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

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Abstract

Background & Aims

Gut microbiota has been gaining increasing 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 as 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 conjugated linoleic and linolenic acids are even scarcer.

Methods

By using an *in vitro* human fermentation model, we assessed the effect of omega-3, CLA isomers, and punicic acid on microbiota modulation.

Results

Fish oil, Omega-3, and CLA samples positively impact *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 samples were able to increase the concentration of GABA, alanine, tyrosine, phenylalanine, isoleucine, and leucine between 12 and 24 h of fermentation.

Conclusions

The impact of the assessed polyunsaturated fatty acids in gut microbiota has been observed in its impact on key bacteria (*Akkermansia*, *Bifidobacterium*, and *Roseburia*) as well as their metabolic byproducts, including butyrate and amino acids, which could potentially play a role in modulating the gut-brain axis.

Keywords

Omega-3; Conjugated linoleic acid; Conjugated linolenic acid; Microbiota modulation; Gamma-aminobutyric acid; *in vitro* fecal fermentation

Abbreviations

α-Linolenic acid (ALA); Arachidonic acid (AA); Blood-brain barrier (BBB); Cardiovascular Disease (CVD); Central Nervous System (CNS); Conjugated fatty acids (CFAs); Conjugated linoleic acid (CLA); Conjugated linolenic acid (CLNA); Docosahexaenoic acid (DHA); Docosapentaenoic acid (DPA); European Food Safety Authority (EFSA); Eicosapentaenoic acid (EPA); Fatty acids (FAs); Fatty acids methyl esters (FAMEs); Fructooligosaccharides (FOS); Gamma-aminobutyric acid/ γ-amino-n-butyric acid (GABA); Gastrointestinal tract (GIT); High-fat diet (HFD); Linoleic acid (LA); Lipopolysaccharide (LPS); Monounsaturated fatty acids (MUFAs); Punicic acid (PUA); Polyunsaturated fatty acids (PUFAs); Punicic acid (PUA); Rabbit gastric extract (RGE); Saturated fatty acids (SFAs); Short-chain fatty acids (SCFAs); T regulatory cells (Treg); Tris-EDTA buffer (TE buffer).

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 gastrointestinal tract (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%). Firmicutes are mainly comprised of Gram-positive bacteria bacilli (*e.g.* *Lactobacillus*), which are obligate or facultative aerobes, and Clostridia classes, anaerobic bacteria. The Bacteroidetes phylum comprises Gram-negative, non-spore-forming, anaerobic bacteria that can tolerate oxygen but cannot use it for growth. Actinobacteria phylum comprises *Bifidobacterium*, which is Gram-positive, non-motile, non-spore-forming and anaerobic bacteria. Lastly, Proteobacteria, which includes *Escherichia*, *Klebsiella* and *Enterobacter* genus, are aerobic or facultative anaerobic Gram-negative and non-spore-forming rods bacteria (3).

The establishment of the gut microbiota occurs early in life, increasing throughout the years from a low level of diversity to a complexity comparable to the adult microbiota by the age of 3 to 5 years (1). Later, 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 nutrients 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, produces vitamins, steroid hormones and neurotransmitters such as gamma (γ)-aminobutyric acid (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, thus, essential elements in gut permeability. Due to this role, 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 (4). 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 (as reviewed by Baptista et al., (7)). One of the most relevant lipids, when considering gut microbiota are short-chain fatty acids (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 T regulatory (Treg) cells (8) and in maintaining the integrity of the epithelial barrier by regulating mucus production and tight junction expression (as reviewed by Melbye et al., (5)). Besides, they also are known to possess immunomodulatory effects since they can promote anti-inflammatory properties. In recent, there has been growing evidence supporting the possibility of SCFAs exerting physiological effects on several organs, including the brain (8). SCFAs can interact with the blood-brain barrier (BBB), by upregulating tight junction proteins and

consequently modulating BBB permeability and through T-cell differentiation towards regulatory subtypes (Treg) (Melbye et al., 2019). Besides, SCFAs can cross BBB via monocarboxylate transporters in endothelial cells. In the central nervous system (CNS), SCFAs can also influence neuroinflammation by interacting with glial cells, and consequently modulate brain function (8).They are carboxylic acids containing 2 to 5 carbon atoms, the most abundant acetate (C2), propionate (C3) and butyrate (C4). These molecules are described to be produced by bacterial species within the phyla Firmicutes, Bacteroidetes and Actinobacteria (2).

Nevertheless, knowledge of the role of other lipids in gut microbiota is still very scarce. The known beneficial roles of polyunsaturated fatty acids (PUFAs) in organs both in health and disease are well documented, specifically for omega-3 FAs. However, the impact of this bioactive fatty acid (FA) on the gut microbiota is poorly defined and studies on the role of other PUFAs, such as conjugated fatty acids (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 (9). 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, high-fat diet (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 lipopolysaccharide (LPS)-suppressing bacteria and bifidobacteria and decrease LPS-producing bacteria, enterobacteria, suppressing endotoxemia responsible for a low-grade systemic inflammation (as reviewed by Costantini et al. (9)). Recently in a study aiming to evaluate, among other parameters, the effect of conjugated linoleic acid (CLA) in

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*) (10).

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 (11), by using an *in vitro* human fermentation model, we aim to investigate the effect of different PUFAs, including omega-3, CLA isomers and punicic acid (PUA), a conjugated linolenic acid (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) were 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) was 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 described in a previous study and their use as the studied omega-3 and CFAs sources has been demonstrated (12). Shortly, Fish oil from Menhaden (F8020) was purchased from Sigma-Aldrich and was used as a source of omega-3 FAs (specifically EPA, DHA and DPA). Pomegranate Kernel Oil cold pressed was supplied by All Organic Treasure (Germany) and used as a PUA source. CLA capsules are MEGACLA A95 from Gold Nutrition, with the main ingredient CLARINOL® A-95. Omega-3 Fully concentrated EPA&DHA capsules (here defined as Omega-3 capsules) were purchased from Prozis. As a CLNA isomer source - specifically PUA-, Xanthigen® capsules (here defined as CLNA capsules) from Cellulase were used. We intended to understand the impact of different concentrations and formulations of the same bioactive FAs in microbiota modulation. Consequently, as described in the mentioned study, the capsule content was extracted and submitted to GIT simulation. Moreover, by using both oils and capsules the two main relevant sources of commercially available options for bioactive FAs oral supplementation, were covered.

2.3. *In vitro* GIT tract digestion

The GIT digestion was performed as described in a previous study (12). 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. Briefly, the protocol is divided into oral, gastric and intestinal phases and the correspondent fluid is used to better simulate the *in vivo* conditions. In mouth digestion, the simulated salivary fluid is complemented with salivary α-amylase from human saliva. Afterwards, the oral bolus is diluted in gastric fluid complemented with gastric enzymes, specifically pepsin and lipase from RGE and agitated at 130 rpm and 37 ºC, at pH 3.0 for 2 h. The resultant gastric chyme is subsequently diluted 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 ºC in the orbital shaker.

