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N-acylphosphatidylethanolamines, N-acylethanolamines and Endocannabinoids: dietary sources and fate during digestion in humans

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Chapter 1. General Introduction

1.1 Gut-brain axis

The term “gut-brain axis” (GBA) indicates the communication between the gut and the brain. This communication is guaranteed by neural messages (through vagal and spinal afferent neurons), immune messages (through cytokines) and endocrine messages, through gut hormones (Holzer *et al.*, 2012).

The regulation of energy homeostasis is located in the hypothalamus involved in the control of food intake by humoral and neural signals.

Feeding behavior and body weight are controlled by peripheral signals that can be short- and long-term signals. The short-term signals are generated by pancreas, liver, skeletal muscle or gastrointestinal tract (GIT) as either afferent sensory relays, nutrients or hormones that reach the central nervous system (CNS) through the blood–brain barrier. Long-term signals provide information to the brain about the energy stores and induce responses to maintain energy homeostasis (Havel, 2001).

A finely orchestrated cooperation of short- and long-term signals allows humans to integrate energy intake and expenditure and to maintain the energy balance. Homeostatic regulation of energy balance is also influenced by hedonic and emotional neural signals. Indeed, eating behavior is physiologically regulated over three main phases: a preparatory phase, an eating phase and a post-eating phase (Berthoud *et al.*, 2011).

The GBA influences the mechanisms implicated in the processes of satiety and satiation. Satiety is the mechanism that inhibits further eating from the post-prandial time until the next meal and determines the intermeal interval. Satiation is the mechanism that determines the end of eating during a meal thus influencing the meal size (Berthoud *et al.*, 2011). The mechanisms underlying GBA communication involve immune-neuro-endocrine mediators. This communication includes the CNS, the autonomic nervous system (ANS), the enteric nervous system (ENS) and the hypothalamic pituitary adrenal (HPA) axis (Holzer *et al.*, 2012).

A role of the gut microbiota in both intestinal physiology and in CNS has been demonstrated thus leading to the individuation of a "microbiota-gut-brain-axis" (Duca & Lam, 2014).

The microbiota is the microbial community of several different environments of the body. The intestinal tract has the most abundant population with 1000 different bacterial species which make up the majority of the all 100 trillion micro-organisms inhabiting the gut (Turnbaugh *et al.*, 2007).

Different studies on germ-free animals showed that the bacterial colonization of gut is important to the development and the maturation of both ENS and CNS. The absence of microbiota is linked with the alterations of gut-sensory motor functions involved in delayed gastric emptying and intestinal tract (Carabotti *et al.*, 2015).

Figure 1 shows the mechanism involved in "microbiota-gut-brain-axis".

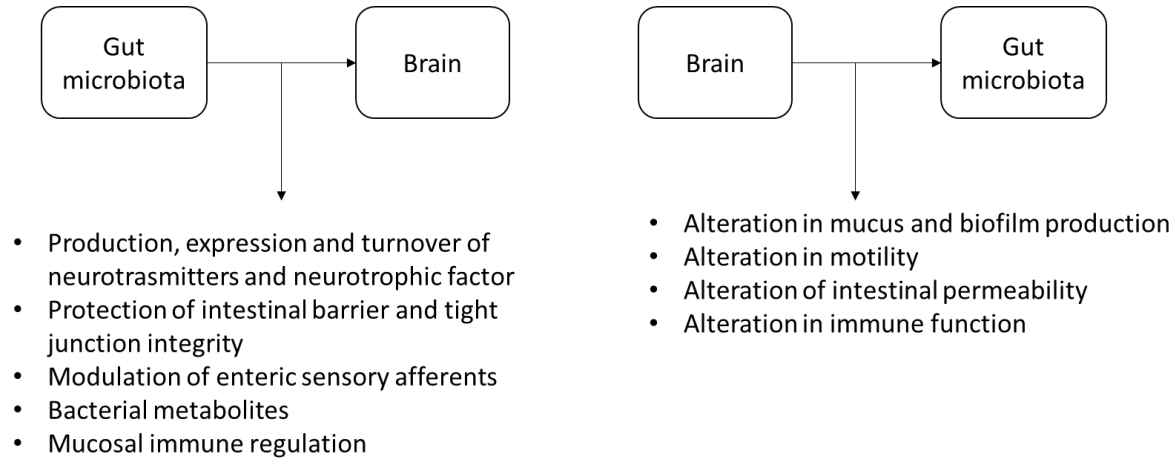

Figure 1.1. Mechanisms of the bidirectional brain-gut microbiota axis (Carabotti *et al.*, 2015).

1.2 Intestinal nutrient sensing

Gustatory system in the oral cavity involves the activity of taste receptors that transmit signals via sensory vagal afferents to the brain informing about the composition of the meal. However, a fine net of taste receptors is also present in the GIT thus constituting a sensory system in the gut. This system allows the GIT to detect the ingested nutrients and inform the CNS by both neural and humoral mechanisms. Indeed, the sensing of nutrients present into the lumen by the taste receptors located on enteroendocrine cells (EECs) induces the secretion of peptides acting as hormones. The GIT endocrine system produces over 20 hormones that influence food intake, insulin release and gut motility (Mace *et al.*, 2015). The action of the hormones is also mediated by vagal afferent fibres which are located in the lamina propria of the intestinal villi where the gut peptides are released (Duca & Lam, 2014).

EECs in the stomach secrete ghrelin (A cells), somatostatin (D-cells), gastrin (G-cells), histamine (L-cells) and leptin (P cells) whereas those in the small intestine and in the colon secrete cholecystokinin (CCK) (I-cells), glucagon like peptide-1 (GLP-1) (L-cells), peptide YY (PYY) (L-cells) and gastric inhibitory peptide (GIP) (K-cells) (Steensels & Depoortere, 2017). Enterochromaffin cells are a type of EECs present in the GIT epithelia and specialized in the secretion of 5-hydroxytryptamine (5-HT3, serotonin) regulating secretory and peristaltic reflexes, and activating vagal afferents through receptors signalling to the CNS (Mace *et al.*, 2015).

The nutrients are detected by a large number of proteins such as nutrient transporters and G-protein-coupled receptors (GPRs). The delivery of dietary nutrient such as carbohydrates, proteins, and fat activates the secretion of hormones by EECs.

The sweet receptors derive from the heterodimerization of TAS_1R_2 and TAS_1R_3, including the family C of GPRs. The sweet receptors are not only expressed on the lingual epithelium, but

they also act as a carbohydrate sensor in the intestine on L-cells and K-cell secreting GLP-1 and GIP respectively (Spreckley & Murphy, 2015).

The glucose-induced GLP-1 secretion is mediated by sweet taste receptor TAS_1R_2-TAS_1R_3, the glucose transporter SGLT1 and ATP-sensitive potassium (K_{ATP}) channels (Steensels & Depoortere, 2017).

The products of breakdown of proteins such as peptides are sensed via lysophosphatidic acid receptor 5 (LPA5R, also known as GPR92/93). The GPR93 is expressed by cells of duodenal mucosa, including L-cells, but it is also present in G-cells and D-cells of stomach. The activation of GPR93 releases CCK and GLP1 (Spreckley & Murphy, 2015; Janssen & Depoortere, 2013).

The aromatic and basic amino acids are sensed by the calcium sensing receptor (CaSR), a receptor expressed in G-cells, I-cells and D-cells promoting the secretion of GLP-1 and PYY. Furthermore, basic amino acids are sensed by GPRC6A, present in gastric antral D-cells and G-cells.

The L-glutamate is an umami tasting stimulus that is sensed by the taste receptor heterodimer TAS_1R_1-TAS_1R_3 expressed in brush cells and endocrine L and X/A cells of gut and also the metabotropic glutamate receptor (Janssen & Depoortere, 2013; Spreckley & Murphy, 2015).

The free fatty acids (FFAs) are delivered from lipid droplets in the small intestine and they are transported to the surface of enterocytes. The transport of FFAs in the intestine occurs via the cluster of differentiation 36 (CD36) and fatty acid transport protein 4 (FATP4) (Steensels & Depoortere, 2017).

The FFAs are sensed by different free fatty acids receptors, depending on their chain length. Medium chain fatty acids (MCFA) and long chain fatty acids (LCFA) are sensed by GPR40 and GPR120 on EECs.

GPR40 is expressed in the pancreatic β-cells influencing the insulin release. This receptor is present also in the cells of GIT expressing GLP-1 and GIP, while GPR120 is expressed in colonic L-cells and in the δ cells of pancreas and promotes the release of GLP-1 (Janssen and Depoortere, 2013; Steensels & Depoortere, 2017).

Short-chain fatty acids (SCFA) are sensed by GPR43 and GPR41. These receptors are located in the human colon and ileum. GPR43 and GPR41 are expressed by enteroendocrine L-cells that secrete PYY. Anyway, GPR41 is more present in L-cells of the small intestine, while GPR43 is expressed in the human colon (Spreckley & Murphy, 2015).

GPR119 detects the fatty acid ethanolamides, such as N-oleoylethanolamine (OEA). This receptor is present in both pancreatic β-cells and intestinal L-cells. The activation in the pancreas of GPR119 mediates the insulin secretion in the presence of glucose. In the gut, the activation of this receptor releases GLP-1 and insulin in a glucose dependent manner (Spreckley & Murphy, 2015; De Luca, 2018).

Table 1.1 summarises the chemosensors present on enteroendocrine cells along GIT.

Table 1.1. Chemosensors in the GIT (Steensels & Depoortere, 2017; Mace *et al.*, 2015).

	Enteroendocrine cells					
	X/A cells	**P cells**	**G-cells**	**D-cells**	**I-cells**	**L-cells**
Gut hormone	Ghrelin	Leptin	Gastrin	Somatostatin	CCK	GLP-1, PYY
Function	Hunger, Adipogenesis, Growth, Hormone release	Influence satiety	Acid secretion	Inhibition of gastrin release	Inhibition of food intake and gastric emptying, stimulation of pancreatic enzyme secretion	Stimulation of insulin release. Inhibition of food intake and transit
Anatomic localization	Stomach	Stomach	Stomach	Stomach, small intestine	Small intestine	Small intestine, colon
Nutrient sensor						
Carbohydrates and sweeteners	TAS_1R_3 SGLT1 K_{ATP} channels		NR	NR	NR	TAS_1R_2, TAS_1R_3, SGLT1, K_{ATP} channels
Proteins	CaSR, GPRC6A, TAS_1R_1-TAS_1R_3		CaSR, GPRC6A, LPA_5R			
Lipids	GPR43, GPR120		GPR41	GPR120	GPR40, GPR41, GPR120, CD36	GPR40, GPR43, GPR41, GPR120 GPR119, FATP4

1.3 Peptides involved in food intake

The enterocytes are absorptive cells that on their apical side contain the microvilli used to enlarge the contact surface of lumen and to express different transporters that regulate the uptake of sugars, amino acids (aa) and fatty acids (FA).

The EECs are considered the most important endocrine organ in the body. These cells secrete a wide range of peptides that influence the regulation of food intake and gastrointestinal motility.

Ghrelin (hunger hormone) is secreted by P/D1-cells (X/A-cells in rodents) from the stomach. The concentration of this hormone increases before the meal and decreases in the post-prandial phase. Ghrelin influences the hunger on a short-term basis, but it also influences the fat storage, resulting in an increased body weight in the long term (Janssen & Depoortere, 2013; Steensels & Depoortere, 2017).

GLP-1 belongs to the family of glucagon peptide. It is secreted after a meal and it induces satiety through the delay of gastric emptying and potentiation of the glucose-induced insulin secretion. GLP-1 is released from intestinal L-cells in the distal gut, it is degraded by dipeptidylpeptidase-IV (DPPIV) (Steensels & Depoortere, 2017).

PYY is a pancreatic polypeptide involved in the mechanisms that control the transit of a meal through the gut to optimise nutrient digestion and absorption. It is released, in the distal gut, from the enteroendocrine L cells in response to a meal and it is degraded by DPPIV (Steensels & Depoortere, 2017).

CKK is a hormone secreted from enteroendocrine I cells of the upper small intestine in response to ingestion of fat and protein. This hormone causes the release of digestive enzyme and bile from pancreas. Furthermore, CCK is also a satiety signal that decreases meal size and delays gastric emptying (Janssen & Depoortere, 2013; Steensels & Depoortere, 2017).

1.4 N-acylphosphatidylethanolamines (NAPEs), N-acylethanolamines (NAEs) and Endocannabinoids (ECs)

N-acylphosphatidylethanolamines (NAPEs), N-acylethanolamines (NAEs) and endocannabinoids (ECs) belong to the class of lipid messengers, present both in prokaryotic and eukaryotic cells (Coulon *et al.,* 2012).

NAEs and 2-monoacylglycerols (2-MAGs) comprise lipids containing a fatty acid, which is linked to either ethanolamine or glycerol, respectively. They belong to a vast group of fatty acid-containing bioactive lipids that can exert a number of physiological functions in the human body through the activation of specific membrane receptors and nuclear receptors.

Biosynthesis of NAPEs

In animals, the synthesis of NAPEs occurs through the enzyme N-acytransferase (NAT). This enzyme has two different activities: NAT Ca^{2+} dependent and NAT Ca^{2+} independent. The enzyme Ca^{2+} is a membrane-bound protein, it is ATP independent and utilizes phosphatidylethanolamine (PE) as acyl acceptor. The formation of NAPEs involves the transfer of an acyl chain from *sn*-1 position of the phospholipid to the amine of PE.

In plants, the synthesis of NAPEs is possible by NAPE synthase activity that promotes the N-acylation of PE by FFAs (Coulon *et al.,* 2012).

Catabolism of NAPEs

In animals, a specific membrane-bound phospholipase D, N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD), catalyses NAPEs hydrolysis. NAPE-PLD belongs to the metallo-β-lactamase family and it is tightly bound to membranes (Ueda *et al.,* 2013). The enzyme is structurally and catalytically different from other PLD family members and it has a specificity for NAPEs among the glycerophospholipids, with similar selectivity for N-acyl species of NAPEs (Blancafort *et al.,* 2014).

Three different alternative ways are note to produce NAEs. The first, after NAPE hydrolysis, is mediated by phospholipase A_2, meanwhile lyso-NAPEs are degraded by a PLD-like activity. The second way is mediated by phospholipase C (PLC) hydrolysis that leads to diacylglycerol and phospho-NAEs that are degraded into NAEs by phosphatase activity.

The thrird biosynthetic way involves the α/β-hydrolase-4 (Abh4) serine hydrolase deacylates NAPEs. The glycerophospho-NAEs (GP-NAEs) thus generated could be hydrolysed by a glycerophosphodiesterase-1 (GDE1) (Hansen & Diep, 2009; Coulon *et al.*, 2012).

In plants, multiple isoforms of PLD are expressed such as PLDβ and PLDγ, that are able to hydrolyse glycerophospholipids, including NAPEs (Coulon *et al.,* 2012; Ueda *et al.,* 2016).

NAEs are hydrolysed in FFAs and ethanolamine by fatty acid amide hydrolase (FAAH) or N-acyethanolamine-hydrolyzing acid amidase (NAAA) (Coulon *et al.,* 2012).

FAAH is a serine hydrolase belonging to the amidase signature family, it is composed of 579 aa. An isozyme of FAAH with ~ 20% sequence identity at the aa level is expressed in humans. The optimal pH for the FAAH activity is 8.5-10 (Ueda *et al.,* 2010).

NAAA is a cysteine hydrolase belonging to the N-terminal nucleophile hydrolase superfamily. The primary structure of NAAA is composed by 359 aa in humans.

The catalytic activity occurs at the optimal pH 4.5-5 (Ueda *et al.,* 2010).

NAEs and ECs receptors

The most well-known NAEs and 2-MAG are the two ECs, anandamide (AEA) and 2-arachidonoylglycerol (2-AG) that bind and activate cannabinoids receptors (CB1 and CB2). The CB receptors are distributed in the GIT and in ENS.

The CB1 receptor is expressed in CNS, in neurons that regulate food intake, energy expenditure and reward responses, as well as in liver, pancreas, muscle and adipose tissue.

The CB1 receptor participates in brain areas to the regulation of energy balance such as the hypothalamus, the corticolimbic circuits, including the nucleus accumbens (NAc) and the ventral tegmental area, and the brainstem (Gatta-Cherifi & Cota, 2015).

The activation of CB1 by ECs increases the consumption of foods. In hypothalamus, the homeostatic appetite regulator, leptin, suppresses the food consumption. This hormone negatively regulates the hypothalamic levels of ECs (Di Marzo *et al.*, 2001).

NAEs and 2-MAGs also have a number of physiological roles targeting others aside the cannabinoid receptors (Hansen & Vana, 2018; Di Patrizio *et al*, 2011; Hillard, 2018).

NAEs activate the vanilloid receptor (TRPV1) which can be also activated by heat, low pH, and capsaicin. This receptor is expressed in the GIT on the terminals afferent fibers and in some immune cells, its activation influences the pain system (Hansen & Diep, 2009).

NAEs link peroxisome proliferator-activated receptor-α (PPAR-α) that is a nuclear receptor involved in fat metabolism. PPAR-α is expressed in the enterocytes in the small intestine and in enteric neurons of myenteric and submucosal plexuses throughout the GIT, but it was also found in vagal afferent fibres in GIT. In rats it was found in all regions of gut including the colon (Izzo & Sharkey, 2010).

All NAEs, particularly OEA, activate PPAR-α. OEA activates this receptor with a half maximal concentration EC_{50} of 120 nM, *in vitro* (Fu *et al.*, 2003).

Moreoveor the NAEs, linoleoylethanolamide (LEA), palmitoylethanolamide (PEA) and OEA link GPR119 and GPR55 that can be also involved in the food intake modulation either by acting in the brain or by signaling from the gastrointestinal system.

In animal studies GPR119 was shown to be expressed in pancreatic islet and in β-cells, on L-cells regulating the release of GLP-1, in duodenum, colon, ileum and CNS (Izzo & Sharkey, 2010; Hansen & Diep, 2009).

In vitro studies, showed that NAEs have agonist activity on GPR119, with an EC_{50} that was found between 3.2 µM and 0.2 µM for OEA, 0.56 µM for LEA and 0.84 µM for PEA (Hansen *et al.*, 2011; Overton *et al.*, 2006).

Table 1.2 reports the localization and function of all NAEs and ECs receptors.

Table 1.2. NAEs and ECs receptors

Receptor	Localization	Function	Compound	Reference
CB1 and CB2	Brain, heart, uterus, leukocytes, spleen, extented muscle, testis, liver, small intestine, pancreas, immune cells	Regulation of energy balance and metabolism, regulation of immune and inflammatory responses	AEA, 2-AG	D'Addario *et al.*, 2014; Gatta-Cherifi & Cota, 2015
TRPV1	Peripheral sensory fibres, immune cells	Regulation of pain system	AEA, OEA, PEA	D'Addario *et al.*, 2014; Hansen & Diep, 2009
GPR119	Pancreas, liver, duodenum, colon, ileum, CNS	Release of GLP-1 and insulin secretion	OEA, LEA, PEA, 2-OG	Hansen *et al.*, *2011;* Izzo & Sharkey, 2010; Hansen & Diep, 2009
PPAR-α	Small intestine, vagal afferent fibres	Lipid metabolism	OEA, PEA	Izzo & Sharkey, 2010

1.5 NAEs, ECs and food intake

ECs have several effects including analgesia, relaxation and appetite stimulation. For this reason, these compounds have been studied to treat various pathologies. Many studies focused on the orexigenic properties of ECs. The oral treatment with a solution of AEA (20 µg/g body weight in soy oil) during lactation in mice increased food intake, body weight and epididymal

fat during adulthood (Aguirre *et al.*, 2012). Furthermore, adult mice daily treated for 150 days with a single oral dose of AEA increased food consumption more than control mice (Aguirre *et al.*, 2017).

NAEs do not activate cannabinoid receptors. They can regulate food intake via activation of PPAR-α, GPR119 (Overton *et al.*, 2006), GPR55 and TRPV1 (Muccioli *et al.*, 2010).

Among NAEs, OEA was shown to prolong the inter-meal time thus decreasing daily food intake (Gaetani *et al.*, 2003). The administration of OEA at an oral dose of 10 mg/kg reduced food intake in rats (Nielsen *et al.*, 2004). The same effect was demonstrated by using OEA (50 mg/kg) in pH-sensitive-enteric coating. That preparation increased the concentration of OEA in small intestine, plasma, liver and adipose tissues, but not in the brain and muscles thus suggesting that the anorectic effect of OEA does not involve the brain (Oveisi *et al.*, 2004). Similarly, a 5 weeks study demonstrated that the oral OEA 100 mg/kg reduced the body weight gain and decreased daily food intake in mice (Thabius *et al.*, 2011).

In humans, we have recently shown that the higher is the content of oleic acid in the meal the higher is the post-prandial response of OEA and the lower is the energy intake at the subsequent meal (Mennella *et al.*, 2015).

Animal studies showed that the intra-peritoneal injection of OEA (10 mg/kg bw) reduced food intake mainly by meal latency and that this effect was stronger in rats fed a 60% high-fat diet (HFD) than in control chow-fed rats (Azari *et al.*, 2014).

Fu & coworkers (2005) suggested that OEA has an important role in the regulation of lipid metabolism. They showed that the intra-peritoneal injection of OEA (5 mg/kg) for two weeks increased the expression of PPAR-α, FAT/CD36, liver fatty-acid binding protein (L-FABP), and uncoupling protein-2 (UCP-2), while decreased the serum cholesterol and triglyceride levels in Zucker rats.

Beside their ability to regulate the food intake and lipid metabolism, NAEs showed also anti-inflammatory effect. In particular, studies focused on PEA showing anti-inflammatory and pain reducing effects via activation of PPAR-α (Petrosino & Di Marzo 2017). Esposito *et al*. (2014) demonstrated that PEA improved macroscopic disease signs as well as decreased neutrophil infiltration and the expression and release of inflammatory cytokines in a mouse model of colitis. On the other hand, in a model of colon cancer the treatment with PEA reduced the angiogenesis via activation of PPAR-α i*n vitro* (Sarnelli *et al.*, 2016).

Furthermore, Borrelli *et al.* (2014) showed in a mouse model of colitis that 1 mg/kg b.w. of PEA reduced the inflammation and intestinal permeability, reduced the weight loss of mice, counteracted DNBS-induced GPR55 and TRPV1 down-regulation and regulated CB1 receptor. The latter study demonstrated that oral PEA could ameliorate intestinal inflammation pharmacological doses.

1.6 NAPEs, NAEs and ECs in foods

About the presence of NAPEs, NAEs and ECs in food matrices very little is known. The concentration of NAPEs in plants is very low except for oat where these compounds represent 10-12% of total phospholipids content.

Table 1.3 shows the concentration of NAPEs and NAEs in different food sources.

Table 1.3. NAPEs and NAEs concentration in dietary sources.

NAPEs

Source	Concentration	Reference
Wheat flour	0.20-0.25 mg/g dry weight	Kotel'nikova, 2011
Wheat flour	0.78 mg/g dry weight	Schaffarczyk *et al.*, 2014
Kidney bean *cultivar* Shchedraya seed	0.35 mg/g dry weight	Kotel'nikova, 2011
Soybean seeds	0.05 mg/g dry weight	Kotel'nikova, 2011
Rye flour	0.20 mg/g dry weight	Kotel'nikova, 2011
Bran	0.15 mg/g dry weight	Kotel'nikova, 2011
Barley seeds	0.27 mg/g dry weight	Kotel'nikova, 2011
Oat seeds	0.22 mg/g dry weight	Kotel'nikova, 2011

NAEs

Source	Concentration	Reference
Pisum sativum *cultivar* Early Alaska seed	3.44 µg/g of fresh weight	Venables *et al.*, 2015
Pisum sativum *cultivar* Taos seeds	0.58 µg/g of fresh weight	Venables *et al.*, 2015
Glycine max seeds	31.8 µg/g of fresh weight	Venables *et al.*, 2015
Phaseolus vulgaris *cultivar* Amarillo del Norte seeds	0.17 µg/g of fresh weight	Venables *et al.*, 2015
Pea seeds	0.45 µg/g fresh weight	Chapman *et al.*, 1999
Glycine max *cultivar* Dare seeds	1.2 µg/g fresh weight	Chapman *et al.*, 1999
Peanut seeds	1 µg/g fresh weight	Chapman *et al.*, 1999
Tomato seeds	0.7 µg/g of fresh weight	Chapman *et al.*, 1999
Corn seeds	1.2 µg/g of fresh weight	Chapman *et al.*, 1999
Toasted sesam oil	443471 ng/mL	Bradshaw & Leishman 2016
Olive oil	6178 ng/mL	Bradshaw & Leishman 2016
Walnut oil	250973 ng/mL	Bradshaw & Leishman 2016
Canola oil	5018 ng/mL	Bradshaw & Leishman 2016
Peanut oil	39581 ng/mL	Bradshaw & Leishman 2016

Sesame oil	2106 ng/mL	Bradshaw & Leishman 2016
Grape seed oil	47578 ng/mL	Bradshaw & Leishman 2016
Safflower oil	2016 ng/mL	Bradshaw & Leishman 2016
Cocoa derived samples	0.01-5.8 µg/g	Di Marzo *et al.*, 1998
Mytilus galloprovincialis	36 ng/g wet tissue weight	Sepe *et al.*, 1997

The NAPEs concentration ranged from 0.25 mg/g dw to 0.78 mg/g dw in wheat flour (Kotel'nikova, 2011; Schaffarczyk *et al.*, 2014), while in the legumes the concentration ranged from 0.35 mg/g dw in Kidney bean *cultivar* Shchedraya to 0.05 mg/g of dw in soybean (Kotel'nikova, 2011).

NAPEs are present in the Solanum tuberosum L. *cultivar* Bintje cells with the content that increased up to 13-folds in anoxia–stressed cells (Rawyler & Braendle, 2001).

The NAEs concentration in legumes ranged from 3.44 µg/g of fresh weight to 31.8 µg/g of fresh weight in Glycine max (Venables *et al.*, 2005).

On the other hand Chapman and coworkers (1999) showed that the highest concentration of these compounds was found in Glycine max *cultivar* Dare and corn seeds (1.2 µg/g fresh weight), whereas the lowest concentration was found in pea (0.45 µg/g fresh weight).

Bradshaw & Leishman (2016) showed the presence of NAEs in different cooking oils such as olive, walnut, canola, peanut, safflower, sesame and grape seed oil. The highest concentration of NAEs was in toasted sesam oil (443471 ng/mL), whereas the lowest concentration was in safflower oil (2016 ng/mL).

NAEs were detected also in cocoa at very low concentrations ranging from 0.01 to 5.8 µg/g, OEA being between 0.17 and 6 µg/g and AEA resulting absent together with 2-AG (Di Marzo *et al*., 1998).

Finally, NAEs were found also in Mytilus galloprovincialis, with a concentration of 41 ng/g wet tissue weight.

1.7 NAEs, ECs and diet

Many studies showed that the NAEs concentration and the function of endocannabinoid system was influenced by the diet controlling different physiological processes (Di Marzo, 2008).

Studies in humans and in animals showed that the concentration of ECs and NAEs can be influenced by diet and, in particular, by its composition in fatty acids.

Pintus *et al.* (2012) showed that the intake of sheep cheese (90g/day) enriched with α-linoleic acid (ALA), conjugated linoleic acid (CLA) and vaccenic acid (VA) modified the plasma lipid and endocannabinoid profile in mildly hypercholesterolemic humans after 3 weeks of treatment.

In particular, the consumption of enriched cheese increased the plasma concentration of CLA, VA, ALA and EPA, whereas a reduction of the endocannabinoid AEA (40%) was shown. The concentration of 2-AG decreased.

In mice, Hansen & coworkers (2009) showed that the ketogenic diet, a low carbohydrate diet, administrated for 4 weeks reduced the levels of NAEs and OEA in the hippocampus, while no significant reduction was observed for AEA and PEA, suggesting that the ketogenic diet can influence the expression of enzymes involved in the biosynthetic and catabolic ways of NAEs.

