixture of both) and the Negative and Positive control (FOS) considering the two different models (CD – Control diet and WD- Western Diet models). The results are the means of three biological replicates. The bars represent the standard deviation.

3.5. Free Amino acids and GABA detection

Besides the production of SCFAs, a healthy gut microbiota has as its core functions the biodegradation of polysaccharides, the enrichment of specific LPS, and the production of vitamins and essential amino acids (94). Amino acids, for instance, can be used to synthesize bacterial cell components or catabolize through different pathways. Indeed, the diversity of amino acid metabolism in gut bacteria may positively or negatively impact the host (95). As already mentioned, SCFAs can also be produced from peptide and amino acid metabolism and not only from dietary fiber fermentation. Although amino acid metabolism is only used by the gut microbiota to a degree of less than 1% (82), this route of SCFA production is usually not considered at all. The most abundant amino acid fermenting bacteria in the human small intestine are bacteria belonging to the *Clostridium* clusters and Proteobacteria (95). Considering the high relative abundance of Proteobacteria, especially in WD samples **(Figure 5.3)**, amino acid fermentation is a process worth considering. In addition to SCFA production, intestinal microbiota is also able to *de novo* synthesize amino acids. However, knowledge regarding amino acid synthesis, production, and use by these bacteria and their impact on host health is still scarce (96).

Considering this, the amino acid concentration was assessed in this study for all the fermented samples and the negative controls at the different fermentation times. The baseline microbiota (Negative control, time 0 h) for both models (CD and WD) already showed some significative differences ($p<0.05$): aspartic acid (16.37±1.06 and 12.75±0.84 mg/L), serine (16.6±1.48 and 1.67±0.22 mg/L) and GABA (4.70±0.71 and 26.78±7.15 mg/L) for CD vs. WD models (**Table S7 Supplementary material**), respectively. Deamination and decarboxylation of certain amino acids, including aspartate and glutamate, threonine, alanine, lysine, and glycine, produce SCFAs which, as mentioned, may have protective effects against certain diseases, enhancing the gut barrier function and immunity (97). In addition, serine metabolism has been pointed out as necessary for macrophage glutathione synthesis to support IL− 1β cytokine production (98). This may explain the lower aspartic acid and serine values observed in the WD model compared to CD. On the other hand, the higher GABA concentration in the WD model is inconsistent with the higher relative abundance of both *Lactobacillus* and *Bifidobacterium* (known GABA producers) observed in CD **(Figure 5.3)**. Interestingly, a study has reported that HFD decreases GABA levels in the frontal cortex and hippocampus of rats, influencing feeding behavior (99). This decreased concentration of GABA in these brain areas in HFD-fed rats compared to control rats may explain the differences observed here in the cecal samples of rats of both CD and WD.

In **Figure 5.9**, it is possible to observe that during the fermentation time, from 6 to 48 h of fermentation, the concentration of aspartic acid, cysteine, asparagine, serine, and threonine decreased except for the FOS samples for both models (CD and WD). In the case of tryptophan, the concentrations decreased for all the samples. Regarding methionine, after 48 h, its concentration decreased and was significantly lower ($p<0.05$) for the Fish oil+Pomegranate oil sample in the WD model and higher for the FOS WD sample. Regarding glutamic acid, its concentration significantly decreased in Negative control, FOS, and Fish oil+Pomegranate oil samples of the WD group. Glycine, threonine, glutamate, lysine, and aspartate can be metabolized to acetate, while threonine, glutamate, and lysine can be used for butyrate synthesis. Propionate is mainly produced from threonine (95). Thus, the aspartic acid decrease may be associated with acetate synthesis, while the threonine decrease may be associated with the synthesis and increase in the concentration of all the analyzed SCFAs (acetate, propionate, and butyrate). In FOS samples, since there seems to be no decrease in the concentration of these amino acids, the synthesis of these SCFAs may be related to a higher extent to the fermentation of FOS, a preferred route for SCFAs synthesis, rather than being due to amino acids metabolism. As mentioned, most of the samples also decreased in serine concentration throughout the fermentation time. Serine is, indeed, an important metabolite and precursor to multiple amino acids in bacteria, such as glycine, cysteine, and tryptophan, and it is also a precursor for the metabolism of sphingolipids, folate, methane, sulfur, cyanoamino acid, and pyruvate (96). Although glutamate can also be a precursor for acetate synthesis, is more often associated with butyrate production (82,95). In this study, there is a significant decrease in the concentration of

glutamate and an increase of butyrate levels in Negative control, FOS, and Fish oil+Pomegranate oil samples of the WD. These results suggest that butyrate production in these samples may result from glutamate fermentation. Glutamate is a key nitrogen/amino group donor for amino acid synthesis and is a major entry point of ammonia into bacterial metabolism. These enzyme pathways are essential for some gut bacteria that rely on ammonia as a nitrogen source, such as several *Bacteroides* spp. (96).

3.5.1. Gut-brain axis modulation prediction

As mentioned, intestinal bacteria are known to be able to *de novo* produce amino acids, although such processes are still poorly understood, this is thought to be highly relevant to compensate for indispensable amino acids deficiency in low-quality protein diets (96). Tyrosine concentrations, for instance, increased in the Fish oil+Pomegranate oil WD sample to higher values than in FOS samples in the CD group. This is highly relevant since tyrosine is a precursor for dopamine. In schizophrenia studies in human subjects, it was demonstrated that such disease is associated with the potential for gut bacterial biosynthesis of tyrosine, and this amino acid is essential in alleviating cognitive dysfunction (100). *Escherichia* spp. and *Bacillus* spp. have been reported to produce both dopamine and norepinephrine (96,101). Considering the increase in tyrosine concentrations and a high relative abundance of the *Escherichia-Shigella* genus, the Fish oil+Pomegranate oil WD samples may present a potential for higher dopamine and norepinephrine production. Recent studies have demonstrated that bacteria isolated from the mammalian gut can synthesize neuroactive compounds, including neurotransmitters, like the mentioned norepinephrine, and many of these result from the catabolism of amino acids. These findings support the hypothesis of a gut-brain axis model, which may be highly relevant in some diseases. Such neuroactive compounds include the already mentioned GABA, which is produced by *Lactobacillus* spp., *Bifidobacterium* spp., *Lactococcus lactic*, norepinephrine which is produced by *Escherichia* spp., and *Bacillus*, dopamine produced by *Bacillus*, histamine and serotonin produced by *Streptococcus* spp., *Escherichia* spp. and *Enterococcus* spp. (96). GABA concentrations increased, especially in WD samples, specifically FOS, Fish, and Pomegranate oil. GABA is microbially produced by the decarboxylation of glutamate via the enzyme glutamate decarboxylase (96). Here, it was observed a considerable decrease in glutamate concentration in WD groups' FOS and Fish oil+Pomegranate oil. This decrease along with a considerable relative abundance of *Bifidobacterium* and *Lactobacillus* may explain the increase in GABA concentration in these samples. This is a significant result considering that HFD is usually responsible for GABA decrease in the frontal cortex (99,102). Notably, it has been reported that GABA signaling has a crucial role in the interplay between the hypothalamus and nucleus accumbens (which play a role in motivation and cognitive processing of aversion) to promote feeding. Interestingly, perinatal maternal consumption of WD presented a long-lasting influence on GABAergic markers, plasticity, and epigenetic markers in both the homeostatic and the reward

pathways implicated in feeding behavior (103). This is highly relevant since GABA, being an inhibitory neurotransmitter, is reported to induce satiation (104).

3.5.2. Branched-chain amino acids

Nonetheless, bacterially-produced amino acids could also present some detrimental consequences in conditions such as insulin resistance in T2DM, where systemic concentrations of amino acids such as aromatic and branched-chain amino acids (leucine, isoleucine, and valine) are elevated (95,96). Here, we observed that Fish oil+Pomegranate oil in the WD sample significantly decreased valine concentration **(Figure 5.9)** after 48 h of fermentation. Moreover, in isoleucine and leucine **(Table S8 supplementary material)** there was a decrease in these amino acids' concentration in the Negative control sample of the WD group but not in all the other samples. Although no decrease was observed, the concentration of these branched-chain amino acids was maintained constant throughout the fermentation time. These observations are important in a WD context: restriction of branched-chain FAs consumption in a WD setting can prevent the development of obesity and insulin resistance in mice. In fact, this is hypothesized to be a promising strategy for mitigating diet-induced obesity (105).

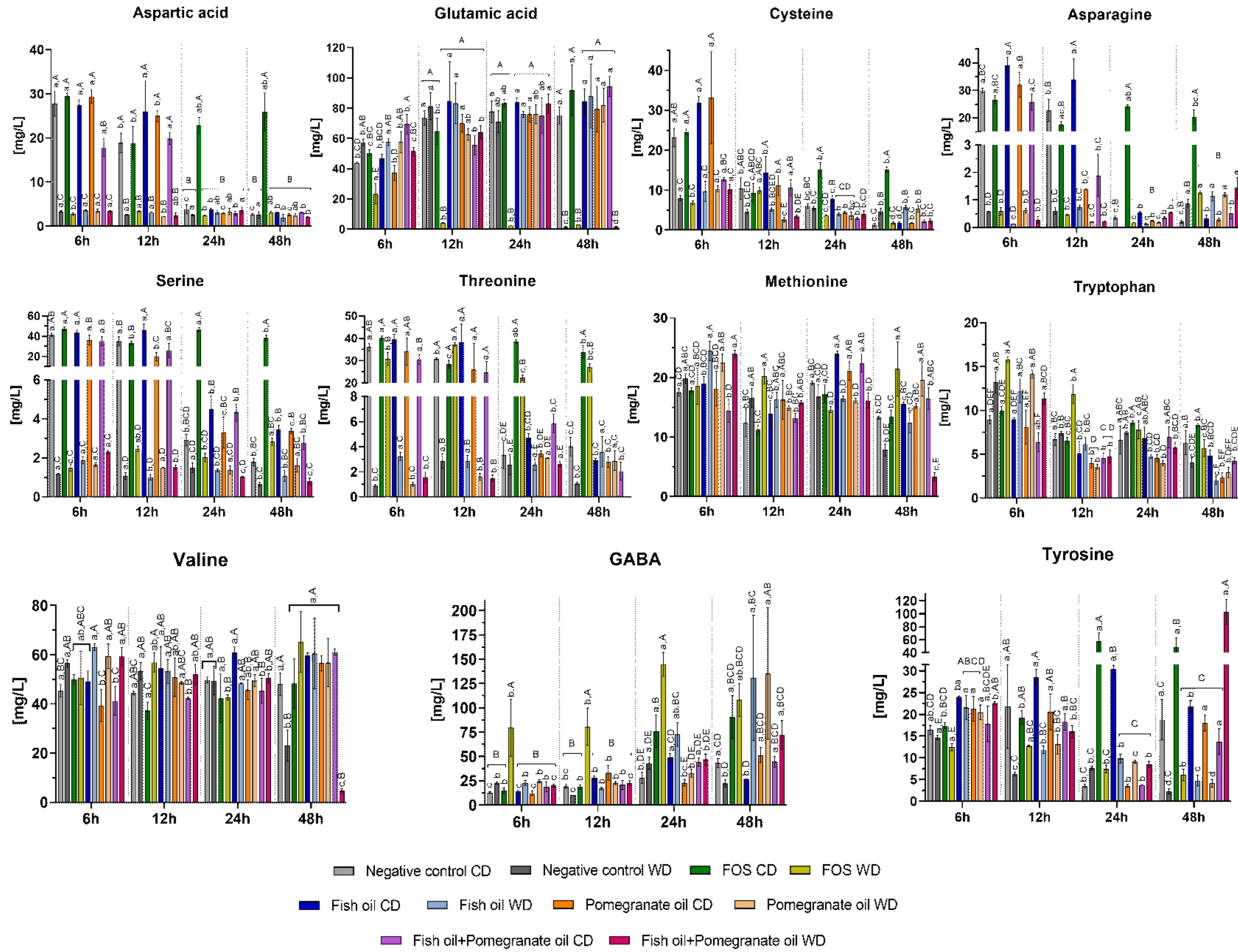


Figure 5.9- Amino acids concentration (mg/L) at different fermentation times (0, 6, 12, 24 and 48 h) for the different bioactive lipids' sources (Fish and Pomegranate oil and a mixture of both) and for the Negative and Positive control (FOS) considering the two different models (CD – Control diet and WD- Western Diet models). The results are the means of three biological and two analytical replicates. The bars represent the standard deviation. Different letters indicate significant differences ($p < 0.05$). The capital letters (A,B,C) indicate the differences among samples - Negative control, Positive control (FOS) and bioactive lipids sources (Fish oil, Pomegranate oil and a mixture of both) - at the same time. The small letters (a,b,c) indicate the differences for the same sample over time

4. Conclusion

The influence of gut microbiota in the onset and development of several metabolic diseases, such as obesity and T2DM, has gained attention over the last few years. Indeed, diet plays an essential role in gut microbiota modulation. It has been reported that diet, and not obesity itself, for instance, is the primary driving force behind gut microbiota alterations (59). Indeed, WD was shown to alter gut microbiome composition, diversity index, microbial relative levels, and functional pathways demonstrated through SCFA and amino acids quantification analysis. These alterations in gut microbiota have been described as predisposing factors to several chronic diseases. In addition to polysaccharides, other bioactive molecules have demonstrated the capacity to modulate gut microbiota. Considering this, the term prebiotic has been changed to include other ingredients that allow not only composition changes but also the metabolic activity of the gastrointestinal microbiome, which may confer beneficial health effects.

In this study, the effect of Fish oil as an omega-3 FAs source (EPA and DHA) and Pomegranate oil as the source of PUA, and a mixture of both oils were subjected to *in vitro* fecal fermentations using as fecal inoculum cecal samples from rats from two different dietary groups: control diet (CD) and the WD (high-fat high-sugar based diet). Compared to the samples before fermentation, it was observed a decrease in the concentration of most bioactive FAs after 6 h of fermentation in all the samples. We hypothesized that this is a result of converting FAs into microbial metabolites and incorporating microbial FA metabolites into bacterial cells. Moreover, this study demonstrated that the initial concentration of the bioactive FAs in the sample may be a relevant parameter to consider when assessing their effect on microbiota modulation.

Afterward, the 16S amplicon metagenomics sequencing approach analyzed the bacterial population's diversity. Using the Chao1 and Shannon diversity indexes, the Fish oil+Pomegranate oil sample, especially in the WD group, was observed as the sample with higher diversity. Considering these results, it was demonstrated for the first time that the mixture of both oils, due to significant bioactive FA content, may have a relevant effect in restoring the WD-induced gut microbiota alterations and possibly have a positive impact on WD-induced diseases. Although WD seems not to affect α-diversity Chao1 and Shannon indexes, it is important to mention that significantly lower α-diversity (Shannon index) is often observed in T2DM and other diseases. Supporting these observations, Fish oil+Pomegranate oil samples in the WD model can also increase the relative abundance of the Firmicutes and Bacteroidetes phylum to similar levels as the control group (CD). This capacity may restore the Firmicutes/Bacteroidetes ratio that is often found to be affected in obesity and T2DM. Besides, this sample can also increase *Akkermansia* and *Blautia* relative mean abundances affected by the WD consumption.

Regarding the microbial metabolites production, acetate was the major SCFA quantified in all the samples. Both groups' fermented samples increased their concentration from 6 to 48 h. On the other hand, butyrate showed considerable production after 48 h for all the samples for the WD model. These are important results to consider since WD decreases SCFA levels, and this

reduction is often associated with the development of chronic diseases. In fact, WD is known to create a specific inflammatory environment in the gut due to the overgrowth of pro-inflammatory Proteobacteria such as *E. coli* along with a significant decrease of SCFAs concentrations like what happens in Chron's disease (84). So, the capacity of Fish oil+Pomegranate oil, especially in the WD group, to decrease the relative abundance of Proteobacteria and *Escherichia-Shigella* group and increase acetate and butyrate concentration is highly relevant.

Nevertheless, SCFAs can also be produced from peptide and amino acid metabolism. Indeed, in our study, we observed a decrease in the concentration of aspartic acid, cysteine, glutamic acid, asparagine, serine, threonine, and tryptophan in most samples. Threonine, glutamate, and aspartate can be metabolized to acetate, while threonine and glutamate can be used for butyrate synthesis. So, the aspartic acid decrease may be associated with acetate synthesis, and the observed threonine concentration decreases with the synthesis of all the analyzed SCFAs. Glutamic acid can be a precursor for both acetate and butyrate.

Amino acids are also precursors of important neuroactive molecules. Indeed, tyrosine concentrations increase in the Fish oil+Pomegranate oil WD sample, which may be significant since tyrosine is a precursor for dopamine and norepinephrine. GABA, an important neurotransmitter, was also increased, especially in WD samples, specifically FOS, Fish oil, and Pomegranate oil samples. These findings may suggest a positive impact on gut-brain axis modulation, which may be highly relevant in some diseases. But further studies are required.

It is important to mention that the lack of standardization of HFD and WD in rodent models makes it difficult to compare different studies. Indeed, diet is such an essential process in gut microbiota modulation that it is easily understood that different fat or carbohydrate (precisely simple sugars) compositions will strongly impact the microbial population. Nevertheless, all these results demonstrate, for the first time, the great potential of using a mixture of both Fish and Pomegranate oil to restore the gut microbiota changes associated with WD consumption and pave the way for further studies focused on the WD-associated diseases such as obesity or T2DM.

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CHAPTER 6

The role of CB1 and GPR120 receptors in omega-3 and punicic acid effects in adipogenesis and intestinal permeability

This chapter intends to uncover the role of CB1 and GPR120 receptors in adipogenesis and of CB1 in the Caco-2 cell permeability model.

This chapter is awaiting submission and is going to be presented as:

Salsinha, A.S., Machado, M., Rodríguez-Alcalá, L.M., Relvas, J.B., Pintado, M. Uncovering the role of CB1 and GPR120 in the effect of Fish oil and Pomegranate oil in adipogenesis and Caco-2 permeability.

Chapter 6

Chapter 6

Preface

Is the CB1 receptor involved in omega-3 (EPA and DHA) and PUA bioavailability using a Caco-2 cell permeability model?

What are the potential effects of Fish oil and Pomegranate oil in adipogenesis? Are those effects mediated by CB1 and GPR120 receptors?

In **Chapter 3** it was demonstrated that through oral supplementation most bioactive FAs, delivered in the form of enriched oils, have a low bioaccessibility index and bioavailability potential. Consequently, it was demonstrated that there is a high availability of these bioactive FAs in the colon, justifying the importance of studying their potential modulatory effect on gut microbiota (as demonstrated in **Chapters 4** and **5**). But the presence of such molecules in the colon is not only going to be available to modulate gut microbiota but importantly, is also going to affect intestinal permeability by altering intestinal cell membrane fluidity, for example.

In consequence, in this last chapter, the effect of Fish oil, Pomegranate oil, and a mixture of both oils in an extensively described and simple model of intestinal permeability – the use of a monolayer of Caco-2 cells - was assessed, and the role of CB1 receptor in mediating these FAs intestinal permeability effects was screened. This is an important study to consider since in this thesis we are assessing the use of omega-3 and PUA-enriched oils in obesity treatment, aiming for a possible systemic approach. So, the knowledge of the molecular mechanisms behind the effects of these bioactive FAs in intestinal permeability is of foremost importance in designing a complete approach targeting gut microbiota, intestinal permeability, adipose tissue, and neuroinflammation modulation for obesity treatment.

Besides, the importance of adipose tissue in obesity progression and the positive role of these bioactive FAs in adipose tissue modulation is extensively described. So, the role of Fish oil, Pomegranate oil, and a mixture of both in adipogenesis was screened, as well as some molecular mechanisms behind their effects. For the first time, the role of both CB1 and GPR120 receptors in mediating Fish oil and Pomegranate oil effects in adipogenesis was studied.

This study intended to pave the way for new lines of investigation and demonstrate that a systemic approach using this kind of enriched oil matrixes may be a potential target therapy to consider in both obesity prevention and treatment.

Chapter 6

Uncovering the role of CB1 and GPR120 in the effect of Fish oil and Pomegranate oil in adipogenesis and Caco-2 permeability

Ana Sofia Salsinha[a,b], Manuela Machado[a], Luís Miguel Rodríguez-Alcalá[a], João B. Relvas[b,c] and Manuela Pintado[a]*

a. Universidade Católica Portuguesa, CBQF - Centro de Biotecnologia e Química Fina –Laboratório Associado, Escola Superior de Biotecnologia, Rua de Diogo Botelho, 1327, 4169-005, Porto, Portugal

b. Instituto de Investigação e Inovação em Saúde and Instituto de Biologia Molecular e Celular (IBMC), Universidade Do Porto – Rua Alfredo Allen, 208, 4200-135, Porto, Portugal

c. Departmento de Biomedicina, Faculdade de Medicina da Universidade do Porto (FMUP), 4200-319 Porto, Portugal

Abstract

The CB1 receptor is a widespread and important endocannabinoid system mediator that is involved in several biological processes: sensory and memory perception, motor activity, adipogenesis, pro-inflammatory processes, and gut permeability. The endocannabinoid system is not limited to this receptor. GPR120 has been pointed out as an important mediator of omega-3 fatty acids, including in adipose tissue development. With chemical agonists and antagonists of both CB1 and GPR120 receptors, their mediatory role in Fish oil and Pomegranate oil effect on adipogenesis and Caco-2 cell permeability was studied. It was demonstrated that despite CB1 modulation, the permeability results for the major bioactive fatty acids (omega-3 – EPA, DPA, and DHA – and the CLNA isomer, punicic acid) were very low or null. Nevertheless, the use of the CB1 agonist and antagonist in combination with the oil samples and alone, demonstrated that CB1 may have a role in intestinal permeability and that different polyunsaturated fatty acids composition will respond differently to CB1. PUFA incorporation in Caco-2 cell membranes negatively impacted their permeability. Some of the mechanisms behind omega-3 EPA, DHA, and DPA bioaccessibility and bioavailability may be mediated through the CB1 receptor, but further studies are required. Fish oil, Pomegranate oil, and Fish oil+Pomegranate oil samples were able to decrease adipogenesis in 3T3-L1 differentiated pre-adipocytes. Upon CB1 activation the capacity of Fish oil and Pomegranate oil to inhibit adipogenesis significantly decreases. Additionally, at the studied concentration GPR120 agonist (TUG-891) functioned as a positive enhancer of the adipogenic process and it was able to counteract the negative effects of the Fish oil and Pomegranate oil samples. In the presence of its antagonist (AH7614) the adipogenesis inhibition potential that all the samples presented was abrogated. This suggests an important role of GPR120 and CB1 in Fish oil and Pomegranate oil adipogenesis effects.