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 (12,14,15).

2.4. Gut microbiota simulation: Fecal fermentations

2.4.1. Fecal samples collection

Human fecal samples were collected fresh on the morning of the assay at the Center for Biotechnology and Fine Chemistry, Catholic University of Porto. All participants received detailed collection instructions and a kit containing one vacuum container, two plastic bags (one cut and one intact), one anaerobiosis sachet (Oxoid™ AnaeroGen™ 3.5L Sachet, Thermo Fisher Scientific), one pair of gloves, and one roll of cling film. The full collection procedure is described in the annexes. Once obtained, the samples were delivered to the study coordinator and kept under anaerobic conditions for no more than two hours before being used in the

fermentation assays. The samples collected were donated voluntarily from five healthy adult donors – 4 females and 1 male 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 weighed inside an anaerobic workstation (Don Whitley Scientific, West Yorkshire, UK) within the two-hour window mentioned earlier, following collection. A subsequent 100 g/L dilution of the fecal samples was made in Reduced Physiological Salt solution (RPS), which consisted of 0.5 g/L cysteine hydrochloride (Merck, Darmstadt, Germany) and 8.5 g/L NaCl (LabChem, Zelienople, USA), adjusting the final pH to 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 (16,17).

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). FOS

was used as the positive control since it is a compound with a known prebiotic effect (18). Indeed, FOS is a well-known carbohydrate that promote positive effects on gut microbiota. Thus, they are marketed worldwide as prebiotics that promote gut health (1). 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). The fecal fermentation samples were stored at −80°C until further analysis.

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). The

279 primer sequences were based on a previous study (2) and the RT-PCR conditions were adapted
280 and optimized from previous studies (16,19).

281 Table 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

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283 **2.5.2.Fatty acid composition analysis by GC-FID**

284 **2.5.2.1. Sample preparation**

285 The FAs profile of the samples after GIT digestion and at the different time points (0, 12, 24
286 and 48 h) of the *in vitro* fecal fermentations was assessed through GC-FID. Briefly, 250 µL of
287 digested samples and 500 µL of fecal fermentation samples were prepared according to previous
288 studies (20,21). For quantification purposes, 200 µL of tritridecanoin acid (1.5 mg/mL) was
289 added before the derivatization process as an internal standard. Afterward, 2.26 mL of
290 methanol, 800 µL of hexane and 240 µL of sodium methoxide (5 M) were added. Samples were
291 vortexed and incubated at 80 ºC for 10 min. After cooling in ice, 1.25 mL of DMF and 1.25 mL
292 of sulphuric acid (3M) were added. Again, samples were vortexed and then incubated at 60 ºC
293 for 30 min. After cooling down, the samples were centrifuged (1250 xg, 18ºC for 5 mins). The

upper layer containing fatty acids methyl esters (FAMEs) was 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 defined as: injector (split 25:1; injection volume 1 µL), injector, and detector temperatures were 250 ºC and 275 ºC, respectively; hydrogen was used as a carrier gas at a flow rate of 1 mL/min. The oven temperature was initially at 60 ºC and then increased to a final temperature of 225 ºC (20,21). Supelco 37 (Sigma, USA) was used for the identification of FAs. GLC-Nestlé36 was assayed for calculation of response factors and detection and quantification limits (LOD: 0.79 ng FA/mL and LOQ: 2.64ng FA/mL).

2.5.3.Short-chain Fatty acids and Lactic acid quantification

SCFA analysis was determined by collecting the fecal fermentation samples supernatant (14,16). 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 6000 rpm, for 15 min at 4 ºC. The supernatant was collected and passed through a 0.22 µm filter (22). The samples were subsequently analyzed by HPLC (23). 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 in analyze 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 PUFAs 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 (24).

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 1. 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, and only the non-absorbed fraction was used to be tested in the microbiota. By performing this analysis, 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 linoleic acid (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 FAs concentration during fermentation (3). To better assess this decrease, the recovery index (RI %) was calculated (Equation 1) for the major FAs detected in each sample (Supplementary Material Table 1).

$$\text{RI (\%)} = \frac{\text{Fatty acid content quantified after fermentation}}{\text{Fatty acid content quantified in the digested sample before fermentation}} \times 100 \qquad \text{(Equation 1)}$$

Regarding the most prevalent saturated fatty acids (SFAs), it was observed that for myristic (C14:0), palmitic (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 fatty acid 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 sample) and C18:0 (0.31±0.007 μg of FA/μL of sample) the RIs were higher - 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 (25). Considering the most prevalent monounsaturated fatty acid (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. In fact, one of these important mechanisms is the process through which LA is converted into CLA isomers. Indeed, it has been reported that *Bifidobacterium* is able to transform LA into different CLA isomers,

including C18:2 *c*9*t*11 and its precursor C18:1 *t*11 (Vaccenic acid) (26). 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 less toxic FAs. Similar results were reported in a recent study but in that study, an increase in Rumenic acid (RA), another CLA isomer, was also reported (27). 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 (27), which may explain the different results obtained here.

Omega-3 FAs, similarly to what was already 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 (28). 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).