Banni *et al.* (2011) studied the influence of intake of 2g/day of either krill oil (KO) or menhaden oil (MO) or olive oil (OO) (control) on the plasmatic levels of ECs in over weight (OW) and obese (OB) subjects for 4 weeks. The intake of KO, but not MO or OO, decreased the level of 2-AG. The decrease of 2-AG is linked to the plasma n-6/n-3 phospholipids long chain and polyunsaturated fatty acid (LCPUFA) ratio. The AEA concentration did not change between diets.

Artmann *et al.* (2008) showed that the composition of diet could influence the NAEs levels in the brain and intestine and modulate the physiopathological function. They administrated 5 different diets to rats for 1 week. The diets were composed with 5 different fats: palm oil (PO),

olive oil (OO), safflower oil (SO), fish oil (FO) and arachidonic acid (AA) containing diet. They showed that the SO diet increased the levels of LEA in the jejunum, liver and in the brain, the OO increased OEA in the liver and it increased the brain concentration of AEA and OEA. The 5 diets reduced the levels of OEA in the jejunum without changes in AEA. Furthermore, the levels of AEA and 2-AG increased in the jejunum, without any changes in the liver.

Meanwhile Joosten *et al.* (2010) showed that in humans the plasmatic levels of NAEs are correlated to both the concentration of serum total FFAs and their specific fatty acid precursors. In rats that had been fed with high fats diet (20-45% of total energy) the intestinal levels of NAEs were reduced but in rats fed with olive oil rich diet as oleic acid caused an increase in the level of OEA (Diep *et al.*,2011).

The oleic acid present in a meal increased the plasmatic concentration of OEA influencing the sensation of appetite and the energy intake at subsequent meals, in humans (Mennella *et al.,* 2015).

1.8 Diet and microbiota

The human body, especially the GIT is colonized about over 1000 different bacterial species. In particular, the highest concentration of bacteria was found in the large intestine (Duca *et al.,* 2014). The most abundant bacterial species that are present in the human intestinal tract belong to the phyla *Firmicutes* and *Bacteroidetes*, while *Proteobacteria*, *Actinobacteria* and *Verrucomicrobia* are less abundant (Mulders *et al.,* 2018; Jeffery & O'Toole, 2013).

The intestinal microbiota is influenced by the age, health, diet and geographical location of the individual, gastrointestinal infections, inflammatory, neurological, immunological, cardiovascular and metabolic disease (Cani *et al., 2*016).

The gut microbiota participates in gut-brain communication influencing the physiology and metabolism in host. The bacteria can modify the phenotype of the individual through the production of metabolites.

Recently, it was demonstrated that diet may influence the microbiota composition (Jeffery & O'Toole, 2013), in fact the consumption of a high fat diet induced changes in the microbiota and increased the obesity (Mulders *et al.*, 2018).

Dietary fats can influence the composition of gut microbiota. Prieto *et al*. (2018) showed that mice fed with diets enriched with different fats had a different profile of gut microbiota that was correlated with many physiological and biochemical parameters.

Blood pressure, insulin, body weight and ghrelin were correlated with bacterial taxa and they increased in mice fed with enriched butter (BT) diet and decreased (except for ghrelin) in mice fed with enriched extravirgin olive oil (EVOO) diet. BT diet increased the values of blood pressure correlating positively with *Desulfovibrio* in faeces. EVOO diet determined lower values of insulin and leptin that were inversely correlated with *Desulfovibrio* and with *Sutterellaceae*, *Marispirillum* and *Mucilaginibacter dageonensis*, respectively.

De Filippo *et al*. (2010) demonstrated that the microbiota of Italian children is different to the one found in children of Burkina Faso. This difference depended by diets of the individuals.

The microbiota composition was related with long term diet and Wu & coworkers (2011) showed that *Bacteirodes* genus was associated with a diet rich in animal products, while genus of *Prevotella* was correlated with a diet rich in plant foods in humans.

The Mediterranean diet (MD) is a dietary pattern characterised by a high daily intake of fruits, vegetables, legumes, nuts and low intake of processed cereals, moderate intake of fish and low intake of saturated fat, meat and dairy products (Martinenz-Gonzalez *et al.*, 2009, Widmer *et al.*, 2015).

On the other hand, the Western diet (WD) is characterised by high intakes of processed meat, butter, fried food, potatoes and high-sugar drinks; it was shown to change the microbiota towards a composition that was associated with several diseases (Myles, 2014; Albenberg & Wu, 2014).

De Filippis & coworkers (2015) showed the existence of a correlation between MD, gut microbiota and metabolites produced by microbiota. MD was associated with an increase of SCFA that were associated with *Bacteroidetes* and *Firmicutes*, while a lower adherence to MD was associated with a high level of urinary trimethylamine oxide (TMAO), a potential factor for cardiovascular disease.

1.9 Microbiota and ECs

In obese humans and rodents the tone of EC system is higher than in normal weight individuals (Bluher *et al.*, 2006; Engeli *et al.*, 2005; Cote *et al.*, 2007; Izzo *et al.*, 2009).

Different compounds that derive from gut microbiota can influence the development of insulin resistance, type 2-diabetes and inflammation. The lipopolysaccharide (LPS) produced by Gram-negative bacteria are the factors that increase the development of metabolic disease (Cani, 2012). The alteration of gut microbiota is linked with the development of obesity and in these mechanisms a role for the ECs system was proposed.

The existence of a link between LPS and EC system, as LPS can influence the production of ECs by immune cells (Liu *et al.*, 2003).

In obese animals and humans the gut microbiota influences the ECs system activity in the colon and in adipose tissues. In obese mice, the ECs system is over-activated in the intestine and in the adipose tissue (Geurts *et al.*, 2011; Muccioli *et al.*, 2010).

The gut microbiota influences the expression of FAAH and MGL in the colon of obese mice (Muccioli *et al.*, 2010).

In the adipose tissue the mRna levels of NAPE-PLD were higher in obese than in lean mice, while the mRna levels of FAAH were higher in lean than in obese mice (Geurts *et al.*, 2011).

Figure 1.2 summarises the interaction between gut microbiota, metabolite and enteroendocrine cell functions.

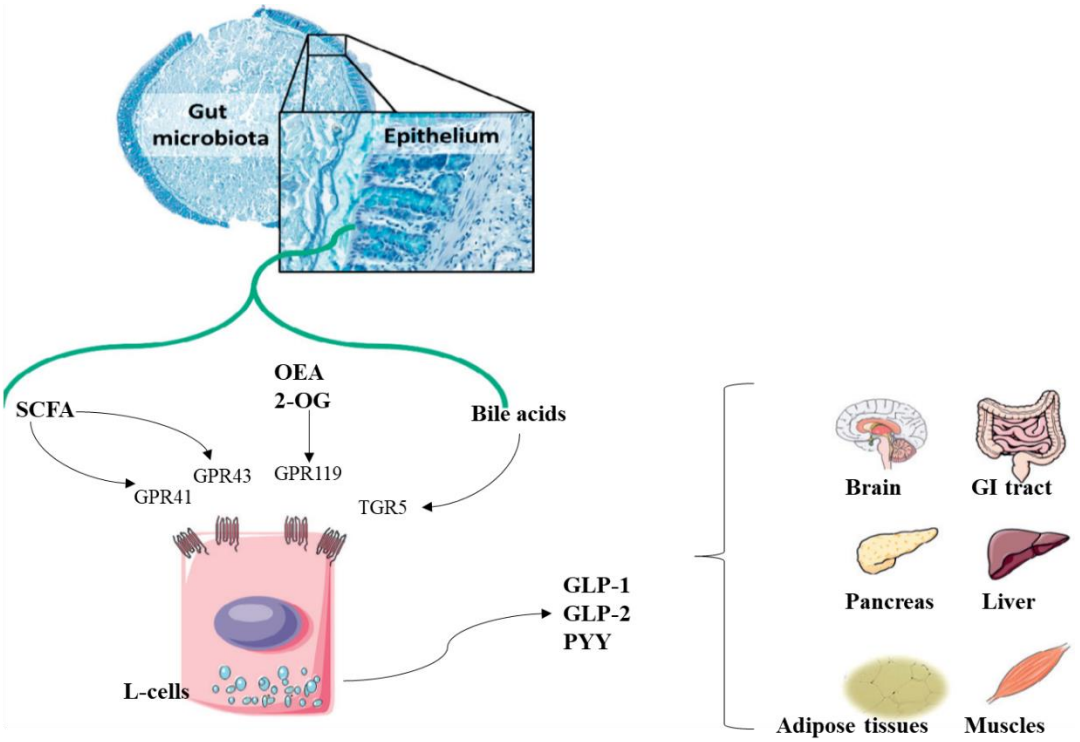

Figure 1.2. Interaction between gut microbiota, metabolites, enteroendocrine L cells functions and specific organs (Cani *et al.*, 2013).

GLP-1 is involved in the control of energy balance and glucose homeostasis via the gut microbial fermentation of dietary fibres and/or non-digestibile carbohydrates. SCFA produced by fermentation promote the release of GLP-1 and PYY by activation of the GPR41 and GPR43 (Delzenne *et al.*, 2011).

The secretion of GLP-1 is stimulated also by the activation of GPR119 by OEA. The activation of GPR119 influences the secretion of GLP-2, a hormone associated with an improvement of mucosal barrier function, improved tight junctions and decrease of the plasmatic concentration of LPS. The production of GLP-2 is regulated by nutritional status of host (Cani *et al.*, 2009; Cani *et al.*, 2013).

NAEs are modulated by gut microbiota and the interaction with GLP-1 can be involved in the cross-talk between the gut microbiota and regulation of host glucose metabolism (Everard &

Cani, 2014). Therefore, ECs system or NAEs controlled by gut microbiota could contribute to control the glucose, lipid and energy homeostasis.

Anyway, in humans a relationship between modified gut microbiota and ECs system tone is not yet demonstrated.

1.10 Abbreviations

GBA: gut-brain axis; GIT: gastrointestinal tract; CNS: central nervous system; EECs: enteroendocrine cells; ANS: autonomic nervous system; ENS:enteric nervous system; HPA: hypothalamic pituitary adrenal; CCK: cholecystokinin; GLP-1: glucagon like peptide-1; PYY: peptide YY; GPRs: G-protein-coupled receptors; FFAs: free fatty acids; CD36: cluster of differentiation 36; FATP4: fatty acid transport protein 4; MCFA: medium chain fatty acids; LCFA: long chain fatty acids; SCFA: Short-chain fatty acids; aa: amino acids; FA: fatty acids; OEA: N-oleoylethanolamine; DPPIV: dipeptidylpeptidase-IV; NAPEs: N-acylphosphatidylethanolamines; NAEs: N-acylethanolamines; ECs: endocannabinoids; 2-MAGs: 2-monoacylglycerols; NAT: N-acytransferase; PE:phosphatidylethanolamine; NAPE-PLD: N-acyl phosphatidylethanolamine phospholipase D; PLC: phospholipase C; Abh4: α/β-hydrolase-4; GP-NAEs: glycerophospho-NAEs; GDE1:glycerophosphodiesterase-1; FAAH: fatty acid amide hydrolase; NAAA: N-acyethanolamine-hydrolyzing acid amidase AEA: anandamide; 2-AG: 2-arachidonoylglycerol; CB: cannabinoids receptors; PPAR-α: peroxisome proliferator-activated receptor-α; L-FABP: liver fatty-acid binding protein; UCP-2: uncoupling protein-2; LEA: linoleoylethanolamide; PEA: palmitoylethanolamide; ALA:α-linoleic acid; EVOO: extravirgin olive oil; CLA: conjugated linoleic acid; VA:vaccenic acid KO: krill oil; MO: menhaden oil; OO:olive oil; OW:over weight; OB:obese; LCPUFA: long chian polyunsaturated fatty acid; PO: palm oil (PO); OO: olive oil; SO: safflower oil; FO:fish oil; AA: arachidonic acid; MD: Mediterranean diet; WD: Western diet; SCFA: short chain fatty acids; TMAO: trimethylamine oxide; LPS: lipopolysaccharide.

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Chapter 2. N-acylphosphatidylethanolamines, N-acylethanolamines and endocannabinoids food database and daily intake upon a Western, a Mediterranean and a Vegetarian dietary pattern

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Abstract

This study aimed to create a food database of N-acylphosphatidylethanolamines (NAPEs), N-acylethanolamines (NAEs) and endocannabinoids (ECs) and to assess their daily intakes on consumption of Mediterranean (MD), Vegetarian (VD) and Western (WD) diet.

Data obtained from 43 foods showed that NAPEs and NAEs were more abundant in vegetal than in animal food products, NAPEs were in the ranges of 0-4032 *vs* 4-398 µg/g dw and NAEs were in the ranges 0-35 *vs* 0.1-0.7 µg/g dw, respectively; conversely, ECs were in the ranges of 0-0.1 *vs* 0-34 µg/g dw.

NAPEs and NAEs daily intakes were higher in MD (263 mg/day and 0.25 mg/day) and VD (242 mg/day and 0.28 mg/day) than in WD (163 mg/day and 0.08 mg/day); conversely, ECs intake was higher in WD and MD (0.17 mg/day) than in VD (0.01 mg/day).

Food contents and daily intakes suggested a potential physiological relevance of dietary NAPEs, NAEs and ECs.

Keywords

Food bioactive compounds; nutrient sensing; gastro-intestinal receptors; food database; phospholipids; lipid mediators.

Chemical compounds studied in this article

Oleoylethanolamide (PubChem CID: 5283454); Palmitoylethanolamide (PubChemCID: 4671); Linoleylethanolamide (PubChem CID: 5283446); Anandamide (PubChem CID: 5281969); 2-Arachidonylglycerol (Pubchem CID: 5282280).

2.1 Introduction

N-acylphosphatidylethanolamines (NAPEs), N-acylethanolamines (NAEs), and the endocannabinoids (ECs) (**Figure 2.1**) are lipids present in animals, plants, bacteria and yeasts.

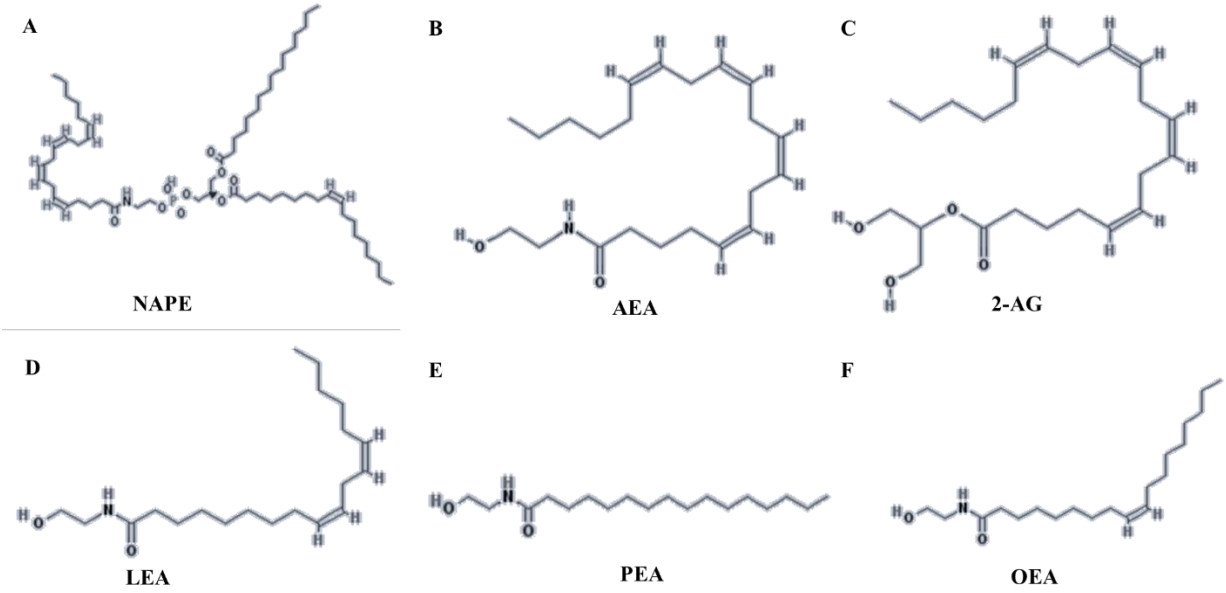

Figure 2.1. Structures of a typical NAPE (16:0/18:1(9Z)/20:4(5Z,8Z,11Z,14Z) (A) and of arachidonoylethanolamide (AEA) (B), 2-arachidonoylglycerol (2-AG) (C), Linoleoylethanolamide (LEA) (D), Palmitoylethanolamide (PEA) (E) and Oleoylethanolamide (OEA) (F).

NAPEs are the precursors of NAEs. In animal tissues, formation of NAEs is catalysed by the NAPE-specific phospholipase D (NAPE-PLD) whereas in plant tissues NAEs can derive from phospholipase-β (PLD-β). In animals NAEs can be hydrolysed in free fatty acids and ethanolamine by fatty acids amide hydrolase (FAAH) or NAE-hydrolyzing acid amidase (NAAA), whereas, in plant tissues, only FAAH is present. In both animal and plant tissues NAEs can be oxygenated by different pathways (Coulon, Faure, Salmon, Wattelet & Bessoule, 2012).

The role of NAPEs in plants, bacteria and yeasts is to protect and stabilize membranes, indeed their content may vary during the biological phases and physical processing (Coulon *et al.*, 2012; Sandoval, Huang, Garrett, Cage & Champan, 1995).

In animals, mounting evidence shows that the concentration of NAEs and ECs in the tissues may be associated with the types of diet consumed. Hansen *et al*. (2009) showed that a 4-week-administration of a ketogenic diet reduced the concentration of NAEs and ECs in the hippocampus of mice. Similarly, NAEs concentration within intestinal cells decreased after feeding rats with a high fat diet for periods lasting from 1 week to 14 weeks (Aviello *et al*., 2008; Di Marzo *et al.*, 2008; Diep *et al.*, 2011). Interestingly, that effect was evidenced sooner with diets providing the highest content of fats and was accompanied by the down-regulation of mRNA levels of NAPE-PLD and unchanged expression of FAAH (Diep *et al.*, 2011).

These findings supported the theory that dietary fats are implicated in NAEs biosynthesis within the cells with a certain specialization (Joosten, Balvers, Verhoeckx, Hendriks & Witkamp, 2010). An olive oil-rich diet providing oleic acid as the predominant fat increased the levels of oleoylethanolamide (OEA) in liver whereas a fish oil-enriched diet decreased the levels of all the NAEs, except for docosahexaenoylethanolamide (DHEA) which remained unchanged (Artmann *et al*., 2008). Similarly, Mennella, Savarese, Ferracane, Sacchi & Vitaglione (2015) showed in humans that a meal providing higher amounts of oleic acid increased post-prandial plasma concentration of OEA which might be responsible of the reduced energy intake at subsequent meal.

In vitro and animal studies showed that NAEs/ ECs are involved in several biological pathways by working as agonists of receptors such as: cannabinoid receptor-1 (CB1) and cannabinoid receptor-2 (CB2) that are present in the enteric nervous system (ENS), in liver and in cardiac, vascular and adipose tissues (Duncan, Davison & Sharkey, 2005); transient receptor potential ion channel of the vanilloid type 1 (TRPV1) that is expressed in the gastro-intestinal tract (GIT)

on the terminals of extrinsic primary afferent fibres and in some immune cells in the mucosa (Izzo & Sharkey, 2010); the peroxisome proliferator-activated receptor-α (PPAR-α) that is highly expressed in kidneys, liver, heart, brown adipose tissue and intestine (Witkamp, 2018). Moreover, NAEs are also able to link G-protein coupled receptor 119 (GPR119) which is expressed on cells in the small intestine, colon and stomach (Hansen & Diep, 2009). Those receptors are involved in food intake and in blood glucose control by regulating the secretion of glucagone-like peptide 1 (GLP-1) from the GIT (Izzo & Sharkey, 2010; Overton, Fyfe & Reynet, 2008).

OEA may also exert its effects on food intake control, visceral pain and intestinal permeability *in vitro* through TRPV1 whereas PEA modulated intestinal permeability via PPAR-α (Karwad *et al.*, 2017).

Interestingly, OEA was suggested to regulate body weight also by modulating the peripheral lipid metabolism because the application of OEA to the enterocytes *in vitro* increased fatty acids uptake by increasing FAT/CD36 mRNA expression (Yang, Chen, Georgeson & Harmon, 2007).

On the other hand, the elegant study by Gillum and co-workers (2008) demonstrated that also NAPEs can influence food intake by signalling the central nervous system after injections in the duodenum, in the peritoneum and in the brain (Gillum *et al.*, 2008).

Although NAPEs, NAEs and ECs are recognised as bioactive compounds and are present in nature, very little is known about their concentration in the food matrices. Only few and sparse studies reported their content in some foods (Chapman, Venables, Markovic, Blair, Bettinger & 1999; Di Marzo *et al.*, 1998; Venables, Waggoner & Chapman, 2005).

This study aimed at creating a database of NAPEs, NAEs and ECs in common foods and at estimating individual daily dietary intake. To this purpose a LC-HRMS analytical method was set up and 43 food products, of which 31 were of vegetal and 12 of animal origin, were

analysed. The database was used to assess the daily dietary intake of those compounds in three dietary plans that were built on the basis of the principles of the Western diet (WD), Mediterranean diet (MD) and Vegetarian diet (VD).

2.2 Materials and methods

2.2.1 Foods

The 43 food products analysed in this study were purchased from a local market and included: beans, lentils and chickpeas (Agria SPA; S. Giuseppe Vesuviano, Italy), brown pasta (Di Martino, Gragnano, Italy), pasta (Antonio Amato, Salerno, Italy), whole wheat flour (Barilla SPA, Parma, Italy), flour (Divella, Rutignano, Italy), parboiled rice (Colussi, SPA, Milano, Italy), whole wheat bread and refined wheat bread (Italy), breakfast whole cereals (Nestlè Italia SPA, Assago, Italy), Gorgonzola cheese (Si Invernizzi Srl, Trecate, Italy), parmigiano cheese (Parmareggio SPA, Modena, Italy), eggs (Maddaloni, Ercolano, Italy), lettuce (Fresco dall'Orto Berna, Battipaglia, Italy), whole milk (Parmalat SPA, Milano, Italy), red wine (Tavernello, Faenza, Italy), codfish (Findus, Roma, Italy), coffee powder (Kimbo SPA, Melito di Napoli, Italy), coffee (Kimbo SPA, Melito di Napoli, Italy), natural tuna (Rio Mare, Bolton Group Srl, Milano, Italy), cocoa (Nestlè Italia SPA, Assago, Italy), salami (Di.A SRL, Caserta, Italy), extravirgin olive oil (Monini Gran Fruttato, Perugia, Italy), butter (Prealpi, Varese, Italy), coconut oil (Food For All snc, Pescantina, Italy), hemp oil (Italy) and palm oil (Italy).

The red meat, potatoes, orange, tomato, apples, anchovies, chicken breast, broccoli and cauliflower were fresh products purchased from local supermarket.

All the foods were analysed in the form they are usually consumed. Pasta, brown pasta, rice, potatoes, broccoli, cauliflower and codfish were boiled in the water and then dripped into a colander. Anchovies were cooked in a pan whereas meat and chicken were grilled. Eggs were hard-boiled. Coffee using 11 g of coffee powder and a volume of 80 mL of water by using a moka machine was prepared.

2.2.2 Extraction of NAPEs and NAEs from foods

Freeze dried food samples (100 mg) were added with 25 µL of the internal standard 2 µg/mL solution of arachidonoylethanolamide d8 (AEA d8) (Cayman Chemical, Ann Arbor, MI).

A solution of $CHCl_3/CH_3OH$ (2:1) (4.5 mL) was used for the extraction. After 30 seconds of shaking, the mixture was centrifuged at 14800 rpm for 10 minutes at 10 °C.

The supernatants were collected in a glass tube and dried under a nitrogen flow. The extract was suspended in a solution of $CH_3CN/C_3H_8O/H_2O$ (60:35:5) before the HRMS analysis.

2.2.3 Liquid Chromatography – High Resolution Mass Spectrometry (LC-HRMS) analysis

LC-HRMS analysis was adapted from Gregory *et al.* (2012). The analyses were performed on an Accela U-HPLC system coupled to an Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The Accela system consisted of a quaternary pump, a thermostated autosampler (8 °C). A Kinetex 2.6 µ C18 100 A column (100 mm × 2.1 mm) (Phenomenex, Torrance, CA) thermostated at 45 °C was used. The mobile phase consisted of 40:60 CH_3CN/H_2O with 5 mM ammonium formate and 0.1% formic acid (A) and 90:10 C_3H_8O/CH_3CN with 5 mM ammonium formate and 0.1% formic acid. (B). Gradient elution was linearly programmed as follows: 32 % B (0-1.5 min), 32–45 % B (1.5-4) min, , 45–52 % B (4-5 min), 52-58 % B (5-8 min), 58-66 % B (8-11 min), 66-70 % B (11-14 min), 70-75 % B (14-18 min), 75-97 % B (18-21 min), costant at 97% B (21-25 min) (Gregory *et al.,* 2012)

The flow rate was set at 200 µL/min and the injection volume was 10 µL.

The U-HPLC system was coupled to an Exactive Orbitrap MS operating with a heated electrospray interface (HESI), in positive and negative ionization mode, in the mass range of *m/z* 120–1200. Mass resolving power was set to 50,000 full width at half-maximum (FWHM, *m/z* 200), scan time was 1 s and the maximum injection time was 100 ms. The interface parameters were optimised using reference compounds of N-acylphosphatidylethanolamine, N-acylethanolamines and endocannabinoids using the following parameters: spray voltage 3.5

kV (positive mode) and 3.0 kV(negative mode), capillary voltage 30V, heater temperature 300 °C, capillary temperature 50 °C, sheath gas 35 arbitrary units, and auxiliary gas 15 arbitrary units.

The instrument was daily calibrated by following Thermo Exactive calibration procedure in positive and negative ions mode.

The Xcalibur software (Thermo Fisher Scientific, San Jose, USA) was used for the chromatographic data acquisition and peak integration.

N-Arachidonoylphosphatidylethanolamine standard was purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Inc., USA).

The concentrations of different NAPEs were quantified as N-Arachidonoylphosphatidylethanolamine equivalents by using the external calibration curve with the linear range (0.2-5µg/mL) and expressed as µg per g of dry food.

ECs (2-arachidonylglycerol, 2-AG; anandamide, AEA; AEAd8) and NAEs (OEA: linoleoylethanolamide, LEA; palmitoylethanolamide, PEA) standards were purchased from Cayman (Cayman Chemical, Ann Arbor, MI). The concentrations were calculated by isotope dilution using calibration curve with the linear range (1-1000 ng/mL) and expressed as ng per g of dry food.

In the chromatographic region between 19-23 min the NAPEs were detected in negative mode as $[M-H]^-$.

In the chromatographic region between 6-9 min the NAEs were detected in positive mode as $[M+H]^+$.

The compounds identification was performed by using the exact mass values up to the fifth decimal digit with a ± 5 ppm mass tolerance. In the **Table 2.1** molecular formula, theoretical mass, experimental mass and mass accuracy have been shown.

Table 2.1. High-Resolution Mass Spectrometry identification of ECs, NAEs and NAPEs.