Keywords: Fish oil; Pomegranate oil; adipogenesis; Caco-2 permeability; CB1; GPR120

Graphical Abstract

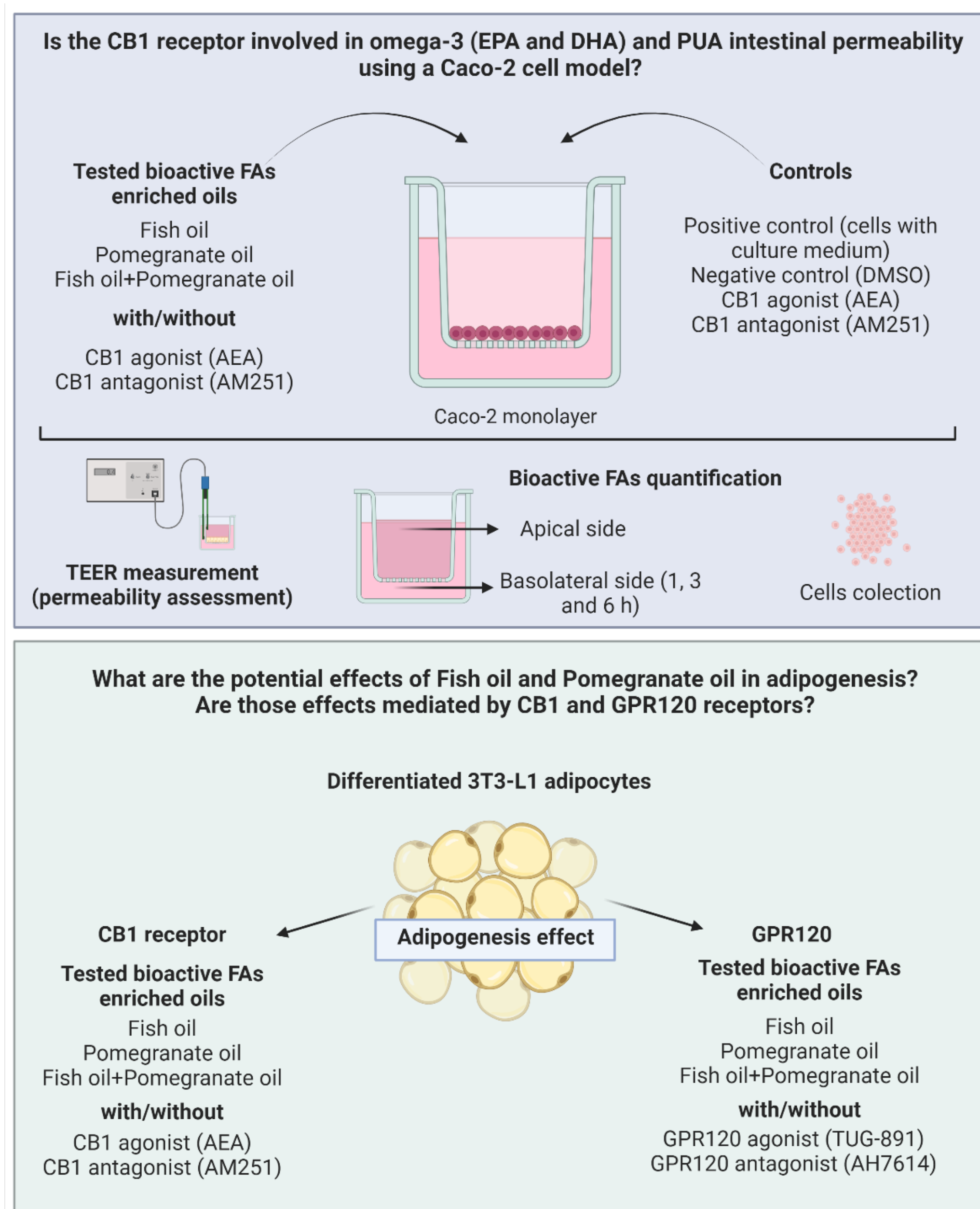

Figure 6.1 – Graphical Abstract. The involvement of CB1 receptor in omega-3 (EPA, DPA and DHA) and PUA in intestinal permeability using a Caco-2 cell model was assessed. For that it was used a chemical agonist (AEA) and antagonist (AM251) of CB1 receptor. The bioactive FAs were quantified through GC-FID in the apical side, basolateral side at the different time-points (1, 3 and 6 h) and in the collected cells. In addition, the role of CB1 and GPR120 receptors in Fish oil, Pomegranate oil and a mixture of both oils in adipogenesis was determined by using the same chemical agonist/antagonist reported for CB1 and the GPR120 TUG-891 agonist and AH7614 antagonist.

1. Introduction

The endocannabinoid system comprises different ligands, analogs, and enzymes that are present in different organs and tissues, such as the brain, liver, adipose tissue, muscle, pancreas, and gut microbiota. This system and its enzymes are among the most potent immunoregulatory compounds since they can regulate the function of several cell sub-systems of either innate or adaptative immunity, mostly exhibiting anti-inflammatory activity (1). As mentioned in **Chapter 1 Section 4.2.1**, AEA and 2-AG are two of the most studied endocannabinoid ligands and activate GPCRs CB1 and CB2 receptors. Unlike classical neurotransmitters or other intracellular molecules, AEA and 2-AG are synthesized "on demand" from AA in a cell and time-specific manner in the membrane of different cell types such as neurons, adipocytes, and skeletal muscle, in response to increased intracellular Ca^{2+} concentration, membrane depolarization, and/or receptor stimulation (2). CB1 is the most abundant and widespread GPCR in the mammalian CNS, being mostly abundant in distinct areas of the brain and peripheral nerve terminations, inhibiting the release of neurotransmitters involved in processes such as sensory perception, memory processing, and motor activity. It is also expressed in non-neuronal peripheral tissues, including the gut, where it is involved in nociception, adipogenesis, and pro-inflammation processes. CB2 is mostly expressed in peripheral organs, lymphoid tissue, and myeloid cells participating in the immune response through β and T lymphocytes. CB2 is also prevalent in other immune cells such as microglia, and CB2 activation seems to be the main target for inflammation-dependent neurodegeneration (1,2). Considering that there is a high abundance of endocannabinoid receptors and enzymes in central and peripheral nervous systems, these mediators can rapidly react to disturbances in the gut to maintain homeostasis according to its physiological needs. Moreover, PEA, an endocannabinoid system analog, has been demonstrated to have a protective effect in several models of neurogenic inflammation, neuropathic pain, and neuroprotection in Parkinson's and Alzheimer's disease. In addition, an altered endocannabinoid signaling has been associated with changes in intestinal permeability, inflammation, and incretin release in human obesity. Considering all of this, targeting this system may be a promising therapy to modulate the microbiome, the gut-brain axis, and potentially CNS alterations (1). In obesity, for instance, the use of CB1 antagonists in mouse models of diet-induced obesity increased *A. muciniphila* and decreased Lachnospiraceae and Erysipelotrichaceae levels, attenuated the associated inflammatory state, and improved hyperglycemia and insulin resistance (2,3). Moreover, CB1 blockade was found to reduce body weight and alleviate obesity-related metabolic disorders via central and/or peripheral effects (4). Moreover, WD (high-fat high-sugar consumption) causes an immediate decrease in the expression of ileal CB2, which is suggested to play a protective role against insulin resistance and inflammation, and PPAR-γ. So, it has been suggested that the WD-induced increase in endocannabinoidome mediators may result in preferential binding to CB1 over CB2 and PPAR-γ (5).

Intestinal permeability refers to the barrier properties of the intestinal mucosa that prevent harmful substances from penetrating the mucosa (6). CB1 agonists AEA, LPS, and cytokines have been described as "gate openers" since they increase gut permeability (4). Some studies have tried to explore the effects of cannabinoids on intestinal permeability in Caco-2 *in vitro* models. One such study reported that the apical application of two phytocannabinoids (Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD)) increased permeability (6,7).

Moreover, the role of the endocannabinoid system in the regulation of BAT and thermogenesis has been proposed to be dependent on CB1 (as reviewed by Cani et al. (4)). Indeed, in obesity rodent models and obese humans, it has been reported a close association between the development of obesity with a simultaneous overactivation of the endocannabinoid system, as shown, among other factors, by overactivation of CB1 receptor in several peripheral tissues involved in metabolic processes such as adipose tissue (8). Overactivation of CB1 has also been demonstrated in cultured adipocytes under conditions of hyperinsulinemia (9) Moreover, a similar upregulation of the endocannabinoid signaling has also been observed: significantly higher levels of 2-AG, but not AEA, have been detected in the visceral fat depot of obese patients (9). Adipocytes not only express CB1 receptors but are also endowed with the full biochemical machinery to synthesize and degrade endocannabinoids. Although the presence of the CB1 receptor in adipocytes is well documented, its functional role is still not well understood (8).

PUFAs are precursors of endocannabinoid molecules, in the brain, for instance. Omega-3 PUFAs have been hypothesized to serve as substrates for docosahexaenoyl ethanolamide and eicosapentaenoyl ethanolamide, which are also able to bind CB1 and CB2 but with lower affinity than AEA. Due to competition between these ligands, they might decrease the activation of endocannabinoid receptors by AEA (4). Interestingly, a diet enriched with DHA has been shown to increase the expression of both CB1 and CB2 receptors in muscles. It has been suggested that in the musculoskeletal system, dietary PUFAs could affect both endocannabinoid levels and the regulation of the proteins of the endocannabinoid system. Moreover, a diet with 5% krill oil (rich in EPA and DHA) enhanced the activity of CB1 receptors in mice (10).

Importantly, the endocannabinoid system is not limited to the activity of specific endocannabinoids on the mentioned CB1 and CB2 receptors, indeed endocannabinoids also interact with PPARs (PPAR-α and -γ), as well as with GPR, like GPR-55 and GPR-119 (4). Moreover, several studies have demonstrated that there is a key role of GPR120 in adipose development including both white and brown adipocytes (11). Indeed, in GPR120 knockdowns the adipogenesis process is inhibiting indicating that GPR120 might act as an adipogenic receptor in 3T3-L1 cells (12–15).

Considering the effects of these receptors in adipogenesis and gut permeability, and considering the possible mediatory effect of PUFAs, namely omega-3, the role of CB1 and GPR120 receptors in these processes and their influence not only in omega-3 but also in CFAs action were assessed.

2. Material and Methods

2.1. Reagents

C18:1 anandamide, in this work, simply defined as AEA, (870432P) was obtained from Sigma-Aldrich (Missouri, USA). AM251 (cat. No. 1117) was from Tocris Bioscience (Bristol, UK). The Adipogenesis kit (ab133102) was from Abcam (Cambridge, UK). The Ethanol absolute anhydrous was purchased from Carlo-Erba (Barcelona, Spain). Both TUG-891 (ortho-biphenyl ligand 4-{[4-fluoro-4′-methyl(1,1′-biphenyl)-2-yl]methoxy}-benzene propanoic acid, SML2025) and AH7614 (4-methylN-9H-xanthen-9-yl-benzenesulfonamide, SML1914), were purchased from Sigma-Aldrich (Missouri, USA).

For the cell culture, DMSO was obtained from Sigma-Aldrich. DMEM and non-essential amino acids were from Gibco (Thermo Scientific, Massachusetts, USA). FBS was purchased from Biowest (France), Penicillin-Streptomycin-Fungizone was obtained from Lonza (Belgium) and Calf-bovine serum iron Fortified was obtained from ATCC (USA).

For the FA analysis, Tritridecanoin was obtained from Larodan (Sweden); HPLC grade Methanol, Hexane, and DMF were obtained from VWR (USA), and Sodium methoxide was purchased from Acros Organics (USA). The standards Supelco 37 and Sulfuric acid were obtained from Sigma-Aldrich (Missouri, USA).

Regarding the western-blot reagents, both RIPA buffer (R0278), Protease inhibitor cocktail (P8340), and Ponceau S dye (78376) were from Sigma-Aldrich. The GPR120 (extracellular) Polyclonal Antibody (PA5-111778) was from Sigma-Aldrich. The 0.45 µm nitrocellulose blotting membranes (10600002) were purchased from Amersham Protran. The SuperSignal West Pico Plus Chemiluminescent Substrate (34580) was from ThermoFisher.

2.2. Bioactive fatty acid sources

The samples used in this study are thoroughly described in **Chapter 3 in Section 2.2**. For this study, only Pomegranate oil, Fish oil, and a mixture (1:1) of both oils were selected considering the results obtained from the previous works (**Chapters 2, 3, 4, and 5**). The samples were previously digested, as described, by using the INFOGEST 2.0 protocol to stimulate the GIT digestion.

2.3. Caco-2

Like what was described in **Chapter 3 Section 2.6.1**, the Caco-2 (Caucasian colon adenocarcinoma) cell line is established for the study of intestinal permeation of bioactive molecules. Caco-2 (ECACC 86010202) cell line, obtained from the European Collection of Authenticated Cell Cultures, was grown in DMEM (4.5 g/L glucose, 4 mM L-glutamine without pyruvate) supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) penicillin and streptomycin

and 1% (v/v) of non-essential amino acids. Cells were maintained at 37 °C under a 5% CO_2 water-saturated atmosphere. Upon 70-80% confluence, cells were collected using trypsin. The Caco-2 cell seeding in Transwells (Milicell hanging cell culture insert, PET 0.4µm, 12-well, 48/pk, Merck) was performed in 3×10^5 cells/well.

2.3.1. FAs transepithelial diffusion across intestinal (Caco-2) cell layer

The permeability assay was carried out using Transwell inserts and 12-well plates. Caco-2 cells were seeded into the inserts to mimic the absorptive epithelia of the human intestine. For culture medium replacement, the medium was removed from the wells and 0.5 and 1.5 mL of fresh culture medium were added to the apical and basolateral sides, respectively. The cells were incubated for 21 days. On the day of the study, the culture medium was removed. Medium in the basolateral side (receptor part) was replaced with 1.5 mL of fresh medium. The medium on the apical side (donor part) was replaced with the corresponding samples. Briefly, 100 µL of digested samples were added to 900 µL of the medium, fully dissolved by vortexing, and further diluted at 1:100 in the culture medium. This concentration was determined based on previous tests on cell cytotoxicity. 500 µL of each sample solution was added in duplicate to the plate. The agonists and antagonists of both CB1 and GPR120 receptors were added according to the concentrations described in the next sections. DMSO (30% v/v) was used as the negative control and culture medium as the positive control. 500 µL aliquots were withdrawn from the basolateral side at 0, 1, 2, 3, and 6 h. After 6 h the apical content was completely removed, and cells were collected using 500 µL of NaOH 0.1 M solution.

2.3.2. Cell layer integrity

TEER was used to assess the Caco-2 cell layer integrity and permeability prediction. TEER was determined at different time points (0, 1, 3, and 6 h) during the permeability assay, to assess the membrane viability and permeability after contact with tested samples. For TEER measurement, it was used a Millicell® ERS-2 Voltohmmeter (Merck, Germany) (16). During permeability experiments, TEER values were always above 250 $\Omega\cdot cm^2$, indicating that the cells were viable along the assay and the cell layer integrity was not compromised (17).

2.3.3. FAs content analysis with gas chromatography

To calculate the apparent permeability (Papp, **Equation 6.1**), the analysis of FAs content in both apical and basolateral sides, and cells was assessed through gas-chromatography. A derivatization process was applied as described in **Chapter 3 Section 2.5.1.1** and in the end, the FAMEs extract was collected and further analyzed in a gas chromatograph Agilent 8860 (Agilent,

USA), equipped with a flame ionization detector and a BPX70 capillary column (60 m x 0.25 mm x 0.25 µm; SGE Europe Ltd, Courtaboeuf, France).

Consequently, the Papp is calculated as follows (18):

$$Papp\left(\frac{cm}{h}\right) = \frac{dQ}{(dt(A \times C_0))} \qquad \textbf{(Equation 6.1)}$$

Where dQ is the total amount of permeated fatty acid (µg of FA/µL of the sample), A is the diffusion area (cm^2), C_0 is the initial concentration of fatty acids (µg of fatty acid /µL of the sample), and dt is the time of the experiment (h). The coefficient dQ/dt represents the flux of FAs across the monolayer.

2.4. 3T3-L1

Pre-adipocytes 3T3-L1 (ATCC CL-173) were acquired from the American Type Culture Collection. Pre-adipocytes were cultured in DMEM with 10% (v/v) of calf bovine serum, Iron Fortified, and 1% (v/v) of Penicillin-Streptomycin-Fungizone. The cells were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO2.

2.4.1. Adipogenesis determination

The effect of the bioactive FAs and the role of CB1 and GPR120 receptors in 3T3-L1 cells adipogenesis was performed using the adipogenesis assay kit according to the manufacturer's instructions. Briefly, cells were seeded at 3.0×10^4 cells/mL in 96-well tissue culture plates and allowed to grow until confluence in DMEM supplemented with 10% calf serum, 1% (v/v) penicillin, and streptomycin. Two days post confluence the media were replaced by the differentiation induction media provided in the kit (comprised of IBMX - 3-isobutil-1-metilxantina-, insulin, Dexamethasone, and 10% FBS). Following induction, the media were replaced every three days by insulin media until more than 80% of the cells were differentiated. The samples were added to the cells in a final dilution of 1:100 and the CB1 and GPR120 respective agonists and antagonists were added as described in **Sections 2.4.2 and 2.4.3**, respectively, and incubated for 24 h. For the lipid Droplet Staining and Quantification assay, the medium was removed from the cells, and 75 µL of fixative buffer (provided in the kit) was added to the cells and further incubated for 15 min. The cells were washed 2x with wash buffer (provided in the kit). Afterward, the plate was dried off and 75 µL of red oil dye was added and incubated for 20 min at 37 °C. A washing step was performed and after the washing buffer was dried off 100 µL of Lipid Droplets Assay Dye Extraction Solution was added to each well. The plate was gently mixed for 15-30 min and the absorbance was read at 490-520 nm in a microplate reader (Synergy H1, Biotek Instruments, USA).

2.4.2. CB1 receptor effect in Caco-2 permeability and 3T3-L1 adipogenesis

To determine the role of the CB1 receptor in Caco-2 cell permeability and 3T3-L1 adipogenesis it was used a described agonist and antagonist of the CB1 receptor. The agonist used was C18:1 anandamide (herein defined as AEA) and AM251 was used as a CB1 antagonist. According to suppliers' information, AM251 was dissolved in DMSO and C18:1 anandamide in ethanol absolute to a final concentration of 10 mM (stock solution). The stock solutions were maintained at -20 °C. AM251 solution was applied to Caco-2 cells to a final concentration of 10 µM and anandamide to a final concentration of 1 µM. The selection of these molecules and the concentrations applied to the cells were based on previous studies aiming to study these molecules' effects on Caco-2 cell permeability (7,19).

2.4.3. GPR120 receptor effect in 3T3-L1 adipogenesis

TUG-891 was the chosen selective agonist for the GPR120 receptor. The stock solution was prepared at 20 mg/ml (≈55mM) in DMSO. It was added to the cells as a final concentration of 30 µM. This concentration was chosen considering previous studies (20–22). AH7614 was the selected negative allosteric modulator of the GPR120 receptor (23). The stock solution was prepared at 100 mM in DMSO, and it was added to the cells at a final concentration of 100 µM. Indeed, *in vitro* studies reported AH7614 GPR120 antagonistic effect on concentrations ranging from 10 to 100 µM (22,24,25). The stock solutions were maintained at -20 °C.

2.4.3.1. GPR120 expression in 3T3-L1 cells confirmation by Western blot

To confirm the expression of GPR120 transmembrane receptor in the 3T3-L1 cell lysates from 3 independent differentiated cultures were collected in RIPA Buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) containing 1:1000 of a phosphatase inhibitor and DTT (Dithiothreitol – Promega, V3151, 1M) and 1:100 of a protease inhibitor. Protein concentrations were determined using a BCA kit (Pierce BCA Protein Assay Kit, Thermo Scientific). The samples were stored at -80 ºC until use. The cell lysates (10 µg) were processed using SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and western Blot. For the SDS-PAGE, 1% DTT and 1X GLB (Gel loading buffer, 150 mM Trizma Base – Fisher Scientific, BP152 –, 6% SDS – Sigma Aldrich, L4390 –, 0.05% Bromophenol Blue – Alfa Aesar, A18469 –, 30% glycerol – Merck, 1.04092.1000 – and 6nM EDTA pH 8.8 – Merck, 1.08452.1000) were added to each cell lysate and a 10 min 95 ºC denaturation process was performed, before the addition of the samples to a 10% polyacrylamide electrophoresis gel. After, the proteins were blotted from the gel into a nitrocellulose blotting membrane by a semidry transfer process, using the Trans-Blot Turbo System (Biorad). The protein transfer was confirmed by

Ponceau S staining. The membranes were blocked with 5% milk and were further incubated overnight with the GPR120 primary antibodies. After incubation with appropriate secondary antibodies, the immunodetection was performed with a Chemiluminescent Substrate solution and using a ChemiDoc XRS+ (Biorad).