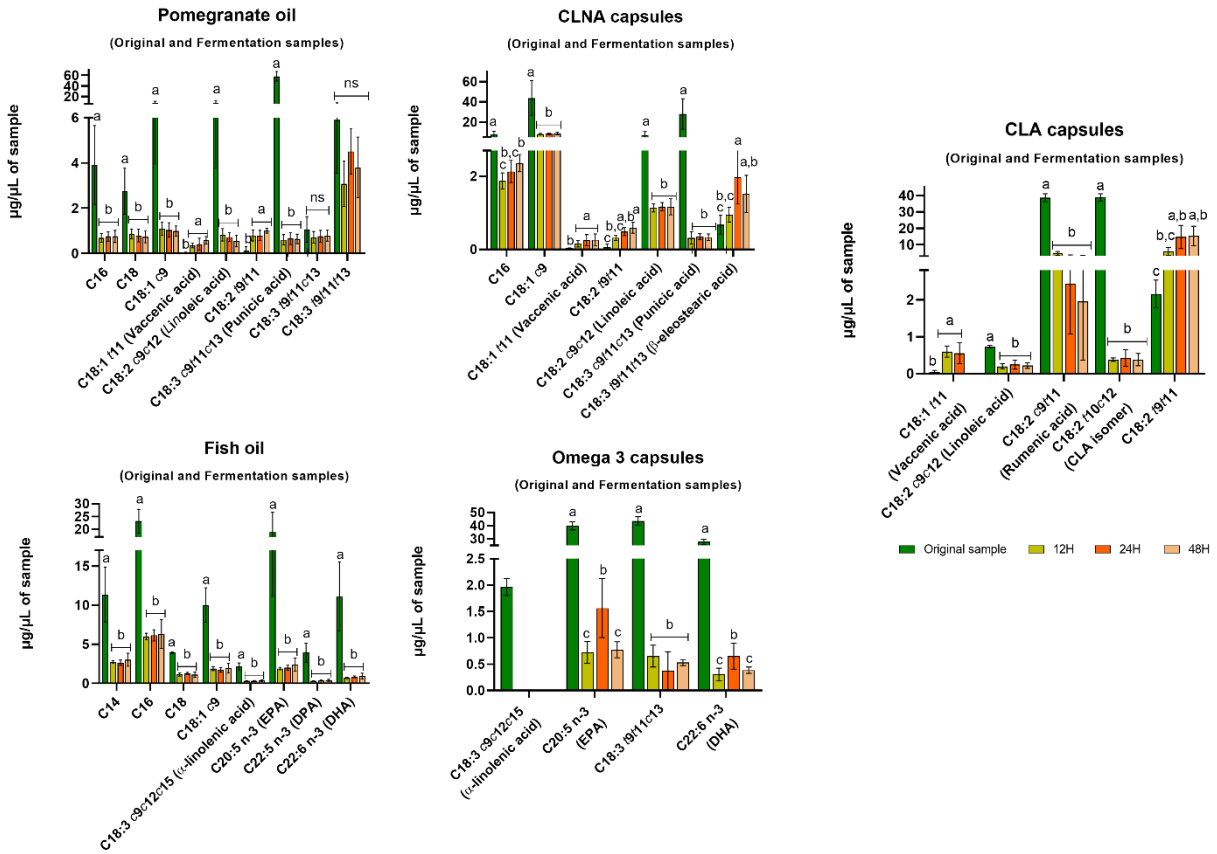

Figure 1- Total fatty acid analysis of the original samples (after gastrointestinal tract digestion) and the corresponding samples after *in vitro* fermentation after 12, 24 and 48 h. Results are the means of five donors and the error bars represent the standard deviation. Different letters indicate statistically significant differences ($p < 0.05$).

437

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 (29) they are also known to possess an effect in metabolizing omega-3 FAs (26). Indeed, a positive effect of omega-3 on *Bifidobacterium* growth has been previously reported (30). In addition, *Roseburia* and *Akkermansia* have been associated with positive effects in several inflammatory diseases, such as inflammatory bowel disease (31) and obesity (32), 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. 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 is in agreement 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) (16,19,33).

In Figure 2 and Supplementary Table 2 the log 16S rRNA gene copies/ng of DNA are illustrated for the different groups of bacteria analyzed in this work. Moreover, in Figure 3 the relative differences (RD (%)) in reference to the negative control were calculated as demonstrated in Equation 2.

$$RD\ (\%) = \frac{\mathrm{SMC}-\mathrm{CMC}}{CMC} \times 100 \qquad \text{(Equation 2)}$$

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 3 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 2) 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

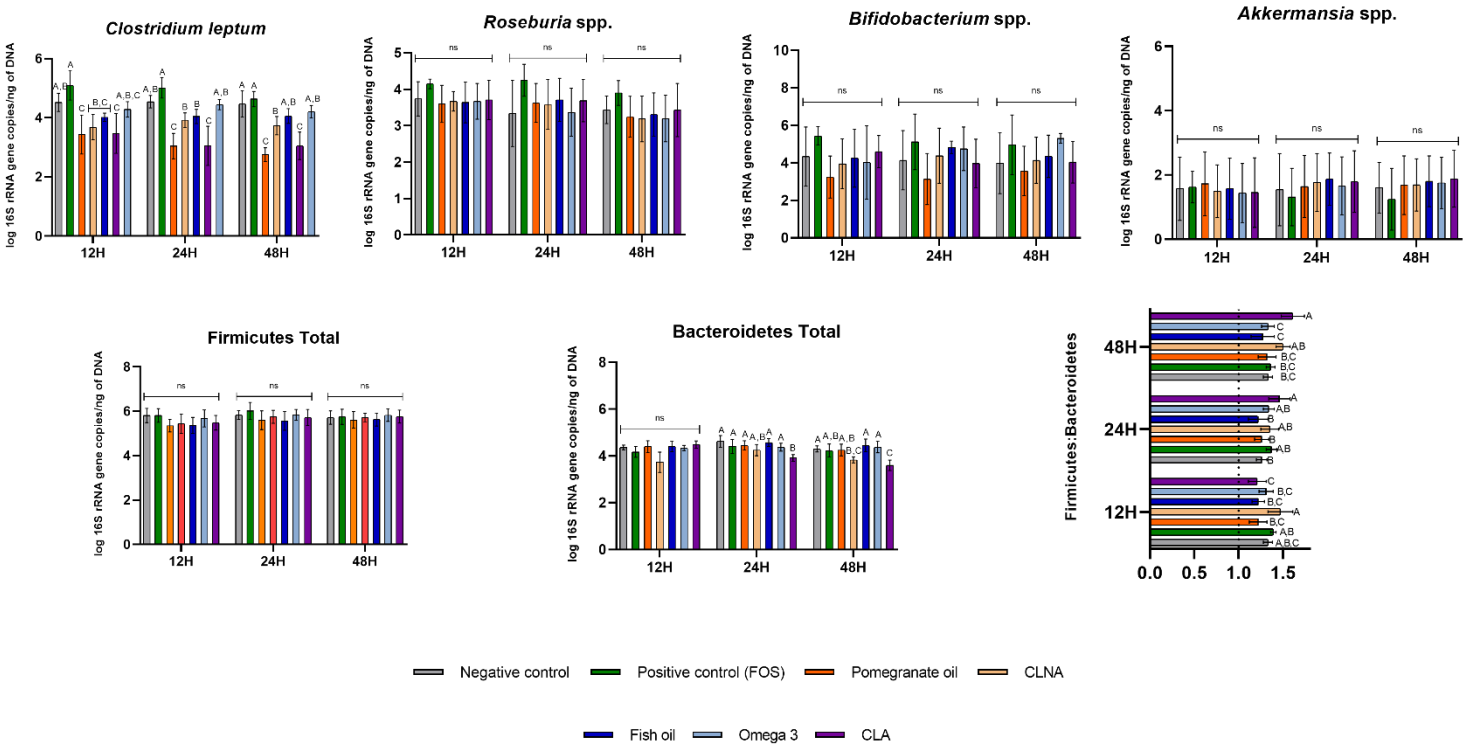

Figure 2 - Values of log 16S rRNA gene copies/ng of DNA of gut bacterial population after 12, 24 and 48 h of *in vitro* fecal fermentation of different bioactive lipids sources (Fish and Pomegranate oil, CLA, CLNA and Omega-3 capsules. Results are the means of five donors and the bars represent standard 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.