Compound	Molecular formula	Theoretical mass $[M+H]^+$ *m/z*	Theoretical mass $[M-H]^-$ *m/z*	Experimental Mass	Mass accuracy (ppm)
ECs					
AEA	$C_{22}H_{37}NO_2$	348.28971		348.28877	-2.70
2-AG	$C_{23}H_{38}O_4$	379.28429		379.28436	0.18
NAEs					
OEA	$C_{20}H_{39}NO_2$	326.30536		326.30499	-1.13
LEA	$C_{20}H_{37}NO_2$	324.28971		324.28995	0.74
PEA	$C_{18}H_{37}NO_2$	300.28971		300.28976	0.17
NAPEs					
16:0-18:2-N16:0	$C_{55}H_{104}NO_9P$		952.73759	952.73984	2.36
16:0-18:2-N18:2	$C_{57}H_{104}NO_9P$		976.73759	976.73890	1.34
18:2-18:2-N16:0	$C_{57}H_{104}NO_9P$		976.73759	976.73890	1.34
18:2-16:0-N18:2	$C_{57}H_{104}NO_9P$		976.73759	976.73890	1.34
16:0-18:2-N18:1	$C_{57}H_{106}NO_9P$		978.75324	978.75163	-1.64
16:0-18:1-N18:2	$C_{57}H_{106}NO_9P$		978.75324	978.75163	-1.64
18:2-18:2-N18:2	$C_{59}H_{104}NO_9P$		1000.73759	1000.74099	3.40
18:1-18:3-N18:2	$C_{59}H_{104}NO_9P$		1000.73759	1000.74099	3.40
18:2-18:1-N18:2	$C_{59}H_{106}NO_9P$		1002.75324	1002.75533	2.08
18:0-18:3-N18:2	$C_{59}H_{106}NO_9P$		1002.75324	1002.75533	2.08
18:3-18:2-N18:2	$C_{59}H_{102}NO_9P$		998.72194	998.72536	3.42
18:2-18:2-N18:3	$C_{59}H_{102}NO_9P$		998.72194	998.72536	3.42
18:1-18:2-N18:1	$C_{59}H_{108}NO_9P$		1004.76889	1004.77163	2.73
18:3-18:0-N18:1	$C_{59}H_{108}NO_9P$		1004.76889	1004.77163	2.73
18:3-16:0-N20:1	$C_{59}H_{108}NO_9P$		1004.76889	1004.77163	2.73
18:0-18:3-N18:1	$C_{59}H_{108}NO_9P$		1004.76889	1004.77163	2.73
18:2-18:1-N20:1	$C_{61}H_{112}NO_9P$		1032.80019	1032.80093	0.72
18:2-18:2-N20:0	$C_{61}H_{112}NO_9P$		1032.80019	1032.80093	0.72
16:0-18:2-N18:0	$C_{57}H_{108}NO_9P$		980.76889	980.77113	2.28
18:1-16:0-N18:1	$C_{57}H_{108}NO_9P$		980.76889	980.77113	2.28
18:2-18:1-N18:0	$C_{59}H_{110}NO_9P$		1006.78454	1006.78540	0.85
18:2-18:0-N18:1	$C_{57}H_{108}NO_9P$		1006.78454	1006.78540	0.85
18:0-18:1-N18:1	$C_{59}H_{112}NO_9P$		1008.80019	1008.80127	1.07
16:0-18:2-N20:0	$C_{59}H_{112}NO_9P$		1008.80019	1008.80127	1.07
18:1-18:0-N20:4	$C_{61}H_{110}NO_9P$		1030.78454	1030.78635	1.76
18:2-18:0-N20:1	$C_{61}H_{108}NO_9P$		1028.76889	1028.77166	2.69

2.2.4 NAPEs identification in food matrix

Chromatographic separation was performed using a HPLC apparatus equipped with two Micropumps Series 200 (PerkinElmer, Shellton, CT, USA).

The chromatographic conditions used for the analysis were the same present in the paragraph 2.3.

Mass spectrometry analysis was performed on an API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Canada) equipped with a TurboIonSpray source working in the negative ion mode. Acquisition was performed in scan in the range *m/z* 120-1200 and in MRM (Multiple Reaction Monitoring).

2.2.5 NAPEs, NAEs and ECs content in typical diets

Three diets of 2000 kcal were developed on the basis of the frequency of consumption of vegetal and animal food products typical of the dietary patterns defined as Western, Mediterranean and Vegetarian.

Western diet (WD) is characterised by high intakes of red and processed meat, butter, fried foods, high-fat dairy products, eggs, refined grains, potatoes, and high-sugar drinks (Myles, 2014).

Mediterranean diet (MD) is characterised by daily intakes of whole grain pasta and bread, high intakes of legumes and fish, low intake of poultry, eggs and dairy products instead of red and processed meat as source of animal proteins, low intake of potatoes and unique use of virgin olive oil as food condiment (Martinenz-Gonzalez, Bes-Rastrollo, Serra-Majem, Lairon, Estruch & Trichopoulou, 2009).

Vegetarian diet (VD) is characterised by the absence of meats, seafoods or products containing these food but inclusion of dairy products and eggs (Craig & Mangels, 2009).

The Terapia Alimentare Dietosystem® (DS Medica, Milano, Italy) software was used to develop the three diets and the daily intake of NAPEs, NAES and ECs for each diet using the developed database was assessed.

The MD was developed considering a daily consumption of fresh and dried fruit, legumes, vegetables, whole wheat bread, brown pasta, breakfast whole cereals, milk, coffee and red wine, while it was considered a weekly consumption of fish, meat and eggs.

The VD was different to MD for the higher daily consumption of fresh fruit and vegetables, for the higher weekly consumption of egg and dairy products and for the absence of meat and fish consumption.

However, in both the diets the extravirgin olive oil was considered as the unique condiment for the preparation of meals.

On the other hand, the WD was devolped considering a weekly consumption of fresh fruit and nuts, legumes and vegetables (mostly potatoes), eggs and daily consumption of bread, pasta, meat, milk and dairy products, coffee and red wine. The extravirgin olive oil and butter were considered as the condiments in the preparation of meals.

2.2.6 Statistical analysis

Results were expressed as means ± standard error of means (SEM). Statistical analyses were performed with the statistical package SPSS for Windows (version 20, SPSS Inc. Chicago, IL, USA).

Levene's test was used to evaluate the normality of distribution and the homogeneity of variance of all monitored variables. The difference between means were tested using Student's t-test. Statistical significance was set at level of $p<0.05$.

2.3 Results

2.3.1 Identification of NAPEs

NAPEs identification in food matrices was carried out by considering chemical structures of compounds and their retention times as obtained by HRMS analysis.

In HRMS analysis NAPEs were characterised by exact mass. However, since several NAPEs species can have the same molecular weight due to the combination of different acyl residues on the ethanolamine bone, NAPEs were tentatively identified using an API 3000 triple quadrupole mass spectrometer. The analyses were firstly performed in scan using cone-induced fragmentation to detect the deprotonated molecules of NAPEs and the carboxylate ions in *sn*-2 and *sn*-1 position. The acquisition was then performed in MRM, to confirm the position of fatty acids by considering the information obtained in scanning experiments.

The position of fatty acid was assigned studying the fragmentation patterns of NAPEs in accordance with the method described by Hansen, Hansen, Bjørnsdottir & Hansen, (1999) and Sandoval *et al.,* (1995). They reported that the *sn*-2 fatty acid carboxylate anion $[R_2CO_2]^-$ appeared at higher intensities than the *sn*-1 fatty acid carboxylate anion $[R_1CO_2]^-$, thereby the positioning of individual acyl groups along the glycerol backbone of diacyl-NAPE was facilitated. MS/MS fragmentation pattern showed fragment ions correspondent to neutral loss of fatty acids from the *sn*-2 $[M\text{-}H\text{-}R_2CO_2H]^-$ and the *sn*-1 $[M\text{-}H\text{-}R_2CO_2H]^-$ position and fragment ions correspondent to neutral loss of dehydrate of fatty acids from the *sn*-2 $[M\text{-}H\text{-}R_2CH{=}CO]^-$ and the *sn*-1 $[M\text{-}H\text{-}R_1CH{=}CO]^-$ position.

Furthermore, the fragment ion $[R_3CONHCH_2CH_2OPO_3H]^-$ was specific of NAPEs species and excluded any confusion with acylphospatidylglicerol because it corresponded to the phosphorylated N-fatty amide head group (Coulon & Buré, 2015).

R_1, R_2, and R_3 refer to the hydrocarbon chains of the acyl groups at *sn*-1, *sn*-2, and N-acyl position.

NAPEs identification by mass spectrometry is shown in **Table 2.2.**

Table 2.2. NAPEs identification by mass spectrometry.

NAPE *sn*-1, *sn*- 2	$[M\ H]^-$ *m/z*	$[R_2CO_2]^-$	$[R_1CO_2]^-$	$[R_3CO_2]^-$	$[M\text{-}H\text{-}R_2CO_2H]^-$	$[M\text{-}H\text{-}R_1CO_2H]^-$	$[M\text{-}H\text{-}R'_2CH{=}CO]^-$	$[M\text{-}H\text{-}R'_1CH{=}CO]^-$	$[R_3CONHCH_2CH_2OPO_3H]^-$
16:0-18:2-N16:0	952.7	279.0	255.0	255.0	672.7	696.7	690.7	714.7	378
16:0-18:2-N18:2	976.7	279.0	255.0	279.0	696.7	720.7	714.7	738.7	402
18:2-18:2-N16:0	976.7	279.0	279.0	255.0	696.7	696.7	714.7	714.7	378
18:2-16:0-N18:2	976.7	255.0	279.0	279.0	720.0	696.0	738.0	714.0	402
16:0–18:2-N18:1	978.7	279.0	255.0	281.0	698.7	722.7	716.7	740.7	404
16:0-18:1-N18:2	978.7	281.0	255.0	279.0	696.7	722.7	714.7	740.7	402
18:2–18:2-N18:2	1000.7	279.0	279.0	280.0	720.7	720.7	738.7	738.7	402
18:1-18:3-N18:2	1000.7	277.0	281.0	279.0	718.7	718.7	736.7	736.7	402
18:2-18:1-N18:2	1002.7	279.0	281.0	279.0	722.7	720.7	740.7	738.7	402
18:0-18:3-N18:2	1002.7	277.0	283.0	279.0	724.7	718.7	742.7	736.7	402
18:3-18:2-N18:2	998.7	279.0	277.0	279.0	718.7	720.7	736.7	738.7	402
18:2-18:2-N18:3	998.7	279.0	279.0	277.0	718.7	718.7	736.7	736.7	400
18:2 18:2-N20:0	1032.7	279.0	279.0	311.0	752.7	752.7	770.7	770.7	434

18:2 18:1-N20:1	1032.7	279.0	281.0	309.0	752.7	750.7	770.7	768.7	432
18:1-18:2-N18:1	1004.7	281.0	279.0	281.0	722.7	724.7	740.7	742.7	404
18:0-18:3-N18:2	1004.7	283.0	277.0	281.0	720.7	726.7	738.7	744.7	404
18:2 18:1-N18:1	1004.7	279.0	281.0	281.0	724.7	722.7	742.7	740.7	404
18:3 18:0-N18:1	1004.7	279.0	279.0	283.0	724.7	724.7	742.7	742.7	406
18:0-18:2-N20:1	1004.7	255.0	277.0	309.0	748.7	726.7	766.7	744.7	432
18:3-16:0-N20:1	1004.7	255.0	277.0	309.0	748.7	726.7	766.7	744.7	432
18:2-18:0-N-18:2	1004.7	279.0	283.0	279.0	724.7	720.7	742.7	738.7	402
16:0–18:2-N18:0	980.7	279.0	255.0	283.0	700.7	724.7	718.7	742.7	406
18:1-16:0-N18:1	980.7	281.0	255.0	281.0	698.7	724.7	716.7	742.7	404
18:2-18:1-N18:0	1006.7	281.0	279.0	283.0	724.0	726.0	742.0	744.0	406
18:2-18:0-N18:1	1006.7	283.0	279.0	281.0	722.7	726.8	740.7	744.8	404
18:0-18:1-N18:1	1008.7	281.0	283.0	281.0	726.0	724.0	744.0	742.0	404
16:0-18:2-N20:0	1008.7	279.0	255.0	311	728.8	752.8	746.8	770.8	434
16:0-18:2-N20:1	1030.7	279.0	255.0	309.0	748.0	772.0	766.0	790.0	432

18:1-18:0-N20:4	1030.7	283.0	281.0	303.0	746.7	748.7	764.7	766.7	426
18:2-18:0-N18:1	1028.7	283.0	279.0	281.0	744.0	748.0	762.0	766.0	404

* R_1, R_2, and R_3 refer to the hydrocarbon chains of the acyl groups at *sn*-1, *sn*-2, and N-acyl position

In the **Figure 2.2** the extracted ion chromatograms of NAEs and NAPEs identified in the walnuts are shown.

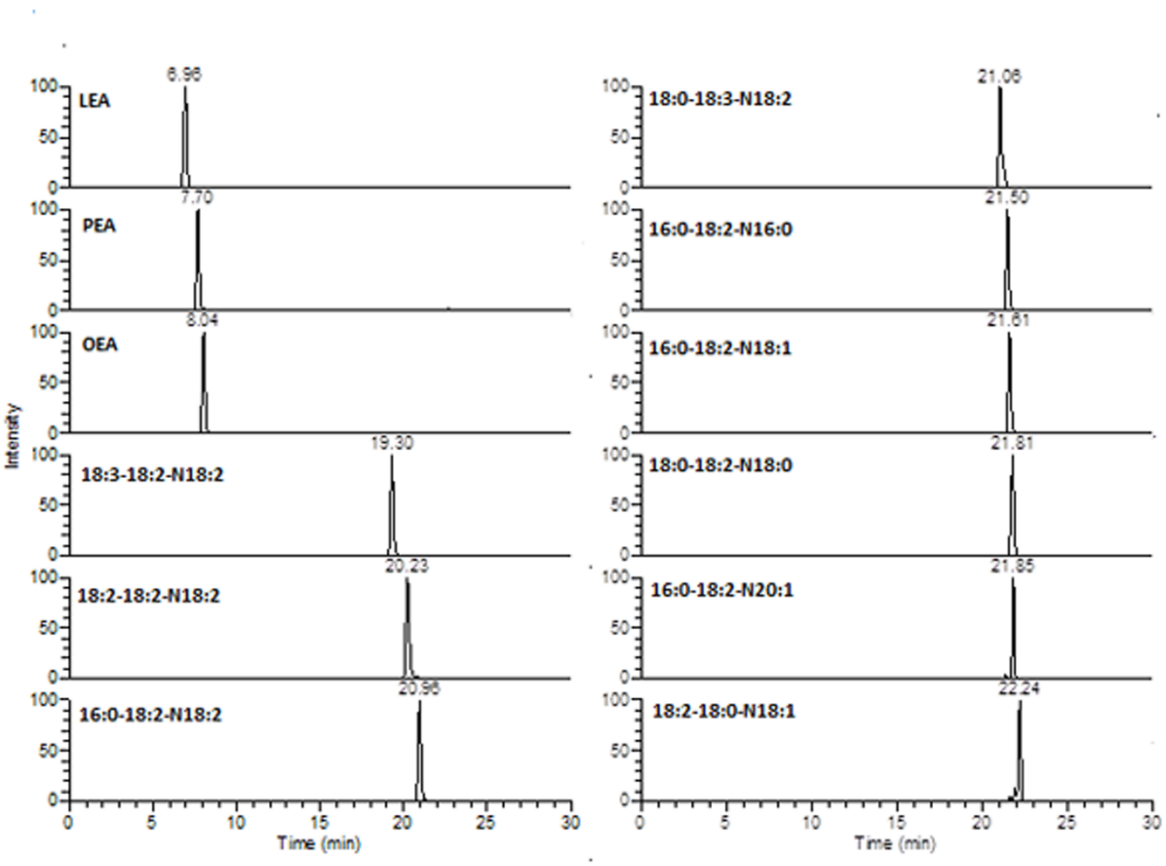

Figure 2.2 Extracted ion chromatograms (XICs) of NAEs and NAPEs present in the walnuts obtained using the high-resolution mass spectrometry in positive and negative ions.

2.3.2 NAPEs content of food products

The concentration of the total NAPEs in foods is shown in **Figure 2.3.**

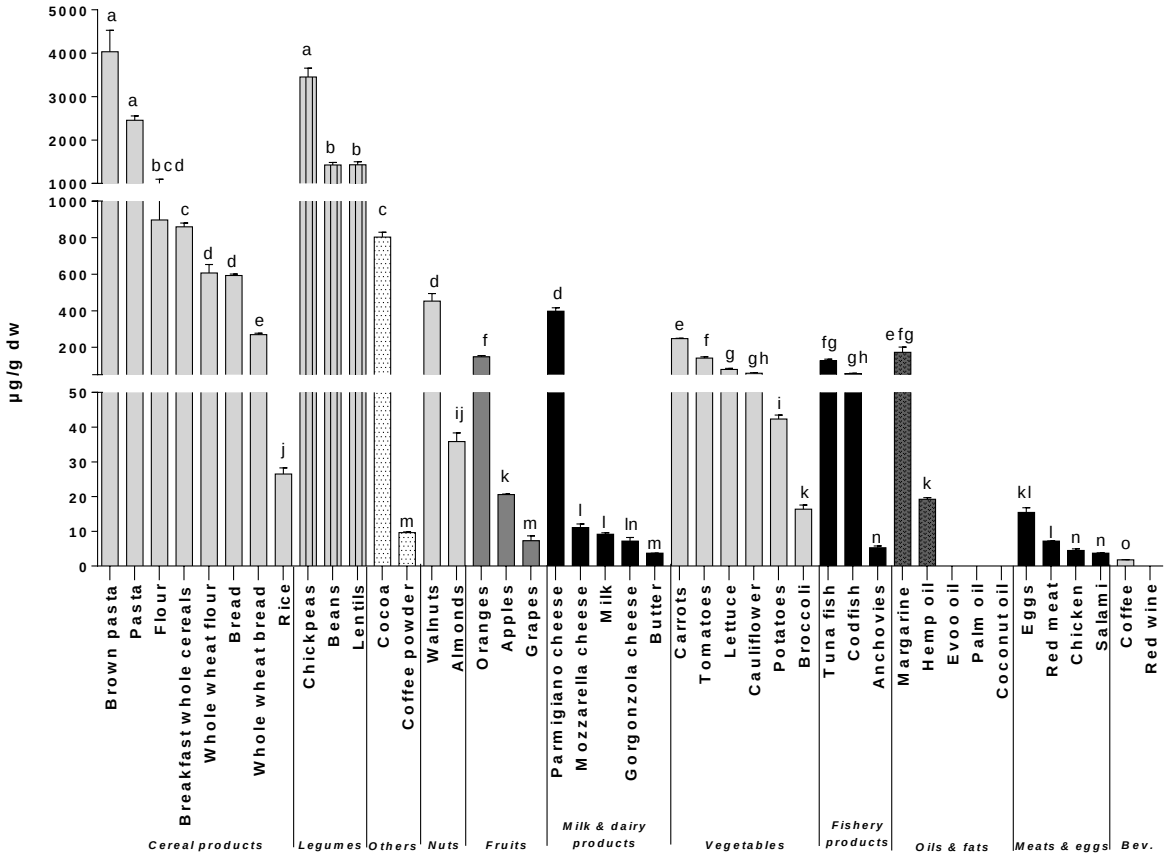

Figure 2.3. Total NAPEs concentration in foods of animal and vegetable origin. Student's t test was used and different letters on the bars indicate $p<0.05$ (number of replicates=3).

Figure 2.3 shows the concentration of the total NAPEs in foods. They were more abundant from vegetal products than in animals. The highest concentration of total NAPEs was found in chickpeas and in brown and white pasta which belong to the food categories of legumes and cereal products, respectively. Those food sources were followed by beans and lentils as well as refined flour whose content of NAPEs was 2.8, 2.8 and 4.5 folds lower than brown pasta, respectively. Breakfast cereals and cocoa powder showed a similar content and were immediately followed by whole wheat flour, bread and walnuts. The latter showed a NAPEs content similar to parmigiano cheese that was the richest source in animal products followed

by tuna fish and codfish. On the other hand meats and eggs were the poorest source of NAPEs among animal products, indeed their mean concentration was 5.5 and 7.6 folds lower than fishery and dairy products, respectively. A similar content of NAPEs, between 58 µg/g dw and 248 µg/g dw, was found in 4 out of 6 analysed vegetables whereas potatoes and broccoli had a 10 folds lower content. Between oils and fats category, margarine was the richest source of NAPEs, followed by hemp oil whereas no traces of NAPEs in EVOO, palm oil and coconut oil were found. In beverages NAPEs were found only in coffee at a concentration of 1.81 µg/g dw. In a selection of foods such as flour, walnuts, parmigiano cheese, eggs, tomatoes and chickpeas, exact NAPEs, as precursors of LEA, PEA, OEA and AEA, were identified and quantified. Data are reported in **Table 2.3.**

Table 2.3. Total concentration of LEA, OEA, PEA precursors in foods. Student's t test was used and different letters in the column indicate $p<0.05$ (number of replicates=3).

Food	**LEA precursors (µg/g dw)**	**OEA precursors (µg/g dw)**	**PEA precursors (µg/g dw)**
	Means ± SEM	**Mean ± SEM**	**Mean ± SEM**
Flour	353.71 ± 37.30^{b}	231.9 ± 3.38^{b}	4.06 ± 0.46^{c}
Walnuts	232.64 ± 9.05^{b}	105.67 ± 75.46^{c}	24.47 ± 1.07^{b}
Chickpeas	1701.1 ± 135.23^{a}	1598.12 ± 75.46^{a}	150.53 ± 7.80^{a}
Parmigiano cheese	28.42 ± 0.78^{d}	235.51 ± 8.12^{b}	20.74 ± 1.23^{b}
Tomato	78.61 ± 3.81^{c}	43.59 ± 1.28^{d}	NF ± NF
Eggs	1.27 ± 0.17^{e}	9.72 ± 0.87^{e}	0.88 ± 0.05^{d}

The highest concentrations of NAEs precursors were found in chickpeas, while the lowest concentrations were found in eggs. Specifically, the concentration of LEA, OEA and PEA

precursors were the 49%, 46% and 4% of total NAPEs in the chickpeas, and the 8%, 63% and 6% of total NAPEs in eggs, respectively.

2.3.3 NAEs and ECs content of foods

Figure 2.4A shows the concentration of LEA in foods. Cereal products showed the highest mean concentration of LEA (65.0-30292.9 ng/g dw), refined flour was the richest product followed by whole wheat flour and by brown pasta, breakfast whole cereals and bread which had a similar content. After cereal products the highest content of LEA was found in cocoa and coffee powder (809.5-3167.0 ng/g dw), nuts (17.6-1326.7 ng/g dw) and vegetables (1.5-1866.7 ng/g dw) which showed a mean LEA content that was ~ 3.1, 9.2 and 14.4 folds lower than cereals, respectively. Among oil and fats, margarine and hemp oil were the richest sources while 10 and 11 folds lower amount of LEA were found in EVOO and palm oil, respectively. Among animal products, eggs were the richest source of LEA being about 2.8 folds higher than butter, 4.8 folds higher than chicken 5.5 folds higher than salami and 5.7 folds higher than codfish as well as 8.9 folds higher than gorgonzola and parmigiano cheese.

Figure 2.4B shows the concentration of PEA in foods. Coffee powder is the richest source of PEA (897.7 ng/g dw). A similar content was found in refined flour (803.7 ng/g dw) which was the richest source of PEA in cereal products followed by whole wheat flour. Breakfast whole cereals and brown pasta showed a similar content of PEA that was about 16 folds lower than that of flour. Margarine and walnuts were the richest source of PEA in their categories (302.3 ng/g dw and 253.3 ng/g dw, respectively) with a concentration similar to whole wheat flour. Among animal products, similar amounts in fishery and meat products as well as in eggs were found whereas milk and dairy products were a bit less abundant being respectively 2.8, 2.1, and 5.7 folds lower than in cereal products. PEA was not found in coffee and red wine.

Figure 2.4C shows the concentration of OEA in foods. The highest concentration of OEA was found in refined flour followed by cocoa, whole wheat flour and coffee powder which showed

a content of OEA that was 1.3, 1.5 and 3.6 folds lower than flour, respectively. Interestingly, for OEA compared to LEA and PEA concentrations, animal products were ranked higher than nuts, legumes and vegetables. In particular, fishery and meat products, such as codfish and read meat, showed an OEA content that was 6 and 12 folds lower than refined flour, respectively. OEA in legumes and vegetables was found at a mean concentration similar to EVOO and palm oil. On the other hand, margarine and hemp oil were found the best sources of OEA in oils and fats category with a content that was similar to walnuts and parmigiano cheese, respectively.

Thus looking at the sum of all NAEs (**Figure 2.4D**) the highest concentration of NAEs was found in flour and whole wheat flour followed by coffee and cocoa powders (34757.4 ng/g dw, 18146.0 ng/g dw, 5086 ng/g dw and 3700.2 ng/g dw, respectively). Brown pasta, breakfast cereals and bread showed a similar content of NAEs (~ 1351.3 ng/g dw) that is very close to that found in carrots, walnuts and margarine, which are the richest sources of NAEs in vegetables, nuts and oils and fats, respectively. In general, among vegetal products legumes were the poorest source of NAEs (177.53 ng/g dw) with chickpeas and beans being 5.3 folds higher than lentils and showing a concentration level similar to cauliflower, tomatoes and lettuce. Among animal products codfish (674.7 ng/g dw) was the richest source of NAEs and it was immediately followed by eggs and red meat. On the other hand milk and dairy products were the poorest animal sources of NAEs (mean concentration of 46.6-229.6 ng/g dw) with butter and parmigiano cheese showing the highest concentration. Beverages and fresh fruits were at the bottom of the concentration ranking list being at a level below 100 ng/g dw.

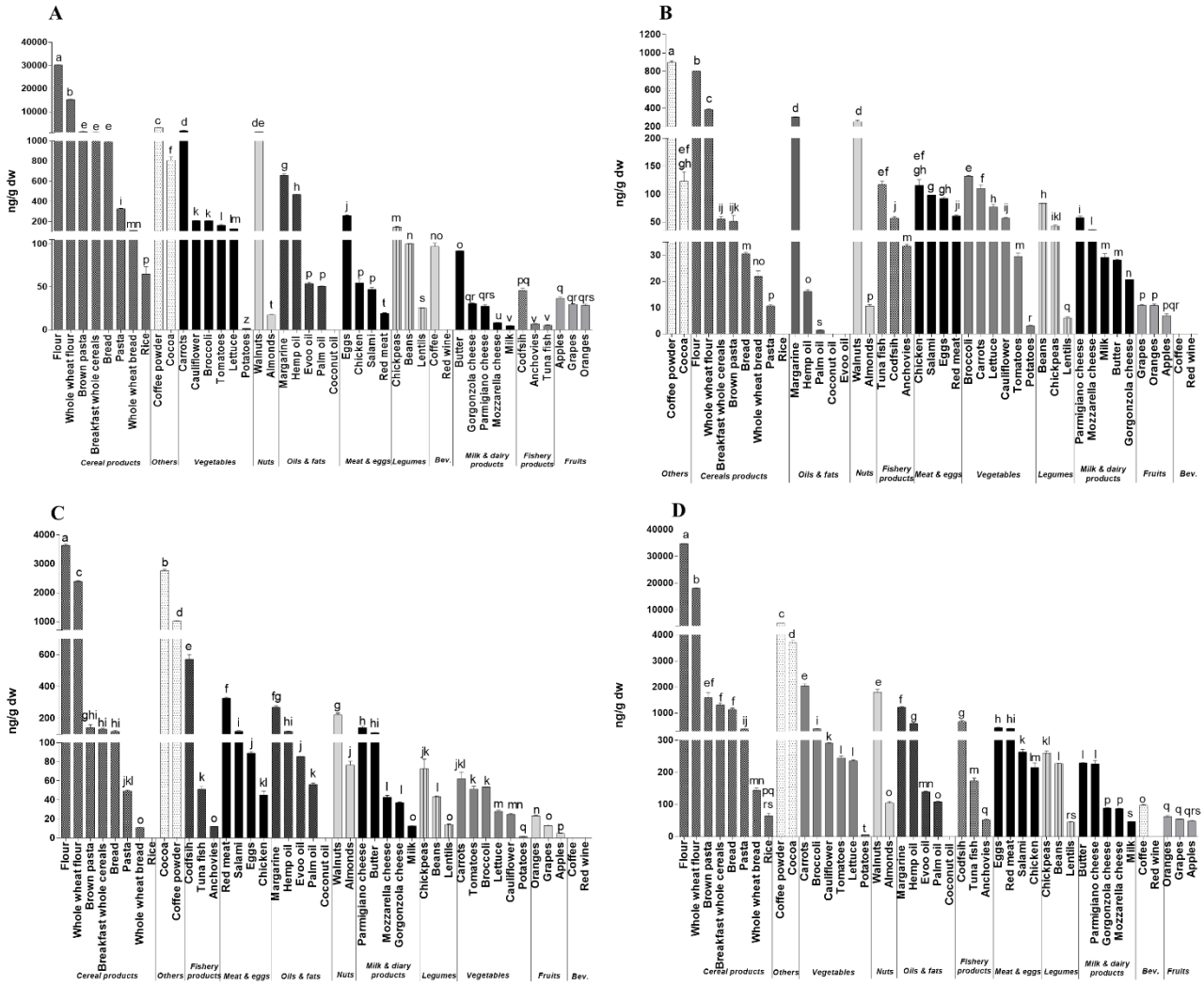

Figure 2.4. LEA (A), PEA (B) and OEA (C) and total NAEs (D) concentration in foods of animal and vegetable origin. Student's t test was used and different letters on the bars indicate $p<0.05$ (number of replicates=3).