2.8. Statistical analysis

Table and figure results are reported as mean values of three biological replicates ± standard deviation. The statistical analysis was performed using IBM SPSS Statistics 28 (SPSS Inc., IBM Corporation, NY, USA). First, data was analyzed for normal distribution using Shapiro-Wilk's test. The homogeneity of variances was verified using Levene's test. To compare the means of three or more groups, was used One-way ANOVA. Tukey's post-hoc test was used to determine differences among groups. The level of significance (*p*) was set at 0.05.

3. Results and Discussion

3.1. The role of CB1 in omega-3 and punicic acid Caco-2 permeability

The CB1 receptor is known to be expressed in Caco-2 cells: an early study demonstrated that CB1 receptors are expressed in normal human colon and colonic epithelium is responsive biochemically and functionally to cannabinoids. CB1- and CB2-receptor expression was present on plasma cells in the lamina propria, whereas only CB2 was present on macrophages. CB2 immunoreactivity was seen in the epithelium of colonic tissue characteristic of inflammatory bowel disease (26). Thus, the effect of the CB1 receptor in the major bioactive FAs (omega-3 EPA, DHA and DPA, and PUA) permeability in Fish oil, Pomegranate oil, and a mixture of both oils was screened for the first time, as far as we know, using an *in vitro* permeability model of a Caco-2 cell monoculture. Some studies have demonstrated that the effect of THC and CBD on TEER was significantly inhibited by the CB1 antagonist (AM251) but not by CB2 antagonism. On the other hand, when endocannabinoids were applied basolaterally, they enhanced the recovery of EDTA-induced increased permeability. Additionally, apical application of AEA and 2-AG exacerbated the EDTA-mediated decrease in TEER in Caco-2 cells and this effect is abrogated by the mentioned CB1 antagonist (6). Moreover, another study reported that apical administration of AEA and 2-AG worsened the permeability effect of hypoxia via CB1 (7). So, the TEER values were determined and are displayed in **Figure 6.2** and **Supplementary material Table S9**. The most direct observation is that the application of AEA alone (the selected CB1 agonist) on the apical side decreased permeability by increasing TEER values ($p<0.05$). Regarding the Fish oil samples it was observed that the combination of both Fish oil and AEA was able to significantly increase TEER values to similar values as AEA alone. On the other hand, the application of AEA in combination with Pomegranate oil resulted in statistically lower TEER values and consequently increased permeability when compared to AEA. Similar results were obtained for the Fish oil+Pomegranate oil sample. It has been reported that competition between PUFAs, specifically omega-3, and AEA might decrease the activation of CB1 and CB2 receptors by the latter (4). So, this may suggest that Pomegranate oil PUFA content, probably its major bioactive FAs (PUA, catalpic, α- and β-eleostearic acids) content may compete with AEA and decrease its activation effect on CB1. This is a plausible explanation for the opposite effect observed for the AEA alone and in combination with Fish oil+Pomegranate oil or Pomegranate oil. In the case of Fish oil, it has been reported that although they may also have similar competitive effects, EPA and DHA-rich diets enhanced the activity of CB1 receptors in mice (10), which may explain the similar TEER values between AEA and Fish oil+AEA. These results are relevant since they demonstrate that different PUFA compositions may have different effects on CB1-induced permeability. Moreover, it was observed that AM251 application (alone) compared to the remaining samples (Fish oil+Pomegranate oil, [Fish oil+Pomegranate oil]+AEA, and [Fish oil+Pomegranate oil]+AM251) significantly decreased the TEER values, increasing permeability. Similar observations were also

made for Fish oil samples, at 1 h the AM251 TEER values were significantly lower than Fish oil+AEA and AEA alone, at 3 h than Fish oil+AM251 and Fish oil+AEA, and at 6 h than Fish oil. So, it seems that CB1 activation by the application of its agonist (AEA) on the apical side, decreases permeability by increasing TEER values. In the presence of its antagonist (AM251) permeability increases by decreasing TEER values. These results seem contradictory considering the results obtained in the previously mentioned study: apical application of endocannabinoids caused increased permeability (lower TEER values) sensitive to cannabinoid CB1 receptor antagonism. This may be explained in part by the AEA and AM251 concentrations applied in the mentioned study: 10 and 30 μM for AEA and 100 nM, 1 and 10μM for AM251. Indeed, the effect on the permeability seems to be dose-dependent. In fact, in our study, lower concentrations of AEA were used (1 μM). Accordingly, in the mentioned study higher TEER values were reported for AEA at lower concentrations (100 and 300 nM) (27). On the other hand, in our work, a similar concentration of AM251 solution (10 μM) was applied to Caco-2 cells compared to the mentioned study. Although statistically significant lower values were obtained for AM251 compared to AEA, for instance, as it is possible to observe in **Figure 6.2**, the values were still high and comparable to the ones reported in the literature.

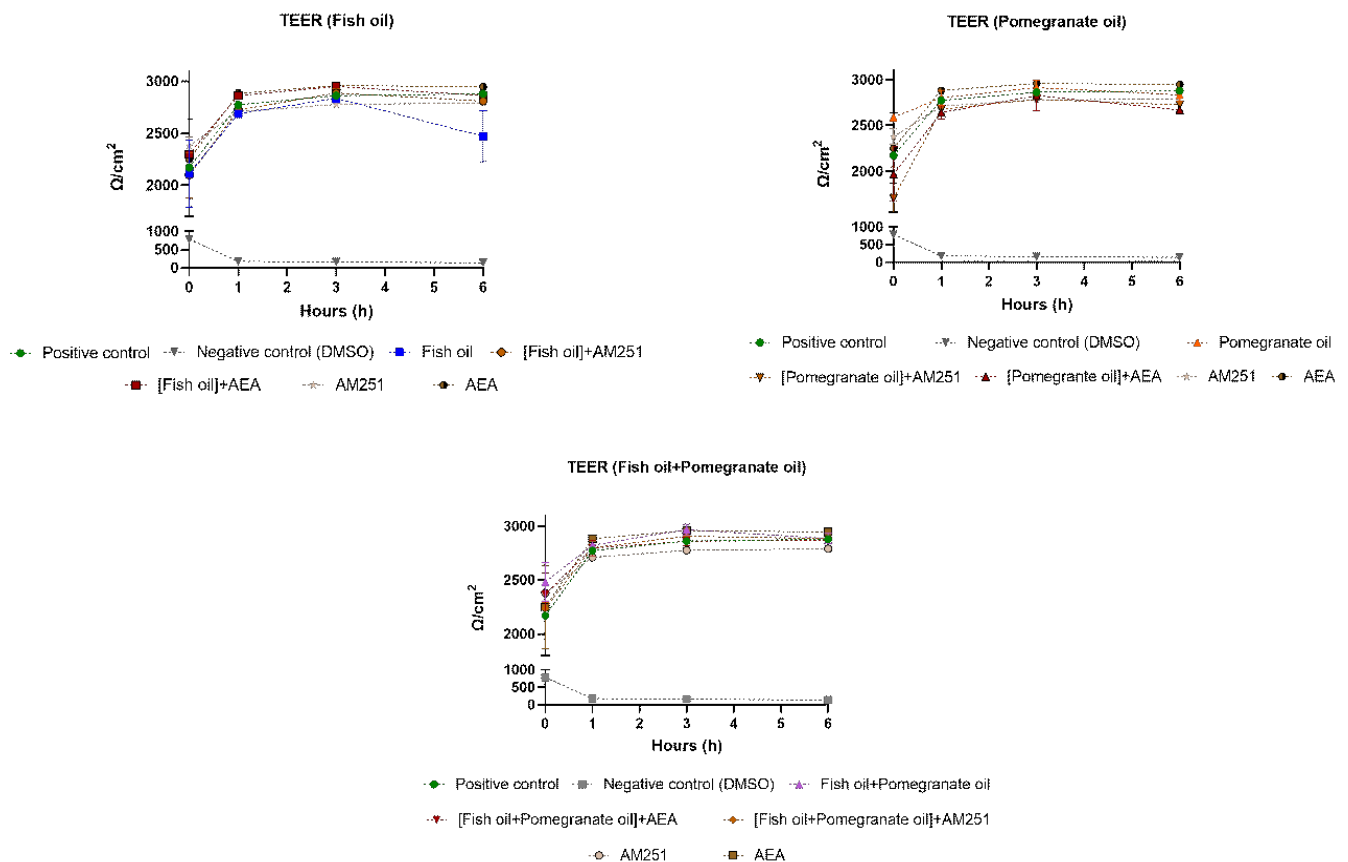

Figure 6.2 – TEER values (Ω/cm2) for the different samples at different time-points (0, 1, 3 and 6 h) and corresponding controls, AM251 CB1 antagonist, AEA (C18:1 anandamide), CB1 agonist. DMSO was used as the negative control and culture medium as the positive control.

Besides TEER measures, the major bioactive FAs were quantified for all the samples in the collected cells and apical side at the end of the experimental time and in the basolateral side at the different time points (1, 3, and 6 h). This assay was performed in the presence and the absence of CB1 agonist and antagonist, AEA and AM251 respectively (**Figure 6.3** and **Table 6.1**). To determine if the FAs were incorporated in the cells, the cells after the experimental time were collected. **Figure 6.3** depicts the FAs profile after normalization with the positive control (cells with culture medium) to eliminate the FFAs naturally present in the culture medium and cells. After this quantification, the RI (%) in **Table 6.1** was calculated according to **Equation 6.2.** The RI was calculated taking into consideration the concentration of a certain FA after the permeability assay in relation to the FA content quantified in the digested sample that was applied to the apical side.

$$\mathrm{RI}\ (\%) = \frac{\text{Fatty acid content quantified after permeability assay}}{\text{Fatty acid content quantified in the digested sample applied to the apical side}} \times 100 \qquad \textbf{(Equation 6.2)}$$

Interestingly, in the Fish oil sample, the presence of CB1 antagonist (AM251) increased the concentration of EPA, DPA, and DHA in the collected Caco-2 cells and in the case of EPA on the apical side. These results are consistent with the TEER values obtained: at 6 h Fish oil TEER values (**Supplementary material Table S9)** are statistically lower than the ones measured for the Fish oil+AM251 sample. This means that the application of Fish oil+AM251 decreases permeability if compared to Fish oil alone. Nevertheless, very low RI was observed regarding these samples (2.5-10%). This may be due to the bioconversion processes of these exogenous omega-3 PUFAs. As described in **Chapter 3 Section 3.2** omega-3 PUFAs can alter and modulate the membrane FA composition (28), which is consistent with the increased presence of these FAs in the cells (compared to positive control without omega-3 addition). So, the decreased permeability observed here may be related to the fact that EPA and DHA can improve barrier integrity in Caco-2 cells. Indeed, very low concentrations of these omega-3 FAs were found in the basolateral side, which is consistent with higher cell incorporations, as discussed. Nevertheless, at 6 h the presence of the CB1 agonist AEA seems to significantly increase the DPA and DHA concentration on the basolateral side compared to Fish oil and Fish oil+AM251, the CB1 antagonist. Similar observations were performed for C14:0, C16:0, C18:0, C18:1 *c*9, and C18:2 *c*9*c*12: the cell concentrations of these FAs were higher ($p<0.05$) in the presence of AM251, especially if compared to Fish oil+AEA sample (**Table 6.1**). So, all these results suggest that at least some of the mechanisms behind omega-3 EPA, DHA, and DPA bioavailability are mediated through CB1.

Regarding the Pomegranate oil sample, it seems that in the presence of a CB1 agonist, the concentration of PUA significantly decreases ($p<0.05$) in the collected cells. No major bioactive FAs – PUA, catalpic, α-eleostearic, and β-eleostearic acids – were detected in the basolateral side. As discussed in **Chapter 3 Section 3.2,** previous studies demonstrated that 24 h incubation with these isomers was shown to induce a cellular accumulation of these FAs. Interestingly, the authors also demonstrated that these FAs can be converted into CLA. Indeed, several CLA

isomers were shown to alter the distribution of tight junction proteins occluding and ZO-1 in a Caco-2 cell model increasing the paracellular permeability (29–32). Thus, these results may explain the low permeability observed in our study.

In the case of the Fish oil+Pomegranate oil sample, the sample without CB1 agonist or antagonist presented higher EPA and DPA concentrations in the collected cells.

The Papp was calculated according to **Equation 6.1** for the detected fatty acids in the basolateral side and is presented in **Table 6.2.** The permeability values obtained for this permeability assay using Fish oil, Pomegranate oil, and a mixture (1:1) of both oils in the absence and in the presence of a chemical agonist (AEA) and antagonist (AM251) of the CB1 receptor demonstrated that despite CB1 activation the Papp for the major bioactive FAs (omega-3 EPA, DPA and DHA and the CLNA isomer PUA) were very low or null. Such results agree with what was previously demonstrated in **Chapter 3.** At 3 h most of the PUFAs detected in the basolateral side presented lower chain length. At 6 h higher chain length PUFAs, namely DPA and DHA are detected in some Fish oil samples, probably due to higher concentrations of these FAs in this sample.

Importantly, it has been reported that a monoculture of Caco-2 does not represent accurately duodenum epithelia due to the presence of tight junctions typical of the colon but not of the small intestine, leading to limited absorption of hydrophilic molecules (33). This may explain the low permeability observed for these molecules. Nevertheless, it is important to point out that in **Chapter 3** similar values were obtained with a co-culture model of Caco-2 and HT29-MTX cells. Moreover, the colonic cell line Caco-2 also presents some limitations as the overestimation of TEER compared to *in vivo* conditions (34). Nevertheless, most studies reporting the effect of the CB1 receptor were only conducted in Caco-2 cells. Since there is a lack of studies on the role of PUFAs in CB1-mediated intestinal permeability and this work intended to function as a screening study of a possible effect of CB1 in omega-3 EPA and DHA and PUA, we restricted the permeability model to Caco-2 cells since it is the simplest and most described model. Although these limitations are important to point out, as it was mentioned this work intended to be used as a first screening. So, further studies with different permeability models assessing CB1 expression and role, such as the co-culture model of Caco-2/HT29-MTX, described in **Chapter 3**, need to be considered in the future.

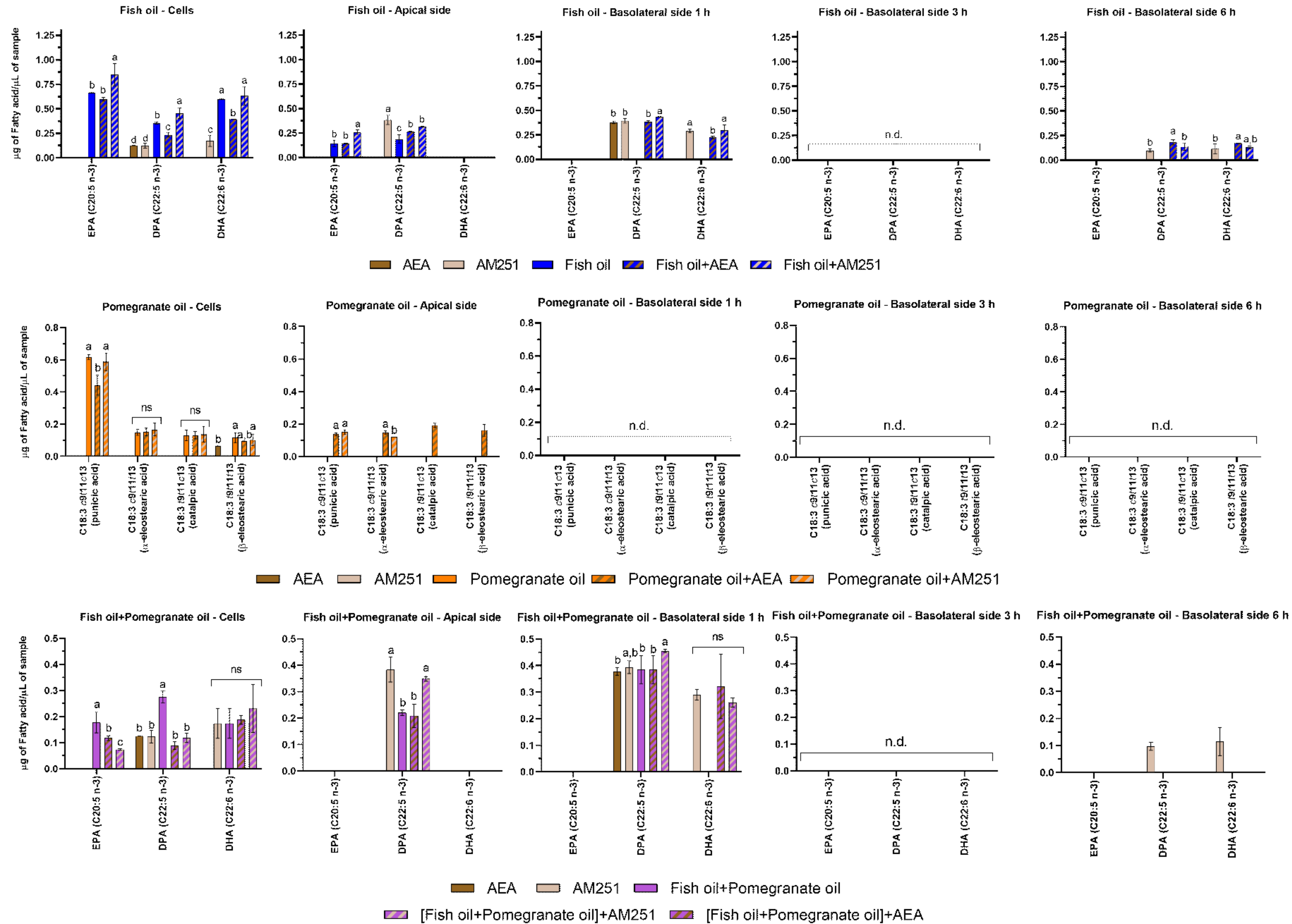

Figure 6.3 - Quantification (µg of fatty acid/µL of sample) of the major bioactive FAs in the different samples (Fish oil, Pomegranate oil, and Fish oil+Pomegranate oil) with and without the presence of CB1 agonist (AEA) and antagonist (AM251). The results are means of two biological replicates and two analytical replicates and are represented as mean±standard deviation. Different letters (a, b) indicate statistically significant differences ($p < 0.05$). The RI (%) was calculated according to **Equation 6.2** for each FA considering the corresponding FA in the original digested sample that was applied to the cells. n.d.= not detected.

Table 6.1 – Quantification (µg of fatty acid/µL of the sample) of the major detected FAs in the different samples (Fish oil, Pomegranate oil, and Fish oil+Pomegranate oil) with and without the presence of CB1 agonist (AEA) and antagonist (AM251). The results are means of two biological replicates and two analytical replicates and are represented as mean± standard deviation. The RI (%) was calculated for each FA considering the corresponding FA in the original digested sample that was applied to the cells on the apical side. n.d.= not detected.