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 3). 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 (27). It has been reported that the bacterial lag phase was dependent on LA concentrations and was proportional to the FA concentration (26). Indeed, along with CLNA (7.17±3.48 µg/µL of sample), Pomegranate oil is the sample with higher LA concentrations (8.21±4.42 µg/µL of sample), which is consistent with the negative RD observed. Thus, considering the other source of punicic acid (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 $p<0.05$) 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 (25,34), 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 (35). Here, at 24 h Pomegranate oil and CLNA showed a positive RD index for *Roseburia* spp. group. 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. Similar results were observed for CLA capsules, which presented a lower concentration of LA (0.73±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 (10). 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. Importantly, *Roseburia* has been described to metabolize LA by the same pathway found in ruminal bacteria (36), 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 (28). 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 (9). As already discussed, it has been demonstrated that, in general, omega-3 PUFAs are able to restore eubiosis in gut

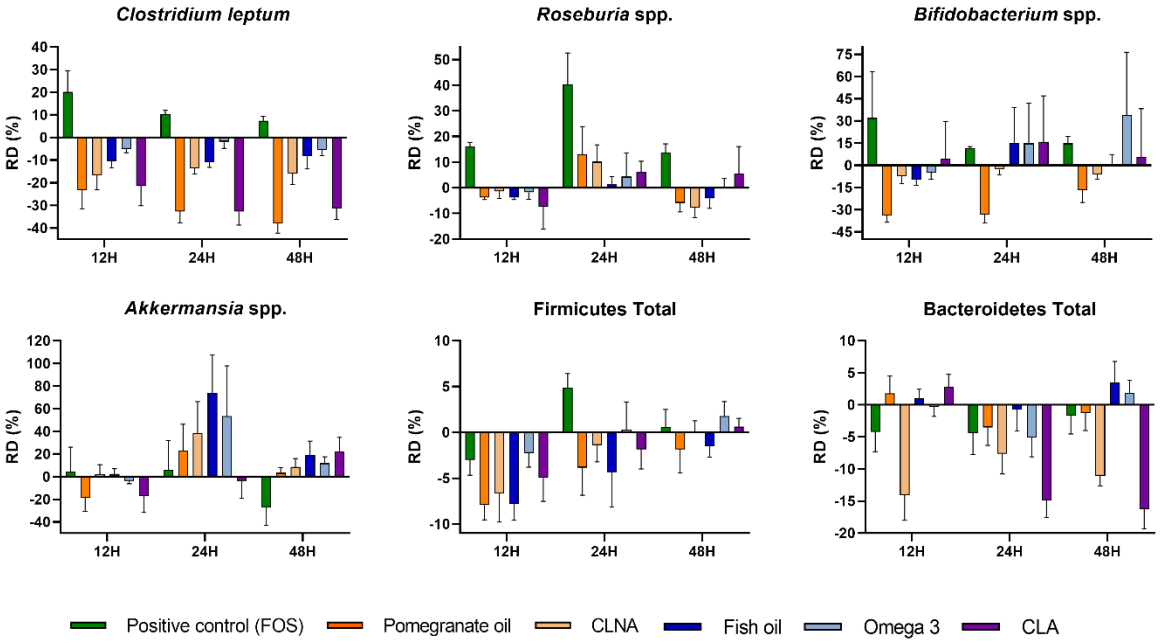

Figure 3 – Relative difference (RD;%) values of gut bacterial population after 12, 24 and 48 h of *in vitro* fecal fermentation of different bioactive lipids sources (Fish and Pomegranate oil, CLA, CLNA and Omega-3 capsules). Results are the means of five donors and the bars represent standard error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

520 microbiota by restoring Firmicutes: Bacteroidetes ratio, increasing Bacteroidetes, butyrate-
521 producing bacteria belonging to the Lachnospiraceae family (*e.g. Roseburia*) and LPS-
522 suppressing bacteria, such as *Bifidobacterium* (9). Here, in the Fish oil and the Omega-3
523 capsules, the sources of EPA and DHA, it was not observed any statistically relevant negative
524 effect on all the bacteria groups analyzed in this work. Through the analysis of the RD, alone, it
525 seems that both omega-3 and Fish oil may have a positive effect upon *Bifidobacterium* spp
526 growth after 24 h fermentation, but further studies are required to validate this result. Although
527 a recent study assessing the effect of omega-3 α-linolenic acid demonstrated that oral
528 supplementation with blended omega-3-rich oils, highly increased the relative abundance of *C.*
529 *leptum* in volunteers with borderline hypercholesterolemia (37), here we observed that both Fish
530 oil and omega-3 present a negative RD. This may be explained by the different omega-3 FAs
531 used. Considering the Bacteroidetes group, after 48 h there was an inhibition of this group of
532 bacteria by CLA and CLNA samples, but not by Omega-3 and Fish oil.

533 This study also evaluated the effect of the assessed bioactive lipids sources on *Akkermansia* spp.
534 *Akkermansia* has been associated with a positive impact on obesity by modulating glucose
535 metabolism and low-grade inflammation (38,39). Importantly, a recent study has demonstrated
536 that a high relative abundance of *Akkermansia* is associated with a low risk of obesity and this
537 association declines with aging (32). It seems that when analyzing the RD of the studied
538 samples and comparing them with FOS, there is a positive effect of all the samples on
539 *Akkermansia spp.* bacteria, translated by a positive RD, especially after 24 h for Pomegranate
540 and Fish oil, CLNA and Omega-3 and after 48 h for CLA.

541 Another highly relevant parameter to consider when assessing a possible prebiotic effect of a
542 bioactive compound in the gut microbiota is the ratio between Firmicutes and Bacteroidetes. It
543 has been described that healthy people display ratios of Firmicutes to Bacteroidetes near 1:1 and
544 an alteration in this ratio is associated with several diseases, particularly obesity. Omega-3,
545 CLA and CLNA (specifically PUA) FAs have been demonstrated to be able to attenuate the
546 increase of the Firmicutes: Bacteroidetes ratio associated with obesity (9,10,28,35,40).

547 Relevantly, in this study, the samples tested have not altered the Firmicutes: Bacteroidetes
548 (Figure 2) ratio and no statistically different effect was observed when compared to a positive
549 control (FOS), except for CLA at 48 h (p<0.05).

550 ### 3.3. Short-chain Fatty acids and Lactic acid production

551 SCFAs are metabolites produced by gut microbiota in the colon by fermentation of food
552 components that are unabsorbed/undigested, such as dietary fibers and resistant starch (41).
553 Acetate, propionate and butyrate are the main SCFAs produced. Although anaerobic
554 fermentation of these dietary products is the largest source of these SCFAs, they can also be
555 produced from amino acid metabolism, however less than 1% of gut microbiota uses these
556 metabolic pathways to produce SCFAs (8,41).