Figure 2.5 shows the concentration of AEA, 2-AG and total ECs. Interestingly, ECs were not found in the foods of plant origin but in cocoa, where AEA was 6.6 ng/g dw, and in cauliflower, where 2-AG was 74.8±2.9 ng/g dw. 2-AG was at a concentration level 1500 folds higher than AEA. Thus total ECs in foods mirrored their 2-AG content. The highest concentration of 2-AG was found in tuna fish (33466.3 ng/g dw), followed by eggs (2403.9 ng/g dw) and anchovies (416.9 ng/g dw). The highest concentration of AEA was found at a similar level in eggs,

chicken and red meat (mean value ~ 16.27 ng/g dw) followed by parmigiano cheese at 8.92 ng/g dw and anchovies at 1.21 ng/g dw.

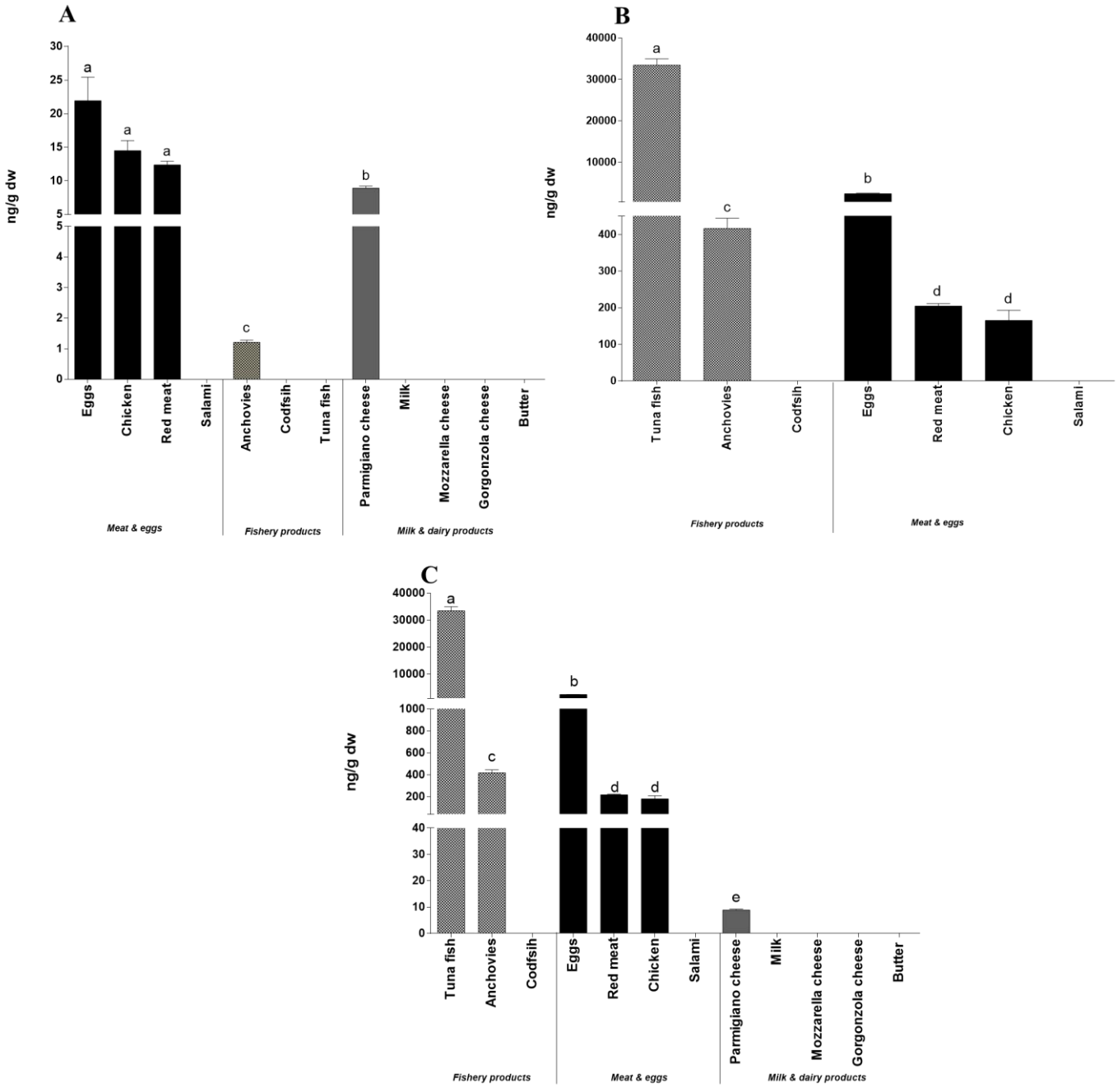

Figure 2.5. AEA (A), 2-AG (B) and total ECs (C) in the animal origin food. Student's t test was used and different letters on the bars indicate $p<0.05$ (number of replicates=3).

2.3.4 NAPEs, NAEs and ECs daily intake in Western, Mediterranean and Vegetarian diet

The **Table 2.4** shows the daily intake of NAPEs, NAEs and ECs upon consumption of a MD, a VD and a WD.

Table 2.4. Daily intake of NAPEs, NAEs and ECs by a Western Diet, a Mediterranean Diet and Vegetarian Diet providing 2000 kcal/day.

	Western Diet	**Mediterranean Diet**	**Vegetarian Diet**
NAPEs (mg/day)	163	263	242
NAEs (mg/day)	0.08	0.25	0.28
ECs (mg/day)	0.17	0.17	0.01

Data showed that the daily intake of NAPEs and NAEs was higher in VD and MD than in WD (**Figure 2.6A** and **Figure 2.6B**, respectively), while the daily intake of ECs in MD and WD was similar and higher than in VD (**Figure 2.6C**).

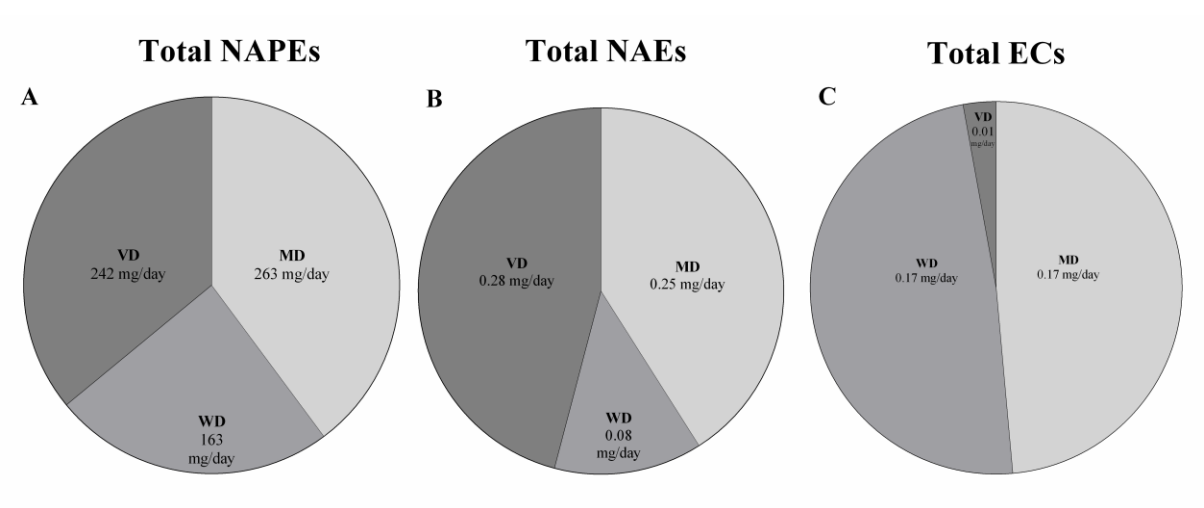

Figure 2.6. Daily intake of NAPEs (A), NAEs (B) and ECs (C) in Mediterranean diet, Western diet and Vegetarian diet.

2.4 Discussion

For the first time in this study, the content of NAPEs, NAEs and ECs in 43 food products was assessed. Data showed that both NAPEs and NAEs were present at higher concentration in vegetal than animal food products whereas ECs were mainly found in animal products.

The concentration of NAPEs in wheat flour was in accordance with previous studies showing concentrations in the range of 250 µg/g dw and 896.7 µg/g dw (Kotel'nikova, 2011; Schaffarczyk, Østdal & Koehler, 2014). On the other hand NAPEs content of legumes was about 4 folds higher than that reported by Kotel'nikova (2011) showing concentration range of 50-350 µg/g dw depending from the beans cultivar. The different analytical method used for quantification, a possibly different cultivar of legumes as well as the fact that in this study legumes were cooked might determine the differences with Kotel'nikova's study.

In the animal food products the highest concentration of NAPEs was found in parmigiano cheese (397.7±19.04 µg/g dw), and the lowest concentration was in salami (3.71±0.14 µg/g dw). Only one study is present in the literature reporting NAPEs content in five species of bivalve mollusks (Sepe, De Petrocellis, Montanaro, Cimino & Di Marzo, 1998).

Data showed that wheat flour is the best source of NAEs (mainly LEA) among the plant origin products. Previous evidence of NAEs in vegetal products is limited to seeds and showed that cotton seeds had the highest concentration (1600 ng/g fresh weight) while pea had the lowest one (450 ng/g fresh weight) with LEA and PEA being the most abundant compounds (Chapman *et al.,* 1999).

In the present study the individual contribution of each compound to the total content of NAEs in beans was in the order LEA>PEA>OEA, in the chickpeas it was LEA>OEA>PEA and in the lentils LEA>OEA>PEA. The difference in the pattern composition of NAEs as well as the

concentration levels in legumes were generally in accordance with previous findings by Venables and colleagues (2005).

Similarly, the concentration of total NAEs in cocoa powder and a concentration level in the order OEA>LEA>PEA>AEA was partially in agreement with previous studies by Di Marzo *et al.* (1998) and di Tomaso, Beltramo & Piomelli (1996).

Regarding animal food products, the highest concentration of NAEs was found in the codfish (674.70±34.46 ng/g dw), while the lowest concentration was found in milk (46.63±1.26 ng/g dw). No previous papers about NAEs content in fishery products were published whereas some data on NAEs content only in human milk exist (Di Marzo *et al.*, 1998).

Data showed that among vegetable oils, hemp oil provided the highest amount of NAEs whereas among animal products tuna fish was the best food source of ECs. Within oils and fats, LEA concentration in EVOO was 53.9 ng/g dw and OEA concentration was 85.7 ng/g dw. Those concentrations were pretty lower than those reported by Bradshaw & Leishman (2016). Different analytical methods, olive cultivars and processing might explain the differences.

Regarding the ECs in animal products, tuna fish was the best source of 2-AG whereas AEA was not found. Interestingly, other NAEs were present in tuna fish with PEA being the most abundant followed by OEA and LEA and the same pattern was found by Sepe and co-workers (1998) in molluscs.

AEA was only found in meat and eggs, in fishery and in milk and derivative products, the highest concentration being in eggs, and the lowest in anchovies. Sepe and co-workers (1998) showed the presence of AEA in different species of molluscs in the range of 2.6-4.9 ng/g wet tissue weight.

In 2010 MD was recognized by UNESCO as an intangible heritage of humanity. Meta-analyses of observational studies and randomised controlled trials showed that the MD reduces the risk of chronic disease and mortality and it modulates the anthropometrical, metabolic and

inflammatory parameters thus aiding in reduction of glycaemia, insulin-resistance and metabolic syndrome as well as in prevention of some types of cancer (Dinu, Pagliai, Casini & Sofi, 2017).

On the other hand VD was associated with a better control of blood pressure and lipids as well as a lower risk of cardiovascular diseases and type 2 diabetes compared with an omnivorous diet (Craig & Mangels, 2009; Kwok, Umar, Myint, Mamas, & Loke, 2014).Very recently Sofi *et al.* (2018) showed that 3 months of a MD or VD in overweight subjects were both effective on reduction of body weight and fat mass but MD also decreased blood triglycerides while VD decreased blood LDL-cholesterol.

On the contrary, WD is well known to be positively associated with obesity and metabolic disease as well as with a modification of human gut microbiota towards endotoxin-producing bacteria (Zinöcker & Lindseth, 2018).

By using the food database of NAPEs, NAEs and ECs here developed, the content of those compounds from a MD, VD and WD was calculated. Data showed that MD and VD provide a similar intake per day of NAPEs and NAEs that is higher than WD. In particular, NAPEs and NAEs in MD and VD exceeded the 250 mg and the 300 µg per day while they were around two and four folds lower in WD, respectively. On the other hand ECs were at a concentration level around 200 µg per day in MD and WD while they were twenty folds less in VD. Moreover, we have found that an amount of NAPEs ranging between the 4% and 63% may act as NAEs precursors. That observation means that more than half of NAPEs present in foods may deliver NAEs when hydrolysed by NAPE-PLD once ingested.

All in all these data could suggest that even if NAPEs and NAEs are subjected to biotransformation processes due to degrading enzymes along the GIT, a certain amount of NAEs and NAPEs travel along the GIT inside the foods and may be released during the digestive processes and exert biological activity through receptors and nervous system

terminals lining in the GIT mucosa or may reach the colon and interact with microbiota. Looking at the diet content it is possible to think that food NAPEs, NAEs and ECs may be physiologically relevant. Indeed, *in vitro* studies, showed that NAEs have agonist activity on GPR119, with an EC_{50} that was found between 65 µg/L and 1.0 mg/L for OEA, 180 µg/L for LEA and 250 µg/L for PEA (Hansen *et al.*, 2011; Overton *et al.*, 2006). Furthermore, Fu *et al.*, (2003) showed that OEA can activate PPAR-α with an EC_{50} of 39 µg/L.

Thus, NAPEs and NAEs travelling in the GIT upon food consumption may contribute to nutrient metabolism, to the regulation of food intake and body weight as well as to the gut permeability and GIT motility through the nutrient sensing in the gut. Future studies should be performed to demonstrate this hypothesis.

2.5 Conclusions

In conclusion the present study provide a database of NAPEs, NAEs and ECs in food products highly consumed worldwide. Data demonstrated that the concentrations of NAPEs and NAEs are higher in the products of vegetal origin than in animal ones, whereas the ECs (AEA and 2-AG) are almost exclusively present in the products of animal origin.

Furthermore, it was shown that dietary patterns inspired on principles of a MD, a VD and a WD provide different daily intakes of NAPEs, NAEs and ECs that may be in a sufficient amount to activate receptors along the GIT. Further studies are warranted to evaluate the contribution of those compounds on health benefits associated with MD and VD.

2.6 Abbreviations

GIT: gastrointestinal tract; GPRs: G-protein-coupled receptors; NAPEs: N-acylphosphatidylethanolamines; NAEs: N-acylethanolamines; ECs: endocannabinoids; NAPE-PLD: N-acyl-phosphatidylethanolamine-specific phospholipase D; NAAA: N-acylethanolamine-hydrolyzing acid amidase; FAAH: fatty acids amide hydrolases; PLD-β: phospholipase-β; CB1: cannabinoid receptor-1; GLP-1: glucagone-like-peptide-1; TRPV1: transient receptor potential vanilloid 1; PPAR-α: peroxisome proliferator-activated receptor-α; LC-HRMS: liquid chromatography–high resolution mass spectrometry; AEAd8: anandamide-d8; LEA: linoleoylethanolamide; PEA: palmitoylethanolamide; OEA: oleoylethanolamide; AEA: anandamide; 2-AG: 2-arachidonylglycerol; MD: Mediterranean diet; VD: Vegetarian diet; WD: Western diet; Bev: Beverages.

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Chapter 3 . Salivary concentration of N-acylethanolamines upon food mastication and after meal consumption: influence of food dietary fiber

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Abstract

The primary objective of this study was to evaluate whether the amount and type of food dietary fiber influenced salivary concentrations of N-acylethanolamines (NAEs) and glucose upon food mastication and in the post-prandial phase.

Three types of biscuits enriched with 3% barley β-glucan (βGB) or whole-wheat bran (WWBB) or without dietary fiber (control, CB) were developed. A crossover randomised human study with 18 healthy and fasting participants collecting saliva samples in a resting condition, upon mastication of parafilm and one of the three biscuits was carried out. Subsequently, the amount of biscuits consumed in an *ad-libitum* breakfast was measured and post-prandial saliva samples, blood glucose, appetite, and food liking were collected over the following two hours. Salivary concentration of oleoylethanolamide (OEA) and linoleoylethanolamide (LEA) significantly increased upon all biscuits mastication compared to non-food conditions, OEA increasing more (~138 folds) than LEA (~7 folds). Subjects consumed always 75g of biscuits at breakfast. Salivary OEA peaked at 15min and returned to baseline concentration at 60 min after consumption of all types of biscuits whereas LEA peaked only after WWBB. Fifteen minutes after βGB consumption all NAEs were significantly lower than after WWBB. No difference of biscuit type on post prandial blood glucose was recorded.

Data demonstrated that NAEs were released in saliva during biscuit mastication, independently from dietary fiber composition. The type of dietary fiber could influence the persistence of NAEs in saliva over 30 min after consumption. Future studies will clarify the mechanisms behind these findings and the role of salivary NAEs in food liking and appetite cues after food consumption.

3.1 Introduction

Satiation is the satisfaction of appetite developing during eating and eventually resulting in the termination of eating (Slavin & Green, 2007; Suzuki, Jayasena, & Bloom, 2012). Satiation is influenced by a series of fine physiological factors comprised in the cephalic phase response to eating and including the action of homeostatic and tonic signals from the gastro-intestinal tract and adipose tissue as well as the reward signals (Hansen, 2014).

Food composition and structure can modulate satiation by influencing chewing time and individual hedonic value of the food (Blundell & Halford, 1994; De Graaf, Blom, Smeets, Stafleu, & Hendriks, 2004). Among food constituents, fats and sugars are positively associated with food palatability and overeating (D'Addario *et al.,* 2014; Ifland *et al.*, 2009) whereas dietary fibers can increase satiation through their bulking and textural properties thus possibly beneficing the control of energy balance (Howarth, Saltzman, & Roberts, 2001; Slavin & Green, 2007). This is well documented in short-term studies where reduced appetite feelings and energy intake at the meal containing dietary fiber and to that following dietary fiber consumption were associated with the amount of fiber consumed and, sometimes, to its viscosity (Slavin & Green, 2007).

However, the reduced palatability of dietary fiber rich foods is often an issue for long-term consumption of these products thus failing the possibility to work for weight management (Alfieri *et al.,* 1995; Hess, Birkett, Thomas, & Slavin, 2011; Howarth *et al.*, 2001). A better understanding of the physiological factors underpinning sensory mechanisms may help to develop new foods being both satiating and palatable.

In this context we have recently demonstrated that tasting of a food (before swallowing) influences plasma levels of the endocannabinoids (ECs) - 2-acylglycerol (2-AG) and arachidonylethanolamide (AEA) -, of the congeners NAEs - OEA, linoleoylethanolamide (LEA) and palmitoylethanolamide (PEA) – and of gut peptides in a manner that is dependent

from the individual liking of the tasted food (Mennella, Ferracane, Zucco, Fogliano, & Vitaglione, 2015). This finding was in line with the well-known role of gustatory system on the cephalic phase of eating and suggested that some mediators could act during mastication and elicit the plasma response.

Post-prandial variation of plasma ECs and NAEs were also reported in the literature with different effect on appetite: ECs being mainly associated with an increase and NAEs with a decrease of appetite (Hansen, 2014; Nielsen, Petersen, Astrup, & Hansen, 2004). In addition, intestinal levels of NAEs and AEA were correlated with the levels of the constituting fatty acids (Hansen, 2014).

Matias *et al.* (2012) demonstrated that ECs and NAEs are present in human saliva at a concentration dependent from people nutritional status: higher is individual body mass index, higher is the salivary ECs and NAEs level. Moreover they showed no variation of salivary ECs and NAEs concentration one hour after meal consumption.

In this study the hypothesis that NAEs could be formed in the mouth during food mastication and might influence satiation upon eating biscuits with different content and types of dietary fiber was tested. Moreover, the appetite sensations and liking of the different biscuits over two hours after food consumption was monitored together with salivary NAEs and blood glucose. To this purpose, biscuits enriched with 3% gel-forming barley β-glucan (βGB), 3% insoluble whole wheat bran (WWBB) and without dietary fiber (CB) were developed and a crossover randomised design protocol was performed in healthy normal weight subjects.

3.2 Materials and methods

3.2.1 Foods

Three types of biscuits containing 3.0% barley β-glucan (βGB), 3.0% whole wheat bran (WWBB), or without dietary fiber (CB) were developed using a traditional recipe for biscuits. All the biscuits were prepared with the following ingredients purchased by local supermarket:

flour (Divella, Bari, Italy), sugar (Eridania, Bologna, Italy), margarine (Vallé, Milan, Italy), yeast (PaneAngeli, Brescia, Italy). To produce βGB, 3% wheat flour were replaced by a barley β-glucan concentrate (Glucagel^{TM}, containing more than 77.5% dietary fiber) purchased from DKSH (Miribel Cedex, France); whereas for WWBB, a whole-wheat bran concentrate (VITACEL, containing 97% dietary fiber) purchased from ITALI (Reggio Emilia, Italy) was used.

The dough was prepared and after layering, circular biscuits with a diameter of 3.5 cm were formed and baked at 190°C for 15 minutes.

3.2.2 Subjects selection

The recruitment was performed among the students of the Department of Agricultural Sciences of University of Naples, who were interviewed about their medical status, subjective eating habits and food preferences (100 recipes, scores from 1 to 9). The selected subjects were healthy, they were not undergoing any medication or drug therapy, they usually had breakfast, they were not on a restrictive diet and had a normal eating behavior as assessed by the TFEQ (Stunkard & Messick, 1985).

Eligible subjects signed an informed written consent before entering this study. They were advised not to vary their physical activity during all the period of the study, always avoiding it the day before the experimental days.

3.2.3 Study design

The study design and protocol were approved by the Ethics Committee of University of Naples. The protocol had a crossover, single blind, randomised design. It was characterised by three treatments per each subject that were conducted on separate days with a 1-week washout period from each other (**Figure 3.1**). Each subject participated to three tests and to a training session that was performed two days before the first test and was finalized to train subjects on detailed procedures to collect saliva samples, to rate their appetite feelings and food liking on VAS

questionnaires and to set chewing time and rate for the mastication protocol. The subjects were instructed to consume a standardized dinner in the evening before the experimental days within the 22:00 h. On the experimental days fasting subjects reached the nutritional laboratory of the Department of Agricultural sciences at 08:30 h and after 10 minutes of rest, baseline blood glucose was measured and they were asked to collect baseline non-stimulated and mechanically stimulated (by parafilm and by food) saliva samples. Immediately after, participants were offered a breakfast comprising 150 g of the same type of biscuit they had just masticated and were asked to eat biscuits until they felt satisfied within 15 min. A glass of water (125 mL) was also offered. The remaining biscuits in subject plates were weighted and energy intake consumed was calculated. After breakfast and 15, 30, 60 and 120 min after breakfast, subjects were asked to collect saliva for 5 min, and to rate appetite feelings and actual liking of the biscuit on VAS questionnaires. Blood glucose at the same time points was also measured.

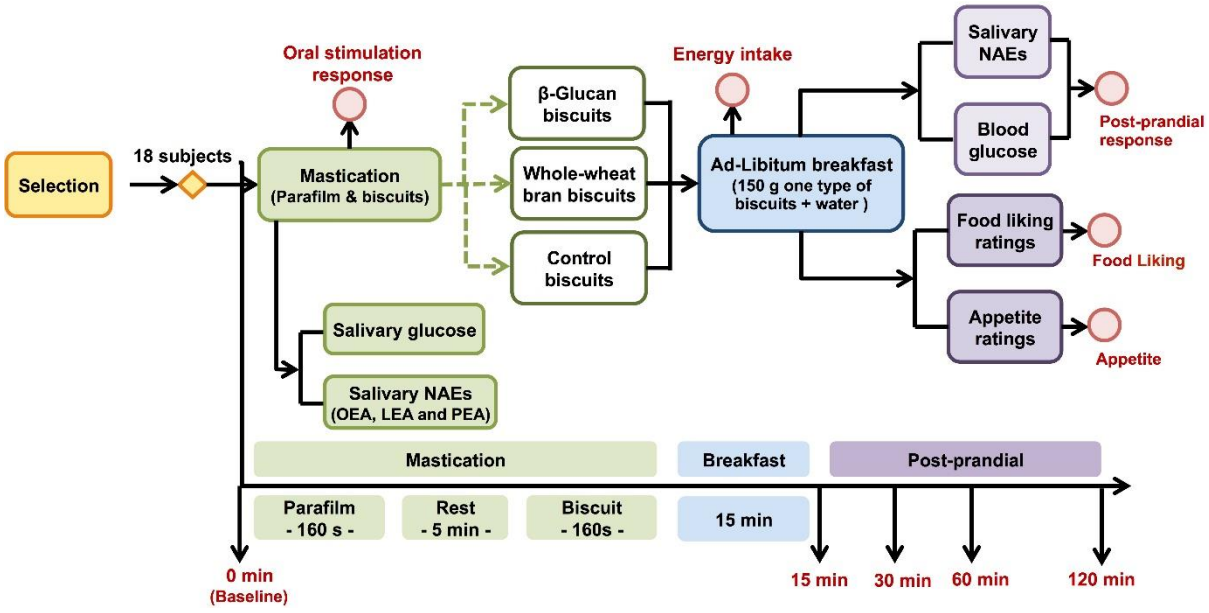

Figure 3.1. Study design

3.2.4 Saliva sample collection and preparation for analysis

Resting drooling was used to collect non-stimulated saliva from the oral cavity. Participants were asked to sit comfortably in an upright position, to have their heads down slightly to pool saliva in the mouth and to let saliva fall into a pre-labeled sterile container for 5 minutes.

To collect mechanically stimulated saliva samples, the participants were asked to chew onto a piece of inert and tasteless paraffin film (0.29 g; PARAFILM purchased by Sigma-Aldrich, St Louis, USA) for 160s at a speed of one mastication per second (every 40s saliva were collected as performed with real biscuits, rhythm was given by a metronome) and then expectorate only the saliva into a pre-labeled sterile container.

Once collected both types of sample were immediately placed on ice to minimize degradation of components until further processing. Saliva samples were aliquoted in pre-labeled Eppendorf tubes (2 mL) and frozen (-40°C) for storage until analysis. Saliva was separated from the bolus immediately after collection by centrifuging samples at 4000 rpm/min per 10 min at 4 °C. Then the supernatant saliva was collected and treated as above.