µg of fatty acid/µL of sample		Myristic acid		Palmitic acid		Stearic acid		Oleic acid		Linoleic acid		α-linolenic acid		Punicic acid		α-eleostearic acid		Catalpic acid		β-eleostearic acid		EPA		DPA		DHA	
		C14:0		C16:0		C18:0		C18:1 c9		C18:2 c9c12		C18:3 c9c12c15		C18:3 c9t11c13		C18:3 c9t11t13		c18:3 t9t11c13		C18:3 t9t11t13		C20:5 n-3		C22:5 n-3		C22:6 n-3	
		MEAN±SD	RI (%)	MEAN±SD	RI (%)	MEAN±SD	RI (%)	MEAN±SD	RI (%)	MEAN±SD	RI (%)	MEAN±SD	RI (%)	MEAN±SD	RI (%)	MEAN±SD	RI (%)	MEAN±SD	RI (%)	MEAN±SD	RI (%)	MEAN±SD	RI (%)	MEAN±SD	RI (%)	MEAN±SD	RI (%)
Cells	AEA	1.02±0.13	-	0.37±0.01	-	2.10±0.24	-	0.50±0.06	-		-	0.28±0.03	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	0.12±0.00	-	n.d.	-
	AM251	0.09±0.04	-	1.10±0.27	-	0.74±0.29	-	2.10±0.46	-	0.20±0.03	-	0.08±0.03	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	0.12±0.02	-	0.17±0.06	-
	Fish oil	0.59±0.01	3.35	2.48±0.04	7.1023	1.18±0.04	20.96	3.05±0.14	20.88	0.39±0.04	14.58	0.26±0.08	11.22	n.d.		n.d.		n.d.		n.d.		0.66±0.00	2.55	0.35±0.01	7.85	0.60±0.00	4.65
	[Fish oil]+AEA	0.56±0.04	3.16	2.00±0.16	5.7063	0.89±0.02	15.81	2.40±0.10	16.44	0.32±0.01	11.85	0.32±0.03	7.45	n.d.		n.d.		n.d.		n.d.		0.60±0.02	2.30	0.23±0.02	5.19	0.39±0.00	3.06
	[Fish oil]+AM251	0.76±0.10	4.31	2.95±0.65	8.4445	1.29±0.33	22.89	3.29±0.77	22.55	0.47±0.09	17.36	0.18±0.01	13.67	n.d.		n.d.		n.d.		n.d.		0.85±0.11	3.26	0.45±0.06	10.0836	0.63±0.09	4.92
	Pomegranate oil	0.16±0.01	0.16	1.69±0.06	1.6851	1.10±0.09	1.10	2.98±0.10	2.98	0.51±0.10	0.51	0.19±0.00	0.19	0.62±0.01	0.62	0.15±0.02	0.15	0.13±0.03	0.13	0.12±0.03	0.12	0.15±0.03	0.15	0.22±0.01	0.22	0.22±0.01	0.22
	[Pomegranate oil]+AEA	0.15±0.04	0.15	1.55±0.47	1.5483	1.17±0.48	1.17	2.74±0.55	2.73	0.44±0.01	0.43	0.16±0.08	0.16	0.44±0.06	0.44	0.15±0.02	0.15	0.13±0.02	0.13	0.09±0.00	0.09	n.d.		0.14±0.03	0.14	0.26±0.09	0.26
	[Pomegranate oil]+AM251	0.15±0.02	0.15	1.38±0.04	1.3805	0.92±0.01	0.91	2.44±0.01	2.44	0.42±0.01	0.42	0.16±0.04	0.16	0.59±0.06	0.59	0.17±0.04	0.17	0.14±0.05	0.14	0.10±0.03	0.10	n.d.		0.11±0.01	0.11	0.17±0.01	0.17
	Fish oil+Pomegranate oil	0.19±0.02	0.19	1.76±0.24	1.7552	1.19±0.09	1.19	3.04±0.42	3.04	0.33±0.02	0.33	0.2±0.01	0.20	n.d.		n.d.		n.d.		n.d.		0.18±0.04	0.14	0.27±0.02	0.18	0.27±0.02	0.27
	[Fish oil+Pomegranate oil]+AEA	0.15±0.00	0.15	1.23±0.06	1.2286	0.81±0.05	0.81	2.24±0.04	2.24	0.29±0.01	0.28	0.16±0.01	0.16	n.d.		n.d.		n.d.		n.d.		0.12±0.01	0.12	0.09±0.02	0.09	0.19±0.02	0.19
	[Fish oil+Pomegranate oil]+AM251	0.16±0.01	0.16	1.49±0.34	1.4868	1.00±0.35	1.00	2.64±0.77	2.64	0.32±0.04	0.32	0.18±0.04	0.18	n.d.		n.d.		n.d.		n.d.		0.07±0.00	0.07	0.12±0.02	0.12	0.23±0.09	0.23
Apical side	AEA	0.23±0.08	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-
	AM251	n.d.	-	0.26±0.01	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	0.38±0.05	-	n.d.	-
	Fish oil	0.15±0.19	0.83	0.19±0.25	0.5461	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		0.14±0.03	0.56	0.19±0.04	4.14	n.d.	
	[Fish oil]+AEA	0.06±0.01	0.36	0.49±0.00	1.4137	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		0.14±0.00	0.55	0.26±0.00	5.86	n.d.	
	[Fish oil]+AM251	0.15±0.19	1.03	0.84±0.08	2.4064	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		0.26±0.02	0.99	0.31±0.00	6.97	n.d.	
	Pomegranate oil	n.d.		0.54±0.09	0.5435	n.d.		0.30±0.13	0.30	0.39±0.12	0.39	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
	[Pomegranate oil]+AEA	n.d.		0.26±0.02	0.2572	n.d.		n.d.		n.d.		n.d.		0.14±0.01	0.14	0.15±0.01	0.15	0.19±0.01	0.19	0.16±0.04	0.16	n.d.		0.32±0.03	0.32	n.d.	
	[Pomegranate oil]+AM251	n.d.		0.14±0.19	0.1359	n.d.		n.d.		n.d.		n.d.		0.15±0.01	0.15	0.12±0.00	0.12	n.d.		n.d.		n.d.		0.21±0.06	0.21	n.d.	
	Fish oil+Pomegranate oil	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		0.22±0.01	0.22	n.d.	
	[Fish oil+Pomegranate oil]+AEA	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		0.21±0.04	0.21	n.d.	
	[Fish oil+Pomegranate oil]+AM251	n.d.		0.18±0.09	0.1834	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		0.35±0.01	0.35	n.d.	
Basolateral side 1 h	AEA	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	0.38±0.01	-	n.d.	-
	AM251	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	0.39±0.02	-	0.29±0.02	-
	Fish oil	n.d.		0.03±0.04	0.0012	n.d.		0.06±0.04	0.42	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
	[Fish oil]+AEA	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		0.38±0.01	8.48	0.22±0.01	1.74
	[Fish oil]+AM251	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		0.43±0.00	9.61	0.3 ±0.06	2.30
	Pomegranate oil	n.d.		0.04±0.03	0.0350	0.03±0.06	0.03	0.07±0.0006	0.07	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	

	[Pomegranate oil]+AEA	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		0.35±0.01	0.35	0.21±0.00	0.21
	[Pomegranate oil]+AM251	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		0.26±0.08	0.26	0.43±0.39	0.43
	Fish oil+Pomegranate oil	n.d.		n.d.		n.d.		0.05±0.04	0.0496	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		0.39±0.05	0.38	n.d.	
	[Fish oil+Pomegranate oil]+AEA	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		0.39±0.05	0.38	0.32±0.12	0.32
	[Fish oil+Pomegranate oil]+AM251	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		0.45±0.01	0.45	0.26±0.02	0.26
Basolateral side 3 h	AEA	0.06±0.02	-	n.d.	-	0.03±0.04	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-
	AM251	n.d.	-	0.11±0.05	-	0.09±0.05	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-
	Fish oil	n.d.		0.61±0.03	1.75	0.37±0.06	6.49	0.37±0.01	2.50	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
	[Fish oil]+AEA	n.d.		0.64±0.01	1.83	0.36±0.05	6.31	0.34±0.01	2.35	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
	[Fish oil]+AM251	n.d.		n.d.	1.92	0.41±0.01	7.24	0.43±0.04	2.97	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
	Pomegranate oil	n.d.		0.67±0.05	0.67	0.45±0.04	0.45	0.47±0.03	0.47	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
	[Pomegranate oil]+AEA	n.d.		0.71±0.06	0.71	0.45±0.02	0.45	0.44±0.04	0.44	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
	[Pomegranate oil]+AM251	n.d.		0.56±0.03	0.56	0.39±0.06	0.39	n.d.	n.d.	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
	Fish oil+Pomegranate oil	n.d.		n.d.	0.69	0.39±0.08	0.39	0.41±0.01	0.41	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
	[Fish oil+Pomegranate oil]+AEA	n.d.		0.64±0.06	0.64	0.38±0.07	0.38	0.38±0.01	0.38	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
	[Fish oil+Pomegranate oil]+AM251	n.d.		0.48±0.20	0.48	0.28±0.10	0.28	0.28±0.15	0.28	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
Basolateral side 6 h	AEA	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	0.17±0.05	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-
	AM251	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	0.1±0.02	-	0.11±0.05	-
	Fish oil	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
	[Fish oil]+AEA	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		0.18±0.03	1.73	0.17±0.00	0.50
	[Fish oil]+AM251	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		0.13±0.03	2.99	0.13±0.01	1.00
	Pomegranate oil	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
	[Pomegranate oil]+AEA	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
	[Pomegranate oil]+AM251	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
	Fish oil+Pomegranate oil	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
	[Fish oil+Pomegranate oil]+AEA	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
	[Fish oil+Pomegranate oil]+AM251	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	

Table 6.2 – Apparent permeability (Papp) (cm/h) for the detected fatty acids in the different samples (Fish oil, Pomegranate oil, and Fish oil+Pomegranate oil) with and without the presence of CB1 agonist (AEA) and antagonist (AM251). The results are means of two biological replicates and two analytical replicates and are represented as mean± standard deviation.

Sample	Fatty acid	Palmitic acid	Stearic acid	Oleic acid	α-linolenic acid	EPA	DPA	DHA
		C16:0	C18:0	C18:1 *c*9	C18:3 *c*9*c*12*c*15	C20:5 n-3	C22:5 n-3	C22:6 n-3
		cm/h						
Papp 3 h								
Fish oil	Fish oil	0.0048±0.0002	0.0180±0.0029	0.0070±0.0001	n.d.	n.d.	n.d.	n.d.
	[Fish oil]+AEA	0.0051±0.0001	0.0175±0.0027	0.0065±0.0001	n.d.	n.d.	n.d.	n.d.
	[Fish oil]+AM251	0.0053±0.0000	0.0201±0.0005	0.0083±0.0009	n.d.	n.d.	n.d.	n.d.
Pomegranate oil	Pomegranate oil	0.0409±0.0031	0.0370±0.0021	0.0155±0.0008	n.d.	n.d.	n.d.	n.d.
	[Pomegranate oil]+AEA	0.0433±0.0038	0.0371±0.0024	0.0145±0.0017	n.d.	n.d.	n.d.	n.d.
	[Pomegranate oil]+AM251	0.0340±0.0014	0.0320±0.0036	n.d.	n.d.	n.d.	n.d.	n.d.
Fish oil+Pomegranate oil	Fish oil+Pomegranate oil	0.0501±0.0091	0.1025±0.0352	0.0490±0.0100	n.d.	n.d.	n.d.	n.d.
	[Fish oil+Pomegranate oil]+AEA	0.0459±0.0041	0.0978±0.0018	0.0448±0.0082	n.d.	n.d.	n.d.	n.d.
	[Fish oil+Pomegranate oil]+AM251	0.0338±0.0080	0.0705±0.0144	0.0318±0.0108	n.d.	n.d.	n.d.	n.d.
Papp 6 h								
Fish oil	[Fish oil]+AEA	n.d.	n.d.	n.d.	n.d.	n.d.	0.0056±0.0013	0.0019±0.0001
	[Fish oil]+AM251	n.d.	n.d.	n.d.	n.d.	n.d.	0.0041±0.0007	0.0014±0.0000

3.2. The role of CB1 and GPR120 receptors in omega-3 and punicic acid effect on adipogenesis

3.2.1. CB1 receptor

The increase in the size of adipose tissue, which is one of the main observations in obesity, can happen by two major processes: hypertrophy, and hyperplasia. Hypertrophy is the increase in the size of existing differentiated adipocytes while hyperplasia is the formation of new adipocytes (adipogenesis) through the differentiation of resident precursors, the preadipocytes. During prolonged caloric excess intake, new adipocytes can emerge from the differentiation of preadipocytes and then contribute to adipose tissue expansion (35).

CB1 receptor has been associated with adipokine synthesis and production, cell growth and differentiation of adipocytes, and lipogenesis stimulation. Indeed, CB1 receptor chemical blockage has been demonstrated to increase adiponectin in WAT and 3T3-F44A cells (8). Moreover, this CB1 blockage by rimonabant can interfere with adipocyte differentiation, cell growth, and lipogenesis, often inducing an opposite effect. This is particularly important in an obesity context (8). It was demonstrated that in genetically obese mice, there is an upregulation of the CB1 receptor compared with lean mice. In addition, the same CB1 upregulation is observed in cultured adipocytes under conditions of hyperinsulinemia (36).

As demonstrated in **Figure 6.4** and considering the relevant role of adipogenesis in obesity development, a possible therapeutical role of omega-3 EPA and DHA and CLNA isomer, PUA, and the role of these FAs in adipogenesis was evaluated. Furthermore, the possible mediatory role of the CB1 receptor in these FAs' effects was evaluated, using the mentioned AEA chemical agonist and an antagonist (AM251). The % of lipid accumulation was calculated in relation to the positive control. Lipid droplet accumulation is used as an indicator for the estimation of adipogenesis. It was observed that the % of lipid accumulation was decreased in Fish oil, Pomegranate oil, and Fish oil+ Pomegranate oil samples. Although not statistically significant, it seems that the mix of both oils has a lower capacity for adipogenesis reduction than the individual samples. CLNA isomer, PUA, has been demonstrated to decrease adipogenesis and preadipocyte differentiation in 3T3-L1 cells (37). Adipogenic differentiation is controlled by various transcription factors, including PPAR γ. CLA isomers, for instance, are described to suppress preadipocyte differentiation in animal and human preadipocytes, by attenuating the expression and activity of adipogenic transcription factors namely PPAR γ and these are dependent on inflammatory signaling (38). Similar results have been described for Pomegranate seed oil where there is inhibition of adipogenesis and differentiation and a decrease of the protein level of PPAR γ in 3T3-L1 preadipocytes (38). So, the PUA capacity to regulate PPAR γ expression may explain its role in adipogenesis inhibition. A recent study has reported that in primary mouse pre-adipocytes and *in vivo* Fish oil treatment resulted in increased proliferation potential compared to control and HFD groups. Moreover, similar observations were made for cultured differentiated primary pre-adipocytes from the eutrophic mice and 3T3-L1 pre-adipocytes treated *in vitro* with

EPA and DHA alone or in a 5:1 ratio (39). These results may be relevant if considering that there is a distinction between a pathologic WAT expansion and a healthy WAT expansion in obesity. The authors stated that the pathological WAT expansion corresponds to the rapid growth of fat mass through hypertrophy of existing fat cells (hypertrophic obesity). This hypertrophy is often associated with a high degree of macrophage infiltration. So, there is the development of an inflammatory state, limited development of blood vessels, and fibrosis due to hypoxia. The healthy expansion occurs when there is adipocyte hyperplasia by *de novo* differentiation. So, adipocyte differentiation is accepted to be important in preventing hypertrophic obesity (a risk factor for T2DM). Adipogenesis is reduced in subcutaneous stromal cells in hypertrophic obesity (39). Thus, the healthy WAT expansion by adipocyte hyperplasia instead of hypertrophy is seen as a promising strategy to prevent obesity-associated metabolic dysfunction. In HFD-induced obesity mice models, induction of adipocyte hyperplasia by increasing adipogenesis alleviates glucose intolerance and hepatic steatosis (40). Considering these observations, in the context of obesity treatment, where obesity is already installed, Fish oil+Pomegranate oil mixture may have a more promising effect, since it presents lower adipogenesis reduction. On the other hand, if considering a preventive treatment for obesity, Fish oil or Pomegranate oil alone seem to have a more promising potential since they seem to prevent adipogenesis, as reported for example for tyrosol (41).

Interestingly, in the presence of AEA, the % of lipid droplet accumulation increased even in the presence of the mentioned samples. These results suggest that upon CB1 activation the capacity of Fish oil and Pomegranate oil to inhibit adipogenesis significantly decreases. This is easily explained by the role of CB1 activation in adipogenesis. In the presence of a CB1 antagonist, it seems that the % of lipid droplet accumulation increases in Fish oil, Pomegranate oil, and Fish oil+Pomegranate oil samples. This may suggest that at least some of these sample's effects are mediated by the CB1 receptor. Nevertheless, the increase in % of lipid droplet accumulation compared to the sample without the chemical antagonist (AM251) presence was only statistically significant in the Pomegranate oil sample. In the presence of AM251 alone, the adipogenesis decreases, as expected. Nevertheless, when assessing the role of AEA there seems to exist an effect on adipogenesis. This effect may be partly explained by the presence of ethanol (0.1% v/v) as an AEA vehicle. As observed in **Figure 6.4**. ethanol (0.1% v/v) control also seems to influence adipogenesis, and this effect cannot be disregarded.

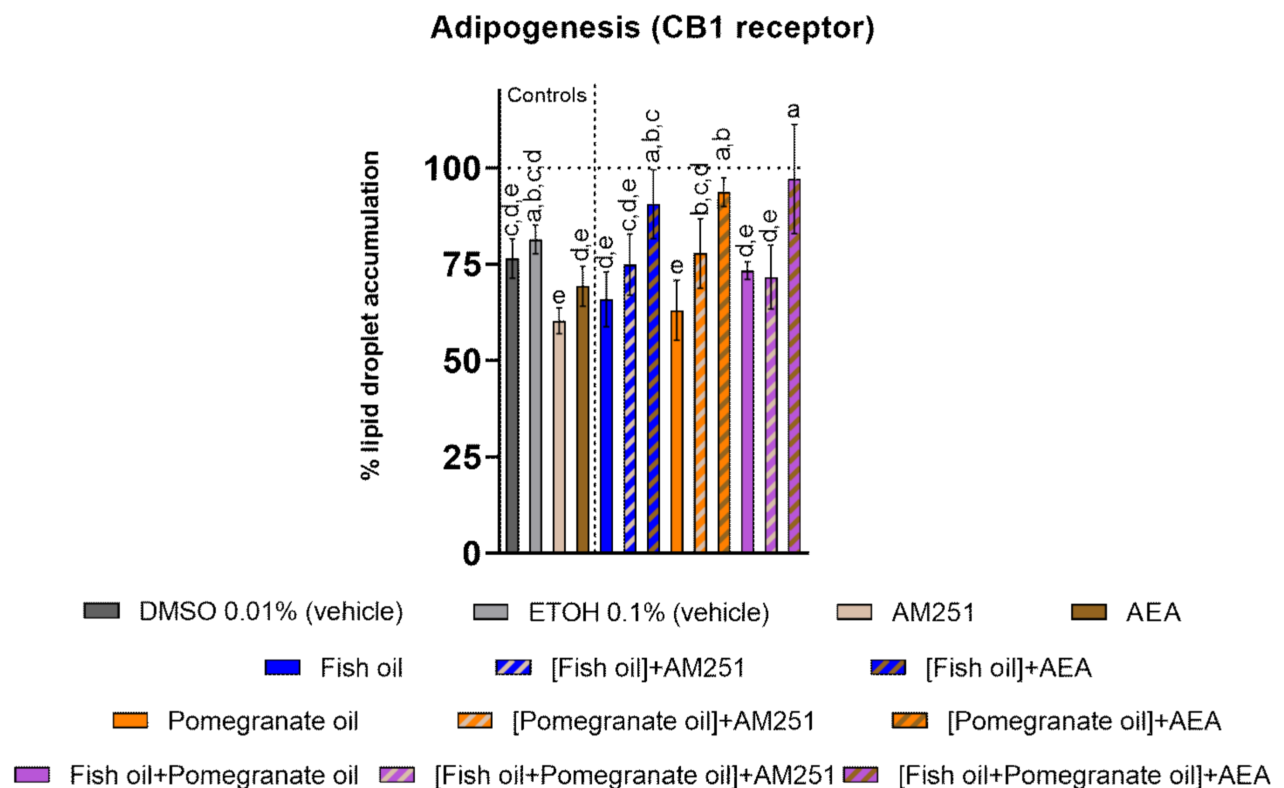

Figure 6.4 – **Effect of CB1 receptor in adipogenesis and its role on the effects of Fish oil, Pomegranate oil and the mixture of both oils**. AM251 was the selected chemical antagonist of CB1 receptor and C18:1 anandamide (AEA) the chemical agonist. DMSO at 0.01% (v/v) and EtOH 0.1% (v/v) were assayed as controls since they were used as AM251 and AEA vehicles, respectively. The resultsare means of two biological replicates and two analytical replicates and are represented as mean±standard deviation. Different letters (a, b) indicate statistically significant differences ($p < 0.05$).

3.2.2. GPR120 receptor

GPR120 receptor has been associated with several biological mechanisms, such as regulation of adipogenesis, inflammation, glucose uptake, and insulin resistance. So, as already discussed and demonstrated in **Chapter 2** GPR120 represents a promising target for the treatment of obesity and obesity-related disorders (42). High expression of GPR120 was detected in several adipose tissues, including subcutaneous, perinephric, mesenteric, and epididymis tissue from HFD-fed mice (43), and a similar pattern was observed in obese humans compared to lean humans (12).

GPR120 expression is dependent on the maturation state of 3T3-L1 adipocytes. Indeed, it cannot be detected in preadipocytes, while showing abundant expression in mature 3T3-L1 cells (11). Therefore, before the adipogenesis experiment, the expression of GPR120 in 3T3-L1 mature adipocytes was first assessed by western blot. Indeed, the expression of GPR120 in differentiated 3T3-L1 was confirmed by the detection of a band of approximately 70 kDa (**Figure 6.5**), which agrees with previous studies reporting molecular weight bands ranging from 42 kDa to 90 kDa (44). These results agree with the results obtained in **Chapter 2** in the Western blot of the HMC3 cell line.

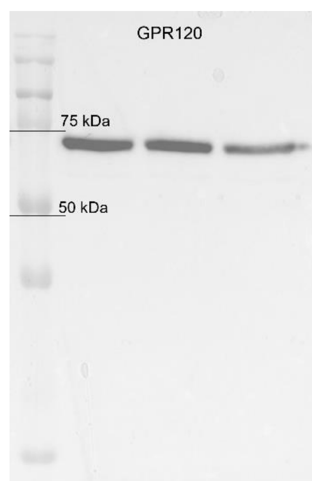

Figure 6.5 –- Western Blot analysis of GPR120 from 3 independent differentiated 3T3-L1 cell line cultures. Precision Plus Protein Standards Dual Color (161-0374, Bio-Rad) was the standard used. Membranes were probed with antibody to GPR120 and a band corresponding to a 70 kDa protein was detected.