557 Considering this, the production of lactic acid and the major SCFAs acetate, propionate and
558 butyrate was assessed as it is displayed in Figure 4. It was observed that lactic acid was the
559 major metabolite produced during the entire fermentation of FOS (Figure 4), which agrees with
560 the increase of lactate-producing bacteria such as *Bifidobacterium* spp., demonstrated by a
561 positive RD (Figure 3). This rise in lactic acid production was translated by a decrease in pH
562 (Figure 5). It was observed an accumulation of lactic acid in FOS samples, which may indicate
563 that the first was not used as a substrate during fermentation. Such results are in agreement with
564 previous studies using similar *in vitro* fermentations models and using a human fecal slurry as
565 inoculum (16,19). Since this study was not conducted using a pH control, the significative
566 decrease of pH in the FOS samples after 12 h may have not allowed the growth of lactate-
567 utilizing bacteria (*e.g. Eubacterium hallii, Anaerostipes caccae, Propionibacterium avidum and*
568 *Veillonella ratti*), leading to an accumulation of lactic acid during the fermentation (42,43).
569 Inhibition of these bacteria growth has been reported previously in pHs lower than 5.2, as
570 detected here (42). For all the other samples – Pomegranate and Fish oil, Omega-3, CLA and
571 CLNA-, it was detected a decrease of lactic acid throughout the fermentation time which is
572 consistent with the higher pH in these samples compared to FOS (Figure 5). Although Fish oil
573 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 and Fish oil and Omega-3 on different lactic acid bacteria may explain the differences observed here between these samples regarding lactic acid production. Besides, lactic acid is an organic compound which is produced via fermentation using different carbohydrate sources. Here, the only carbohydrate source used was FOS (44). Besides, in Pomegranate and Fish oil, CLNA, CLA and Omega-3 samples, the maintenance of the pH ≈6 allowed the use of lactic acid bacteria 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.

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 (45). 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 (19). The increasing concentration of acetate along with the decreasing concentration of lactic acid, in Fish oil, Omega-3 and CLNA may be due to

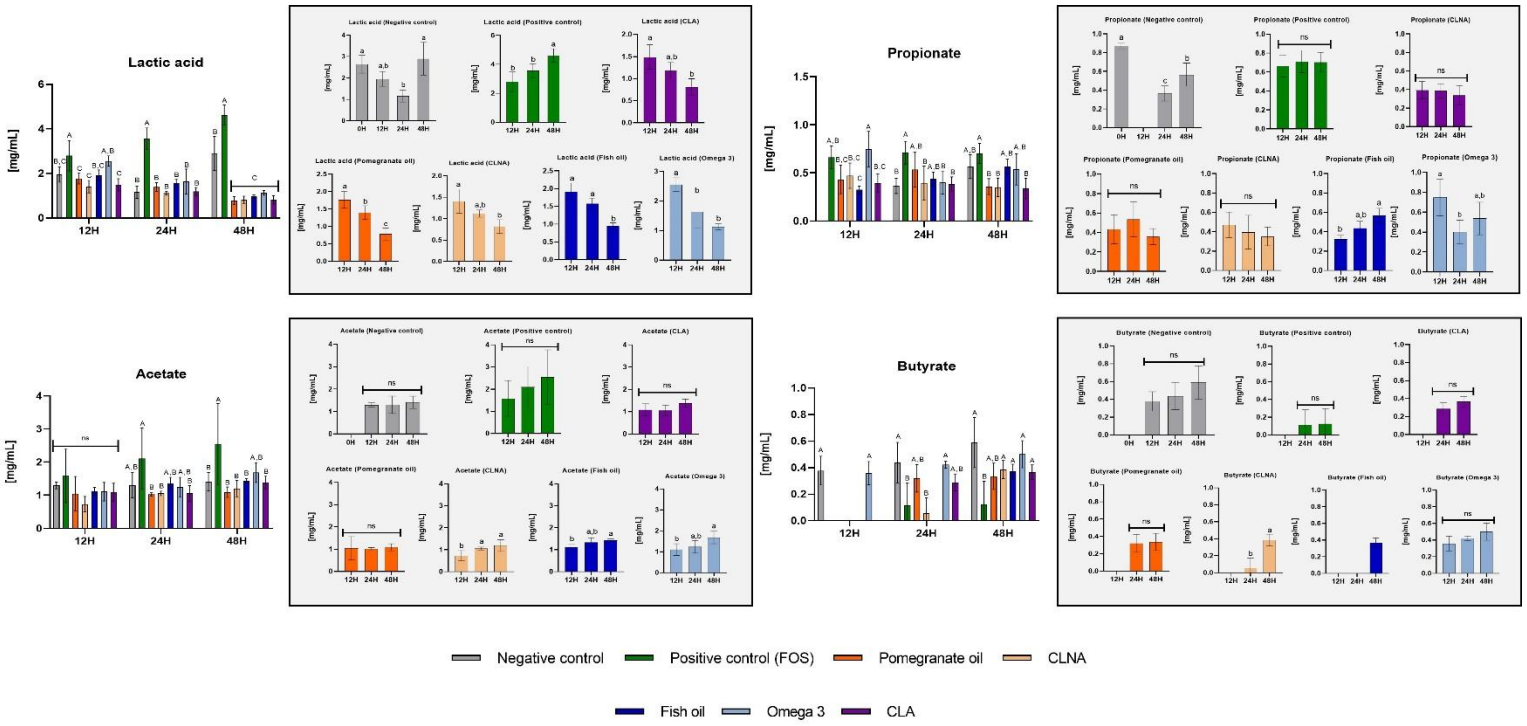

Figure 4 – Concentrations of short-chain fatty acids (mg/mL) – Acetate, Propionate and Butyrate - and Lactic acid after 12, 24 and 48 h of the *in vitro* fecal fermentation of different bioactive lipids sources (Fish and Pomegranate oil, CLA, CLNA and Omega-3 capsules). The values presented are the means 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.