3.2.5 Salivary N-acylethanolamines measurement

NAEs (OEA, LEA, and PEA) were simultaneously quantified in saliva samples prepared as described above by LC/MS/MS. Extractions were performed using the solid-phase method. Saliva samples (1 mL) were centrifuged at 16000×g for 5 min at 4°C. The supernatants were collected and 1 mL were separated in another tube and spiked with 200 μg/mL of AEA-d8 internal standards. Oasis HLB 1 cc, 30 mg cartridges (Waters) were preconditioned using 1 mL methanol and 1 mL H_2O under a vacuum manifold. Samples were introduced onto the cartridges and drawn under gentle vacuum at a flow rate of approximately 1 mL/min. The cartridges were washed with 1 mL 40% aqueous methanol and NAEs were eluted in 1 mL acetonitrile. The eluents were dried under a stream of nitrogen before reconstitution in acetonitrile/water (50:50 v/v) (100 μL) for HPLC/MS/MS analysis. To estimate the extraction

efficiency, peak areas obtained for deuterated internal standards extracted from saliva were compared with non-extracted controls in 100 µL acetonitrile:water (50 : 50) (Lam, Marczylo, & Konje, 2010).

The analysis was performed using an HPLC apparatus equipped with two micropumps Perkin-Elmer series 200 (Norwalk, CT, USA). A Synergi Max RP 80 column, 50 × 2.1 mm (Phenomenex, USA) was used with flow rate set to 0.2 mL/min. Injection volume was 20 µL, as well as mobile phases and the gradient program were the same as reported by Lam *et al.* (2010). MS/MS analyses were performed by an API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Canada) equipped with a TurboIonSpray source. The acquisition was carried out in Multiple Reaction Monitoring (MRM) in positive ion mode for each compound. Data acquisition and processing were performed using Analyst software v. 1.4. Acquisition parameters (Mennella, Savarese, Ferracane, Sacchi, & Vitaglione, 2015).

3.2.6 Appetite and food liking questionnaires

In the appetite VAS questionnaires, three main questions (‘How satiated do you feel?’, ‘How full do you feel?’ and ‘How hungry do you feel?’) were asked and subjects indicated “satiety”, “fullness” and “hunger” on 100 mm VAS, anchored to a minimum on the left as “not at all” and a maximum on the right as “extremely”, the point corresponding to their sensations (Green, Delargy, Joanes, & Blundell, 1997).

Similarly, in the food liking VAS subjects were asked to evaluate the hedonic value for sweetness, salty, fatty perception, consistency and overall palatability of the biscuit they had for breakfast (‘How do you like for sweetness?’, ‘How do you like for saltiness?, ‘How do you like for fatness?’, ‘How do you like for consistency?’ and ‘How do you like for overall palatability?’).

3.2.7 Blood and salivary glucose

Blood glucose by finger pricking and using a bedside glucometer (One Touch Ultra Easy; LifeScan Inc., Milpitas, CA) was measured. Accuracy of the glucometer has been evaluated by the manufacturer using least-squares linear regression analysis and found to be 97% "clinically accurate" when compared with reference (YSI2700) results.

Salivary sugar concentration was measured using the same glucometer as previously reported (Neyraud, Prinz, & Dransfield, 2003). One sensor strip for each saliva sample (10 μL) was used.

3.2.8 Statistical analysis

Results were expressed as means ± SEM. Statistical analyses were performed with the statistical package SPSS for Windows (version 16). The biochemical analyses were analysed and expressed as the absolute variations from the baseline to eliminate the possible effects of inter-subject fasting variability. The total AUC for hunger, fullness and satiety (from baseline over 2 h from breakfast consumption) were estimated using the linear trapezoidal rule.

Kolmogorov-Smirnov and Shapiro tests as well as Levene's test were used to evaluate the normality of distribution and the homogeneity of variance of all monitored variables, respectively. Logarithmic transformation was applied to non normally distributed data. Differences of subjective appetite and food liking recorded after the consumption of the three types of biscuit and the response curves of salivary glucose and NAEs were tested by ANOVA for repeated measures in combination with Bonferroni test; $p < 0.05$ was considered statistically significant; $p < 0.1$ was considered as a trend.

Pearson correlation coefficients were calculated to assess bivariate associations between data sets ($p<0.05$ was considered significant).

3.3 Results

3.3.1 Foods

The nutritional compositions of the three types of biscuit are reported in **Table 1**.

They differed only for the content and type of dietary fiber being the soluble and gel-forming barley β-glucan or the insoluble whole-wheat bran.

Table 3.1: Nutritional composition of 100 g biscuits. CB: control biscuits; βGB: β-glucan biscuits; WWBB: whole wheat bran biscuits.

	CB	**βGB**	**WWBB**
Energy (kcal)	419	395	396
Protein (g)	6.5	6.0	6.0
Fat (g)	8.5	8.3	8.2
Carbohydrate (g)	79	74	74
β-glucan (g)	0	3.0	0
Whole-wheat bran (g)	0	0	3.0

3.3.2 Subjects

Eighteen volunteers (7 M/11 F), with mean age of 27 ± 1 years (range 20 – 37) and with mean body mass index (BMI) of 23.3 ± 0.58 kg/m^2 (range 19.5-28.0 kg/m^2), participated in this study. Among them, 5 subjects were overweight with BMI ≥ 25 kg/m^2.

3.3.3 Salivary NAEs upon mastication

The concentration of NAEs in saliva samples collected upon mastication is reported in **Figure 3.2**.

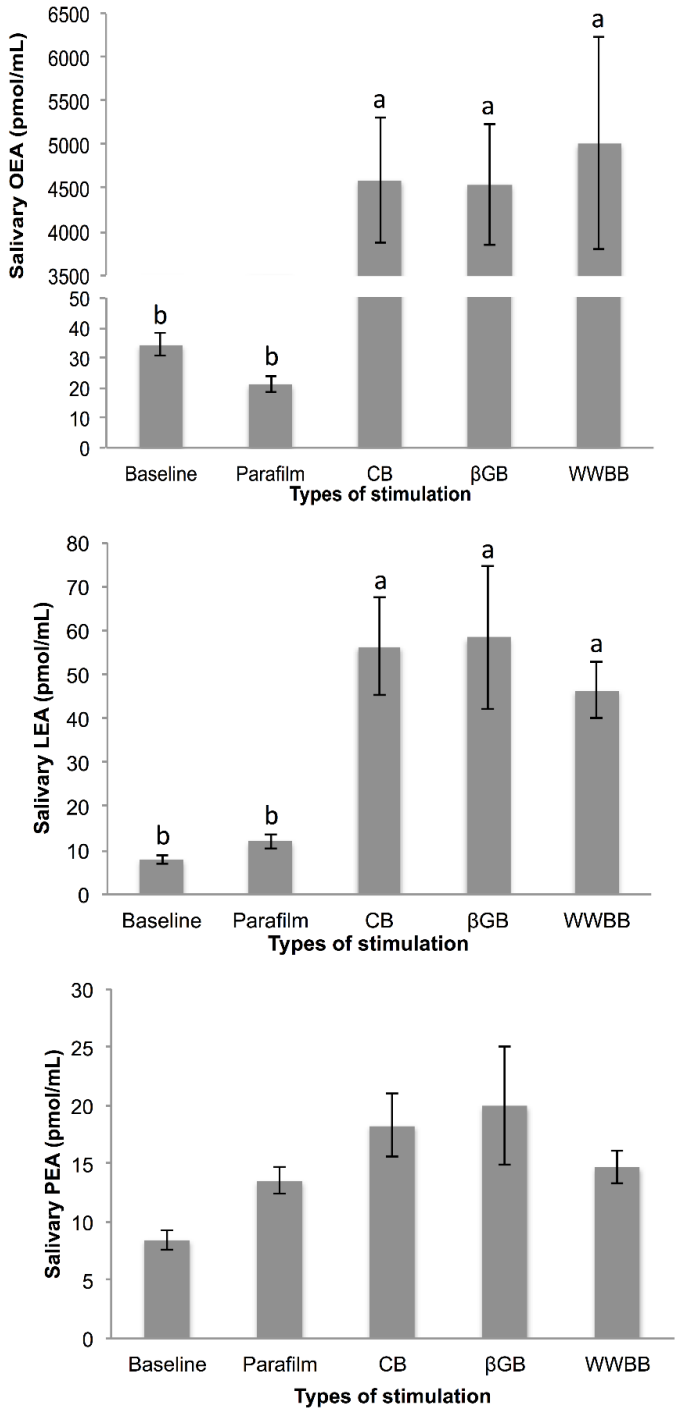

Figure 3.2. Concentration of salivary NAEs from fasting subjects at baseline (resting condition) and upon mastication of parafilm or control biscuits (CB), barley β-glucan-enriched biscuits (βGB) or whole wheat bran–enriched biscuits (WWBB). Different letters on the bars indicate $p<0.05$

The concentrations of OEA and LEA in saliva samples collected upon food mastication were significantly higher than in non-stimulated (baseline) and parafilm-stimulated saliva samples, whereas no difference between the last two was found. No differences nor trends were found for salivary PEA between samples.

3.3.4 Salivary glucose upon mastication

Concentration of salivary glucose in baseline and non-stimulated saliva samples was below 20 mg/dL. It was significantly different from glucose concentration of food-stimulated saliva being about 303.7 mg/dL. No significant difference between salivary glucose released upon different food mastication was found because it was 374.3 ± 44.7 mg/dL upon βGB, 282.3 ± 20.9 mg/dL upon WWBB and 261.9 ± 31.1 mg/dL upon CB ($p > 0.05$).

Interestingly, significant positive correlations between the concentrations of OEA and glucose in saliva from chewed CB (R^2=0.682; p=0.015) and WWBB (R^2=0.742; p=0.002) and a tendency towards a negative correlation in saliva from βGB (R^2=-0.403; p=0.154) were found.

3.3.5 Food intake

No significant different food intake was recorded upon the three food conditions as energy intake at breakfast was 282 ± 40 kcal, 313 ± 33 kcal and 307 ± 40 kcal when subjects were offered βGB, CB and WWBB, respectively.

3.3.6 Postprandial salivary NAEs

The variation from baseline of salivary NAEs concentration over 2 hours following biscuit consumption and the AUC of each NAE are reported in **Figure 3.3**.

Salivary concentration of all NAEs, except PEA following βGB, peaked at 15 minutes post-breakfast compared to baseline, this increase being significant for OEA after all types of biscuits and for LEA only after WWBB. From 15 min to 60 min, a general trend towards reduction of mean concentrations following consumption of all three types of biscuit was

found. At 60 min, concentrations of LEA and OEA returned to the baseline values (**Figure 3.3**).

Fifteen minutes after the consumption of βGB lower concentrations of OEA ($p = 0.017$), LEA ($p = 0.001$) and PEA ($p = 0.038$) than after WWBB were observed. The AUCs of NAEs did not significantly change after the consumption of the different biscuits (although the mean magnitude was always in the order WWBB > CB > βGB) (**Figure 3.3**).

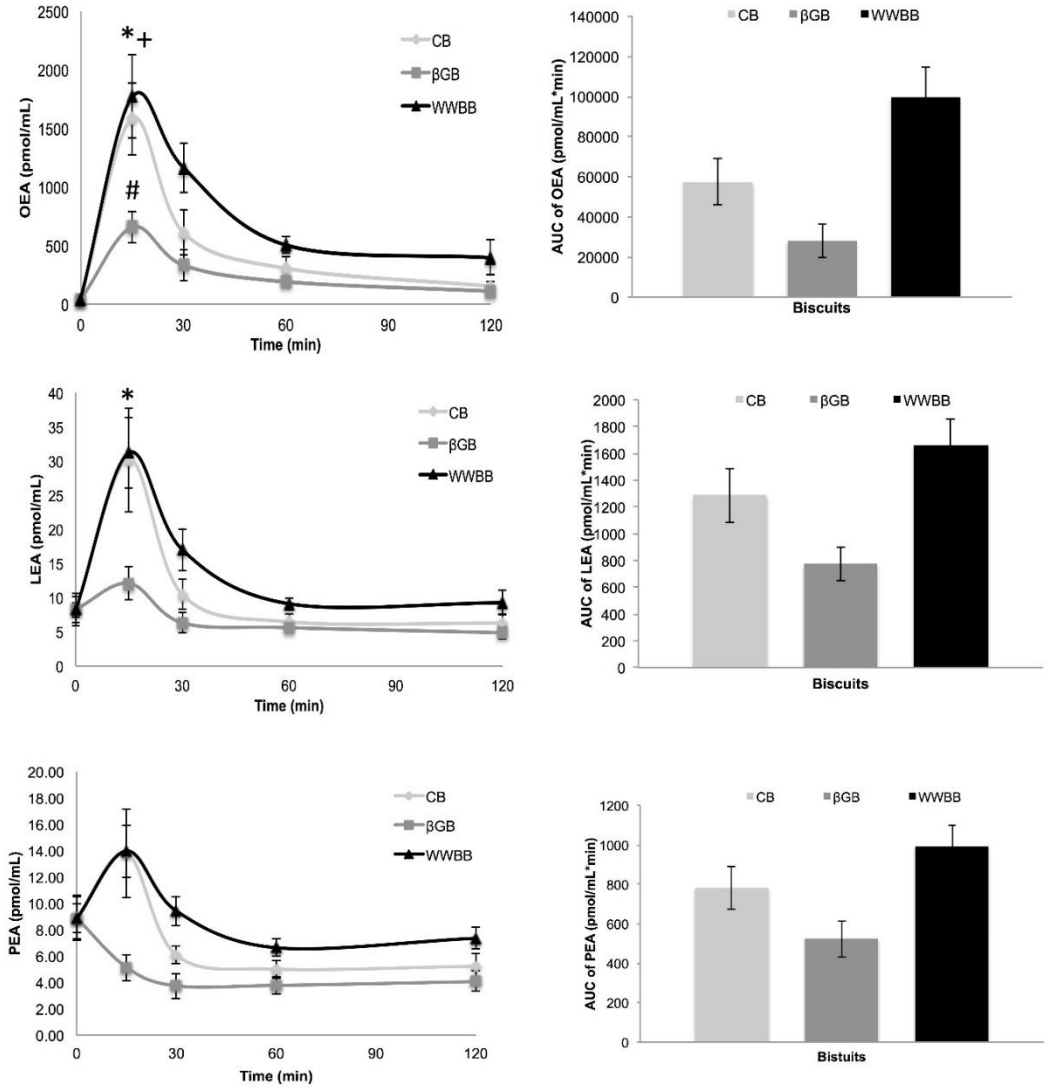

Figure 3.3. Concentration-time curves and AUC $_{(0\text{-}120)}$ of postprandial salivary NAEs. $^{*, +, \#}p < 0.05$ *vs* baseline.

3.3.7 Postprandial appetite and food liking

Table 3.2 shows mean appetite and food liking scores recorded over the 2 hours post-breakfast. Data showed no effect of the types of biscuit on postprandial appetite sensations as well as on liking of biscuits for sweetness, salty, fat perception, consistency and overall palatability.

Interestingly, fullness and satiety sensations positively correlated with salivary OEA concentration at 30 min post breakfast ($R=0.424$; $p=0.031$ and $R = 0.451$; $p=0.021$, respectively).

In addition, an inverse correlation between liking of biscuits for sweetness and salivary OEA concentration at 15 min ($R = -0.55$; $p = 0.002$) was found.

Table 3.2: Appetite and food liking scores (means ± SEM) collected over 120 min after consumption of the three types of biscuits (CB: control biscuits; βGB: β-glucan biscuits; WWBB: whole wheat bran biscuits).

	Baseline			15 min			30 min			60 min			120 min		
Appetite	**CB**	**βGB**	**WWBB**	**CB**	**βGB**	**WWBB**	**CB**	**βGB**	**WWBB**	**CB**	**βGB**	**WWBB**	**CB**	**βGB**	**WWBB**
Hunger	70±7	57±6	52±8	25±4	22±5	17±3	38±7	29±4	24±4	45±6	34±5	28±4	60±7	64±6	57±5
Fullness	24±7	29±7	36±8	53±7	66±6	59±8	60±6	54±6	57±7	51±6	48±6	48±6	30±6	28±6	30±5
Satiety	29±8	32±7	28±7	50±6	66±5	49±7	51±7	56±6	50±7	49±6	46±5	45±7	32±5	27±6	29±6
Liking															
Sweetness	49±6	44±7	48±5	44±7	42±6	38±6	43±6	41±6	34±5	40±7	38±6	33±5	38±7	35±6	33±6
Saltiness	31±5	26±6	37±8	24±5	26±6	28±6	26±5	26±5	30±6	25±5	23±4	32±6	30±6	22±4	31±6
Fatness	40±7	36±7	34±8	36±7	34±7	29±6	32±7	26±6	33±7	36±7	28±6	29±7	34±7	26±5	30±7
Consistency	33±7	30±6	28±6	24±5	26±5	24±5	27±6	24±5	28±6	24±5	30±5	28±6	27±5	31±5	24±5
Overall palatability	39±6	43±6	39±6	37±7	33±5	37±5	40±6	36±4	39±6	38±6	39±5	39±6	40±7	46±6	33±4

3.3.8 Blood glucose response

Blood glucose concentration peaked at 15 min after βGB and CB, and at 30 min after WWBB consumption (**Figure 3.4**). In particular, considering concentration values measured at peak point, a mean concentration of 133 ± 8 mg/dL *vs* a mean baseline value of 93 ± 5 mg/dL was found when βGB was consumed, while concentrations of 136 ± 6 mg/dL *vs* 93 ± 3 mg/dL, and 143 ± 6 mg/dL vs 93 ± 2 mg/dL, respectively, were elicited by WWBB and CB.

The AUC of blood glucose was 13835 ± 546 mg min/dL, 14231 ± 604 mg min/dL and 13824 ± 372 mg min/dL after βGB, WWBB and CB, respectively and no significant difference among the types of biscuit was found (glycaemia × treatment interaction; $p > 0.05$). Pearson's analysis showed a negative correlation between salivary OEA concentration and blood glucose at 30 min (-0.480, $p = 0.044$) and a tendency at 15 min (-0.456, $p = 0.066$).

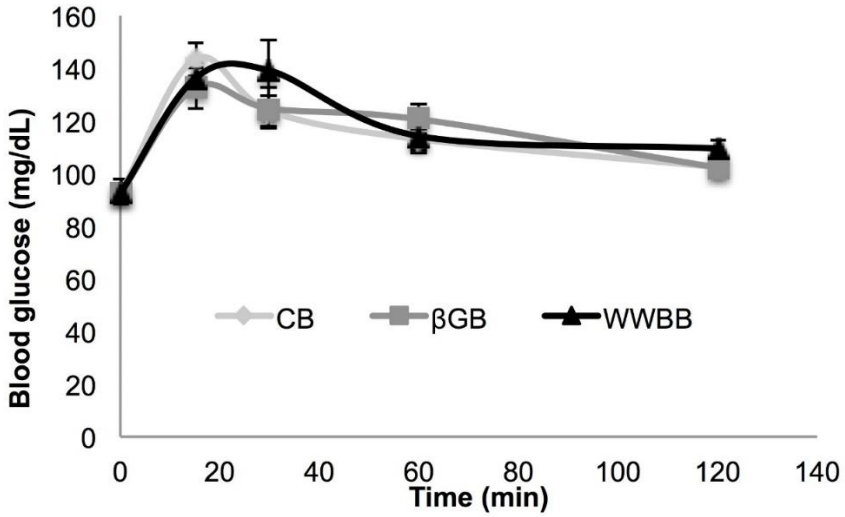

Figure 3.4. Concentrations of blood glucose (Means ± SEM) over 2h following consumption of CB, βGB or WWBB.

3.4. Discussion

For the first time in this study, the concentration of NAEs was measured in stimulated saliva samples and it was demonstrated that mastication itself did not cause variation of these compounds compared to baseline resting saliva in healthy normal weight subjects. Indeed the salivary concentrations of all NAEs meanly increased in saliva only upon food mastication, independently from the type of biscuit, compared to non-food (parafilm) condition mastication. In particular data showed that only OEA and LEA significantly increased upon all biscuits mastication, OEA increasing more (~138 folds) than LEA (~7 folds) and PEA (~2 folds).

These findings strongly suggested that NAEs directly derived from the food. So, in attempt to demonstrate this hypothesis biscuits were analysed for NAEs content and data showed that all NAEs were present in the 3 types of biscuit with mean concentrations of OEA of 14.0 nmol/g, LEA 1.2 nmol/g and PEA 0.1 nmol/g. Moreover OEA and LEA were in CB at a double concentration compared to βGB and WWBB whereas PEA was at a similar concentration in all the biscuits.

The presence of NAEs in the biscuits was in line with previous literature reporting that NAEs can be endogenously formed in the intestine (Artmann *et al.*, 2008; Hansen & Diep, 2009; Hansen, 2014; Petersen *et al.*, 2006; Sarro-Ramirez, 2013), brain (Artmann *et al.*, 2008; Hansen, 2014; Simon & Cravatt, 2010; Tsuboi *et al.*, 2013), liver (Artmann *et al.*, 2008) and other mammalian tissues (Hansen & Diep, 2009; Hansen, 2014), and they are also largely diffused, together with their natural precursors NAPEs, in both plant and animal kingdoms being found in plants, yeast, slime molds, insects, and mammals (Coulon, Faure, Salmon, Wattelet, & Bessoule, 2012; Fezza *et al.*, 2003; Hansen & Diep, 2009; Hansen, 2014; Hayes, Stupak, Li, & Cox, 2013; Muccioli, Sia, Muchowski, & Stella, 2009; Bomstein, 1965). However, we could not exclude that some NAEs could also come from the biotransformation of their NAPE precursors during mastication upon the action in the mouth of NAPE-PLD

(Matias *et al.*, 2012). Future studies should verify this hypothesis.

The lacking differences of salivary OEA and LEA upon mastication of different types of biscuits, although a double concentration of these NAEs was found in CB compared to βGB and WWBB, suggested that the oral factors and processes that might underpin the release of NAEs from the biscuits and their persistence in the mouth could be majorly influenced from the chewing time and speed (that was fixed in the present study) compared to other factors such as the amplitude of muscular activity or non-chewing processes such as the tongue movement (Yven *et al.*, 2012). In this respect, the fixed rate of bites and time during mastication protocol could be considered as a limitation of the study because it did not allow us to evaluate whether normal chewing behavior of subjects could led to salivary concentrations of NAEs more closely mirroring their content in the biscuits.

A further study specifically focused on the mechanisms and individual factors behind the formation and fate of salivary NAEs with freely chewing subjects should be performed.

However, the chewing speed and time in the mastication protocol was established on the basis of a preliminary experiment conducted in the training session (*data not shown*) where participants' behavior and possibility to chew the biscuits and spit the chewed food without swallowing were evaluated. In other words, the mastication protocol was tailored on the participants and the best conditions to collect all chewed food avoiding any loss due to unwanted swallowing were used.

So, it was assumed that individual way of eating biscuits during the protocol to assess satiation at breakfast was similar to that adopted during mastication protocol and caused similar concentrations of NAEs in the food boluses that were ingested. Data showed that no effects of the different types of biscuits on satiation was present. This feature was in line with the similar palatability showed by the three types of biscuit and could be addressed to the level of dietary fiber enrichment of the biscuits thus indicating that a 3% increase of a viscous or insoluble

dietary fiber in a bakery food product did not create the physiological conditions responsible for a sooner meal termination in normal weight and fasting subjects (Howarth *et al.,* 2001; Burton-Freeman, 2000). Interestingly, the types of dietary fibers in the biscuits influenced immediate post-prandial salivary concentration of NAEs.

To the best of our knowledge no one before us studied saliva composition in the immediate post-prandial phase. Matias and co-workers (2012) collected saliva after 1h from meal consumption and demonstrated that ECs and NAEs (AEA, 2-AG, OEA and PEA) did not change in the post-prandial compared to the pre-prandial phase. On the contrary, data of the present study clearly showed that in the immediate post-prandial phase NAEs formed from the food during mastication was still present in the mouth and did not reduce equally after eating the different types of biscuits. In particular, a faster and dramatic decrease of all salivary NAEs after βGB consumption than CB or WWBB was observed. This finding might depend from the physical properties of the βGB bolus. In fact, it could be hypothesised that the βGB bolus, thanks to its content of barley β-glucan having a gel-forming capacity and binding ability (Hughes & Swanson, 1989; Mudgil & Barak, 2013), could better entrap compounds and let the mouth free from NAEs upon swallowing determining a better effect of mouth cleaning compared to WWBB and CB. This hypothesis well fits with the food origin of salivary NAEs and it is in accordance with the recommendation to study saliva composition by avoiding eating just before the collection because residues of foods present in the mouth can influence results (Kaufman & Lamster, 2002; Yoshizawa *et al.,* 2013). However, it should be noticed that together with biscuits, participants drank a glass of water that caused a rinsing of their mouths as evidenced by the fact that the post-prandial saliva samples appeared free from food residues at all the time points. Another hypothesis might be that even small β-glucan residue in the mouth after βGB swallowing might differently influence the activity of oral NAPE-PLD or FAAH, the enzyme degrading NAEs (Matias *et al.,* 2012), compared to WWBB and CB, and

this effect could be evident in presence of few food residue that might remain in the mouths even after drinking.

Further studies should test these hypotheses and clarify the mechanisms behind the present findings.

Worth of notice were the positive associations found between post-prandial salivary OEA with fullness and satiety sensations as well as the inverse associations with liking of biscuits for sweetness. From these findings it could be speculated that the increase of OEA in saliva during eating and in the immediate post-prandial phase might have modulated anandamide (AEA) concentration in saliva. Indeed OEA was shown to reduce AEA degradation by substrate competition for FAAH (Maurelli *et al.,* 1995; Jonsson *et al.,* 2001). Survival of AEA could in turn selectively enhance sweet taste, and consequent liking of biscuits for sweetness, as previously demonstrated in rats (Yoshida *et al.,* 2010). In general those associations also suggested that salivary OEA may be implicated in the sensory mechanisms occurring in the post-ingestive phase and influencing the flavor-consequence learning that underpin individual food preference and choice (Yeomans, 2006). In this study fasting subjects were asked to eat the biscuits until they felt comfortably satisfied and no other food was available. However, in a normal life condition it may also be possible that the salivary OEA may have a role on the amount or type of foods people can eat when exposed to different food cues, as it happens for example in a buffet-style meal.

Appropriate studies should be performed to test these hypotheses.

Finally, in this study no effect of βGB consumption on the postprandial blood glucose was found compared to WWBB and CB. The amount of biscuits eaten by subjects (75g on average) could explain this result. In fact considering the amount of biscuits consumed and the 3% enrichment with the dietary fiber, βGB provided 2g of barley β-glucans corresponding to a 3.9% of the dietary fiber by available carbohydrates. In these conditions the lacking effect of

βGB in modulation of blood glucose was in line with a previous study from our research group showing that a meal providing 3g of barley β-glucans (2.3% by available carbohydrates) did not influence blood glucose response compared to the control meal (Barone Lumaga, Azzali, Fogliano, Scalfi, & Vitaglione, 2012). On the contrary meals providing 3g of barley β-glucans (5.2% by available carbohydrates) (Vitaglione, Lumaga, Stanzione, Scalfi, & Fogliano, 2009) or 6.2g - 7.3g β-glucans (12.4% or 14.6% by available carbohydrates) (Jenkins, Jenkins, Zdravkovic, Würsch, & Vuksan, 2002) showed the hypoglycaemic effect of the barley β-glucans-enriched meal compared to the control.