The role of GPR120 as a mediator of omega-3 anti-inflammatory effects is well described in both **Chapter 1** and **Chapter 2**. A possible mediatory mechanism of Nf-κB inhibition of CLA and CLNA (PUA) isomers through GPR120 was demonstrated for the first time in **Chapter 2**. Indeed, regarding CFAs, most effects of these FAs, including anti-inflammatory and adipogenic are thought to be mediated by PPAR α and γ. As mentioned, the % of lipid accumulation was decreased in Fish oil, Pomegranate oil, and Fish oil+Pomegranate oil samples. Although not statistically significant, it seems that the mix of both oils has a lower capacity for adipogenesis reduction than the individual samples. Although PUA has been described to have a negative effect on adipogenesis, the opposite has been widely reported for omega-3 FAs. Nevertheless, some reports are showing that other bioactive FAs namely ALA, rather than DHA improve adipogenesis in 3T3-L1 cells in a GPR120-dependent pathway. A DHA-dose dependent (10-200 μM) decrease in lipid droplet % was observed, while ALA treatment resulted in more abundant lipid accumulation with the correspondent concentration increase (45). The existence of a dose-dependent mechanism for omega-3 action on adipogenesis may explain the different results reported and may be extremely relevant in designing therapies for obesity or preventive strategies.

To determine if these FAs' effects on adipogenesis were somehow mediated through GPR120, TUG-891 was selected in this study as the synthetic agonist of this receptor. Indeed, TUG-891 has been described to activate GPR120 in 3T3-L1 preadipocytes and influence adipogenesis (45). An early study demonstrated that there is a dose-response in 3T3-L1 cells regarding TUG-891: at low concentrations (0.5 μM) this chemical agonist functions as a positive enhancer in the adipogenic process, and higher concentrations (5 μM) induces the opposite effect: osteogenesis (46). It was observed a statistically significant increase in adipogenesis for all the samples in the presence of TUG-891 **(Figure 6.6)**. Despite the higher concentration of 30 μM used in this study, this chemical agonist functioned as a positive enhancer of the adipogenesis process and alone it was able to counteract the negative effects of the Fish oil and Pomegranate

oil samples. The increase in adipogenesis was expected since the activation of GPR120 has been widely reported to induce adipogenesis.

There are several studies demonstrating that GPR120 plays a key role in adipose tissue development, namely white and brown adipocytes. Indeed, GPR120 knockdown by siRNA or GPR120-/- embryonic mice models, was found to inhibit adipogenesis (43,45,47). In fact, in the presence of the antagonist AH7614 (alone), adipogenesis was inhibited. However, it is important to mention that this was not statistically different from the TUG-891 sample or the vehicle control (DMSO 0.01%), so no conclusion can be undoubtedly made about this sample. Nevertheless, in the presence of AH7614 the adipogenesis inhibition potential that all the samples presented was abrogated. This suggests an important role of GPR120 in Fish oil and Pomegranate oil adipogenesis inhibitory effect. So, considering that both TUG-891 and Fish oil and Pomegranate oil seem to present opposite roles in adipogenesis through GPR120, it seems that GPR120 can have a dual role in adipogenesis mediation, which depends on the ligand and is dose-dependent.

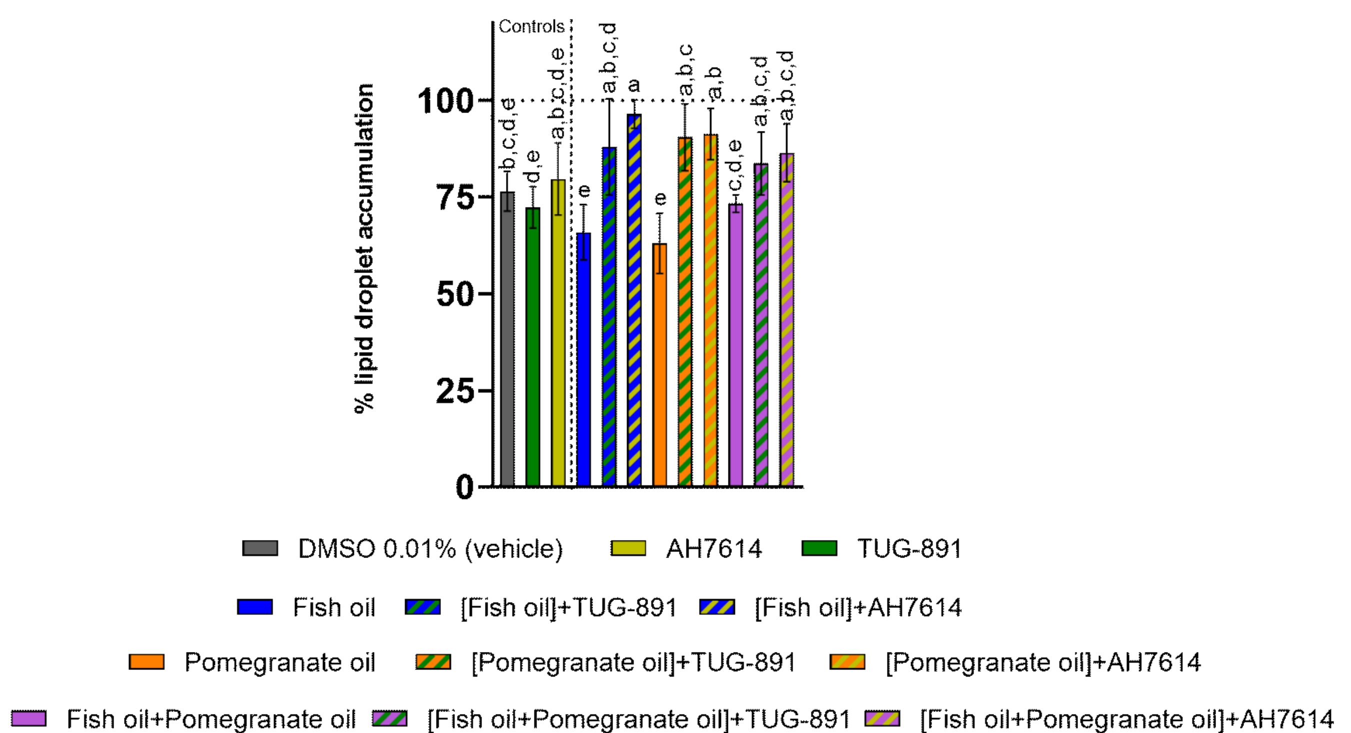

Figure 6.6 – Effect of GPR120 receptor in adipogenesis and its role on the effects of Fish oil, Pomegranate oil and the mixture of both oils. AH7614 was the selected chemical antagonist of GPR120 receptor and TUG-891 the chemical agonist. DMSO at 0.01% (v/v) was assayed as a control since it was used as AH7614 and TUG-891 vehicle. The results are means of two biological replicates and two analytical replicates and are represented as mean±standard deviation. Different letters (a, b) indicate statistically significant differences ($p < 0.05$).

4. Conclusion

Considering TEER values, there is an opposite effect of AEA alone and in combination with Fish oil+Pomegranate oil or Pomegranate oil. This may suggest that Pomegranate oil PUFA, probably its major bioactive FAs (PUA, catalpic, α- and β-eleostearic acids) content may compete with AEA and decrease its activation effect on CB1. The same was not verified for Fish oil, which in combination with AEA showed similar TEER values as AEA alone. Although they may also have similar competitive effects, EPA and DHA-rich diets enhanced the activity of CB1 receptors in mice, which may explain the similar TEER values. So, it has been demonstrated that different PUFA compositions may have different effects on CB1-induced permeability. PUFAs, such as omega-3 and CLNA isomers, incorporation in cell membranes will negatively impact the permeability and may explain the results obtained. Collectively these results suggest that at least some of the mechanisms behind omega-3 EPA, DHA, and DPA bioaccessibility and bioavailability may be mediated through the CB1 receptor. Nevertheless, further studies with different permeability models assessing CB1 expression and role, such as the co-culture model of Caco-2/HT29-MTX, described in **Chapter 3**, need to be considered in the future.

Regarding the adipogenesis assays, the % of lipid accumulation was decreased in Fish oil, Pomegranate oil, and Fish oil+Pomegranate oil samples, suggesting a potential of these samples for adipogenesis reduction. Although not statistically significant, it seems that the mix of both oils has a lower capacity for adipogenesis reduction than the individual samples. Contradictory theories have been emerging regarding the potential role of adipogenesis in obesity progression. Thus, in the context of obesity treatment, where obesity is already installed, Fish oil+Pomegranate oil mixture may have a more promising effect, since it presents lower adipogenesis reduction. On the other hand, if considering a preventive treatment for obesity, Fish oil or Pomegranate oil alone seem to have a more promising potential since they seem to prevent adipogenesis. Regarding the CB1 receptor role on Fish and Pomegranate oil in adipogenesis, in the presence of AEA, the % of lipid droplet accumulation increased. These results suggest that upon CB1 activation the capacity of Fish oil and Pomegranate oil to inhibit adipogenesis significantly decreases. On the other hand, regarding the GPR120 receptor, it was observed a statistically significant increase in adipogenesis for all the samples in the presence of TUG-891 (GPR120 agonist). At the studied concentration this chemical agonist functioned as a positive enhancer of adipogenesis and alone it was able to counteract the negative effects of the Fish oil and Pomegranate oil samples. Moreover, in the presence of the antagonist AH7614 (alone), the adipogenesis process was inhibited. On the opposite, in the presence of AH7614 the adipogenesis inhibition potential that all the samples presented was abrogated. This suggests an important role of GPR120 in Fish oil and Pomegranate oil adipogenic inhibitory effect. In addition, these results seem to demonstrate that the role of GPR120 in adipogenesis is ligand- and dose-dependent.

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CHAPTER 7

Final remarks

1. Conclusions

The major results and conclusions from the work reported in this thesis are demonstrated in **Figure 7.1.** It has been demonstrated the great potential of using omega-3, EPA and DHA, CLA isomers, RA and C18:2 *t*10*c*12, and the CLNA isomer PUA, in a systemic approach targeting different organs and tissues to promote and restore the negative effects induced by WD (or WD-like nutrients) consumption.

Indeed, all the PUFAs studied showed promising anti-inflammatory potential by reverting the NF-κB activation by PA and Fructose in a microglia cell line. Importantly, this action was demonstrated to be mediated by the GPR120 receptor. Besides, omega-3 and CLA showed antioxidant potential by inhibiting ROS production, also induced by PA and Fructose. Omega-3 and CLNA isomer PUA demonstrated an inhibiting capacity regarding Src activation, which is highly relevant in a neuroinflammation context. Importantly, although the anti-inflammatory role of omega-3 FAs in the hypothalamus through NF-κB pathway inhibition (by GPR120 activation) has been demonstrated before in rodent models (1–3), their impact in microglia activation and specifically on its markers, such as Src activation and ROS production, was overlooked. Besides, the possible beneficial impact of CFAs, namely CLA and CLNA isomers, in obesity is often associated with reducing body fat and with their anti-inflammatory properties in peripheral tissues. This capacity is often described to be mediated through PPAR activation (4–7). As demonstrated in **Chapter 1 Sections 3.1.2 and 3.1.3,** very few studies have addressed the impact of CLA and CLNA isomers in the brain, if considering more specifically the impact of these PUFAs in microglia activation in an obesity context even fewer (*in vitro* or *in vivo)* studies are available. In addition, most studies on CFAs have been reporting a PPAR-mediated anti-inflammatory action. We have demonstrated, for the first time, that in microglia cells the PA and Fructose-induced NF-κB pathway activation can be inhibited by CLA (RA and C18:2 *t*10*c*12) and CLNA (PUA) isomers and this process may be mediated by GPR120. These results are extremely relevant since *i*) they pave the way to further *in vivo* studies to assess and demonstrate the full potential of CFAs in the brain; *ii*) they demonstrated that GPR120 plays an important role not only in omega-3 FAs effects but also in other PUFAs, such as CFAs; being a relevant therapeutical target; *iii*) they demonstrate that other PUFAs, besides omega-3, may offer a systemic therapeutical approach for obesity treatment/prevention.

Despite these promising results, it is important to consider that the more accessible route to administer these bioactive PUFAs is through oral consumption. Considering this, the effect of GIT was simulated using the standardized INFOGEST protocol. It is relevant to mention that there are very few studies addressing the bioaccessibility of similar matrixes, and most *in vitro* studies available use different protocols for GIT simulation, which makes the accurate comparison of results extremely challenging. All the bioactive FAs provided through commercial sources - Fish oil and omega-3 capsules content, Pomegranate oil and CLNA capsules content, and CLA capsules content – showed low RI and bioaccessibility indexes, which compromises the desired

effect in CNS. So, further studies are required to find effective ways of delivering these bioactive molecules to promote health effects in a neuroinflammation context. It is important to mention that some contradictory results have emerged regarding the bioaccessibility and bioavailability of such PUFAs, with higher indexes being reported (8) compared to the ones presented in this work. Nevertheless, the doses accessed here (3 g), although higher than the daily recommended doses (250-500 mg for omega-3 EPA and DHA and ≈2 g for CLA and CLNA isomers), are lower than the doses tested in several of the published clinical trials (8). Moreover, in most of these studies, PUFA supplementation was a part of a more complex diet. This factor will also impact both the bioaccessibility and bioavailability of these bioactive FAs. Thus, further studies are required to fully address these issues and for a complete understanding of the most effective approach for omega-3 and CFAs delivery. Nevertheless, in the study presented in this thesis important conclusions were drawn, summing up its relevance: *i*) there is a need to use standardized protocols, either *in vitro* or *in vivo* (including clinical trials) when assessing both bioaccessibility and bioavailability of bioactive molecules aiming for a more accurate comparison of results; *ii*) the concentration of a given PUFA, the matrix in which it is incorporated, the daily dose provided and the dietary context, are important considerations to be made when studying its bioaccessibility and bioavailability; *iii*) the low bioaccessibility observed in this study and the retention of a significative bioactive PUFA content in the colon paved to way to further studies aiming to assess these PUFAs modulatory effects in gut microbiota.

The importance of gut microbiota modulation in several diseases, like obesity, T2DM, IBD, and food allergies, among others, has been widely established and diet is a key target to consider. Prebiotics, for instance, are extensively described to modulate the intestinal microbiota and for a long time, the prebiotic action was mainly attributed to polysaccharides. Nevertheless, nowadays, the prebiotic term has been revised and other ingredients, including omega-3 FAs, have been included since they have been shown to allow changes in the composition and metabolism of the gut microbiome resulting in important health benefits (9–11). Taking into consideration the observations made in the previous study and the possible beneficial effect of these PUFAs in gut microbiota, the Fish and Pomegranate oil and Omega-3, CLA, and CLNA capsules content were subjected to *in vitro* human fecal fermentations. It was demonstrated that these bioactive FAs may present a prebiotic potential by modulating the bacterial community, promoting the growth of important bacteria in disease-like contexts such as *Akkermansia* spp. Moreover, they can also modulate bacterial metabolism by increasing butyrate synthesis, promoting an anti-inflammatory environment. Besides, they promote the synthesis of several important amino acids, including GABA and tyrosine, important in gut-brain axis communication. This study demonstrated that *i)* indeed, omega-3 FAs present important prebiotic activity, which was markedly demonstrated by metabolic changes in bacterial communities, *ii)* besides omega-3, there are other relevant PUFAs, in this case, CFAs, that may present an important modulatory role in gut microbiota, *iii)* the gut microbial changes induced by these FAs may be extremely relevant in an obesity/metabolic

syndrome context and *iv)* these PUFAs may have a positive role in gut-brain axis modulation through SCFAs and amino acids synthesis.

Considering the third point mentioned in the previous paragraph – *iii*) the gut microbial changes induced by these FAs may be extremely relevant in an obesity/metabolic syndrome context – the next objective of this work was to demonstrate if the selected PUFA sources – Fish oil, Pomegranate oil, and a mix of both – were able to revert the WD-induced alterations, often associated with diseases like T2DM, metabolic syndrome and obesity, after *in vitro* fermentations using cecal samples from rats subjected to a WD-like diet. For the first time, it was demonstrated the synergistic effect of Fish oil and Pomegranate oil in promoting important alterations to restore the gut microbiota negative effects that were induced by WD consumption. Indeed, the mixture of Fish oil and Pomegranate oil was able to increase α-diversity, a crucial parameter that is known to be decreased in several diseases, increase at the phylum level Firmicutes and Bacteroidetes relative abundances as well as *A. muciniphila*. Again, GABA and tyrosine levels were also found to be elevated after fermentation with this sample. All these results suggest *i*) the great potential of using oils enriched in different PUFAs in the treatment of metabolic diseases like obesity or potentially T2DM. Moreover, *ii*) it was demonstrated that despite the great potential that omega-3 FAs show, there are other bioactive FAs that along with omega-3 may promote more relevant synergetic effects.

To uncover some molecular mechanisms behind this bioactive FAs action the last work of this thesis was focused on assessing their role in gut permeability and adipogenesis. First, considering the low bioaccessibility discussed before, the CB1 receptor involvement in the gut permeability modulation by these FAs was assessed by using chemical agonists and antagonists. Importantly, CB1 is the most abundant and widespread GPCR in the mammalian CNS, being expressed in peripheral tissues, including the gut, where it is involved in nociception, adipogenesis, pro-inflammation processes, and gut permeability. We hypothesized that its modulation may affect these FAs' permeability. Interestingly, it was demonstrated that CB1 may have a role in intestinal permeability and different bioactive PUFAs may respond differently to this receptor. So, CB1 may be a possible target to increase bioaccessibility and bioavailability indexes, increasing the absorption of such PUFAs. Secondly, as discussed in **Chapter 1 Section 1.1**, most of the anti-obesity potential of these FAs is due to their capacity for adipose tissue modulation. Considering the positive results obtained for GPR120 regarding their possible role in the anti-inflammatory effect of CLNA in the studied microglia cell line, we intended to understand if there is any involvement of this receptor in CLNA and Omega-3 effects in adipose tissue (here studied by the use of a 3T3-L1 adipocyte cell line). Again, in peripheral tissues, such as adipose tissue, the CFAs effect is often associated with PPAR modulation. Moreover, considering the expression of CB1 in adipose tissue and its involvement in adipogenesis, the involvement of this receptor in Omega-3 (Fish oil) and CLNA (Pomegranate oil) effects in adipogenesis modulation were also determined. Once again, there seems to be an important role of both GPR120 and CB1 (studied separately) in Fish and Pomegranate oil inhibitory adipogenesis effects. These results

demonstrate that the molecular mechanisms behind these bioactive PUFA actions in several tissues and organs are still elusive, and further studies are required. Nevertheless, the targeting of these receptors in obesity to improve existing or new therapies, including possible approaches with Omega-3 and CLNA PUFAs, may not be disregarded.

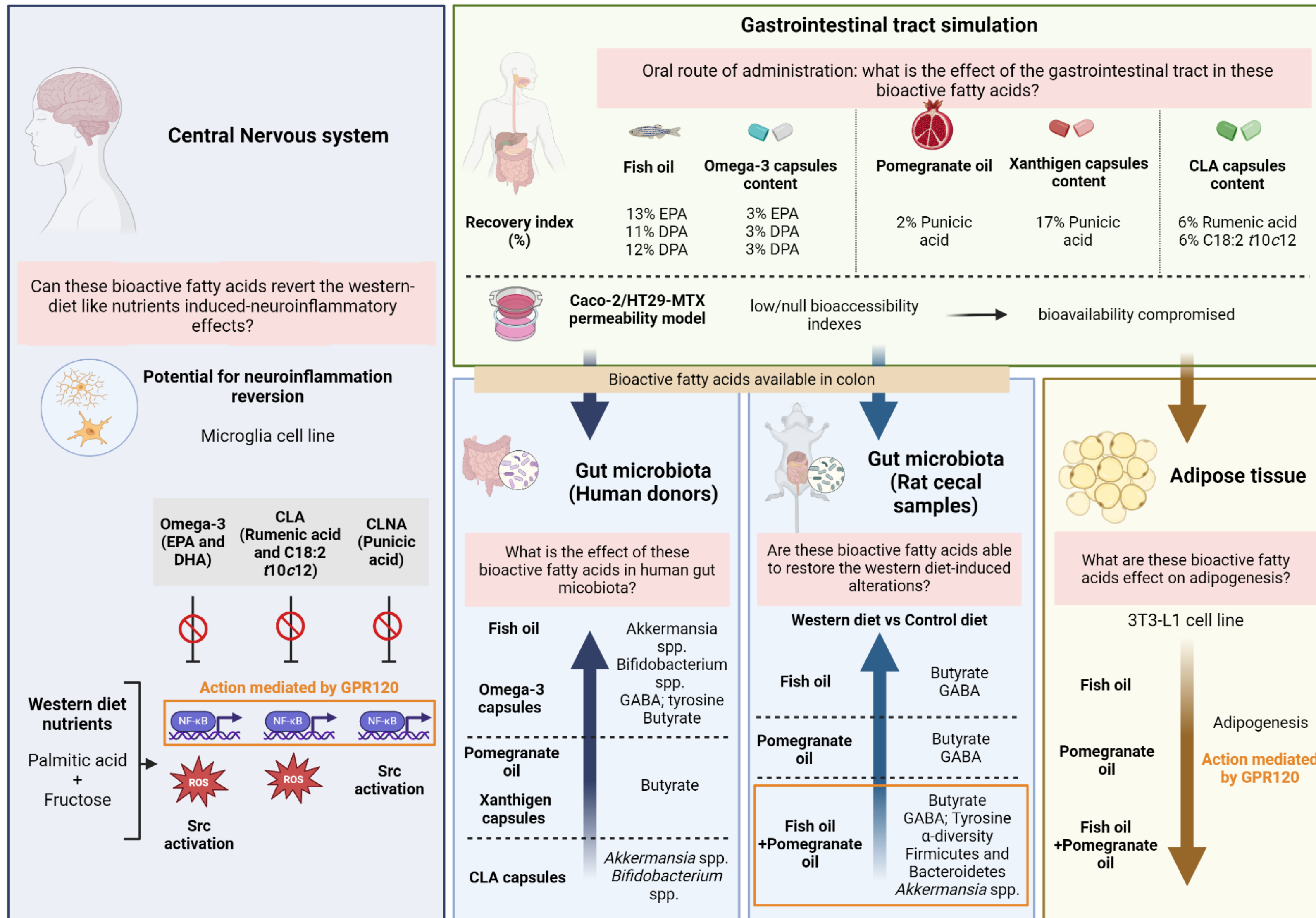

Figure 7.1 – **Major conclusions** of the work developed in this thesis.