596 a cross-feeding effect among intestinal bacteria, using lactic acid as a substrate for the
597 production of acetate as the final metabolite (46,47). This observation was not evident during
598 FOS fermentation, where it was observed an accumulation of lactic acid, as already discussed.
599 At 48 h the concentration of acetate for Pomegranate and Fish oil, CLNA and CLA samples
600 were significatively lower than the FOS sample. Importantly, *Clostridium* bacteria are known
601 producers of acetate (45). Since there is a statistically significant reduction, as discussed in
602 section 3.2, of *C. leptum* bacteria, in Pomegranate oil, CLA and CLNA samples compared to
603 FOS, especially at 48 h, this may explain the lower acetate levels between these samples.
604 Additionally, more and more evidence has been showing that PUFAs, namely omega-3 and
605 CFAs, can attenuate the metabolic syndrome that is closely associated with obesity (11).
606 Interestingly, it has been reported that butyrate is able to mitigate insulin resistance in mice and
607 attenuate inflammation in 3t3-L1 adipocytes, while acetate was demonstrated to promote

metabolic syndrome in HFD-fed mice via parasympathetic activation (48,49). 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 (45). Besides, some Bacteroidetes bacteria and *Roseburia* species have also been described as propionate producers (50). 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). 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, gene expression, reduces oxidative stress, among other relevant functions (45). 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 (45). 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 (28,51). 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 (51).

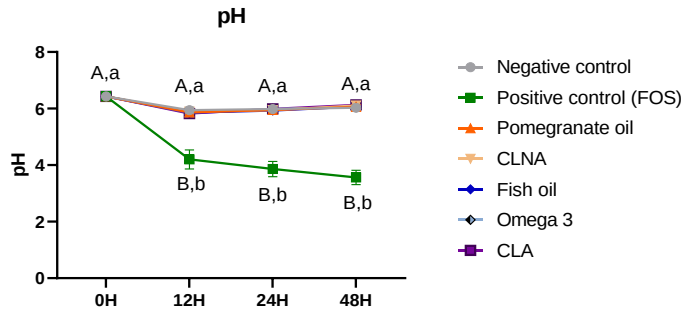

Figure 5 -pH determination at different fermentation times (0, 12, 24 and 48h) for the different bioactive lipids' sources (Fish and Pomegranate oil, CLA, CLNA and Omega-3 capsules) and for the Negative and Positive control (FOS). The results are the means of five different donors. The bars represent 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 (52). Importantly, as mentioned, propionate and butyrate are formed as products, although at low levels, from peptide and amino acid fermentations (50). In fact, protein fermentation usually takes place in the distal large intestine where carbohydrates are already depleted (8). 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 (50,52). 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 6 and in Supplementary Material Table 3.

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 (52). This heightened bacterial protein fermentation is associated with elevated pH and reduced carbohydrate availability in the large intestine (53). Colonic bacteria exhibit a preference for certain amino acid substrates, such as lysine, arginine, glycine, valine, and isoleucine (52). 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 (52). On the other hand, low gut pH and the presence of carbohydrates reduce peptide and amino acid fermentation *in vitro* (53). 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 (52). In fact, it was observed here 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 (50). 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 (52). 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) 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 (50). 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 in (50)). 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 in order to maintain intracellular pH homeostasis in several gut bacteria (54). 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 to pyruvate. And, for example, in *Clostridium propionicum*, alanine fermentation leads to the production of propionate via the pyruvate pathway (50). 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 (52). Interestingly, here it was observed that both Fish oil and Omega-3 samples promote an increase in the concentration of several amino acids from 12 to 24 h, namely GABA, alanine, tyrosine, phenylalanine, isoleucine and leucine (Figure 6). More relevantly, in alanine, tyrosine, phenylalanine and isoleucine (in the case of Omega-3 sample) the raise 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 (55). Moreover, gut microbiota production of tyrosine, a precursor for dopamine, has been positively correlated with cognitive function in Schizophrenia patients (56). 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 (30). In this study, as mentioned, it was observed an increase in GABA production in both Fish oil and Omega-3 samples 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 is able to alter the levels of GABA in the hypothalamus. Here, along with an increase in GABA concentration it was observed, an acetate concentration rise from 12 to 24 h (Figure 4) 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 (57,58). 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 (57). Relevantly, in this study, Omega-3 samples 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

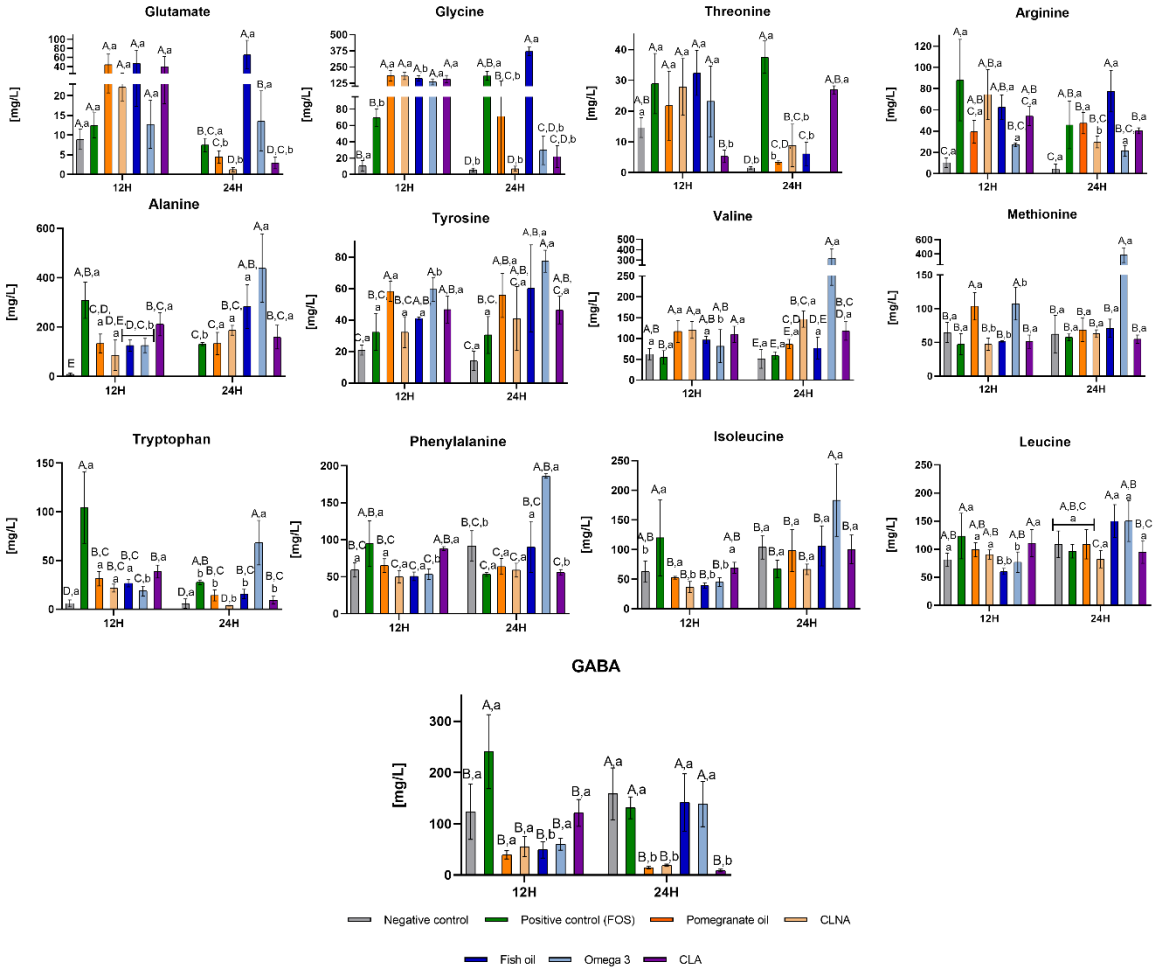

Figure 6 - Concentrations of amino acids (mg/L) after 12 and 24 h of the in vitro fecal fermentation of different bioactive lipids sources (Fish and Pomegranate oil, CLA, CLNA and Omega-3 capsules). The values presented are the means of five donors and the bars represent the standard deviation. Different 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.

associated with the promotion of both differentiation and proliferation of peripheral inflammatory Th1 cells (59). 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 (30). 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 (60).