3.5. Conclusions

Data of this study clearly demonstrated that no salivary response of NAEs is elicited by mastication itself in healthy and normal weight subjects but NAEs can be originated in the mouth from foods upon mastication. Enrichment of biscuits with a 3% dietary fiber did not influence NAEs released in the mouth upon mastication as well as the biscuits-induced satiation in fasting subjects. However, the types of dietary fiber (gel-forming or insoluble) influenced the concentration of these compounds in the mouth within the first hour from food consumption. The associations found between salivary OEA concentration in the immediate post-prandial phase and actual liking of the biscuits consumed as well as fullness and satiety sensations suggested some role of OEA in the sensory mechanisms underpinning food behavior and choice. Further studies are warranted to clarify the mechanisms and the individual factors underpinning NAEs release in the mouth upon eating different types of foods from different types of subjects.

3.6 Abbreviations

βGB: β-glucan biscuits; WWBB: Whole-wheat bran biscuits; CT: Control biscuits; NAEs: N-acylethanolamines; OEA: Oleoylethanolamide; LEA: Linoleoylethanolamide; PEA: Palmitoylethanolamide; ECs: Endocannabinoids; 2-AG: 2-acylglycerol; AEA: Arachidonylethanolamide; TFEQ: Three Factor Eating Questionnaire; LC/MS/MS: Liquid chromatography tandem mass spectrometry; AUC: Area under the curve;

ANOVA: Analysis of variance; NAPEs: N-acylphosphatidylethanolamines;

NAPE-PLD: N-acylphosphatidylethanolamine-phospholipase D.

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Chapter 4. Salivary concentrations of NAPEs and NAEs upon mastication: influence of the individual nutritional status and fat types in the food

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Abstract

N-acylphosphatidylethanolamines (NAPEs) and N-acylethanolamines (NAEs) are bioactive compounds present in human saliva at concentrations depending from individual nutritional status. Recent evidence shows that their concentration in saliva increase upon food mastication and persist depending from the food matrix.

The objective of this study was to evaluate the influence of individual nutritional status on the salivary NAPEs/NAEs upon mastication of biscuits prepared with different fats. Three types of biscuits were prepared with 8% of extravirgin olive oil (EVOB), 8% of palm oil (PALMB) and without added fat (CONB) and a cross-over modified sham feeding protocol was carried out in 25 (12 normalweight, NW, and 13 obese, OB) subjects. Unstimulated (US) and stimulated (by a parafilm piece, SS, and by the three biscuits) saliva samples were collected and analysed by LC-HRMS to assess NAPEs and NAEs concentrations.

Salivary NAPEs concentration was higher in US from OB than from NW (0.28±0.05 µg/mL *vs* 0.12±0.02µg/mL), whereas NAEs concentration in both US and SS was higher in OB than in NW (10.82±1.41 ng/mL *vs* 4.82±0.19 ng/mL for US; 6.15±0.79 ng/mL *vs* 3.79±0.43 ng/mL for SS).

In comparison with SS, concentrations of NAPEs and NAEs (but not PEA) always increased in saliva upon biscuit mastication at a higher extent in NW than in OB. Mastication of EVOB, determined in OB 2.2 folds higher salivary NAPEs than in NW, whereas CONB and PALMB determined in NW a 4.3 and 1.6 folds higher increase of NAEs than in OB, respectively.

Keywords: saliva, NAEs, NAPEs, Body Mass Index, mastication.

4.1 Introduction

Mastication is the first step of digestion preparing the food to be swallowed and the gastrointestinal tract to efficiently digest and absorb nutrients. During mastication saliva allows the humidification of the food matrix, the dissolution of nutrients and their contact with sensory receptors in the mouth as well as the initiation of lipid and starch breakdown through the action of endogenous enzymes such as lipase and α-amylase, respectively (Spielmann & Wong, 2011; Chen, 2009; Yoshizawa *et al.*, 2013).

Saliva is produced by major and minor salivary glands. The major glands are the parotid, submandibular and sublingual which produce >90% of the total volume of saliva, while the minor glands are present in the oral mucosa at lower lip, tongue, palate, cheeks and pharynx. Stimulated saliva (SS) is secreted by the parotid gland upon mastication, while unstimulated saliva (US) is secreted by both the submandibular and sublingual glands even in absence of mastication. SS has higher protein content and lower viscosity than US (Mosca & Chen, 2017). Among other constituents such as proteins, enzymes, mucins, nitrogen compounds, electrolytes, saliva contains N-acylethanolamines (NAEs). They are bioactive lipid compounds present in small concentrations in animals, plants, bacteria and yeasts. They are involved in different physiological processes (Coulon *et al.*, 2012) such as the regulation of feeding behaviour, lipid metabolism, pain regulation system (Hansen, 2014; Witkamp, 2018).

The concentration of NAEs in human saliva from fasting subjects was associated with individual nutritional status because higher levels were present in obese (OB) than in normal weight (NW) subjects (Matias *et al.*, 2012). However, we demonstrated that NAEs in saliva could also derive from foods upon mastication with concentrations depending from food type and composition (Kong *et al.*, 2016; Mennella *et al.*, 2018). That evidence was in line with recent findings that NAEs and ECs are present in many food products at concentrations ranging between 0.1-0.7 µg/g d.w. *vs* 0-35 µg/g d.w. for NAEs and 0-34 µg/g d.w. *vs* 0-0.2µg/g d.w.

for ECs in animal *vs* vegetal products, respectively (De Luca *et al.,* 2018-submitted to Food Chemistry). Strikingly N-acyl-phosphatidylethanolamines (NAPEs) were even 100 times higher than NAEs in foods. Thus, considering that N-acylphosphatidylethanolamines (NAPEs) are known precursors of NAEs through the action of NAPE-specific phospholipase D (NAPE-PLD) and this enzyme is also expressed in salivary glands (Hansen, 2014, Coulon *et al.,* 2012; Matias *et al.,* 2012) we hypothesised that food is a source of NAEs continuously forming along the gastro-intestinal tract and possibly interacting with local receptors that has been highly underestimated until now.

Indeed, NAEs can interact with several types of receptors such as a G-protein-coupled receptor GPR119 (Janssen & Depoorter, 2013; Izzo & Sharkey, 2010), lining on the mucosa of the small intestine, colon and stomach, and influencing food intake as well as insulin secretion in the presence of glucose (Borrelli & Izzo, 2009; Coulon *et al.,* 2012; Spreckley and Murphy, 2015). Moreover, in the area under the gastro-intestinal mucosa NAEs could also interact with cannabinoid receptors type 1 (CB1) and type 2 (CB2), transient receptor potential vanilloid type 1 (TRPV1) and peroxisome proliferator-activated receptor-α (PPAR-α) (Borrelli *et al.,* 2015; Izzo & Sharkey, 2010).

In this frame, the objective of this study was to evaluate the influence of individual nutritional status on the salivary NAPEs and NAEs upon mastication of biscuits with a different fat composition. For this purpose three model of biscuits prepared with 8% of extra-virgin olive oil, 8% of palm oil and without fats were developed and a cross-over modified sham feeding protocol was carried out. Saliva samples were collected and analysed by LC-HRMS-analysis.

4.2 Materials and methods

4.2.1 Foods

Three types of biscuits were developed using a traditional recipe (Kong *et al.*, 2016) and differing for the type and amount of fats.

The biscuits were prepared with the following ingredients that were purchased by local supermarket: flour (Divella, Bari, Italy), extravirgin olive oil (Monini, Spoleto,PG, Italy), palm oil (Italy), paraffin oil (FaDem, Naples, Italy), sugar (Eridania, Italy) and yeast (Pane Angeli, Brescia, Italy).

The recipe of the biscuits included 55% flour, 17.5% sugar, 18.75% oil, 21.5% water, 0.5% yeast.

All biscuits were formed with a diameter of 3.5 cm and were baked at 190°C for 15 minutes in a forced-air circulation oven.

4.2.2 Subjects selection

Twenty five healthy subjects were selected among students and staff of Department of Agriculture of University of Naples. They were not undergoing any medication or drug therapy or presenting diseases.

The participants were divided into two groups depending on their body mass index (BMI): ormal weight (NW; BMI<25 kg/m^2) and obese (OB; BMI>30 Kg/m^2) subjects. The eating behaviour was assessed for the Restraint, Disinhibition, and Hunger factors using the Three Factor Eating Questionnaire (TFEQ) as described by Stunkard & Messick (1985). The preference and the consumption of high fat foods was assessed by the Fat Preference Questionnaire (FPQ) as reported by Ledikwe *et al. (*2007) to adapt to an Italian population. The Power of Food Scale (PFS) was used in order to assess the psychological impact of living in food-abundant environments (Lowe *et al.*, 2009).

All experimental procedures were approved by the Ethics Committee of the University of Naples.

4.2.3 Study design

The study was conducted at the Department of Agricultural Sciences of the University of Naples. It was a randomised, crossover study. The experimental tests consisted in three treatments performed by each subject on two separate days with one week in between. On the experimental days, subjects were instructed not to eat, not to drink beverages (except water) and not to smoke two hours before the test, and were asked to clean their teeth no later than one hour before the outset of the study.

Figure 4.1 shows the study design.

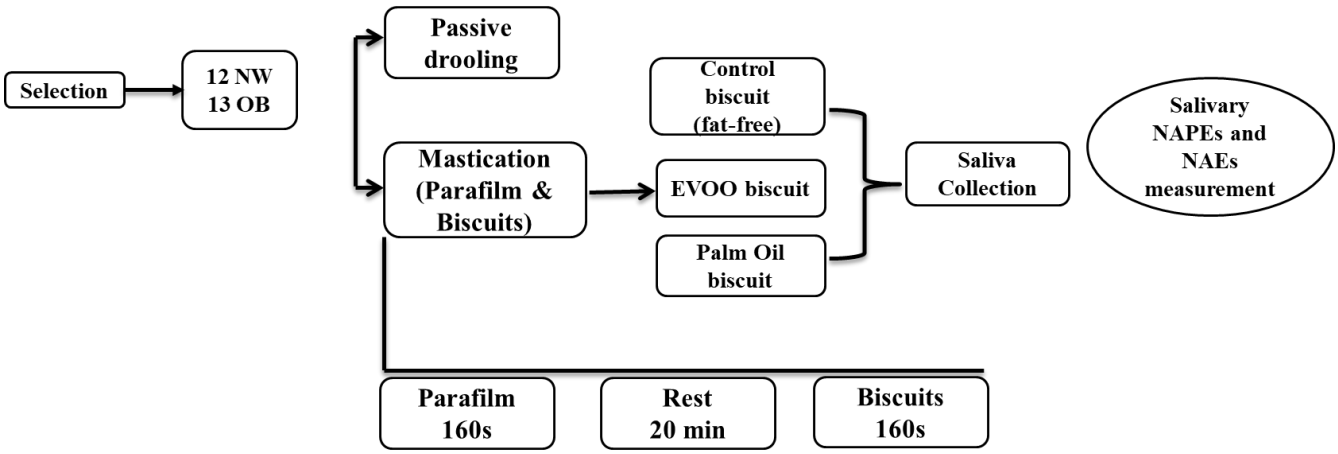

Figure 4.1. Study design

4.2.4 Saliva sample collection and preparation for analysis

Resting drooling was used to collect US from the oral cavity. Participants were asked to sit comfortably in an upright position, to have their heads slightly bent forward in order to pool saliva in the mouth and to let saliva fall into a pre-labeled sterile container for 10 minutes. Then, after drinking some water the subjects were asked to collect SS by chewing a piece of paraffin film (0.29 g; PARAFILM purchased by Sigma-Aldrich, USA) and expectorating the accumulated saliva every 40 sec into sterile tubes for 160 sec. Subsequently with applying the same procedure by replacing parafilm with biscuits, subjects collected the bolus (chewed biscuit).

After collection each type of saliva sample was aliquoted in pre-labeled Eppendorf tubes (2 mL) and frozen (-40°C) for storage until analysis. Bolus samples were centrifuged 4000 rpm/min for 10 min at 4°C and supernatants were stored like US and SS samples for further analysis.

The flow of US and SS was calculated by subtracting the weight of the tube filled with saliva to the weight of the empty tube, divided by collection time and it was expressed as mL/min (Agha–Hosseini *et al.*, 2010).

4.2.5 Determination of salivary NAPEs and NAEs

To the saliva samples (500 µL) were added 25 µL of 2 µg/mL of Arachidonoyl Ethanolamide-d8 (AEA-d8) (Cayman Chemical, Ann Arbor, MI) as internal standard and extracted with 4.5 mL of a solution of $CHCl_3/CH_3OH$ (2:1). After 30 seconds of shaking, the mixture was centrifuged at 14800 rpm for 10 minutes at 10 °C.

The organic phase was dried under nitrogen stream and the dried extract was dissolved in 500 µL of $C_2H_3N/C_3H_8O/H_2O$ (65:30:5) immediately before the LC-HRMS analysis.

4.2.6 Determination of NAPEs and NAEs in biscuits

To the biscuit (100 mg) was added 25 µL of the internal standard 2 µg/mL solution of arachidonoylethanolamide d8 (AEA d8) (Cayman Chemical, Ann Arbor, MI). 4.5 mL of a solution of $CHCl_3/CH_3OH$ (2:1) was used for the extraction. After 30 seconds of shaking, the mixture was centrifuged at 14800 rpm for 10 minutes at 10 °C.

The supernatants were collected in a glass tube and dried under a nitrogen flow. The extract was suspended in a solution of $C_2H_3N/C_3H_8O/H_2O$ (65:30:5) before the HRMS analysis (De Luca *et al.*, 2018 submitted Food Chemistry).

4.2.7 Liquid Chromatography – High Resolution Mass Spectrometry (LC-HRMS) analysis

LC-HRMS analysis was adapted from Gregory *et al.* (2012). Data were acquired on an Accela U-HPLC system coupled to an Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The Accela system consisted of a quaternary pump, a thermostated autosampler (10 °C) and a column oven. A Kinetex 2.6 µ C18 100 A column (100 mm × 2.1 mm) (Phenomenex, Torrance, CA) thermostated at 45 °C was used. The methods and the parameters were the same used by De Luca *et al.* (2018) (submitted Food Chemistry).

N-Arachidonoylphosphatidylethanolamine standard was purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Inc., USA).

The concentrations of different NAPEs were quantified as N-Arachidonoylphosphatidylethanolamine equivalents by using the external calibration curve with the linear range (0.2-5µg/ml) and expressed as µg per mL of saliva.

NAEs (oleoylethanolamide, OEA; linoleoylethanolamide, LEA; palmitoylethanolamide, PEA) standards were purchased from Cayman (Cayman Chemical, Ann Arbor, MI). The concentrations were calculated by isotope dilution using calibration curve with the linear range

(1-1000 ng/ml) and expressed as ng per mL of saliva. In the chromatographic region between 19-23 min the NAPEs were detected in negative mode as $[M-H]^-$.

In the chromatographic region between 6-9 min the NAEs were detected in positive mode as $[M+H]^+$.

The identification of compounds was performed by using the exact mass values up to the fifth decimal digit with a ± 5 ppm mass tolerance.

4.2.8 NAPEs identification in food matrix

Chromatographic separation was performed using a HPLC apparatus equipped with two Micropumps Series 200 (PerkinElmer, Shellton, CT, USA).

The chromatographic conditions used for the analysis were the same as described above.

Mass spectrometry analyses were performed on an API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Canada) equipped with a TurboIonSpray source working in the negative ion mode. Acquisition was performed in scan in the range m/z 120-1200 and in MRM (Multiple Reaction Monitoring).

4.2.9 Salivary lipase activity by spectrophotometric assay

Lipase activity was measured by the method described by Mennella *et al.* (2014). Into 1.5 mL centrifuge tubes 0.1 mL saliva was mixed with 1 mL of 100 mM Tris–HCl buffer (pH 8.3) containing 0.3 mM 5-5′-dithio-bis (2-nitrobenzoic acid) (DTNB). Then 20 μL of phenylmethylsulfonyl fluoride (PMSF) 20 mM in ethanol was added as a non-specific esterase inhibitor and tubes were incubated for 5 min at 37 °C. The ability of 0.4 mM PMSF to inhibit salivary esterases, without modifying lipase activity as described by Kurooka *et al*. (1977) was demonstrated during the set-up of the assay. Finally, 0.1 mL of 20 mM 2,3-dimercapto-1-propanol tributyrate in ethanol was added and samples were incubated for 60 min at 37 °C. Salivary lipase released 2,3-dimercapto-1-propanol that reacted with DTNB to release TNB anions. Tubes were then centrifuged at 14800 rpm for 10 min at 4°C and the absorbance at 412

nm was measured. Colour intensity was proportional to the lipase activity that was quantified in saliva samples using a specific calibration curve. It was done using triacylglycerol lipase from Aspergillus niger (200 U/g, purchased by Sigma, St. Louis, MO, USA) as a standard. A mother solution of 1 U/mL was prepared dissolving the powder in Tris–HCl buffer (pH 8.3) and consecutive dilutions were carried out to obtain a curve ranging from 9.375 to 150 U/L.

4.2.10 Statistical analysis

By considering inter-individual variations for salivary NAEs and ECs found in our previous works (Kong *et al.,* 2016; Mennella *et al.,* 2018), we calculated that a sample size of 12 subjects was sufficient to detect 40% variations of salivary NAEs with an 80% power and an $\alpha = 0.05$. Statistical analysis was performed using IBM SPSS Statistics version 20 (IBM Corporation, Armonk, New York, USA).

Kolmogorov-Smimov and Shapiro tests, as well as Levene's test were used to evaluate the normality of distribution and the homogeneity of variance of all monitored variables, respectively. Logarithmic transformation was applied to non-normally distributed data and mean values were compared through non-parametric Mann-Whitney U test, since normal distribution was not observed.

For correlation analysis, Pearson's was used when appropriate, p values less than 0.05 indicate statistical significance.

Statistical significance was defined as $p<0.05$ and results were expressed as means ± standard error of means (SEM).

4.3 Results

4.3.1 General and psychometric characteristics of subjects

The general and psychometric characteristics of subjects are reported in **Table 4.1.**

Table 4.1. Characteristic of participants. Asterisk indicates p<0.05 for OB *vs* NW.

	NW (mean±SD)	**OB (mean±SD)**	**P-values**
Number (n)	12	13	
Women	9	8	
Men	3	5	
Age (years)	30.4±8.7	35.5±10.7	p=0.318
BMI (kg/m^2)	22.4±2.2	32.6±3.6*	**p=0.000**
Unstimulated Saliva Flow Rate (mL/min)	0.6±0.4	0.4±0.2	p=0.828
Stimulated Saliva Flow Rate (mL/min)	2.0±1.2	1.9±0.8	p=0.807
Lipase (U/L)	13.02±1.2	18.5±2.0	**p=0.04**
TFEQ			
Dietary restraint (score)	6.8±1.0	10.7±1.3*	**p=0.032**
Disinhibition (score)	5.1±0.5	7.6±0.9*	**p=0.020**
Hunger (score)	2.2±0.6	4.5±1.1	p=0.209
PFS			
Food Available (score)	12.6±1.4	14.0±1.5	p=0.498
Food Present (score)	11.0±0.2	12.1±1.2	p=0.540
Food Taste (score)	13.2±0.8	13.6±1.5	p=0.796
FAT PREFERENCE			
Taste (%)	65.0±4.3	56.4±4.7	p=0.192
Frequency (%)	44.5±3.9	40.5±4.57	p=0.519
Difference (%)	24.3±4.8	19.8±4.2	p=0.480

As expected OB had higher BMI and lipase activity than NW. No difference between NW and OB for the taste preference and frequency of consumption of high fat foods compared to the low fat counterparts were found through FPQ.

Similarly, no differences emerged between NW and OB subjects for the appetite for palatable foods at three levels of food proximity (food available, food present, and food tasted) as assessed by PFS questionnaire (Lowe *et al.*, 2009).

TFEQ showed that OB were more restrained than NW (10.7±1.32 *vs* 6.83±1.03, p=0.032) and less disinhibited (5.08±0.5 *vs* 7.62±0.86, p=0.020).

No difference was found for the salivary flow between the two groups of subjects.

4.3.2 Foods

Table 4.2 shows the nutritional composition of the three types of biscuit. The only difference between biscuits was the type of fat or fat replacer used in the recipe: extravirgin olive oil, palm oil and paraffin oil.

Table 4.2. Nutritional composition of 100 g biscuits. CONB: control biscuits; EVOB: Evoo biscuits and PALMB: palm biscuits.

	CONB	**EVOB**	**PALMB**
Energy (Kcal)	344	435	443
Protein (g)	7	7	7
Carbohydrate (g)	79	79	79
Fat (g)	0	10	11
Saturated		1.4	4.7
Monounsaturated		7.0	3.9
Polyunsaturated		0.9	1.3

4.3.3 NAPEs identification in biscuits

NAPEs identification in biscuits was carried out by considering chemical structures of compounds and their retention times as obtained by HRMS analysis

In HRMS analysis NAPEs were characterised by exact mass. However, since several NAPEs species can have the same molecular weight due to the combination of different acyl residues on the ethanolamine bone NAPEs were tentatively identified using an API 3000 triple quadrupole mass spectrometer. The analyses were firstly performed in scan using cone-induced fragmentation to detect the deprotonated molecules of NAPEs and the carboxylate ions in *sn*-

2 and *sn*-1 position. The acquisition was then performed in MRM, to confirm the position of fatty acids by considering the information obtained in scanning experiments (De Luca *et al.*, 2018 submitted *Food Chemistry*). Details for NAPEs identification by mass spectrometry are reported in **Table 4.3**

Table 4.3 NAPEs identification in biscuits by mass spectrometry.

NAPE *sn*-1, *sn*- 2	**[M H]$^-$** *m/z*	**[R_2CO_2]$^-$**	**[R_1CO_2]$^-$**	**[R_3CO_2]$^-$**	**[M-HR_2CO_2H]$^-$**	**[M-H-R_1CO_2H]$^-$**	**[M-H-R'$_2$CH=CO]$^-$**	**[M-H-R'$_1$CH=CO]$^-$**	**[$R_3CONHCH_2CH_2OPO_3H$]$^-$**
16:0-18:2-N16:0	952.7	279.0	255.0	255.0	672.7	696.7	690.7	714.7	378
16:0-18:2-N18:2	976.7	279.0	255.0	279.0	696.7	720.7	714.7	738.7	402
16:0–18:2-N18:1	978.7	279.0	255.0	281.0	698.7	722.7	716.7	740.7	404
18:2–18:2-N18:2	1000.7	279.0	279.0	280.0	720.7	720.7	738.7	738.7	402
18:2-18:1-N18:2	1002.7	279.0	281.0	279.0	722.7	720.7	740.7	738.7	402
18:3-18:2-N18:2	998.7	279.0	277.0	279.0	718.7	720.7	736.7	738.7	402
18:2 18:2-N20:0	1032.7	279.0	279.0	311.0	752.7	752.7	770.7	770.7	434
18:2 18:1-N20:1	1032.7	279.0	281.0	309.0	752.7	750.7	770.7	768.7	432

* R1, R2, and R3 refer to the hydrocarbon chains of the acyl groups at *sn*-1, *sn*-2, and N-acyl position

4.3.4 NAPEs and NAEs concentration in biscuits

Table 4.4 shows the total concentration of NAPEs and NAEs in the biscuits.

Table 4.4. NAEs and NAPEs concentration in control biscuit (CONB), EVO-containing biscuit (EVOB) and palm oil containing biscuit (PALMB). The values are expressed as mean ± error standard deviation. Student's t test was used and different letters in the column indicate $p<0.05$ (number of replicates=3).

	LEA(ng/g)	PEA(ng/g)	OEA(ng/g)	Total NAEs(ng/g)	NAPEs(µg/g)
CONB	806 ± 263^{b}	7.5 ± 2^{b}	86 ± 29^{b}	900 ± 294^{b}	876±42
EVOB	1976 ± 350^{ab}	28.2 ± 6^{ab}	244 ± 44^{a}	2249 ± 400^{ab}	973±76
PALMB	2388 ± 194^{a}	37.0 ± 3^{a}	297 ± 20^{a}	2722 ± 216^{a}	1091±46

No difference between biscuits for the concentration of NAPEs was found.

LEA and PEA concentrations were higher in PALMB than in CONB (2387±193.8 ng/g *vs* 806.0±262.7 ng/g, p=0.017, for LEA and 37.0±3.0 ng/g *vs* 7.5±2.4 ng/g, p=0.003, for PEA).

OEA concentration was higher in both PALMB and EVOB than in CONB (297.0±19.5 ng/g *vs* 86.4±29.0 ng/g, p=0.007, and 244.5±43.5 *vs* 86.4±29.0 ng/g; p=0.049, respectively).

Total NAEs concentration was higher in PALMB than in CONB (2722±19.5 ng/g *vs* 900.6±2.4 ng/g, p=0.015).

4.3.5 NAPEs concentration in saliva

Figure 4.2A shows concentrations of NAPEs in US, SS and upon mastication of the three types of biscuit. NAPEs concentration in US was higher in OB than in NW subjects (0.28±0.04 µg/mL *vs* 0.12±0.025 µg/mL; p=0.015), whereas in SS no difference between OB and NW was found (0.26±0.05 *vs* 0.12±0.02 µg/mL, p=0.049).

Data showed that upon mastication of food the concentration of salivary NAPEs was higher than in a no-food condition (SS) in both NW and OB subjects. Individual effect of each type of biscuit on salivary NAPEs concentration is shown in **Figure 4.2B**.

In NW a 41% higher concentration of salivary NAPEs with EVOB than PALMB was found whereas no difference between PALMB and EVOB compared to CONB was recorded.

In OB, EVOB caused a 82% increase of NAPEs compared to PALMB and 145% increase compared to CONB, with no difference between PALMB and CONB. Only EVOB caused a different increase of salivary NAPEs between NW and OB with a 2.2 folds higher increase in OB than NW.

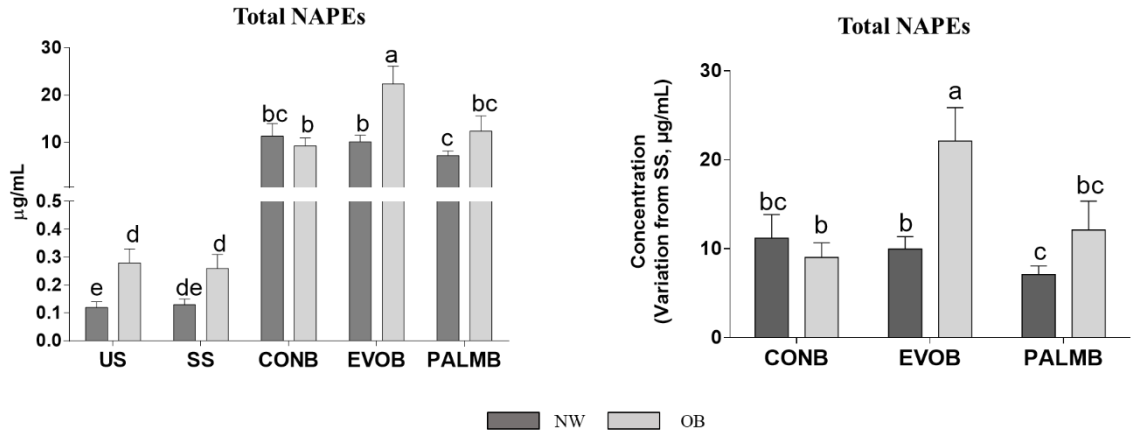

Figure 4.2. Salivary concentration of NAPEs in NW and OB subjects at baseline and upon mastication of parafilm or biscuits. A) NAPEs concentration in unstimulated saliva (US), stimulated saliva (SS) and saliva collected upon mastication of control biscuit (CONB, paraffin oil), extra-virgin olive oil-containing biscuit (EVOB) and palm oil containing biscuit (PALMB). **B**) Effect of biscuit type on salivary concentration of NAPEs. The statistical tests used for analysis were Mann-Whitney and Wilcoxon tests. Different letters on the bars indicate $p<0.05$ (number of replicates=3).

4.3.6 NAEs concentration in saliva

Figure 4.3 shows the concentration of individual and total NAEs in US, SS and in saliva upon mastication of the three types of biscuit as well as the effect of each type of biscuits on the variations of salivary concentration upon mastication.