2. Future Work

This study functioned as an important screening for the potential for a preventive or therapeutical approach of omega-3 and CFAs, using enriched oils, in WD-associated obesity effects. Despite the promising results and the modulatory effect on gut microbiota, there is a great degradation of major bioactive FAs during GIT digestion and all the bioactive FAs in most of the samples demonstrated low bioavailability capacity which will drastically compromise the desired effect on CNS. One solution could be finding encapsulation strategies to improve both GIT resistance and consequently oxidative stability as well as bioavailability. So, further studies are required to find the most promising encapsulation strategy that can comprise microencapsulation, nanoencapsulation, and bigels, among others. The selection of the best strategy will have to take into consideration bioaccessibility, considering that in the ideal formulation, part of the bioactive FAs content must be available for gut microbiota due to its relevant effects, as demonstrated in this work, and part of these bioactive FAs must be absorbed through intestinal epithelium and be able to cross BBB to exert the demonstrated positive effect in the CNS. One important aspect that needs to be further deepened, considering the promising results obtained here, is the gut-brain axis communication and the potential of these FAs to modulate it. Indeed, this interaction can be further assessed using in vitro BBB models for the gut-brain axis (12).

Once the best encapsulation strategy is found, the next step will encompass the validation of Fish oil+Pomegranate oil's effect on obesity therapy and prevention using rodent models. For that a layout of a possible experience will be illustrated: male C57BL/6 mice will be randomly assigned to the control diet (standard chow diet, CD) or WD (high-sugar and high-fat diet). After 12 weeks, WD and CD animals will be randomly distributed into 8 groups (n=8/group) and their diet will be orally supplemented, daily, with fish oil, pomegranate oil, or with a Fish oil+Pomegranate oil blend (1:1) encapsulated with pre-determined daily dietary doses. Once the obesity and metabolic syndrome parameters are established and confirmed, oral supplementation with the mentioned bioactive oils starts. Body weight and food intake will be monitored weekly to calculate weight gain and gross energy intake (13). Glucose and insulin tolerance tests are going to be assessed at the end of the experimental time (week 16), to determine insulin and glucose resistance. At week 16, mice will be sacrificed: brain (hypothalamus, prefrontal cortex, and striatum), liver, and adipose tissue (mesenteric, subcutaneous, and visceral) will be collected. Serum samples are going to be collected and used to determine the postprandial glucose and triglycerides, as described by Nunes et al. (13). Moreover, serum leptin is going to be determined (14). Liver and gastrointestinal tissues (duodenum and colon) are going to be immediately excised, dissected into small pieces, and stored. Ultrasound imaging, macroscopic appearance, ultrastructure analysis, and histological examination will be performed to analyze hepatic changes between experimental groups. Triglyceride contents in the liver samples are going to be quantified. To determine the effects on hepatic mitochondrial function, bioenergetic parameters in isolated hepatic mitochondria are going to be assessed. The junctional complexes, namely tight

junctions, adherens junctions, and desmosomes, are going to be studied in the duodenum and colon sections (13). The adipose tissue of the mesenteric, epididymal, and retroperitoneal regions is going to be weighed and the visceral fat is going to be determined (15). Furthermore, adipocyte size and weight, insulin-stimulated, glucose uptake, and lipolysis in isolated adipocytes will be performed as previously described (16). The fatty acid composition of the collected adipose tissue is going to be determined (17). Gene (mRNA levels) and protein expression from liver and adipose tissue are going to be studied and measured for relevant and previously selected proteins (*e.g.* glucose transporter-1 (GLUT1), glucose-6-phosphatase (G6PC), acetyl-CoA carboxylase 1 (ACC1), fatty acid synthase (FASN), hormone-sensitive lipase (HSL), and other relevant proteins) (16). As described by Socodato et al. (18) the brain is going to be collected, frozen, and cryosectioned in a Cryostat. Confocal images from tissue sections of the prefrontal cortex and hippocampus are going to be acquired and microglia, neurons, TNF, and synapses quantification are going to be performed. Using flow cytometry and specific markers, the characterization of immune cells in the acquired samples is going to be obtained. RNA is going to be extracted for a transcriptomics analysis to be performed.

Finally, cecal samples will be obtained on week 16 (final experimental time) and will be used to assess gut microbiota alterations in the studied diets compared to control animals (SC diet animals).

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Chapter 7.2

SUPPLEMENTARY MATERIAL

Supplementary material

Figures

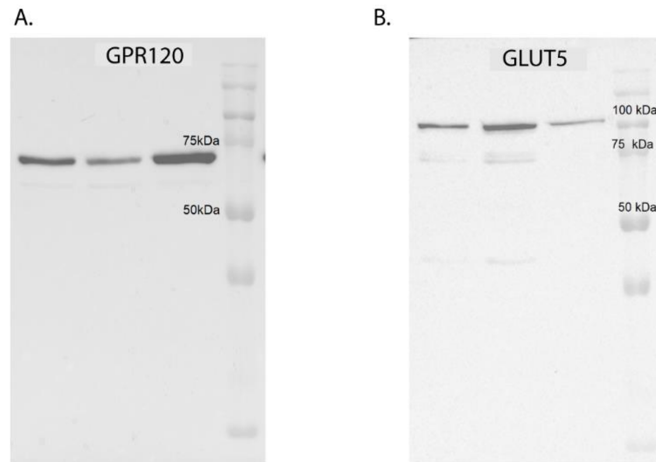

Figure S1 – Western Blot analysis of GLUT5 transporter **(A)** and GPR120 **(B)** from 3 independent HMC3 cell line cultures. Precision Plus Protein Standards Dual Color (161-0374, Bio-Rad) was the standard used. **(A)** Membranes were probed with antibody to GLUT5 and a clear band with approximately 100 kDa was observed; two other bands corresponding to 75 and 45 kDa proteins were detected. **(B)** Membranes were probed with antibody to GPR120 and a band corresponding to a 70 kDa protein was detected.

Tables

Table S1 - Transepithelial electrical resistance (TEER) measures ($\Omega \cdot cm^2$) used to assess the cell layer integrity of the Caco-2/HT29 co-culture.

Sample		0H	1H	3H	6H
Positive control		1110	1030	954	881
Negative control		370	169	180	184
CLA capsules	1	1012	890	830	774
	2	1090	950	877	864
CLNA capsules (Xanthigen)	1	884	789	671	700
	2	859	700	660	710
Omega-3capsules	1	1030	930	680	190
	2	1025	953	740	164
Fish oil	1	1142	1047	980	990
	2	1159	1070	1056	1055
Pomegranate oil	1	1035	745	570	555
	2	1061	630	515	555

Table S2 – Antioxidant activity of Pomegranate oil. Fish oil. CLA capsules. CLNA capsules (Xanthigen). Omega-3 capsules using DPPH and ABTS radicals' methods. The antioxidant activity is presented in mg of Trolox equivalent (TE)/ g of the sample and μmol TE/g of the sample. [a,b] Different superscript letters within a row for significant differences ($p < 0.05$).

Samples		DPPH		ABTS	
		mg TE/g of sample	μmol TE/g of sample	mg TE/g of sample	μmol TE/g of sample
Pomegranate oil	Original	1.48±0.12	**5.93±0.47^a**	0.74±0.08	**4.60±0.40^a**
	Digested	1.02±0.16	**4.09±0.66^b**	2.89±0.20	**2.21±0.19^b**
Fish oil	Original	1.03±0.14	**4.11±0.55^a**	1.15±0.10	**2.97±0.31^b**
	Digested	1.20±0.08	**4.79±0.33^a**	0.55±0.05	**11.55±0.79^a**
CLA capsules	Original	0.16±0.03	**0.42±0.02^b**	0.23±0.02	**0.94±0.09^b**
	Digested	0.14±0.03	**0.57±0.14^a**	0.97±0.14	**4.13±0.29^a**
CLNA capsules	Original	0.07±0.001	**1.15±0.015^a**	1.83±0.09	**7.30±0.38^a**
	Digested	0.05±0.004	**0.83±0.06^b**	1.31±0.08	**5.24±0.10^b**
Omega-3capsules	Original	1.23±0.04	**4.93±0.17^a**	1.19±0.04	**4.74±0.14^a**
	Digested	0.66±0.03	**2.65±0.10^b**	0.90±0.10	**3.60±0.39^b**

Table S3- FAs composition of the original digested sample (Fish oil and Pomegranate oil) before the Caco-2/HT29-MTX permeability assays and of cells. apical side and basolateral side collected after the end of permeability assays (6h) for each of the tested samples. Values are expressed as mean±standard deviation of µg of FAs/ µL of sample. The positive and negative controls were used to normalize the values. The RI (%) was calculated for each phase. Values are expressed as mean±standard deviation of µg of FAs/ µL of the sample. [a.b.c.d]Different superscript letters within a row for significant differences ($p < 0.05$).

µg of fatty acid/ µL of sample		CLA capsules					CLNA capsules (Xanthigen)					Fish oil					Pomegranate oil				
		Digested sample	Cells	RI (%)	Apical side	RI (%)	Digested sample	Cells	RI (%)	Apical side	RI (%)	Digested sample	Cells	RI (%)	Apical side	RI (%)	Digested sample	Cells	RI (%)	Apical side	RI (%)
C14	Myristic acid	n.d.	n.d.		n.d.		n.d.	n.d.		n.d.		8.81±0.02	2.41±0.22	27.41	4.02±0.05	45.61	n.d.	n.d.		n.d.	
C15	Pentadecylic acid	n.d.	n.d.		n.d.		n.d.	n.d.		n.d.		0.54±0.01	0.40±0.05	73.69	0.34±0.00	62.71	n.d.	n.d.		n.d.	
C16	Palmitic acid	0.42±0.01	4.80±0.23	1134.51	0.20±0.01	47.39	3.71±0.00	1.79±0.01	48.21	1.83±0.23	49.29	17.49±0.07	4.16±0.59	23.76	8.95±0.07	51.19	2.28±0.01	5.41±0.41	237.40	0.60±0.07	26.40
C16:1 *c*9	Palmitoleic acid	n.d.	n.d.		n.d.		n.d.	n.d.		n.d.		10.78±0.02	3.53±0.32	32.76	4.07±0.19	37.76	n.d.	n.d.		n.d.	
C18	Stearic acid	0.41±0.05	0.79±0.26	191.10	1.40±0.10	338.75	1.36±0.03	0.91±0.01	67.05	1.62±0.27	119.86	2.82±0.01	0.37±0.17	13.21	1.83±0.12	64.84	1.68±0.05	0.74±0.02	44.16	0.85±0.07	50.75
C18:1 *t*9	Elaidic acid	n.d.	n.d.		n.d.		n.d.	n.d.		n.d.		n.d.	n.d.		n.d.		0.22±0.08	0.05±0.05	22.48	0.31±0.02	143.02
C18:1 *t*11	Vaccenic acid	n.d.	n.d.		n.d.		n.d.	n.d.		n.d.		n.d.	n.d.		n.d.		0.27±0.09	0.43±0.06	155.93	n.d.	
C18:1 *t*12		0.83±0.26	1.14±0.16	136.95	1.00±0.05	120.64	n.d.	n.d.		n.d.		n.d.	n.d.		n.d.		0.32±0.11	0.70±0.09	221.12	0.62±0.01	195.42
C18:1 *c*9	Oleic acid	1.81±0.03	1.02±0.26	56.54	0.75±0.05	41.48	17.54±0.09	8.15±0.13	46.43	4.83±0.82	27.53	7.29±0.06	1.76±0.39	24.12	3.03±0.11	41.54	4.22±0.09	5.82±0.34	137.87	0.86±0.09	20.49
C18:1 *c*11	cis-vaccenic acid	0.23±0.03	0.26±0.08	111.50	0.33±0.01	141.97	0.60±0.00	12.10±0.13	2007.09	n.d.		3.01±0.03	0.55±0.05	18.29	1.38±0.06	45.73	0.35±0.02	0.27±0.07	78.68	n.d.	
C18:2 *c*9*c*12	Linoleic acid	0.42±0.00	0.28±0.05	66.77	n.d.		2.93±0.03	1.36±0.04	46.50	0.59±0.13	19.96	n.d.	n.d.		n.d.		4.67±0.04	1.40±0.12	30.05	0.74±0.09	15.80
C18:2 *c*9*t*11	Rumenic acid	18.47±0.58	3.93±.29	21.29	2.22±0.23	12.01	n.d.	n.d.		n.d.		n.d.	n.d.		n.d.		0.31±0.08	0.41±0.03	133.74	n.d.	
C18:2 *t*10*c*12	CLA isomer	18.22±0.61	4.87±0.44	26.71	1.56±0.16	8.58	n.d.	n.d.		n.d.		2.31±0.04	0.55±0.05	23.87	0.49±0.06	21.38	n.d.	n.d.		n.d.	
C18:3 *c*9*c*12*c*15	α-linolenic acid	0.61±.000	0.38±0.02	62.38	n.d.		n.d.	n.d.		n.d.		1.18±0.01	0.41±0.03	34.61	0.42±0.05	35.79	0.52±0.02	0.35±0.04	67.69	n.d.	
C18:2 *t*9*t*11	CLA isomer	3.01±0.01	1.56±0.15	51.99	1.03±0.05	34.23	0.63±0.10	0.51±0.04	81.75	n.d.		n.d.	n.d.		n.d.		1.23±0.31	1.24±0.12	100.92	n.d.	
C20:5 n-3	EPA	n.d.	n.d.		n.d.		n.d.	n.d.		n.d.		13.01±0.43	1.43±0.20	11.03	1.64±0.43	12.59	n.d.	n.d.		n.d.	
C18:3 *c*9*t*11*c*13	Punicic acid	n.d.	n.d.		n.d.		6.03±0.37	0.74±0.02	12.23	n.d.		n.d.	n.d.		n.d.		31.88±0.72	3.27±0.09	10.25	n.d.	
c18:3 *c*9*t*11*t*13	α-eleostearic acid	n.d.	n.d.		n.d.		1.50±0.05	0.36±0.02	23.65	n.d.		n.d.	n.d.		n.d.		5.32±0.06	1.13±0.20	21.24		
C18:3 *t*9*t*11*c*13	Catalpic acid	n.d.	n.d.		n.d.		1.61±0.09	n.d.		n.d.		n.d.	n.d.		n.d.		5.19±0.12	1.08±0.04	20.72		
C18:3 *t*9*t*11*t*13	β-eleostearic acid	n.d.	n.d.		n.d.		n.d.	n.d.		n.d.		n.d.	n.d.		n.d.		7.62±0.16	1.26±0.05	16.53	0.39±0.03	5.16
C22:6 n-3	DHA	n.d.	n.d.		n.d.		n.d.	n.d.		n.d.		6.44±0.50	0.54±0.08	8.44	0.55±0.17	8.52	n.d.	n.d.		n.d.	

Table S4- RI (%) of the major fatty acids quantified in the study samples after 12, 24 and 48 h of in vitro fecal fermentation of different bioactive lipids sources (Fish and Pomegranate oil, CLA, CLNA and Omega-3 capsules). The values presented result from the means of five donors. n.d = not detected; u.q.l= under quantification limit

Major fatty acids quantified		RI (%)														
		Pomegranate oil			**CLNA**			**CLA**			**Fish oil**			**Omega-3**		
		12 h	24 h	48 h	12 h	24 h	48 h	12 h	24 h	48 h	12 h	24 h	48 h	12 h	24 h	48 h
Myristic acid	**C14**	u.q.l	u.q.l	u.q.l	u.q.l	u.q.l	u.q.l	n.d	n.d	n.d	24.29	23.39	26.65	n.d.	n.d.	n.d.
Palmitic acid	**C16**	17.59	19.23	19.28	23.51	26.72	29.59	24.34	53.90	41.09	25.91	26.55	27.30	n.d.	13.17	16.57
Stearic acid	**C18**	31.09	28.54	26.26	31.85	32.25	34.65	127.70	143.87	94.17	29.60	32.04	29.01	20.84	27.03	21.81
Oleic acid	**C18:1 c9**	14.62	13.84	13.21	18.60	19.42	20.27	80.49	93.27	86.26	18.89	17.29	19.71	4.90	8.95	4.03
Vaccenic acid	**C18:1 t11**	1710.50	1945.37	2788.05	386.93	609.87	650.88	1309.34	1206.36	76.87	n.d	n.d	n.d	n.d	n.d	n.d
Linoleic acid	**C18:2 c9c12**	10.04	8.81	6.70	15.80	16.28	16.24	27.58	34.15	30.21	19.64	17.23	21.18	n.d	n.d	n.d
Rumenic acid	**C18:2 c9t11**	n.d	n.d	n.d	n.d	n.d	n.d	12.15	6.30	5.05	n.d	n.d	n.d	n.d	n.d	n.d
CLA isomer	**C18:2 t10c12**	n.d	n.d	n.d	n.d	n.d	n.d	0.98	1.09	0.99	11.08	11.09	12.03	n.d	n.d	n.d
CLA isomer	**C18:2 t9t11**	645.94	642.52	819.75	511.18	785.86	954.75	274.36	681.51	715.45	n.d	n.d	n.d	n.d	n.d	n.d
EPA	**C20:5 n-3**	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	10.04	10.56	12.72	1.81	3.72	1.94
Punicic acid (PUA)	**C18:3 c9t11c13**	0.69	0.75	0.73	1.12	1.27	1.20	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
catalpic acid	**C18:3 t9t11c13**	67.37	71.05	74.28	9.59	10.31	10.76	n.d	n.d	n.d	n.d	n.d	n.d	1.50	0.87	1.22
β-eleostearic acid	**C18:3 t9t11t13**	40.65	59.63	50.30	101.81	215.95	193.04	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
DPA	**C22:5 n-3**	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	8.14	9.22	10.11	n.d	n.d	n.d
DHA	**C22:6 n-3**	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	6.28	7.71	8.55	1.10	2.36	1.39

Table S5- Values of log 16S rRNA gene copies/ng of DNA of gut bacterial population (Number of copies) after 12, 24 and 48 h of *in vitro* fecal fermentation of different bioactive lipids sources (Fish and Pomegranate oil, CLA, CLNA and Omega-3 capsules). The values presented are the means of five donors ± standard deviation. Different letters indicate significant differences ($p < 0.05$). Relative differences (RD, %) in reference to the Negative control are displayed. The capital letters (A,B,C) indicate the differences among samples Negative control, Positive control (FOS) and bioactive lipids sources for the population of the same microbial genus at the same time. The small letters (a,b,c) indicate the differences for the same sample over time for the same microbial genus population.