3.5. Assessment of the effect of PUFAs 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 7 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 7 (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 one corresponding to the Negative control. Analyzing the loading plot (Figure 7 (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

773 explaining Fish oil differentiation from the remaining samples at both 12 and 24 h of
774 fermentation. So, these two amino acids are also important metabolites to be considered in the
775 fermentation of Fish oil. To the best of our knowledge, a possible relation between glycine and
776 omega-3 FAs was only observed in another study aiming to assess the effect of dietary
777 supplementation with *Lactobacillus* in piglets. In this study, it was found that serum PUFAs
778 C18:2*c*9*c*12 (LA), C18:3*c*9*c*12*c*15 (α-Linolenic acid, ALA), C20:4 n-6 (Arachidonic acid, AA)
779 and relevantly C22:6 n-3 (DHA) were elevated along with serum free amino acids glycine,
780 alanine, valine, isoleucine, asparagine, aspartate, glutamine, methionine, phenylalanine and
781 leucine in the supplemented group compared with the control group (61). Moreover, as
782 mentioned, omega-3 FAs, specifically EPA and DHA are greatly known due to their anti-
783 inflammatory potential. Likewise, intake of diets with high content of glycine has been
784 associated with decreased accumulation of fat mass in rodent studies and glycine was previously
785 associated with anti-inflammatory effects (62). Considering this, the higher content of both
786 glycine along with DHA, EPA and DPA observed in this study in Fish oil after 24 h of
787 fermentation, may be relevant in an anti-inflammatory perspective specifically in diseases where
788 an inflammatory imbalance is observed.

789 On the other hand, along PC2 the dominant parameters are the FAs PUA, Catalpic acid and β-
790 eleostearic. The high concentration of these two FAs explains the differentiation of both
791 Pomegranate oil and CLNA samples. In PC3, combining the analyses of the score and loading
792 plots (Figure 7 (B1 and B2)). it is observable the separation of the Omega-3 sample (24 h) from
793 the remaining samples mainly due to the amino acids alanine (0.909), tyrosine (0.686), valine
794 (0.788), methionine (0.847), phenylalanine (0.912), isoleucine (0.854) and leucine (0.794). By
795 analyzing this score plot is also observable an increase from 12 to 24 h, which is consistent with
796 what was previously discussed in section 3.4. Nevertheless, with this PCA analysis, besides the
797 already discussed amino acids (alanine, tyrosine, phenylalanine, isoleucine and leucine), valine
798 seems to be an important amino acid that needs to be considered in Omega-3 samples.
799 Importantly, a recent study demonstrated that gut microbiota can 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 (22). 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 (63). 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 were shown to attenuate the inflammatory levels observed in Parkinson's disease mice model and reverse motor and non-motor dysfunctions and dopaminergic neuron impairment (64). So, it is highly relevant the capacity of Omega-3 samples, 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 amino acids mentioned, it is also relevant to consider the potential of these samples, especially Fish oil and Omega-3 samples in gut-brain axis modulation.

Further studies are needed to determine the precise mechanisms for the development and prognosis of cardiovascular disease events with regular use of fish oil supplements

Moreover, reflecting on the distinction observed between Fish oil and Omega-3 samples in the PCA, and specifically considering EPA and DHA, it is also important to point out that this omega-3 FAs 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.