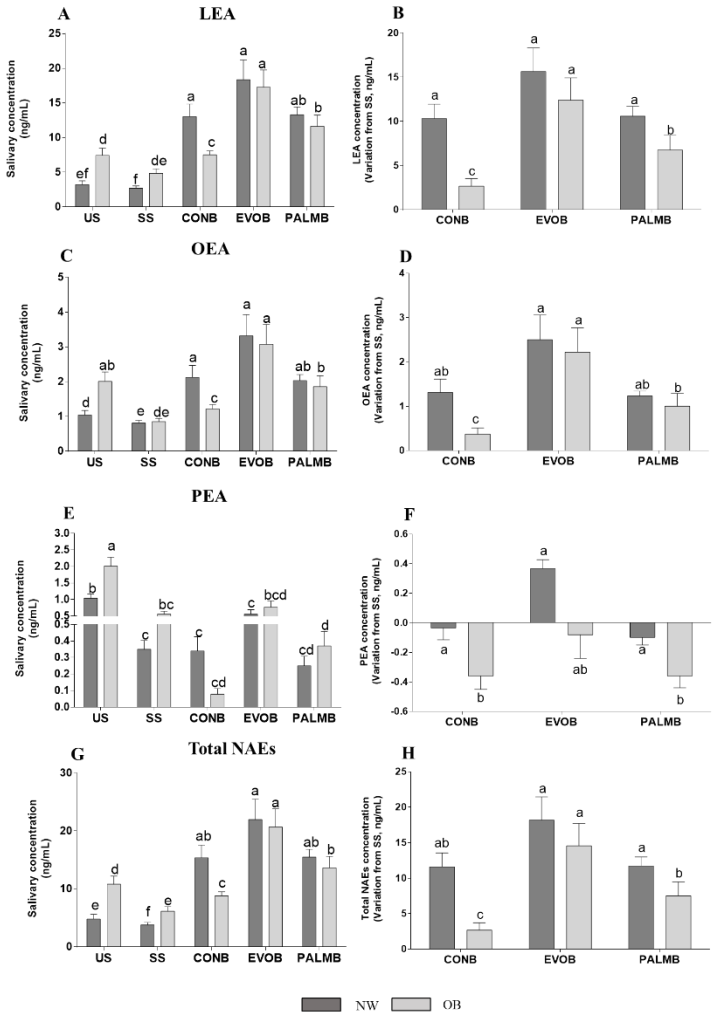

Figure 4.3. Salivary concentration of NAEs in NW and OB subjects at baseline and upon mastication of parafilm or biscuits. A) LEA concentration in unstimulated saliva (US), stimulated saliva (SS) and saliva collected upon mastication of control biscuit (CONB, paraffin oil), extra-virgin olive oil-containing biscuit (EVOB) and palm oil containing biscuit (PALMB). **B)** Effect of biscuit type on salivary concentration of LEA. **C)** OEA concentration in US, SS and saliva collected upon mastication of CONB, EVOB and PALMB. **D)** Effect of biscuit type on salivary concentration of OEA. **E)** PEA concentration in US, SS and saliva collected upon mastication of CONB, EVOB and PALMB. **F)** Effect of biscuit type on salivary concentration of PEA. **G)** Total NAEs concentration in US, SS and saliva collected upon mastication of CONB, EVOB and PALMB. **H**) Effect of biscuit type on salivary concentration of total NAEs. The statistical tests used for analysis were Mann-Whitney and Wilcoxon tests. Different letters on the bars indicate $p<0.05$ (number of replicates=3).

LEA concentration (**Figure 4.3A**) was higher in US and SS of OB than in NW subjects (7.44±1.04 ng/mL *vs* 3.22±0.55 ng/mL, p=0.001, in US and 4.86±0.65 ng/mL *vs* 2.71±0.33 ng/mL, p=0.01 in SS). In NW and OB subjects, no difference was found between US and SS. Data showed that upon biscuit mastication the concentration of LEA was higher than in a no-food condition (SS) in both NW and OB subjects. Individual effect of biscuits on salivary LEA concentration is shown in **Figure 4.3B.**

In NW, no difference between biscuits was recorded. In OB, with EVOB a 83% and 363% higher concentration of LEA compared to PALMB and CONB was found. Moreover, a 153% higher concentration with PALMB than CONB was recorded.

CONB and PALMB caused a different increase of salivary LEA between NW and OB with a 3.8 and 1.6 folds higher increase in NW than in OB respectively.

OEA concentration was higher in US of OB than in NW subjects (2.01±0.27 ng/mL *vs* 1.04±0.13 ng/mL; p=0.008), whereas no difference between OB and NW was found in SS (0.81±0.08 ng/mL *vs* 0.85±0.09 ng/mL) (**Figure 4.3C**).

OEA concentration was higher in US than in SS in NW and OB. Individual effect of each type of biscuit on salivary OEA concentration is shown in **Figure 4.3D.**

In NW, no difference between biscuits was found. In OB, with EVOB a 122% and 500% higher concentration of salivary OEA compared to PALMB and CONB was found. Moreover, a 170% higher concentration with PALMB than CONB was recorded.

Only CONB caused a different increase of salivary OEA between NW and OB with a 3.5 fold higher increase in NW than in OB.

PEA concentration was higher in US of OB than in NW subjects (1.37±0.20 ng/mL *vs* 0.56±0.13 ng/mL; p=0.002), whereas no difference was found in SS between OB and NW (0.56±0.08 ng/mL *vs* 0.37±0.05 ng/mL). PEA was higher in US than in SS of NW and OB subjects (**Figure 4.3E**).

Individual effect of each type of biscuit on salivary PEA concentration is shown in **Figure 4.3F.**

In both NW and OB, no difference between biscuits was recorded.

CONB and PALMB caused a different reduction of salivary PEA between NW and OB with a 10 and 3.6 folds higher reduction in OB than in NW, respectively. No difference was found between NW and OB.

Finally, total salivary NAEs concentration in both US and SS was higher in OB than in NW subjects (10.82±1.41 ng/mL *vs* 4.82±0.79 ng/mL, p=0.002 for US and 6.15±0.79 ng/mL *vs* 3.79±0.43 ng/mL, p=0.031, for SS) (**Figure 4.3G**).

Total NAEs concentration was higher in US than in SS in both NW and OB groups. Individual effect of each type of biscuit on salivary total NAEs concentration is shown in **Figure 4.3H**.

In NW, no difference between biscuits was found. In OB, with EVOB a 94% and 443% higher concentration of salivary total NAEs compared to PALMB and CONB was found. Moreover, a 180% higher concentration with PALMB than CONB was recorded.

CONB and PALMB caused a different increase of salivary total NAEs between NW and OB with a 4.3 and 1.6 folds higher increase in NW than in OB respectively. Only CONB caused a different increase of salivary total NAEs between NW and OB with a 1.7 folds higher increase in NW than in OB.

4.4 Discussion

This is the first study reporting the presence of NAPEs in saliva and their concentrations. NAPEs are membrane phospholipids and they are present in many organs such as brain, spinal cord, testis, spleen and in physiological fluids such as plasma and lymph, at very low concentration (Coulon *et al.,* 2012). Gillum *et al.* (2008) showed that the plasmatic concentration of NAPEs in rat was 2.46 µmol/L while the concentration in lymph was 0.73 µmol/L.

Data showed that NAPEs, LEA, PEA, and OEA were present at higher concentrations in US from OB than NW being positively correlated with BMI ($p=0.03$; $p=0.009$; $p=0.012$; $p=0.018$; respectively). Data of PEA and OEA confirmed those previously found by Matias *et al.* (2012) while no previous literature exist on salivary LEA and NAPEs levels in NW and in OB. Since FAAH and NAPE-PLD are expressed in saliva producing organs (Matias *et al.,* 2012), it was hypothesised that the differences between NW and OB might be due to a down-expression of FAAH and/or a decreased enzyme activity or to an over expression of NAPE-PLD and/or an increased activity as a result of obesity.

Worth of notice is the different concentration of NAEs in US compared to SS, and the finding that NAEs level is reduced over mastication (US>SS). US is secreted by the submandibular and sublingual glands, while SS is secreted by parotid glands; SS has a higher protein concentration and lower viscosity than the US (Prinz *et al.,* 2007). Therefore, it is likely that also hydrolytic enzyme as FAAH is more concentrated and/or active in SS than US thus lowering NAEs concentration.

The increase of NAPEs in saliva upon biscuit mastication as well as their presence in the biscuits were a clear indication those molecules can derive directly from the biscuits.

Data showed that upon EVOB mastication, NAPEs concentration in saliva was higher in OB than in NW subjects and, within the OB group, EVOB determined a higher increase of NAPEs

in saliva than PALMB and CONB. Contrarily, in NW no difference between EVOB and CONB was found whereas PALMB determined the lowest variation of NAEs levels compared to stimulation by parafilm.

The difference between OB and NW for salivary NAPEs upon EVOB mastication might be due to a less active NAPE-PLD or by a different duration/efficiency of mastication in OB compared to NW.

Individuals with higher BMI are known to eat faster (and masticate less) than those with a lower BMI (Zhu & Hollis 2014; Hill & McCutcheon, 1984) but because of the known higher activity of salivary alfa-amylase (Mennella *et al.,* 2014) OB could be more efficient in delivering NAPEs from the biscuit compared to NW. In other word, even if OB masticated less the chemical breakdown of a starchy food matrix (as a biscuit) could happen faster in OB than NW thus delivering a higher amount of NAPEs into the saliva.

In addition, the texture of EVOB could partly explain the findings in OB. Indeed, biscuits containing oil have a hard texture because a low quantity of air during the mixing of ingredients remains entrapped (Jacob & Leelavathi, 2007). Since rheological properties of foods influence the masticatory process and soft or water-rich foods are rapidly swallowed compared with hard foods, in this study OB might masticate EVOB for a longer time than for the other biscuits (Jalabert-Malbos *et al.,* 2007).

On the other hand the increase of NAEs concentration in NW and in OB subjects upon biscuit mastication compared to SS is in agreement with our previous studies where subjects masticated biscuits prepared with different dietary fibers (Kong *et al.,* 2016) or puddings (Mennella *et al.,* 2018).

Regarding the effect of the biscuit type on salivary NAEs, no differences in NW between biscuits were found, whereas in OB salivary NAEs increased upon biscuit mastication following an order of magnitude that was for EVOB>PALMB>CONB.

The differences between OB and NW might result from different activities of alfa-amylase and FAAH. Similarly to what hypothesised for NAPEs from EVOB in OB, also NAES could be better released by OB from the food matrix due to a more efficient mechanical and enzymatic disruption of the food matrix, but a higher activity of FAAH in saliva of OB than NW might account for the low concentrations of NAEs in OB. This hypothesis was in line with the evidence of an over-expression of FAAH in the abdominal adipose tissue from OB compared to NW (Murdolo *et al.,* 2007) and a higher FAAH activity in adipocytes from subjects with higher BMI and waist circumference (Cable *et al.,* 2011). Contrarily, other authors showed that FAAH was under-expressed in adipose tissue from OB compared with NW (Bluher *et al.,* 2006; Lofgren *et al.,* 2007). Future studies should clarify the mechanisms behind the different levels of NAEs in saliva of OB and NW upon food mastication. On the other hand, irrespective from individual nutritional status, data demonstrated a higher contribution of EVOB than PALMB and CONB to salivary NAEs levels. This finding was likely due to the ability of oleic acid (most abundant fatty acid of EVOO) to activate NAPE-PLD and inhibit FAAH. This hypothesis was in agreement with findings showed by Fu *et al.* (2007) and Schwartz *et al.* (2008) on the activity of NAPE-PLD and FAAH in duodenum and jejunum of rats as a response to oleic acid occurring within few minutes from stimuli presentation.

Factors independent from specific fatty acid composition of palm oil and paraffin oil but most probably related to the mastication process such as differences in chewing cycles, bite force, degree of hunger, etc were hypothesised to be implicated in the different salivary NAEs concentrations upon mastication of PALMB and CONB.

4.5 Conclusions

This study demonstrated that NAPEs and NAEs in saliva can derive from food upon mastication. The salivary concentrations of NAPEs and NAEs were higher in OB than in NW subjects. The increase of salivary NAEs upon food mastication was independent from the biscuit type in NW, while in OB the type of biscuits contributed to the salivary NAEs concentration in the following order: EVOB>PALMB>CONB. Future studies should evaluate the physiological role of increased concentration of salivary NAEs and NAPEs upon food mastication and the implications of NAPE-PLD and FAAH activities.

4.6 Abbreviations

NAEs: N-acylethanolamines; NAPEs: N-acylphosphatidylethanolamines; ENC: enteric nervous system; CB: cannabinoid receptors; TRPV1: transient receptor potential vanilloid type 1; PPAR-α: peroxisome proliferator-activated receptor-α; BMI: Body Mass Index: BMI; TFEQ: Three Factor Eating; FPQ: Fat Preference questionnaire; PFS: Power of Food Scale; GI: gastrointestinal; GPR: G-protein-coupled receptor; CB1: cannabinoid receptors type 1; CB2: cannabinoid receptors type 2; TRPV1: transient receptor potential vanilloid type 1; PPAR-α: peroxisome proliferator-activated receptor-α; OB: obese; NW: normal weight; NAAA: N-acylethanolamine-hydrolyzing acid amidase; FAAH: fatty acids amide hydrolase; LC-HRMS: liquid chromatography–high resolution mass spectrometry; AEAd8: anandamide-d8; OEA: oleoylethanolamide; LEA: linoleoylethanolamide; PEA: palmitoylethanolamide; EVOB: Evoo biscuits; PALMB: Palm biscuits; CONB: Control biscuits. US: unstimulated saliva; SS: stimulated saliva.

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Chapter 5. Bioaccessibility of N-acylethanolamines and endocannabinoids from a mediterranean and a western diet in a simulator of the human intestinal tract (SHIME®)

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Abstract

N-acylethanolamines (NAEs) and endocannabinoids (ECs) are bioactive molecules involved in different physiological and biochemical processes in both animal and plant tissues. NAPES, NAEs and ECs have been recently quantified in many foods and some evidence also suggests an interaction between gut microbiota and ECs system. The fate of dietary NAPEs, NAEs and ECs in the gastro-intestinal tract has been underestimated.

In this study the bioaccessibility of NAEs and ECs from a Mediterranean (MD) and a Western diet (WD) along the gastrointestinal tract was assessed *in vitro* by using the simulated model of the human intestinal ecosystem (SHIME®).

To this purpose two different diets such as a Mediterranean (MD) and a Western diet (WD) were prepared and used to feed the SHIME® system. The supernatants deriving from the different phases of the *in vitro* digestion were analysed to assess the concentrations of NAEs and ECs by LC-HRMS and the ability to inhibit FAAH by commercial kit.

Data showed that both the concentration of total NAEs and FAAH inhibition upon a MD and a WD were not influenced by diets but mainly by fecal donor. This was associated with the different composition of the gut microbiota of the two donors.

Keywords: microbiota, diets, N-acylethanolamines, SHIME®

5.1 Introduction

N-acylethanolamines (NAEs) and endocannabinoids (ECs) are lipidic messengers involved in different physiological mechanisms in both animals and plants.

In animals the enzyme involved in the synthesis of N-acylphosphatidylethanolamine (NAPEs) is N-acytransferase (NAT), while in plants the synthesis of NAPEs is possible by the NAPE synthase activity that promotes the N-acylation of phosphatidylethanolamine (PE) by free fatty acids (FFAs) (Coulon *et al.,* 2012). The NAEs and ECs derive from hydrolysis of NAPEs by the N-acyl-phosphatidylethanolamine-specific phospholipase D (NAPE-PLD) enyzme, a specific membrane-bound phospholipase D (Ueda *et al.*, 2010). In plants multiple isoforms of PLD are expressed such as PLDβ and PLDγ which are able to hydrolyse glycerophospholipids (including NAPEs).

NAEs are hydrolysed in free fatty acid and ethanolamine by fatty acid amide hydrolase (FAAH) or N-acylethanolamine-hydrolyzing acid amidase (NAAA) (Coulon *et al.,* 2012, Ueda *et al.,* 2010).

The glycerol ester 2-arachidonoylglycerol (2-AG) is synthesised from diacylglycerol via the enzyme diacylglycerol lipase (DAGL) and subsequently it is hydrolysed by monoacylglycerol lipase (MAGL) in arachidonic acid and glycerol (Witkamp, 2018).

Different natural compounds are natural inhibitors of FAAH *in vitro*. They include some flavonoids such as kaempferol, apigenin, luteolin, quercetin, myricetin and genistein (Thors *et al.*, 2007; Thors *et al.,*2008).

The endocannabinoids (ECs), anandamide (AEA) and 2-AG activate cannabinoids receptors (CB1 and CB2) that are widely distributed into the human body.

NAEs and ECs link various receptors present along the gastrointestinal tract (GIT) such as vanilloid receptors TRPV1, peroxisome proliferator-activated-α (PPAR-α), G protein-coupled receptors, GPR119 and GPR55 that are involved in the modulation of food intake either by

acting in the brain or by signaling from gastrointestinal system (Izzo & Sharkey,2010; Hansen & Diep, 2009).

The activation of GPR119 by NAEs, and in particular by oleoylethanolamide (OEA), leads to enhanced glucose-dependent insulin secretion from pancreatic β-cells and influences the release of intestinal peptide glucagon like peptide-1 (GLP-1), glucose dependent insulinotropic peptide (GIP) and peptide YY (PYY) (Hansen *et al.,* 2012).

The concentration of NAEs and ECs in plasma and tissues of animals and humans and their involvement in the physiological processes are influenced by diet and its fat composition (Di Marzo, 2008; Pintus *et al.,* 2013; Hansen *et al.*,2009; Banni *et al.,* 2011; Artmann *et al*., 2008; Joosten *et al.*, 2010; Diep *et al.,* 2011; Mennella *et al.,* 2015).

NAPEs, NAEs and ECs are bioactive compounds present in nature but very little is known about their concentration in the food products (Chapman *et al.,* 1999; Di Marzo *et al.,* 1998; Venables *et al.,* 2005).

We have recently shown that NAPEs, NAEs and ECs are present in food products and that NAPEs and NAEs are most abundant in vegetables products than in animal products, while the opposite was found for ECs (Kong *et al.,* 2016; Mennella *et al.,* 2018; De Luca *et al.,* 2018-submitted to Food Chemistry).

The gut microbiota is able to influence the ECs system activity, the expression of FAAH in the colon and in other tissues as well as the mRna levels of NAPE-PLD in obese mice (Geurts *et al.,* 2011; Muccioli *et al.,* 2010). On the other hand, diet can influence the function of gut microbiota and dietary fats can induce obesity also through modification of gut microbiota composition (De Filippo *et al.,* 2010; Mulders *et al.,* 2018; Prieto *et al.,* 2018). Lipopolysaccharide (LPS), which role in the development of metabolic disease is well known, can also influence the ECs system by inducing the production of ECs by immune cells (Cani, 2012; Liu *et al.,* 2003).

NAEs are modulated by gut microbiota and the interaction with GLP-1 can be involved in the cross-talk between the gut microbiota and regulation of host glucose metabolism (Everard & Cani, 2014). The ECs system or NAEs controlled by gut microbiota could contribute to control blood glucose, lipids and energy homeostasis.

However, a relation between diet, gut microbiota, NAEs-ECs concentration and ECs system tone in humans has not yet demonstrated.

In this study the fate of NAEs and ECs along the gastrointestinal tract (GIT) was assessed *in vitro* by using the simulated model of the human intestinal ecosystem (SHIME®) mimicking both physiological conditions and the microbial ecosystem found in the ascending (AC), transverse (TC) and descending (DC) colon.

To this purpose two different diets such as a Mediterranean (MD) and a Western diet (WD) were prepared and used to feed the SHIME® system. The supernatants deriving from the different phases of the *in vitro* digestion were analysed to assess the concentration of NAEs and ECs by LC-HRMS and the ability to inhibit FAAH by commercial kit.

5.2. Material and methods

5.2.1 Diets

Two powders including food types and proportion typical of a Mediterranean Diet (MD) or a Western Diet (WD) were prepared.

To prepare the MD based powder the following pattern was considered: a high intake of whole wheat cereals, legumes and fish, a low intake of dairy and poultry products and meat, low intake of potatoes and the use of the virgin olive oil as food condiment (Martinez-Gonzalez *et al.*, 2009). For the WD powder a high intake of red and meat, butter, fried foods, high-fat dairy products, eggs, refined grains were considered (Myles, 2014).

Table 5.1 shows the weight of lyophilised foods included in the MD and WD.

Table 5.1. Weight of foods used to develop Mediterranean (MD) and Western diet (WD).

Dried Food	MD (grams)	WD (grams)
Orange	2.22	0.69
Walnuts	0.75	0
Almond	0.75	0
Beans	0.41	0.05
Chickpeas	0.41	0.05
Lentils	0.41	0.05
Potatoes	0.17	1.7
Tomatoes	1.01	0.1
Cauliflower	0.5	0.05
Broccoli	0.5	0.05
Bread	0	4.19
Whole wheat bread	2.15	0
Pasta	0	4.19
Brown pasta	4.19	0
Breakfast whole cereals	2.15	
Codfish	0.38	0.05
Anchovies	0.38	0.05
Tuna fish	0.38	0.05
Mozzarella cheese	0.40	1.38
Gorgonzola cheese	0.40	1.38
Parmigiano cheese	0.40	1.38
Chicken	0.16	0.5
Eggs	0.15	0.5
Salami	0	1.6
Red meat	0	1.5
Extravirgin olive oil	1.76	0.23

5.2.2 TWIN SHIME® with MD and WD

The SHIME® simulator (simulator of the human intestinal microbial ecosystem, ProDigest, Belgium) was used to mimic the *in vivo* human intestinal fermentation.

Fresh fecal sample was used to inoculate a TWIN SHIME® set-up, consisting of ascending, transverse and descending colon for two different donors. The one male and one female fecal stool donors were non-smoking adults, between 25 and 31 years of age, with no prior history of antibiotic or probiotic use for 6 months and 3 weeks, respectively.

For each donor, 3 double-jacketed vessels were used, simulating one combined stomach/small intestine, ascending (pH 5.6-5.9), transverse (pH 6.15-6.4) and descending (pH 6.6-6.9).

Freshly donated fecal sample was stored in a collection box with an anaerobic AnaeroGen™ bag at 4°C for less than 8 hours. A 20% (w/v) solution of the fecal sample was homogenised with phosphate buffer for 10 min using a stomacher® 400 circulator. The sterilized phosphate buffer consisted of 8.8g/L K_2HPO_4, 6.8g/L KH_2PO_4 and 0.1g sodium thioglycolate in demi water. The pH was adjusted to 7 and 15 mg sodium thionite was added before use. After mixing, the inoculum was centrifuged for 2 min at 500g. Per 100mL vessel volume, 5mL of inoculum supernatant was used. After the two weeks stabilization period, the microbiota was collected and stored with 50% sterilized cryoprotectant (a final concentration of 42% glycerol in demi water, 0.5g/L cysteine HCl, 10g/L trehalose and 3g/L tryptic soy broth) at -80°C until further experiments.

Before the experiment the microbiota was let overnight with pH controlled in the range 6.6-6.9 followed then by a 3-day program with simulated feedings every 8 hours.

Every 8 hours, 70ml fresh liquid feed with pH 2 entered the stomach vessel for each donor with a constant feed composition (1.2g/L arabinogalactan, 2.0g/L pectin, 0.5g/L xylan, 0.4g/L glucose, 3.0g/L yeast extract, 1.0g/L special peptone, 3.0g/L mucin, 0.5g/L l-cysteine-HCL, 4.0g/L starch). After 90 minutes, 30ml of pancreatic juice, prepared with 12.5g/L NaHCO3;

6g/L Oxgall and 0.9g/L pancreatin from porcine ≥ 3 * USP, was added. After 90 minutes of small intestinal phase, the total volume was transferred to the ascending colon (pH 5.6-5.9, V=250mL), in series connected with the transverse (pH 6.15-6.4, V=400mL) and descending colon (pH 6.6-6.9, V=300mL).

The vessel volumes, pH and retention times were kept constant at all times (Van den Abbeele *et al.,* 2010).

The experiment included three treatment days, with daily addition of dried foods per donor to the stomach phase. The experimental procedure consisted of the addition to the stomach phase of 10g/L of dried WD or MD per donor. During fermentation at 0, 1, 2, 4, 6 and 8 hours sample collections in all colon parts were carried out. Immediately after the collection, samples were centrifuged for 10 minutes at 9000rpm at a temperature of 4°C and the obtained supernatants were filtered using a 0.20µM RC syringe filter and stored at -20°C until analysis.

The two donors were used as biological replicates.

5.2.3 Extraction of NAEs and ECs from colon phase

The extraction of NAEs and ECs from each colon phase was performed by using the protocol proposed by Gachet & coworkers (2015) with the addition of some modifications.

500 µL of sample were added to 1 mL of the C_2H_3N solution and 50 µL of internal standard solution of 200 ng/mL arachidonoylethanolamide d8 (AEA d8) (Cayman Chemical, Ann Arbor, MI).

The mixture was placed inside the refrigerator at 6°C for 10 min to facilitate the protein precipitation.

Subsequently, the samples were centrifuged at 14000 rpm at 4°C for 5 min.

The supernatant was transferred to a tube, diluted with mL of H_2O and extracted by solid-phase separation (C18 Sep-Pak cartridge pre-activated with 3 mL CH_3OH and equilibrated with 3 mL

10% C_2H_3N). Cartridges were washed with 3 mL 10% C_2H_3N and eluted with 3 mL of $C_2H_3N/C_4H_8O_2$ (1:1).

The elutes were evaporated to dryness under nitrogen. The samples were reconstituted in 500 µL $C_2H_3N/C_3H_8O/H_2O$ (60:35:5) and filtrated before the LC-HRMS analysis.

5.2.4 Liquid Chromatography – High Resolution Mass Spectrometry (LC-HRMS) analysis

LC-HRMS analysis was adapted from Gregory *et al.* (2012). The analysis was performed on an Accela U-HPLC system coupled to an Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The Accela system consisted of a quaternary pump, a thermostated autosampler (8 °C). A Kinetex 2.6 µ C18 100 A column (100 mm × 2.1 mm) (Phenomenex, Torrance, CA) thermostated at 45 °C was used (De Luca *et al.,* 2008 submitted to *Food Chemistry*).

ECs (2-arachidonylglycerol, 2-AG; anandamide, AEA; AEAd8) and NAEs (OEA: linoleoylethanolamide, LEA; palmitoylethanolamide, PEA) standards were purchased from Cayman (Cayman Chemical, Ann Arbor, MI). The concentrations were calculated by isotope dilution using calibration curve with the linear range (1-1000 ng/mL) and expressed as ng per mL of colon phase.

In the chromatographic region between 6-9 min the NAEs were detected in positive mode as $[M+H]^+$.

The compounds identification was performed by using the exact mass values up to the fifth decimal digit with a ± 5 ppm mass tolerance.

5.2.5 FAAH inhibition assay

To check the ability of the supernatants to inhibit the FAAH a Fatty Acid Amide Hydrolase-Inhibitor Screening Assay (Cayman Chemical, Ann Arbor, MI) was used.

170 µL of assay buffer (125 Mm Tris-HCl, pH 9, containing 1 mM of EDTA) was added with 10 µL of FAAH (human recombinant) and 10 µL of supernatant or inhibitor (20 µM of JZL 195).

The plate was incubated at 37° C for 5 minutes.

Subsequently, 10 µL of substrate (20 µM of Arachidonyl amide) was added and the plate was incubated at 37°C for 30 minutes.

The fluorescence was measured using a Spectramax M5 (Molecular devices, USA) with a wavelenght of 340-360 nm and an emission wavelenght of 450-465 nm.