Samples	**Time (h)**	**Gut microbiota**						
		Phylum Firmicutes			**Phylum Bacteroidetes**	**Phylum Actinobacteria**	**Phylum Verrucomicrobiota**	**Firmicutes:Bacteroidetes**
		Total	*Clostridium leptum*	*Roseburia* spp.	Total	*Bifidobacterium* spp.	*Akkermansia* spp.	
		Number of copies	Number of copies	Number of copies	Number of copies	Number of copies	Number of copies	
	0	**Fecal microbiota composition of volunteer participants.**						
		5,71±0,21^{a}	**4.73±0.33^{a}**	**4.55±0.39^{a}**	**4.05±0.22^{c}**	**3.50±1.74^{a}**	**2.05±0.92^{a}**	**1.41±0.07^{a}**
Negative control	12	5.81±0.32^{A,a}	4.52±0.31^{A,B,a}	3.74±0.47^{A,a,b}	4.36±0.10^{A,b,c}	4.34±1.57^{A,a}	1.57±0.98^{A,a}	1.33±0.05^{A,B,C,a}
	24	5.83±0.19^{A,a}	4.54±0.21^{A,B,a}	3.34±0.91^{A,b}	4.62±0.25^{A,a}	4.14±1.58^{A,a}	1.80±1.12^{A,a}	1.26±0.06^{B,a}
	48	5.72±0.30^{A,a}	4.47±0.44^{A,a}	3.44±0.38^{A,b}	4.30±0.13^{A,c}	3.99±1.63^{A,a}	1.60±0.78^{A,a}	1.33±0.05^{B,C,a}
Positive control (FOS)	12	5.81±0.31^{A,a}	5.09±0.49^{A,a}	4.15±0.13^{A,a}	4.17±0.23^{A,a}	5.44±0.49^{A,a}	1.62±0.49^{A,a}	1.39±0.03^{A,B,a}
	24	6.02±0.38^{A,a}	5.01±0.35^{A,a}	4.25±0.44^{A,a}	4.40±0.30^{A,a}	5.12±1.48^{A,a}	1.31±0.89^{A,a}	1.37±0.06^{A,B,a}
	48	5.75±0.34^{A,a}	4.63±0.26^{A,a}	3.90±0.34^{A,a}	4.23±0.28^{A,B,a}	4.96±1.59^{A,a}	1.24±0.96^{A,a}	1.36±0.05^{B,C,a}
Pomegranate Oil	12	5.35±0.28^{A,a}	3.43±0.66^{C,a}	3.60±0.51^{A,a}	4.40±0.25^{A,a}	3.25±1.12^{A,a}	1.72±0.99^{A,a}	1.22±0.10^{B,C,a}
	24	5.6±0.42^{A,a}	3.04±0.43^{C,a}	3.63±0.53^{A,a}	4.45±0.20^{A,a}	3.14±1.36^{A,a}	1.64±0.97^{A,a}	1.26±0.08^{B,a}
	48	5.61±0.37^{A,a}	2.75±0.23^{C,a}	3.25±0.56^{A,a}	4.25±0.25^{A,B,a}	3.57±1.32^{A,a}	1.68±0.91^{A,a}	1.32±0.10^{B,C,a}
CLNA	12	5.44±0.43^{A,a}	3.68±0.43^{B,C,a}	3.67±0.27^{A,a}	3.73±0.43^{A,c}	3.95±1.33^{A,a}	1.49±0.82^{A,a}	1.47±0.17^{A,a}
	24	5.75±0.29^{A,a}	3.92±0.24^{B,a}	3.59±0.68^{A,a}	4.25±0.24^{A,B,a}	1.38±1.47^{A,a}	1.76±0.9^{A,a}	1.35±0.10^{A,B,a}
	48	5.72±0.19^{A,a}	3.73±0.31^{B,a}	3.19±0.63^{A,a}	3.83±0.13^{B,C,b,c}	4.14±1.23^{A,a}	1.69±0.81^{A,a}	1.5±0.08^{A,B,a}
Fish Oil	12	5.36±0.36^{A,a}	4.02±0.14^{B,C,a}	3.64±0.56^{A,a}	4.40±0.22^{A,a}	4.25±1.55^{A,a}	1.57±0.95^{A,a}	1.22±0.07^{B,C,a}
	24	5.57±0.43^{A,a}	4.04±0.24^{B,a}	3.71±0.59^{A,a}	4.57±0.17^{A,a}	4.83±0.33^{A,a}	1.87±0.81^{A,a}	1.22±0.11^{B,a}
	48	5.63±0.29^{A,a}	4.06±0.25^{A,B,a}	3.31±0.59^{A,a}	4.45±0.27^{A,a}	4.35±1.13^{A,a}	1.8±0.79^{A,a}	1.27±0.13^{C,a}
Omega-3	12	5.68±0.38^{A,a}	4.28±0.26^{A,B,C,a}	3.67±0.49^{A,a}	4.34±0.11^{A,a}	4.02±1.96^{A,a}	1.44±0.92^{A,a}	1.31±0.08^{B,C,a}
	24	5.84±0.24^{A,a}	4.44±0.17^{A,B,a}	3.37±0.66^{A,a}	4.37±0.17^{A,a}	4.75±1.16^{A,a}	1.66±0.90^{A,a}	1.34±0.06^{A,B,a}
	48	5.82±0.28^{A,a}	4.20±0.21^{A,B,a}	3.20±0.64^{A,a}	4.38±0.24^{A,a}	5.31±0.26^{A,a}	1.75±0.80^{A,a}	1.33±0.07^{C,a}
CLA	12	5.48±0.33^{A,a}	3.47±0.67^{C,a}	3.71±0.54^{A,a}	4.48±0.16^{A,a}	4.6±0.86^{A,a}	1.45±1.08^{A,a}	1.21±0.10^{C,b}
	24	5.72±0.36^{A,a}	3.05±0.66^{C,a}	3.69±0.58^{A,a}	3.92±0.14^{B,b}	3.97±1.29^{A,a}	1.79±0.96^{A,a}	1.46±0.12^{A,a}
	48	5.76±0.29^{A,a}	3.05±0.46^{C,a}	3.43±0.73^{A,a}	3.6±0.22^{C,c}	4.04±1.11^{A,a}	1.88±0.88^{A,a}	1.61±0.13^{A,a}

Table S6- Concentrations of the most relevant amino acids identified (mg/L) after 12 and 24 h of the *in vitro* fecal fermentation of different bioactive lipids sources (Fish and Pomegranate oil, CLA, CLNA and Omega-3 capsules). The concentrations of the original samples, after gastrointestinal tract digestion, are present as reference values. The values presented are the means of five donors ± standard deviation. Different letters indicate significant differences ($p < 0.05$). The capital letters (A,B,C) indicate the differences among samples - Negative control, Positive control (FOS), and bioactive lipids sources - for the same amino acid at the same time. The small letters (a,b,c) indicate the differences for the same sample over time for the amino acid. u.q.l= under quantification limit; n.d.= not detected

Samples	Time (h)	Free Amino acid composition												
		Glutamate	Glycine	Threonine	Arginine	Alanine	Tyrosine	Valine	Methionine	Tryptophan	Phenylalanine	Isoleucine	Leucine	GABA
		Free amino acids composition of the starting sample												
Negative control	0	**27.98±5.83**	**135.80±32.1**	**22.52±1.94**	**103.16±38.75**	**91.19±34.88**	**24.49±5.03**	**15.78±0.93**	**25.77±1.15**	**17.07±3.64**	**30.09±2.23**	**16.92±2.79**	**50.93±9.89**	**62.21±14.63**
	12	8.90±2.58^{A,a}	10.48±6.33^{B,a}	14.60±3.19^{A,B,a}	10.30±4.52^{C,a}	8.65±4.67^{E,a}	20.92±3.17^{C,a}	62.23±13.23^{A,B,a}	64.63±14.79^{B,a}	6.03±3.54^{D,a}	59.25±9.70^{B,C,a}	62.84±18.25^{A,B,b}	81.07±12.05^{A,B,a}	123.64±54.19^{B,a}
	24	u.q.l	5.29±2.49^{D,b}	1.53±0.39^{D,b}	4.50±4.50^{C,a}	u.q.l	14.08±6.14^{C,a}	51.37±22.88^{E,a}	62.17±28.14^{B,a}	5.89±4.83^{D,a}	91.90±20.28^{B,C,b}	104.10±20.05^{B,a}	108.55±23.92^{A,B,C,a}	158.45±50.57^{A,a}
Positive control (FOS)	12	12.46±3.19^{A,a}	69.46±10.50^{B,b}	28.92±9.80^{A,a}	72.83±42.39^{A,a}	308.71±73.70^{A,B,a}	32.38±11.65^{B,C,a}	54.86±15.83^{B,a}	47.18±15.45^{B,a}	104.45±36.71^{A,a}	95.10±30.74^{A,B,a}	119.97±64.44^{A,a}	123.25±40.51^{A,a}	240.75±72.17^{A,a}
	24	7.46±1.69^{B,C,a}	180.99±33.55^{A,B,a}	37.55±5.23^{A,a}	45.86±22.39^{A,B,a}	130.90±6.12^{C,b}	30.56±11.73^{B,C,a}	59.18±9.19^{E,a}	57.58±4.82^{B,a}	27.78±2.31^{A,B,b}	53.34±2.78^{C,a}	67.56±14.59^{B,a}	96.28±12.00^{A,B,C,a}	131.08±21.38^{A,a}
Pomegranate oil	12	44.01±23.29^{A,a}	183.39±41.74^{A,a}	21.74±11.23^{A,a}	39.50±10.84^{A,B,C,a}	133.40±38.60^{C,D,a}	58.21±6.42^{A,a}	116.94±26.38^{A,a}	103.61±20.40^{A,a}	31.29±7.34^{B,C,a}	65.04±9.35^{B,C,a}	52.57±2.24^{B,a}	98.99±12.68^{A,B,a}	39.42±8.12^{B,a}
	24	4.41±1.57^{C,b}	71.05±71.18^{B,C,b}	3.42±0.60^{C,D,b}	47.54±10.02^{B,a}	133.28±44.91^{C,a}	55.73±14.09^{A,B,a}	87.06±10.87^{C,D,E,a}	68.43±17.41^{B,a}	14.28±5.53^{B,C,b}	63.94±10.87^{C,a}	98.31±35.75^{B,a}	108.60±26.04^{A,B,C,a}	14.66±2.17^{B,b}
	Original sample (GIT digestion)	67.09	288.73	30.61	16.32	94.53	55.76	59.02	10.58	19.32	22.62	38.04	66.96	u.q.l
CLNA	12	22.18±3.50^{A,a}	179.77±28.28^{A,a}	27.89±9.19^{A,a}	74.51±23.60^{A,B,a}	85.53±61.54^{D,E,a}	32.29±9.90^{B,C,a}	120.76±20.30^{A,a}	47.15±8.96^{B,b}	21.72±4.06^{B,C,a}	49.84±8.09^{C,a}	36.62±9.50^{B,b}	90.31±8.16^{A,B,a}	55.39±20.09^{B,a}
	24	1.20±0.52^{D,b}	6.92±3.16^{D,b}	5.91±8.49^{B,C,b}	29.84±5.50^{B,C,b}	186.97±19.68^{B,C,a}	41.07±20.37^{A,B,C,a}	146.62±19.42^{B,C,a}	62.92±5.80^{B,a}	3.73±0.39^{D,b}	58.91±9.38^{C,a}	66.74±8.84^{B,a}	82.33±15.53^{C,a}	19.16±2.28^{B,b}
	Original sample (GIT digestion)	77.31	264.33	40.07	35.03	84.98	52.45	30.41	11.51	17.33	27.35	48.14	60.23	84.43
Fish oil	12	46.52±29.29^{A,a}	162.71±20.74^{A,b}	32.41±7.31^{A,a}	62.72±11.56^{A,B,a}	125.33±21.99^{D,C,b}	41.12±0.85^{A,B,a}	96.62±8.80^{A,B,a}	51.56±1.19^{B,b}	26.77±3.70^{B,C,a}	50.54±5.84^{C,a}	39.48±4.82^{B,b}	60.22±5.50^{B,b}	49.05±15.85^{B,b}
	24	66.01±31.38^{A,a}	370.46±32.44^{A,a}	6.10±3.85^{C,b}	77.67±19.56^{A,a}	283.51±88.71^{A,B,a}	60.18±27.68^{A,B,a}	77.35±26.32^{D,E,a}	70.84±13.49^{B,a}	15.68±4.78^{B,C,b}	90.25±34.62^{B,C,a}	106.33±33.73^{B,a}	149.84±29.40^{A,a}	141.24±56.16^{A,a}
	Original sample (GIT digestion)	110.91	294.94	41.66	264.65	120.82	69.49	48.07	n.d.	19.41	30.58	53.37	65.53	100.52
Omega-3	12	12.73±6.07^{A,a}	134.29±22.75^{A,a}	23.15±11.49^{A,a}	27.12±1.77^{B,C,a}	125.38±29.05^{D,C,b}	59.58±7.49^{A,b}	81.62±39.65^{A,B,b}	107.51±24.34^{A,b}	18.82±4.96^{C,b}	53.37±6.98^{C,b}	45.13±7.35^{B,b}	76.73±18.23^{B,b}	60.10±11.55^{B,a}
	24	13.58±7.63^{B,a}	29.57±17.56^{C,D,b}	u.q.l	21.34±5.02^{B,C,a}	439.42±139.04^{A,a}	77.43±6.95^{A,a}	319.14±91.65^{A,a}	382.94±102.19^{A,a}	68.37±22.78^{A,a}	186.31±2.83^{A,B,a}	183.26±60.89^{A,a}	150.01±36.30^{A,a}	138.45±44.09^{A,a}
	Original sample (GIT digestion)	94.18	301.39	39.94	247.02	107.43	73.40	46.94	7.46	20.06	31.20	62.90	62.51	100.11
CLA	12	39.91±21.95^{A,a}	156.46±27.51^{A,a}	5.27±2.04^{B,b}	54.21±8.94^{A,B,C,a}	211.65±47.11^{B,C,a}	46.72±8.48^{A,B,a}	110.1±19.21^{A,a}	51.14±9.83^{B,a}	38.73±6.69^{B,a}	88.04±2.64^{A,B,a}	69.41±9.24^{A,B,a}	110.80±24.11^{A,a}	121.24±26.16^{B,a}
	24	2.95±1.50^{C,D,b}	21.85±13.58^{C,D,b}	26.92±1.31^{A,B,a}	40.36±2.77^{B,a}	159.51±47.97^{B,C,a}	46.37±8.74^{A,B,C,a}	118.13±22.35^{B,C,D,a}	54.66±6.18^{B,a}	9.53±3.81^{B,C,b}	55.81±3.93^{C,b}	100.42±24.05^{B,a}	94.90±20.19^{B,C,a}	8.88±2.71^{B,b}
	Original sample (GIT digestion)	68.97	258.50	21.91	314.02	48.58	52.08	25.80	11.06	19.73	30.43	34.85	51.99	78.73

Table S7 - pH values of the different samples at the different fermentation times (0, 6, 12, 24 and 48 h). CD-Control Diet and WD- Western Diet model. The values presented are the means of three biological replicates ± standard deviation. Different letters indicate significant differences ($p < 0.05$). The capital letters (A,B,C) indicate the differences among samples Negative control, Positive control (FOS) and bioactive lipids sources (Fish oil, Pomegranate oil and a mixture of both) at the same time. The small letters (a,b,c) indicate the differences for the same sample over time.

Time (h)	Negative control CD	Negative control WD	FOS CD	FOS WD	Fish oil CD	Fish oil WD	Pomegranate oil CD	Pomegranate oil WD	Fish oil+Pomegranate oil CD	Fish oil+Pomegranate oil WD
0	7.33±0.15^{a}	7.48±0.08^{a}	7.33±0.15^{a}	7.48±0.08^{a}	7.33±0.15^{a}	7.48±0.08^{a}	7.33±0.15^{a}	7.48±0.08^{a}	7.33±0.15^{a}	7.48±0.08^{a}
6	6.69±0.46^{b,A}	6.17±0.01^{b,B}	5.05±0.15^{b,C}	4.92±0.01^{b,C}	6.56±0.03^{b,AB}	6.20±0.01^{c,B}	6.41±0.04^{b,AB}	6.20±0.02^{c,B}	6.33±0.02^{b,AB}	6.19±0.00^{c,B}
12	6.42±0.18^{b,AB}	6.07±0.03^{c,B}	4.45±0.07^{c,E}	4.91±0.03^{b,D}	6.39±0.02^{b,AB}	6.17±0.01^{c,C}	6.29±0.04^{bc,BC}	6.18±0.02^{c,C}	6.20±0.04^{b,C}	6.19±0.01^{c,C}
24	6.28±0.15^{b,A}	6.19±0.01^{b,A}	4.29±0.02^{cd,B}	4.83±0.07^{b,B}	6.18±0.03^{c,A}	6.22±0^{bc,A}	6.21±0.04^{bc,A}	6.25±0.03^{c,A}	6.22±0.06^{b,A}	6.22±0.02^{bc,A}
48	6.28±0.17^{b,ABC}	6.26±0.02^{b,ABC}	4.18±0.02^{d,E}	4.35±0.01^{c,D}	6.12±0.02^{d,C}	6.37±0.01^{b,A}	6.14±0.07^{c,BC}	6.36±0.02^{b,A}	6.14±0.03^{b,BC}	6.31±0.01^{b,AB}

Table S8- Amino acids concentration values (mg/L) of the different samples at the different fermentation times (0, 6, 12, 24 and 48 h). CD-Control Diet and WD- Western Diet model. The values presented are the means of three biological replicates ± standard deviation. Different letters indicate significant differences ($p < 0.05$). The capital letters (A,B,C) indicate the differences among samples Negative control, Positive control (FOS) and bioactive lipids sources (Fish oil, Pomegranate oil and a mixture of both) at the same time. The small letters (a,b,c) indicate the differences for the same sample over time.