824

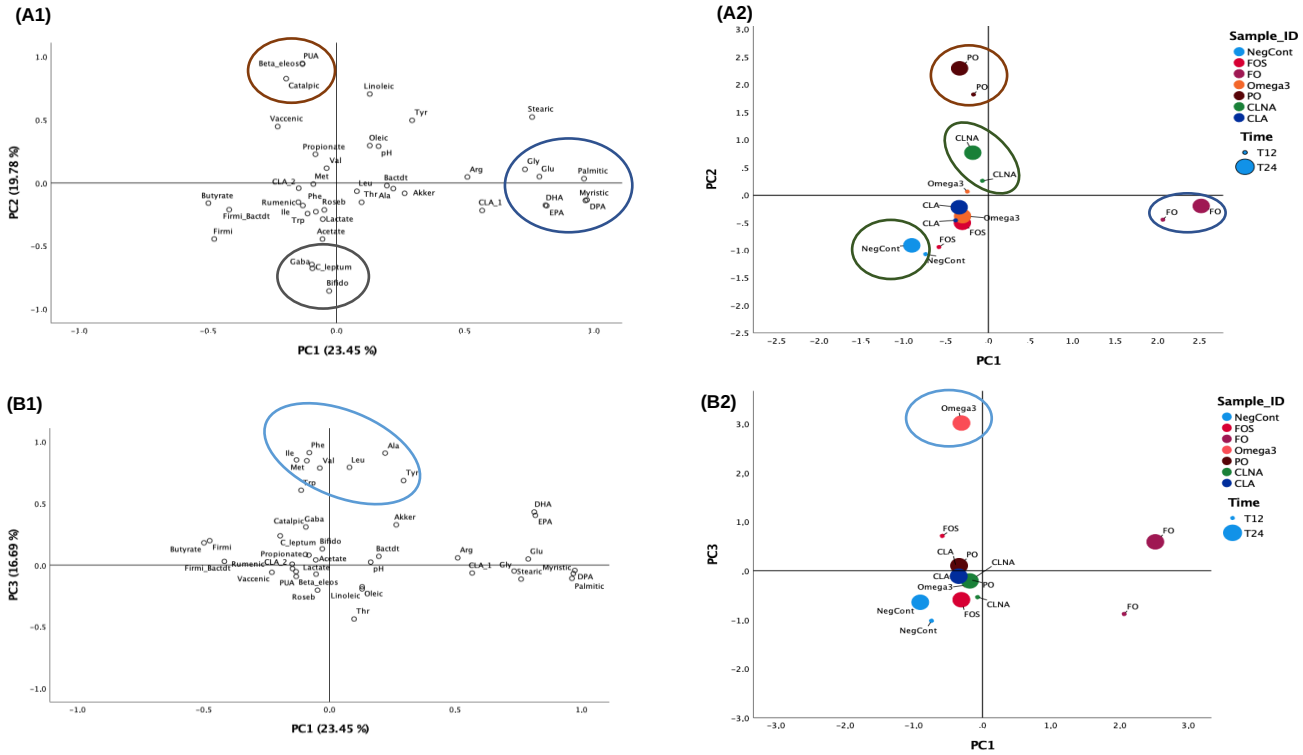

Figure 7 – A. Principal component analysis biplot PC1 vs PC2: (A1) Variables loadings distribution along each component and (A2) data scores along the components, for 12 and 24 h. B. Principal component analysis biplot PC1 vs PC3: (B1) variables loadings distribution along each component and (B2) data 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 *c*9; Vaccenic – C18:1 *t*11; Linoleic – C18:2 *c*9*c*12; Rumenic – C18:2 *c*9*t*11; CLA_1 – C18:2 *t*10*c*12; CLA_2 – C18:2 *t*9*t*11; EPA – C20:5 n-3; PUA – C18:3 *c*9*t*11*c*13; Catalpic – C18:3 *t*9*t*11*c*13; beta_eleos – C18:3 *t*9*t*11*t*13; 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 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 – Rumenic acid- 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 gut microbial population but also considering 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. Moreover, a recent study have demonstrated that DHA-rich Fish oil is able to increase the abundance of *Akkermansia* and *Lactobacillus* and consequently regulate peptide YY (PYY) expression, which can reduce food intake and affect energy metabolism (65). 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 (9,10,28,32,35,40). Besides, the observation at 48 h

852 of an increased production of butyrate higher than FOS, which was accompanied by a decrease
853 in acetate production is highly relevant in obesity since butyrate has been associated with both
854 an anti-inflammatory potential and a beneficial effect on insulin resistance. On the other hand,
855 acetate is known to promote metabolic syndrome (48,49). Besides, it was demonstrated that
856 Fish oil and Omega-3 samples were able to increase the concentration of several amino acids
857 from 12 to 24 h fermentation, namely GABA (a highly relevant neurotransmitter), alanine,
858 tyrosine, phenylalanine, isoleucine and leucine. For instance, alanine has been associated with a
859 therapeutical potential in diseases such as schizophrenia, Alzheimer's disease and renal disease
860 (55). In addition, the cognitive function of patients with schizophrenia is associated with the
861 potential for gut microbiota to biosynthesize tyrosine, a precursor for dopamine (56).

862 Despite all these interesting and promising results, especially in obesity therapy and
863 neuroinflammatory diseases by gut-brain axis modulation, it is important to point out, however,
864 that although fecal fermentations represent a valid approach, several sources of bias have to be
865 considered: intestinal transit and permeability as well as metabolite transportation (8). Besides,
866 studies focusing on regular and/or long-term supplementation of the PUFAs studied are still
867 insufficient. Although limited, most of the studies available are focused on fish oil supplements
868 (enriched in Omega-3) and some controversial results have been observed. Indeed, a recent
869 study demonstrated that a regular use of such supplements was suggested to be a risk factor for
870 atrial fibrillation and stroke among the general population but could be beneficial for the
871 progression of cardiovascular disease (CVD) (66). In fact, European Food Safety Authority
872 (EFSA) has established that 250–500 mg/day of EPA and DHA are the dietary recommended
873 daily dose for European adults, based on CVD risk considerations (67). Moreover, it is
874 important to mention that it was stated that supplemental intakes of EPA and DHA combined at
875 doses up to 5 g/day, and supplemental intakes of EPA alone up to 1.8 g/day, do not raise safety
876 concerns for adults (67). Corroborating these, Casula and collaborators (68) performed a meta-
877 analysis on randomized controlled trials assessing the CVD preventing potential of
878 administrating at least 1 g/ day, for at least 1 year of Omega-3 FAs supplements. It was

879 evidenced that long-term Omega-3 supplementation is beneficial for the onset of cardiac death,
880 sudden death and myocardial infarction among patients with a history of CVD. Additionally,
881 early studies in rodent models have demonstrated a cardioprotective effect against ischemia and
882 reperfusion after long-term supplementation (4 to 5 weeks) of dietary fish oil (69). Similarly,
883 long-term ALA supplementation (for >16 months until >18 months of age) was a well-tolerated
884 nutritional strategy against age-associated platelet hyperreactivity and thrombotic potential (70).
885 The authors demonstrated that at least part of this effect is associated with the beneficial
886 modulatory potential of Omega-3 ALA-rich diet in aged gut microbiota. This effect was
887 hypothesized to be achieved by ALA modulatory role of the inflammatory response and
888 dysregulation of metaorganismal metabolite trimethylamine N-oxide and SCFAs during aging
889 (70). Considering all these observations, although this study highlights relevant evidence of the
890 positive effect of these PUFAs in gut microbiota modulation, it is important to complement
891 these studies with *in vivo* assays and clinical trials. Special emphasis should be placed on the
892 long-term effects of the other studied PUFAs (CLA and CLNA isomers) on overall human
893 health, as well as the long-term impact of all PUFAs on gut microbiota modulation.

894 ## 5. Acknowledgments

895 This work was supported by National Funds from FCT- Fundação para a Ciência e a Tecnologia
896 through project UID/Multi/50016/2020. The author Ana Sofia Salsinha would also like to
897 acknowledge FCT for her PhD grant with the reference SFRH/BD/136857/2018.

898 ## 6. Conflict of interest

899 The authors declare no conflict of interest.

900 ## 7. Author contribution

901 The authors from this article worked on the following CRediT roles: Ana Sofia Salsinha worked
902 in Conceptualization, Methodology, Formal analysis, Investigation, Validation, Data Curation,
903 Writing - original draft, Writing - Review & Editing. The authors Helena Araújo-Rodrigues and
904 Cindy Dias were involved in Conceptualization, Methodology, Investigation, Validation,

Writing - Review & Editing. The author André Cima in Conceptualization, Methodology, Investigation, Validation. Both Luis M. Rodríguez-Alcalá and João B. Relvas were responsible for Validation, Writing - Review & Editing and Supervision. Manuela Pintado was responsible for the following roles: Conceptualization, Resources, Validation, Writing - Review & Editing, Supervision, Funding acquisition and Project administration.

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List of Figures

Figure 1- 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 ($p < 0.05$).

Figure 2 - 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.

Figure 3 – 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.

Figure 4 – 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.

Figure 5 -pH determination at different fermentation times (0, 12, 24 and 48h) 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.

Figure 6 - 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.

Figure 7 – 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.