Sample	Time (h)	Amino acids (mg/L)																
		Aspartic acid	Glutamic acid	Cysteine	Asparagine	Serine	Glycine	Threonine	Arginine	Alanine	GABA	Tyrosine	Valine	Methionine	Tryptophan	Phenylalanine	Isoleucine	Leucine
Negative control CD	0	16.37±1.06	34.49±3.38	10.86±1.89	16.60±1.38	23.65±1.60	10.78±1.80	18.46±1.42	29.67±1.89	29.18±2.74	4.70±0.71	8.87±1.04	27.47±2.02	8.52±0.62	4.42±0.46	25.53±2.24	21.76±1.91	34.33±2.50
	6	27.73±2.70^{a,A}	43.96±0.40^{b,CD}	23.16±2.31^{a,AB}	29.99±0.87^{a,BC}	41.23±1.59^{a,AB}	34.78±2.31^{a,D}	36.05±1.76^{a,AB}	48.00±3.34^{a,A}	39.41±1.25^{c,DE}	12.87±0.83^{c,B}	16.40±1.02^{ab,CD}	45.36±2.46^{a,BC}	17.50±0.65^{a,CD}	8.92±0.46^{a,DEF}	49.16±3.40^{a,DE}	37.49±3.94^{ab,ABC}	53.93±2.93^{ab,BC}
	12	18.84±2.20^{b,A}	73.43±4.74^{A,a}	10.45±2.77^{b,ABC}	22.74±4.04^{b,B}	34.82±4.54^{a,B}	32.29±7.70^{a,B}	30.87±0.48^{b,A}	15.52±2.48^{b,AB}	69.96±5.53^{ab,BC}	19.2±1.95^{bc,B}	21.79±9.63^{a,AB}	44.4±0.73^{a,ABC}	12.37±2.34^{b,BC}	6.67±0.68^{a,BC}	40.52±9.43^{ab,BC}	32.86±5.10^{b,AB}	51.88±0.25^{b,AB}
	24	3.86±0.99^{c,B}	77.72±7.23^{a,A}	5.98±0.58^{bc,BC}	0.36±0.06^{c,B}	2.91±0.29^{b,BCD}	47.8±3.85^{a,ABC}	3.32±1.05^{c,DE}	9.83±4.13^{b,C}	78.95±7.67^{a,A}	27.96±5.73^{b,DE}	3.54±0.41^{b,C}	49.76±1.11^{a,AB}	19.11±0.35^{a,BC}	6.62±1.59^{a,ABC}	34.73±1.92^{b,B}	43.34±1.59^{a,BC}	59.5±2.53^{b,CD}
	48	2.67±0.21^{c,B}	75.20±5.58^{a,A}	1.32±0.34^{c,C}	0.20±0.05^{c,B}	1.78±0.19^{b,BC}	42.75±15.76^{a,ABC}	4.01±0.74^{c,C}	10.97±3.39^{b,ABC}	64.60±4.27^{b,AB}	43.21±4.87^{a,CD}	18.80±4.63^{a,C}	48.00±4.54^{a,A}	13.24±0.24^{b,CD}	6.32±1.37^{a,B}	33.43±3.19^{b,AB}	36.87±2.24^{ab,A}	50.02±4.13^{b,CD}
Negative control WD	0	12.75±0.84	47.59±13.31	10.77±1.26	13.54±3.65	1.67±0.22	18.86±7.92	21.17±4.63	42.69±16.46	48.47±20.14	20.64±12.80	10.22±2.26	33.82±6.95	12.30±3.05	5.54±1.02	29.65±4.69	26.48±6.10	41.01±6.02
	6	3.35±0.19^{a,C}	57.41±2.05^{b,AB}	7.96±0.68^{a,C}	0.59±0.00^{b,D}	1.19±0.02^{a,C}	53.42±2.18^{a,B}	0.9±0.07^{b,C}	14.06±0.52^{a,BC}	50.5±1.43^{b,BC}	22.75±1.08^{b,B}	14.62±0.49^{a,E}	56.57±1.52^{a,AB}	19.80±0.81^{a,ABC}	13.19±1.18^{a,AB}	52.72±3.69^{a,CDE}	46.10±1.57^{a,AB}	65.73±2.87^{a,ABC}
	12	2.63±0.01^{a,B}	81.19±8.89^{a,A}	4.62±0.57^{b,CED}	0.61±0.13^{b,C}	1.07±0.18^{a,D}	39.00±2.00^{b,ABC}	2.86±0.57^{a,B}	11.05±0.88^{ab,BC}	64.43±2.67^{a,BC}	9.97±0.14^{c,B}	6.22±0.44^{c,C}	53.36±3.53^{a,AB}	16.57±1.59^{a,AB}	7.4±0.21^{b,B}	47.75±3.64^{a,ABC}	35.83±1.93^{b,AB}	57.99±3.37^{b,AB}
	24	2.55±0.19^{a,B}	71.03±7.26^{ab,A}	5.42±0.48^{b,C}	<0.05	1.49±0.25^{a,CD}	40.91±1.79^{b,C}	2.56±0.80^{a,E}	12.05±2.40^{ab,BC}	77.59±5.91^{a,A}	43.06±6.33^{a,DE}	7.60±0.45^{b,C}	49.41±5.78^{a,AB}	16.74±2.07^{a,CD}	7.46±0.19^{b,AB}	35.64±2.12^{b,B}	41.65±4.25^{ab,BC}	53.06±3.35^{b,C}
	48	2.53±0.73^{a,B}	1.37±0.38^{c,B}	4.68±0.79^{b,B}	0.88±0.14^{a,B}	0.64±0.11^{b,C}	27.8±6.24^{c,ABC}	1.09±0.10^{b,C}	8.83±1.18^{b,CD}	42.82±0.22^{b,C}	22.34±3.65^{b,D}	2.29±0.60^{d,C}	23.04±6.28^{b,B}	7.81±1.02^{b,DE}	4.04±0.56^{b,CDE}	13.99±0.48^{c,C}	6.27±0.12^{c,B}	9.41±1.05^{c,E}
FOS CD	6	29.52±0.65^{a,A}	50.47±2.08^{c,BC}	24.57±0.71^{a,A}	26.57±1.59^{a,BC}	47.13±1.83^{a,A}	45.81±2.06^{ab,CD}	40.13±0.92^{a,A}	41.1±5.88^{a,A}	44.46±2.64^{d,CD}	14.6±2.7^{c,B}	17.41±0.7^{b,BCD}	49.87±2.07^{ab,ABC}	17.81±0.49^{a,CD}	9.96±0.52^{a,CDE}	47.52±1.60^{a,DE}	40.63±1.90^{a,ABC}	61.98±4.37^{a,ABC}
	12	18.76±3.76^{b,A}	64.69±8.77^{bc,A}	9.23±2.42^{c,ABCD}	17.56±1.13^{ac,B}	33.3±1.90^{b,B}	36.6±3.63^{b,ABC}	28.32±1.83^{c,A}	16.01±1.77^{b,AB}	65.38±1.86^{c,BC}	18.49±2.33^{b,B}	19.25±1.53^{b,AB}	37.23±3.54^{a,C}	11.2±0.73^{b,C}	6.57±0.37^{c,BC}	36.13±5.17^{ab,BC}	29.48±5.07^{b,B}	46.94±1.26^{b,B}
	24	22.89±1.79^{ab,A}	83.19±2.59^{ab,A}	15.07±1.77^{b,A}	24.24±0.64^{ab,A}	46.64±1.92^{a,A}	59.93±3.94^{a,A}	38.43±0.85^{ab,A}	15.8±1.92^{b,AB}	85.38±1.25^{a,A}	75.78±16.51^{a,B}	57.31±13.33^{a,A}	42.27±9.85^{a,B}	17.23±1.49^{a,CD}	8.58±0.25^{b,A}	35.46±1.34^{ab,B}	42.26±0.27^{a,BC}	61.09±0.99^{a,B}
	48	25.94±4.27^{ab,A}	91.97±16.75^{a,A}	15.12±0.62^{b,A}	20.40±2.56^{bc,A}	38.25±2.65^{b,A}	47.99±10.70^{ab,AB}	33.89±2.70^{b,A}	10.58±2.45^{b,BCD}	75.09±4.17^{b,A}	90.43±21.92^{a,BCD}	48.33±14.75^{a,B}	48.31±10.15^{a,A}	13.44±1.08^{b,C}	8.32±0.19^{b,A}	30.36±7.78^{b,B}	39.88±6.54^{ab,A}	57.58±0.08^{a,ABC}
FOS WD	6	2.75±0.30^{b,C}	23.39±6.68^{a,E}	6.89±0.49^{b,C}	0.60±0.14^{b,D}	1.48±0.17^{c,C}	67.65±0.98^{a,A}	30.87±2.93^{b,B}	14.4±1.69^{b,BC}	61.81±2.58^{bc,A}	79.57±29.41^{b,A}	12.45±0.82^{a,E}	50.57±10.88^{ab,ABC}	18.52±3.00^{a,BCD}	15.79±0.32^{a,A}	52.64±9.12^{a,CDE}	40.35±9.58^{a,ABC}	72.28±9.47^{a,A}
	12	3.41±0.16^{a,B}	4.17±0.24^{b,B}	9.9±0.75^{a,ABC}	0.47±0.01^{b,C}	2.47±0.14^{ab,D}	47.73±2.45^{b,A}	37.26±0.74^{a,A}	17.07±1.46^{a,A}	49.47±9.31^{c,C}	80.65±19.12^{b,A}	12.7±0.15^{a,BC}	56.85±3.89^{ab,A}	20.19±1.27^{a,A}	11.82±1.08^{b,A}	58.42±3.12^{a,A}	43.77±1.23^{a,A}	62.31±1.39^{ab,A}
	24	2.38±0.05^{b,B}	2.35±0.14^{b,B}	3.73±0.09^{c,CD}	0.18±0.01^{c,B}	2.02±0.22^{b,CD}	43.38±3.08^{b,BC}	22.42±1.21^{c,B}	9.87±0.73^{c,C}	76.47±2.05^{a,AB}	144.86±12.06^{a,A}	7.40±0.91^{b,C}	42.61±1.04^{b,B}	14.61±0.49^{a,D}	7.77±1.02^{c,AB}	31.72±1.22^{b,BC}	34.86±1.19^{a,D}	49.67±1.71^{b,CD}
	48	3.18±0.24^{ab,B}	2.97±0.04^{b,B}	1.73±0.35^{d,C}	1.27±0.06^{a,B}	2.84±0.19^{a,BC}	26.71±5.14^{c,BC}	26.99±1.84^{bc,B}	3.40±0.55^{d,E}	66.68±0.11^{ab,AB}	108.00±17.17^{ab,BCD}	6.07±1.33^{b,C}	65.25±12.42^{a,A}	21.43±4.55^{a,A}	5.71±0.96^{c,BC}	37.46±2.45^{b,AB}	43.19±6.71^{a,A}	54.78±3.91^{b,ABC}
FO CD	6	27.41±1.11^{a,A}	46.53±3.23^{b,BCD}	31.90±1.61^{a,A}	39.08±2.89^{a,A}	43.70±2.13^{a,A}	37.98±0.92^{b,D}	39.72±1.99^{a,A}	16.69±0.38^{a,BC}	40.33±2.81^{b,DE}	14.04±0.93^{c,B}	23.95±0.29^{ba,ABCD}	49.21±4.12^{ab,ABC}	18.94±1.23^{b,BCD}	8.97±0.21^{a,DEF}	55.72±3.59^{a,BCD}	40.24±4.14^{b,ABC}	57.01±5.83^{b,BC}
	12	25.99±7.08^{a,A}	84.64±26.07^{a,A}	14.32±4.04^{b,A}	33.84±7.58^{a,A}	46.06±6.38^{a,A}	40.2±6.36^{ab,ABC}	38.26±8.38^{a,A}	17.19±2.92^{a,A}	77.35±19.88^{a,AB}	28.21±1.53^{b,B}	28.63±1.77^{a,A}	54.54±8.78^{a,AB}	13.87±2.22^{c,BC}	5.10±1.34^{b,CD}	48.48±7.67^{ab,ABC}	41.44±8.37^{ab,A}	64.33±10.41^{ab,A}
	24	3.78±0.23^{b,B}	83.83±3.16^{a,A}	7.87±0.83^{c,B}	0.55±0.04^{b,B}	4.49±0.69^{b,B}	50.16±2.28^{a,ABC}	4.7±0.33^{b,CD}	11.96±1.27^{b,BC}	82.59±11.79^{a,A}	49.11±3.86^{a,CD}	30.41±0.89^{a,B}	60.81±2.27^{a,A}	24.02±0.43^{a,A}	6.83±1.25^{ab,ABC}	47.47±1.39^{ab,A}	53.93±0.91^{a,A}	77.19±2.58^{a,A}
	48	3.09±0.13^{b,B}	84.36±8.34^{a,A}	1.72±0.34^{d,C}	0.32±0.13^{b,B}	3.45±0.23^{b,B}	49.47±5.65^{ab,A}	2.91±0.16^{b,C}	10.7±1.47^{b,BCD}	66.08±0.08^{ab,AB}	26.57±0.39^{b,D}	21.83±1.37^{b,C}	59.65±1.12^{a,A}	15.5±0.40^{c,BC}	4.85±0.62^{b,BCD}	42.11±2.66^{b,A}	45.08±2.91^{ab,A}	63.45±2.68^{ab,A}
Fish oil WD	6	3.56±0.15^{a,C}	57.55±2.24^{a,AB}	9.68±2.47^{a,C}	0.13±0.00^{c,D}	1.88±0.18^{a,C}	53.38±11.34^{ab,BC}	3.23±0.33^{a,C}	21.57±1.73^{a,B}	52.14±1.47^{b,ABC}	22.88±2.69^{b,B}	21.55±2.71^{a,ABCD}	63.08±1.35^{a,A}	24.45±1.63^{a,A}	12.21±1.36^{a,BC}	72.76±3.71^{a,A}	48.93±2.03^{a,A}	71.22±1.99^{a,A}
	12	3.17±0.02^{a,B}	83.17±13.37^{a,A}	5.12±0.41^{b,BCED}	0.73±0.07^{b,C}	1.01±0.14^{b,D}	47.92±6.93^{ab,A}	2.85±0.48^{a,B}	15.68±1.75^{b,AB}	59.85±8.92^{a,BC}	17.14±1.38^{b,B}	11.86±0.82^{b,BC}	53.23±4.88^{a,AB}	16.28±1.27^{b,ABC}	6.16±0.74^{b,BC}	51.49±1.14^{b,AB}	37.61±2.14^{a,AB}	62.31±1.15^{b,A}
	24	3.08±0.42^{ab,B}	75.94±2.00^{a,A}	3.97±0.40^{b,CD}	0.14±0.03^{c,B}	1.36±0.07^{b,CD}	57.26±1.97^{a,A}	2.54±0.45^{a,E}	18.45±0.82^{ab,A}	79.99±1.73^{a,A}	72.74±12.25^{ab,BC}	9.82±0.99^{b,C}	48.31±0.14^{a,AB}	16.43±0.44^{b,D}	4.71±0.20^{b,CD}	33.51±1.67^{c,BC}	41.86±1.03^{a,BC}	57.94±1.47^{b,BC}

	48	1.93±0.78^{b,B}	87.93±21.09^{a,A}	5.62±0.52^{b,B}	1.14±0.16^{a,B}	1.07±0.27^{b,BC}	33.98±6.62^{b,ABC}	3.59±1.21^{a,C}	15.71±2.20^{b,A}	74.3±4.15^{b,A}	130.73±64.23^{a,BC}	4.77±1.23^{c,C}	60.48±14.35^{a,A}	12.41±1.68^{c,CD}	2.03±0.46^{c,F}	36.02±2.01^{c,AB}	45.37±8.90^{a,A}	52.15±1.92^{c,BCD}
Pomegranate oil CD	**6**	29.38±1.46^{a,A}	37.27±4.87^{b,D}	33.12±11.54^{a,A}	32.08±4.48^{a,B}	36.12±4.93^{a,B}	34.46±4.11^{b,D}	34.08±6.05^{a,AB}	16.59±2.70^{a,BC}	33.26±2.30^{b,E}	12.14±2.21^{c,B}	21.3±2.79^{a,ABCD}	39.35±6.44^{b,C}	18.12±2.90^{a,BCD}	8.09±1.91^{a,EF}	49.29±7.36^{a,D}	29.77±5.14^{a,C}	51.45±0.16^{a,C}
	12	24.99±1.28^{b,A}	70.02±5.73^{a,A}	11.14±2.47^{b,A}	1.40±0.02^{b,C}	19.92±4.08^{b,C}	34.49±4.76^{b,ABC}	26.17±11.69^{a,A}	9.28±0.79^{c,C}	65.17±5.47^{a,BC}	33.09±7.37^{b,B}	20.57±4.09^{a,AB}	50.89±7.90^{ab,AB}	16.24±3.30^{a,ABC}	3.98±0.53^{b,D}	40.51±6.27^{a,BC}	40.78±8.41^{a,AB}	58.86±8.00^{a,AB}
	24	2.91±0.12^{c,B}	76.02±5.06^{a,A}	4.38±0.29^{b,CD}	0.26±0.01^{b,B}	3.30±0.79^{c,BC}	54.73±2.70^{a,AB}	3.43±0.23^{b,DE}	11.79±1.36^{b,BC}	67.87±4.22^{a,AB}	22.83±3.25^{bc,E}	3.51±0.35^{b,C}	45.83±4.04^{ab,B}	21.13±1.60^{a,AB}	4.56±0.42^{b,CD}	42.46±4.78^{a,A}	39.57±2.17^{a,CD}	61.90±3.72^{a,B}
	48	2.61±0.30^{c,B}	79.31±14.73^{a,A}	1.79±0.14^{b,C}	0.29±0.07^{b,B}	3.38±0.13^{c,B}	44.8±6.46^{ab,AB}	2.78±0.42^{b,C}	13.77±0.92^{ab,AB}	66.85±6.83^{a,AB}	51.49±7.87^{a,BCD}	18.00±1.86^{a,C}	56.55±3.10^{a,A}	15.20±0.39^{a,BC}	2.37±0.56^{b,EF}	39.75±2.48^{a,AB}	42.52±0.48^{a,A}	60.99±2.23^{a,AB}
Pomegranate oil WD	**6**	3.46±0.38^{a,C}	57.54±6.85^{b,AB}	10.3±0.76^{a,C}	0.62±0.08^{b,D}	1.65±0.11^{a,C}	61.89±6.11^{a,AB}	1.00±0.15^{b,C}	20.78±1.18^{a,B}	52.45±3.26^{b,ABC}	24.47±1.44^{b,B}	20.47±1.67^{a,ABCD}	59.37±5.03^{a,AB}	22.50±1.47^{a,AB}	14.13±0.47^{a,AB}	65.6±6.88^{a,ABC}	46.33±3.40^{a,AB}	68.56±5.56^{a,AB}
	12	2.2±0.04^{b,B}	62.71±3.80^{ab,A}	2.58±0.52^{c,E}	0.20±0.01^{c,C}	1.5±0.01^{a,D}	47.72±0.48^{b,A}	1.58±0.27^{b,B}	14.49±0.54^{b,AB}	65.9±6.19^{b,BC}	22.58±1.51^{b,B}	13.09±2.2^{b,BC}	48.56±0.64^{a,ABC}	14.89±0.39^{b,BC}	3.57±0.25^{b,D}	38.89±6.89^{b,BC}	33.58±0.96^{a,AB}	53.38±2.25^{b,AB}
	24	3.32±0.71^{ab,B}	76.03±6.29^{ab,A}	3.59±0.92^{bc,cd}	0.19±0.03^{c,B}	1.38±0.21^{a,CD}	58.53±2.37^{a,A}	3.1±0.02^{a,DE}	19.11±0.57^{a,A}	83.47±5.10^{a,A}	32.72±3.94^{b,DE}	9.09±0.34^{c,C}	49.52±2.43^{a,AB}	16.12±0.38^{b,D}	3.95±0.32^{b,D}	28.33±0.31^{b,C}	44.38±1.06^{a,BC}	60.02±0.59^{ab,B}
	48	2.5±0.29^{ab,B}	82.01±11.09^{a,A}	5.32±0.82^{b,B}	1.19±0.09^{a,B}	1.61±0.35^{a,BC}	20.74±1.26^{c,C}	2.88±0.77^{a,C}	11.40±1.03^{c,ABC}	61.41±6.48^{b,B}	135.17±67.34^{a,AB}	4.14±0.88^{d,C}	56.73±9.75^{a,A}	19.62±2.76^{a,AB}	2.91±0.58^{b,DEF}	33.33±3.51^{b,AB}	43.62±9.19^{a,A}	44.09±5.62^{c,D}
Fish oil+Pomegranate oil CD	**6**	17.79±2.03^{a,B}	69.42±6.64^{b,A}	12.71±0.51^{a,BC}	25.78±2.91^{a,C}	34.94±4.27^{a,B}	43.71±4.98^{a,CD}	30.58±2.16^{a,B}	11.80±3.34^{a,C}	59.58±8.93^{a,AB}	18.65±4.80^{b,B}	17.82±4.06^{a,BCDE}	41.27±5.71^{b,C}	14.41±1.90^{b,D}	6.39±1.09^{ab,F}	39.79±5.22^{a,E}	33.50±5.49^{b,B}	54.06±8.21^{a,BC}
	12	19.84±1.31^{a,A}	55.47±6.29^{b,A}	10.56±2.02^{a,AB}	1.89±0.76^{b,C}	25.93±6.95^{a,BC}	27.19±5.10^{b,C}	24.75±4.83^{a,A}	8.91±2.18^{ab,C}	51.2±7.49^{a,C}	20.75±4.58^{b,B}	18.28±1.92^{a,B}	42.35±0.51^{b,B}	13.21±0.77^{b,BC}	4.56±0.59^{b,CD}	40.04±5.28^{a,BC}	31.92±0.68^{b,AB}	51.81±3.94^{a,AB}
	24	2.84±0.50^{b,B}	75.17±11.83^{ab,A}	2.90±0.23^{b,D}	0.37±0.04^{b,B}	4.32±0.44^{b,B}	44.14±6.95^{a,BC}	5.83±0.74^{b,C}	8.95±0.45^{ab,C}	56.33±14.78^{a,B}	44.17±4.15^{a,DE}	3.73±0.03^{b,C}	45.29±5.03^{b,B}	22.40±1.49^{a,AB}	6.93±1.20^{a,ABC}	48.11±1.44^{a,A}	39.24±2.28^{ab,CD}	62.53±3.17^{a,B}
	48	3.13±0.12^{b,B}	94.39±6.58^{a,A}	2.20±0.33^{b,C}	0.52±0.20^{b,B}	2.78±0.36^{b,BC}	41.56±6.02^{ab,ABC}	2.05±0.70^{b,C}	6.03±0.53^{b,D}	75.44±4.06^{a,A}	44.64±5.65^{a,BCD}	13.74±2.97^{a,C}	61.09±1.13^{a,A}	16.47±1.82^{b,ABC}	4.25±0.35^{a,ABC}	42.5±4.08^{a,A}	46.81±2.37^{a,A}	61.60±3.93^{a,A}
Fish oil+Pomegranate oil WD	**6**	3.42±0.14^{a,C}	51.56±2.74^{c,BC}	10.29±1.15^{a,C}	0.28±0.12^{b,D}	2.29±0.07^{a,C}	45.18±1.58^{a,CD}	1.55±0.41^{b,C}	19.04±1.76^{a,BC}	47.02±3.39^{c,CD}	20.22±1.57^{c,B}	22.55±0.31^{b,AB}	59.3±3.76^{a,AB}	23.91±0.66^{a,A}	11.34±0.64^{b,CDE}	70.39±4.52^{a,AB}	48.14±4.90^{a,A}	67.14±0.005^{a,AB}
	12	2.47±0.50^{ab,B}	64.16±4.47^{b,A}	3.38±0.33^{b,DE}	0.23±0.01^{b,C}	1.51±0.11^{b,D}	41.44±2.34^{a,AB}	1.49±0.25^{b,B}	12.27±0.74^{b,ABC}	66.97±4.20^{b,BC}	22.64±2.22^{c,B}	16.02±1.45^{b,BC}	52±4.02^{ab,AB}	15.82±0.30^{b,ABC}	4.72±0.72^{b,CD}	34.94±1.03^{b,C}	34.42±1.69^{b,AB}	52.61±0.31^{c,AB}
	24	3.55±0.75^{b,B}	82.92±6.23^{a,A}	3.96±0.90^{b,CD}	0.55±0.02^{b,B}	1.03±0.03^{c,D}	51.02±8.92^{a,ABC}	2.61±0.19^{a,E}	12.68±1.69^{b,BC}	81.71±5.10^{a,A}	46.82±5.91^{b,DE}	8.51±0.69^{b,C}	50.49±1.91^{b,AB}	16.13±1.30^{b,D}	5.74±0.64^{b,BCD}	28.26±1.04^{c,C}	45.21±0.30^{b,B}	63.39±0.65^{b,B}
	48	2.05±0.08^{b,B}	1.74±0.26^{d,B}	2.27±0.50^{b,C}	1.44±0.38^{a,B}	0.81±0.17^{c,C}	25.76±3.23^{b,BC}	<0.05	8.78±0.23^{c,CD}	13.68±0.33^{d,D}	72.19±14.63^{a,BCD}	103.07±19.32^{a,A}	4.61±0.98^{c,B}	3.35±0.69^{c,E}	<0.05	34.68±2.96^{b,AB}	44.49±8.15^{a,A}	52.15±1.92^{c,BCD}

Table S9 – TEER values (Ω/cm2) used to assess the cell layer integrity of the Caco-2 culture, for the different samples at different time-points (0, 1, 3 and 6 h) and corresponding controls, AM251 CB1 antagonist, AEA (C18:1 anandamide), CB1 agonist. DMSO was used as the negative control and culture medium as the positive control.

Hour (h)	Positive control	Negative control (DMSO)	AM251	AEA	Fish oil	[Fish oil] +AM251	[Fish oil] +AEA	Pomegranate oil	[Pomegranate oil] +AEA	[Pomegranate oil] +AM251	Fish oil+ Pomegranate oil	[Fish oil+ Pomegranate oil] +AEA	[Fish oil+ Pomegranate oil] +AM251
0	2170	780	2379.00±118.79^{ab}	2250.00±545.89^{ab}	2112.50±453.26^{ab}	2098.50±309.01^{ab}	2299.00±5.66^{ab}	2588.00±19.80^{a}	1967.50±423.56^{ab}	1707.00±480.83^{b}	2483.50±255.27^{a}	2368.50±273.65^{ab}	2237.50±78.49^{ab}
1	2773	177	2709.50±34.65^{bcd}	2882.50±24.75^{a}	2686.50±17.68^{cd}	2722.50±51.62^{bcd}	2861.50±9.19^{a}	2802.50±113.84^{ab}	2639.50±105.36^{d}	2674.50±70.00^{cd}	2822.50±40.31^{ab}	2796.50±102.53^{abc}	2799.50±6.36^{abc}
3	2861	159	2775.50±10.61^{b}	2958.50±40.31^{ab}	2836.50±58.69^{ab}	2886.50±60.10^{ab}	2950.00±35.36^{ab}	2912.00±118.79^{ab}	2825.00±237.59^{ab}	2785.50±27.58^{ab}	2968.00±70.71^{a}	2858.50±57.28^{ab}	2906.50±14.85^{ab}
6	2878	138	2790.50±16.26^{ab}	2946.00±1.41^{a}	2471.50±345.78^{c}	2808.50±6.36^{ab}	2863.50±17.68^{ab}	2833.50±94.05^{ab}	2669.50±55.86^{bc}	2723.00±60.81^{ab}	2884.00±67.88^{ab}	2867.50±10.61^{ab}	2883.50±2.12^{ab}