5.2.6 Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics version 20 (IBM Corporation, Armonk, New York,USA).

Kolmogorov-Smimov and Shapiro tests as well as Levene's test were used to evaluate the normality of distribution and the homogeneity of variance of all monitored variables, respectively. Logarithmic transformation was applied to non-normally distributed data and mean values were compared through non-parametric Mann-Whitney U and Wilcoxon tests, since normal distribution was not observed.

Statistical significance was defined as $p<0.05$ and results were expressed as means ± standard error of means (SEM).

5.3 Results

Concentration of NAEs and ECs in stomach and small intestine

Table 5.2 shows the concentration of NAEs and ECs in the stomach and small intestine phase.

The values were expressed as means ± SEM.

Table 5.2. NAEs and ECs in stomach and small intestine (SI) upon MD and WD in donor 1, in donor 2 and the mean concentration. The data are expressed as mean ± SEM. No difference between donors within stomach or SI was found for each monitored analyte.

	MD					WD				
	ECs ng/mL	LEA ng/mL	OEA ng/mL	PEA ng/mL	NAEs ng/mL	ECs ng/mL	LEA ng/mL	OEA ng/mL	PEA ng/mL	NAEs ng/mL
Stomach 1	0.10±0.08	0.31±0.20	1.07±0.55	2.07±0.55	3.45±0.88	16.21±7.50	1.37±0.23	16.30±1.19	3.08±2.11	20.70±14.80
Stomach 2	0.28±0.26	0.20±020	1.32±0.67	3.10±0.46	4.60±0.37	18.95±4.01	1.25±0.69	0.45±0.78	0.31±0.42	2.02±1.94
Stomach (mean)	0.18±0.13	0.25±0.13	1.20±0.40	2.58±0.40	4.02±0.50	17.60±3.87	1.30±0.32	8.38±6.40	1.70±1.14	11.40±7.61
Small intestine 1	3.16±2.41	19.71±10.7	23.50±1.95	1.53±0.76	44.75±13.11	16.18±7.64	13.69±5.59	33.69±1.14	2.96±1.05	50.30±5.74
Small intestine 2	1.24±0.55	17.04±3.51	23.91±2.86	2.98±0.98	43.94±1.03	18.90±3.92	17.09±5.09	44.50±7.59	2.38±0.47	63.90±12.20
Small intestine (mean)	2.19±1.19	18.37±5.09	23.69±1.55	2.25±0.65	44.3±5.90	17.60±3.90	15.40±3.47	39.10±4.19	2.70±0.53	57.20±6.70

The concentration of ECs was higher in WD than in MD in stomach and in the small intestine.

The concentration of LEA was similar in both the diets with an increase of concentration in the small intestine.

The concentration of OEA, PEA and total NAEs was higher in WD than in MD, furthermore the concentration of OEA and total NAEs was higher in small intestine than in stomach for both the diets.

Concentration of ECs and NAEs in ascending colon (AC)

The concentration of ECs in small intestine was 2.19 ng/mL in MD, while it was 17.6 ng/mL in WD.

In order to highlight the effect of diets on bioaccessibility of NAEs and ECs, the baseline (before adding the diets to the small intestine) concentration at each colon tract was subtracted to those found at each time point and data were reported as variations from baseline.

Figure 5.1 shows the mean of variation from baseline concentrations of the ECs in AC (**A**), as well as the individual variation from baseline in AC of donor 1 (AC1) (**B**) and donor 2 (AC2) (**C**) upon MD and WD.

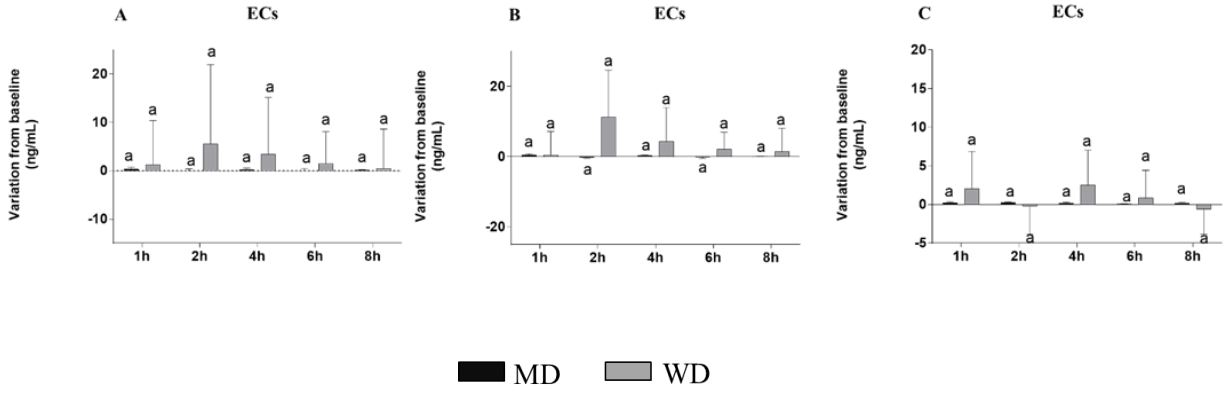

Figure 5.1. Variation from baseline of ECs in AC (mean of the two donors) (A), in AC1 (donor 1) (B) and in AC2 (donor 2) (C). The statistical tests used for analysis were Mann-Whitney and Wilcoxon tests.Different letters on the bars indicate significant differences ($p<0.05$) within the same diet. (number of replicates=3).

No difference was found between two diets (**Figure 5.1A**). Looking at the concentration profile in individual donors (**Figure 5.1B-C**) no difference was found in AC1 and in AC2.

Compared to the concentration in SI, the concentration of ECs in AC was reduced by a minimum of 91% after 1h to a maximum of 100% after 6h of treatment with MD, whereas the reduction ranged between 69% (after 1h) and 97% (after 8h) upon the WD.

Figure 5.2 shows the variation from baseline of LEA in AC (**A**), LEA in AC1 (**B**), LEA in AC2 (**C**), OEA in AC (**D**), OEA in AC1 (**E**), OEA in AC2 (**F**), PEA in AC (**G**), PEA in AC1

(**H**), PEA in AC2 (**I**), total NAEs in AC (**J**), total NAEs in AC1 (**K**), total NAEs in AC2 (**L**), upon MD and WD. Panels A, D, G and J refer to mean concentrations of the two donors; panels B, E, H, K refer to concentrations in donor 1; panels C, F, I, L refer to to concentrations in donor 2.

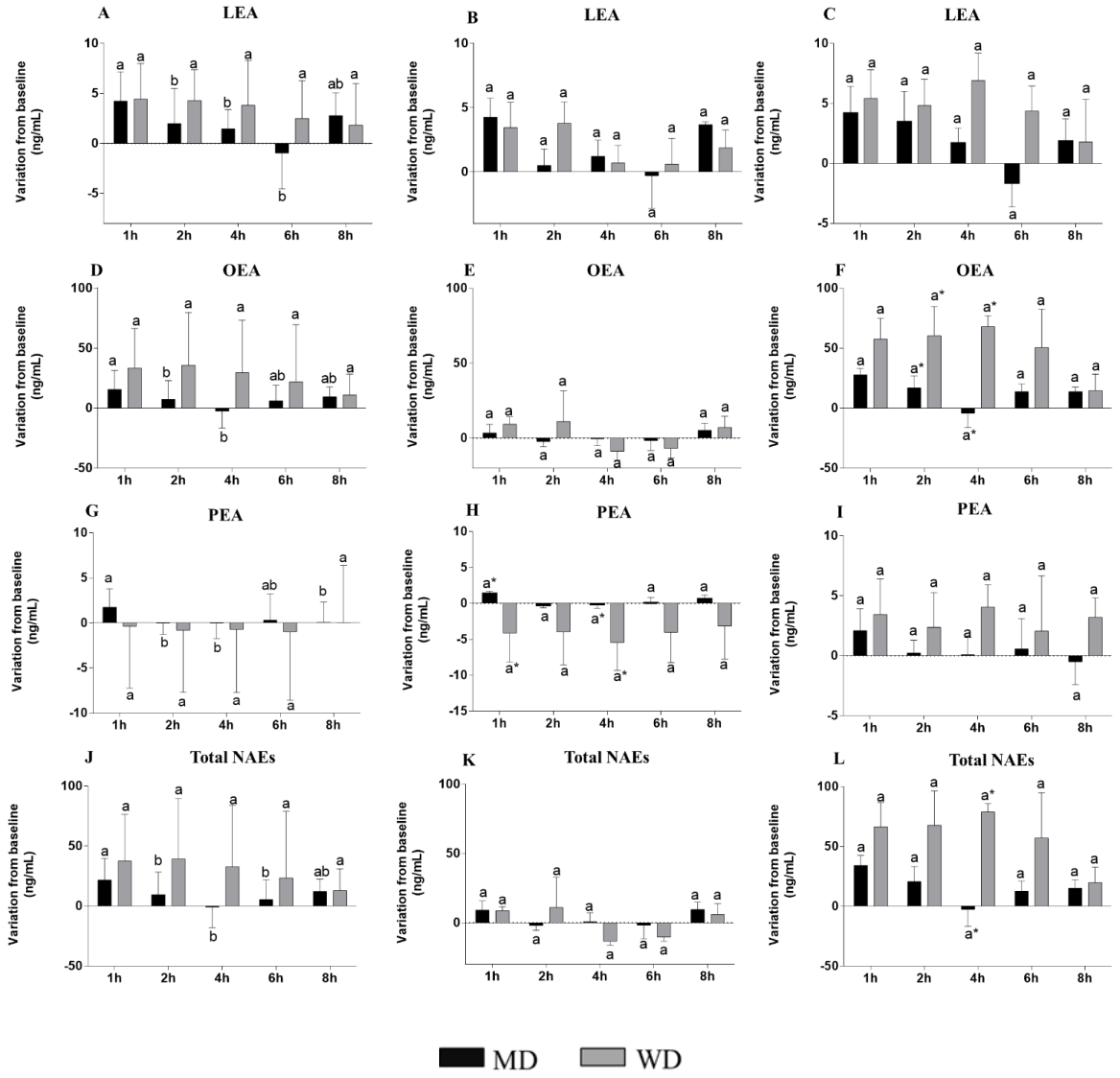

Figure 5.2. Variation from baseline of LEA (A) in AC, LEA in AC1 (B) and LEA in AC2 (C), OEA in AC (D), OEA in AC1 (E) and OEA in AC2 (F), PEA in AC (G), PEA in AC1 (H), PEA in AC2 (I), total NAEs in AC (J), total NAEs in AC1 (K), total NAEs in AC2 (L). Panels A, D, G and J refer to mean concentrations of the two donors; panels B, E, H, K refer to concentrations in donor 1; panels C, F, I, L refer to to concentrations in donor 2. The statistical tests used for analysis were Mann-Whitney and Wilcoxon tests. Different letters indicate significant differences ($p<0.05$) within the same diet. Asterisks indicate significant differences ($p<0.05$) between MD and WD (number of replicates=3).

No difference between the diets for LEA bioaccessibility in AC was found. A reduction of mean concentration of LEA at 2h, 4h and 6h *vs* 1h with MD was observed (p=0.028; p=0.028; p=0.028) (**Figure 5.2A**), whereas in WD no difference was found.

No significant effect of time in individual donors was evidenced (**Figure 5.2B-C**). However, WD determined a higher bioaccessibility of LEA in AC of donor 2 than of donor 1 after 4h (p=0.049), whereas with MD no difference between donors was observed.

Compared to the concentration in SI, the concentration of LEA in AC was reduced by a minimum of 79% after 1h to a maximum of 105% after 6h of treatment with MD, whereas the reduction ranged between 71% (after 1h) and 88% (after 8h) upon the WD.

Similarly to LEA, no difference between the diets for OEA bioaccessibility in AC was found but reduction over time of OEA at 2 h and 4h *vs* 1h with MD was observed (p=0.046) (**Figure 5.2D**).

Looking at OEA profile in individual donors (**Figure 5.2E-F**) it is evident that donor 2 (**Figure 5.2F**) showed a higher bioaccessibility of OEA from both diets than donor 1. Specifically, a higher bioaccessibility in donor 2 *vs* donor 1 was found with MD after 1h (p=0.049) and with WD after 1h, 4h and 6h (p=0.049) of fermentation. Moreover, at 2h and 4h fermentation OEA concentration in AC of donor 2 from WD was higher than from MD (p=0.049).

Compared to the concentration in SI, the concentration of OEA in AC was reduced by a minimum of 34% after 1h to a maximum of 110% after 4h of treatment when the system was fed with MD, whereas the reduction ranged between 9% (after 2h) to 72% (after 8h) upon WD.

PEA was the least bioaccessible NAE. No difference between diets was found but a trend towards a reduction over time of PEA at 2h, 4h 6h, 8h *vs* 1h with MD was observed (p=0.028, p=0.046, p=0.028 and p=0.046, respectivey) (**Figure 5.2G**).

That difference was exclusively due to the donor 1 (**Figure 5.2H**) who also showed a smaller reduction of baseline concentration with MD than with WD after 1h and 4h fermentation (p=0.049; p=0.049).

A higher bioaccessibility in donor 2 *vs* donor 1 was found with WD after 4h (p=0.049) Compared to the concentration in SI, the concentration of PEA in AC was reduced by a minimum of 44% after 1h to a maximum of 101% after 4h fermentation with MD, whereas the reduction ranged between 99% minimum (after 8h) to 137% (after 6h) upon the WD.

Similarly to OEA (that was the most abundant NAE in AC), no difference was found between the two diets for bioaccessibility of total NAEs (**Figure 5.2J**) which mainly reflected those of donor 2.

Specifically a significantly higher bioaccessibility of total NAEs was recorded from WD than from MD after and 4h fermentation (p=0.049) in donor 2 (**Figure 5.2L**); no difference between diets in donor 1 was found. Moreover, a higher bioaccessibility in donor 2 *vs* donor 1 was found with MD after 1h (p=0.049) and with WD after 1h, 4h and 6h (p=0.049) of fermentation.

Compared to the concentration in SI, the concentration of total NAEs in AC was reduced by a minimum of 54% after 1h to a maximum of 102% after 4h fermentation with MD, whereas the reduction ranged between 32% (after 2h) to 78% after (8h) upon the WD.

Concentration of ECs and NAEs in TC

Figure 5.3 shows the variation from baseline of ECs in TC (**A**), ECs in transverse colon of donor 1 TC1 (**B**), ECs in transverse colon of donor 2 TC2 (**C**).

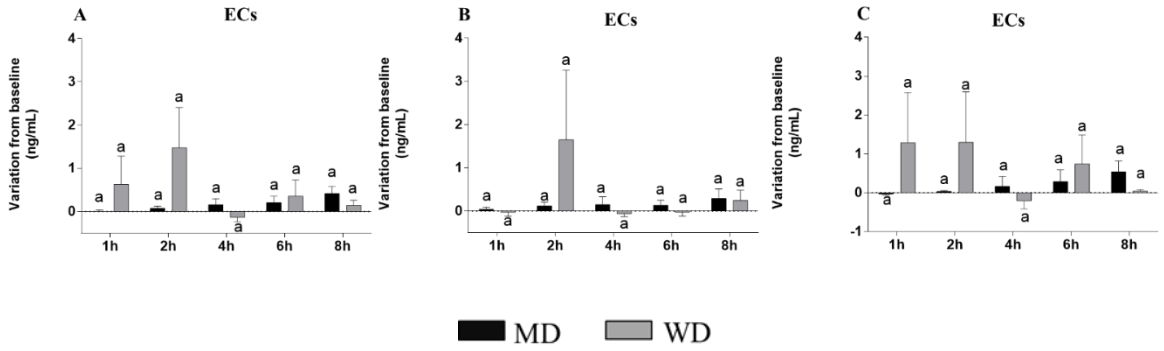

Figure 5.3. Variation from baseline of ECs in TC (mean of the two donors) (A), in TC1 (donor 1) (B) and in TC2 (donor 2) (C). The statistical tests used for analysis were Mann-Whitney and Wilcoxon tests. Different letters indicate significant differences ($p<0.05$) within the same diet. (number of replicates=3).

No difference was found for the variation from baseline of the ECs in TC between diets and within diet over time. No difference was found in TC1 and TC2 (**Figure 5.3B-C**) and between donors with the same diet.

Compared to the concentration in SI, the concentration of ECs in TC was reduced by a minimum of 89% after 8h to a maximum of 100% after 1h of treatment with MD, whereas the reduction ranged between 92% (after 2h) and 101% (after 4h) upon the WD.

Figure 5.4 shows the variation from baseline of LEA in TC (**A**), LEA in TC1 (**B**), LEA in TC2 (**C**), OEA in TC (**D**), OEA in TC1 (**E**), OEA in TC2 (**F**), PEA in TC (**G**), PEA in TC1 (**H**), PEA in TC2 (**I**), total NAEs in TC (**J**), total NAEs in TC1 (**K**), total NAEs in TC2 (**L**), upon MD and WD.). Panels A, D, G and J refer to mean concentrations of the two donors; panels

B, E, H, K refer to concentrations in donor 1; panels C, F, I, L refer to to concentrations in donor 2

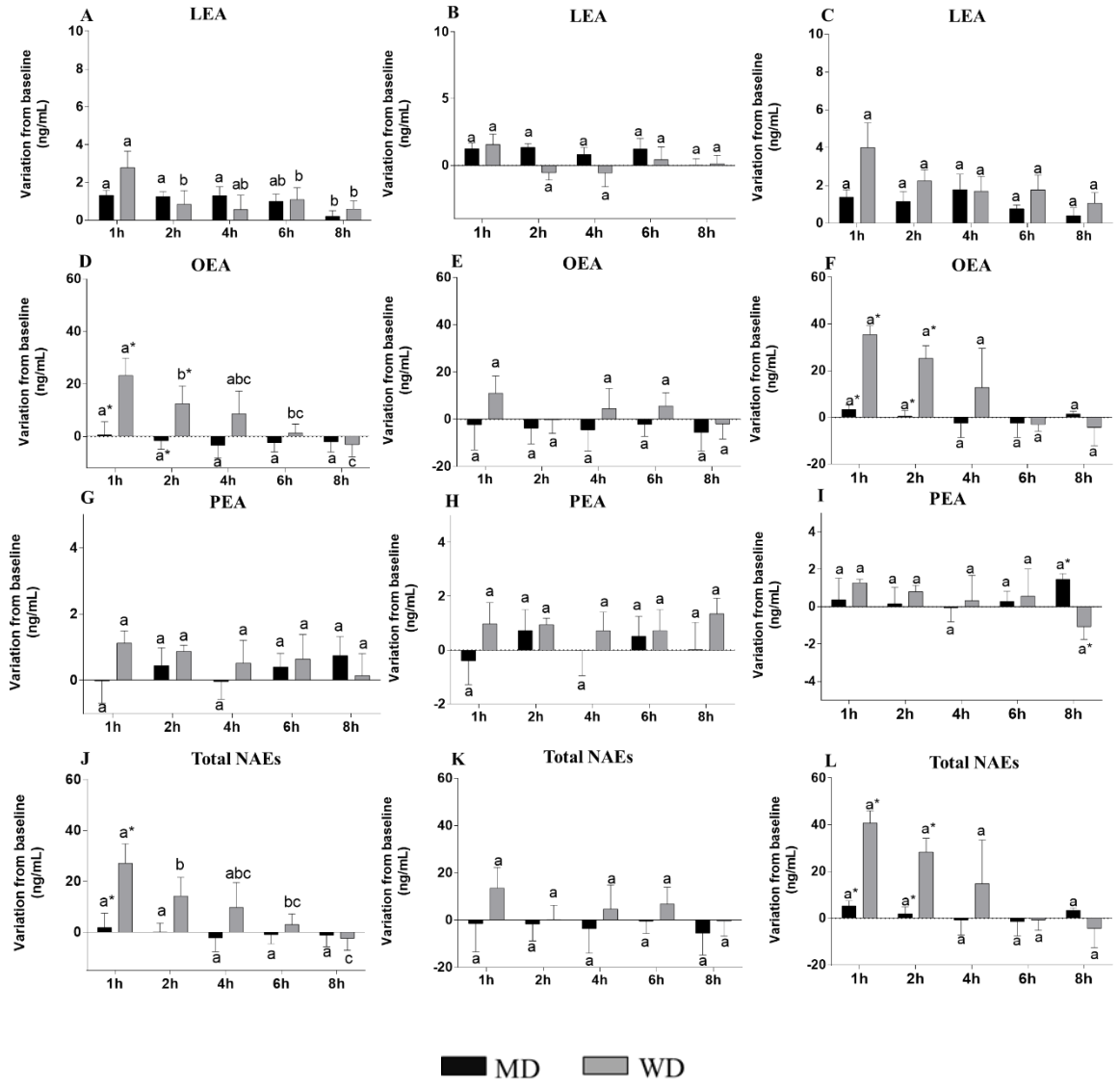

Figure 5.4. Variation from baseline of LEA (A) in TC, LEA in TC1 (B) and LEA in TC2 (C), OEA in TC (D), OEA in TC1 (E) and OEA in TC2 (F), PEA in TC (G), PEA in TC1 (H), PEA in TC2 (I), total NAEs in TC (J), total NAEs in TC1 (K), total NAEs in TC2 (L). Panels A, D, G and J refer to mean concentrations of the two donors; panels B, E, H, K refer to concentrations in donor 1; panels C, F, I, L refer to concentrations in donor 2. The statistical tests used for analysis were Mann-Whitney and Wilcoxon tests. Different letters indicate significant differences ($p<0.05$) within the same diet. Asterisks indicate significant differences ($p<0.05$) between MD and WD (number of replicates=3).

No difference between the diets for LEA bioaccessibility in TC was found. A reduction of mean concentration of LEA at 8h *vs* 1h, 2h and 4h with MD (p=0.027; p=0.026; p=0.027) and at 2h, 6h and 8h *vs* 1h with WD was observed (p=0.028; p=0.028; p=0.028) (**Figure 5.4A**).

Looking at LEA profile in individual donors (**Figure 5.4B-C**) it is evident that donor 2 (**Figure 5.2C**) showed a higher bioaccessibility of LEA from both diets than donor 1 with a significant difference from WD. Compared to the concentration in SI, the concentration of LEA in TC was reduced by a minimum of 93% after 1h to a maximum of 99% after 8h of treatment with MD, whereas the reduction ranged between 82% (after 1h) and 97% (after 4h) upon the WD

The bioaccessibility of OEA in TC was higher from WD than from MD at 1h (p=0.049) and 2h (p=0.049). When the system was fed with WD a reduction of mean concentration of OEA at 2h, 6h and 8h *vs* 1h (p=0.027;p=0.046; p=0.028), at 8h *vs* 2h (0.028) and 8h *vs* 4h (p=0.046) was observed. (**Figure 5.4D**).

Considering the OEA profile in individual donors it is evident that donor 2 showed a higher bioaccessibility of OEA.

In particular donor 2 showed a higher bioaccessibility of OEA than in donor 1 from WD after 1h (p=0.049) and 2h (p=0.049). Furthermore, in donor 2 the bioaccessibility of OEA was higher in WD than in MD at 1h (p=0.049), at 2h (p=0.049) (**Figure 5.4F**).

Compared to the concentration in SI, the concentration of OEA in TC was reduced by a minimum of 98% after 1h to a maximum of 115% after 4h of treatment when the system was fed with MD, whereas the reduction ranged between 41% (after 1h) to 108% (after 8h) upon WD.

Considering PEA bioaccessibility in TC no difference was found between diets (**Figure 5.4G**). In donor 1 the bioaccessibility was not influenced by diet (**Figure 5.4H**), while in donor 2 the bioaccessibility was higher from MD than from WD after 8h (p=0.049) (**Figure 5.4I**).

The bioaccessibility of this compound with WD it was higher in donor 1 than in donor 2 after 8h ($p=0.046$).

Compared to the concentration in SI, the concentration of PEA in TC was reduced by a minimum of 76% after 8h to a maximum of 102% after 4h of fermentation with MD, whereas the reduction ranged between 58% (after 1h) to 95% (after 8h) upon WD.

Considering total NAEs, the bioaccessibility in TC was higher from WD than from MD at 1h ($p=0.046$). Total NAEs bioaccessibility from WD was reduced at 2h, 6h and 8h *vs* 1h, ($p=0.028$; $p=0.046$; $p=0.028$) and at 8h *vs* 2h ($p=0.028$) (**Figure 5.4J**).

For the total NAEs profile in individual donors (**Figure 5.4K-L)** it is evident that donor 2 (**Figure 5.4L**) showed a higher bioaccessibility than donor 1 particulary with significant effect after 1h and 2h ($p=0.049$; $p=0.049$) of feeding the system with WD.

Furthermore, in donor 2 the bioaccessibility of total NAEs was higher from WD than from MD at 1h ($p=0.049$), at 2h ($p=0.049$).

Compared to the concentration in SI, the concentration of total NAEs in TC was reduced by a minimum of 96% after 1h to a maximum of 105% after 4h using the MD, whereas the reduction ranged between 53% (after 1h) to 104% (after 8h) upon WD.

Concentration of ECs and NAEs in DC

Figure 5.5 shows the concentration of the ECs in DC (**A**), in DC of donor 1 (DC1) (**B**) and donor 2 (DC2) (**C**) upon MD and WD.

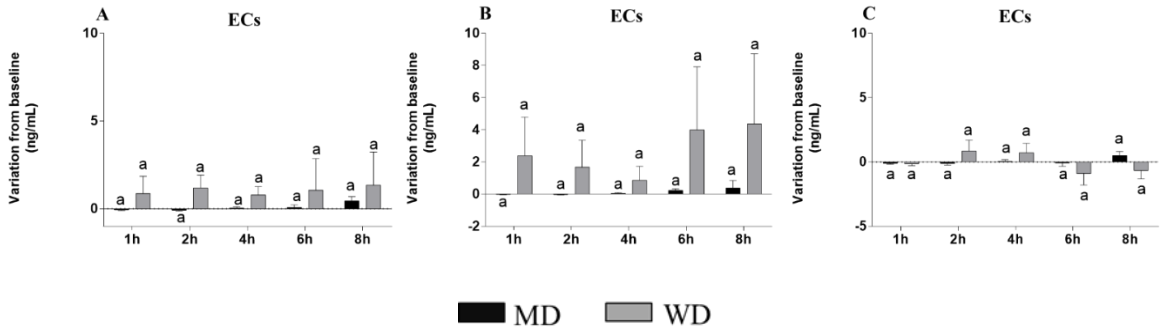

Figure 5.5 Variation from baseline of ECs in DC (mean of the two donors) (A), in DC1 (donor 1) (B) and in DC2 (donor 2) (C). The statistical tests used for analysis were Mann-Whitney and Wilcoxon tests.Different letters indicate significant differences ($p<0.05$) within the same diet. (number of replicates=3).

No difference was found for the variation from baseline of the ECs in DC between diets and within diet over time. No difference was found in donor 1 and donor 2 (**Figure 5.5B-C**), but donor 1 showed a higher bioaccessibility than donor 2 that was significant after 6h ($p=0.046$), of feeding system with WD, whereas no difference with MD was found.

Compared to the concentration in SI, the concentration of ECs in DC was reduced by a minimum of 88% after 8h to a maximum of 102% after 2h of treatment with MD, whereas the reduction ranged between 92% (after 2h) and 96% (after 4h) upon the WD.

Figure 5.6 shows the variation from baseline of LEA in DC (**A**), LEA in DC1 (**B**), LEA in DC2 (**C**), OEA in DC (**D**), OEA in DC1 (**E**), OEA in DC2 (**F**), PEA in DC (**G**), PEA in DC1 (**H**), PEA in DC2 (**I**), total NAEs in DC (**J**), total NAEs in DC1 (**K**), total NAEs in DC2 (**L**), upon MD and WD. Panels A, D, G and J refer to mean concentrations of the two donors; panels B, E, H, K refer to concentrations in donor 1; panels C, F, I, L refer to to concentrations in donor 2

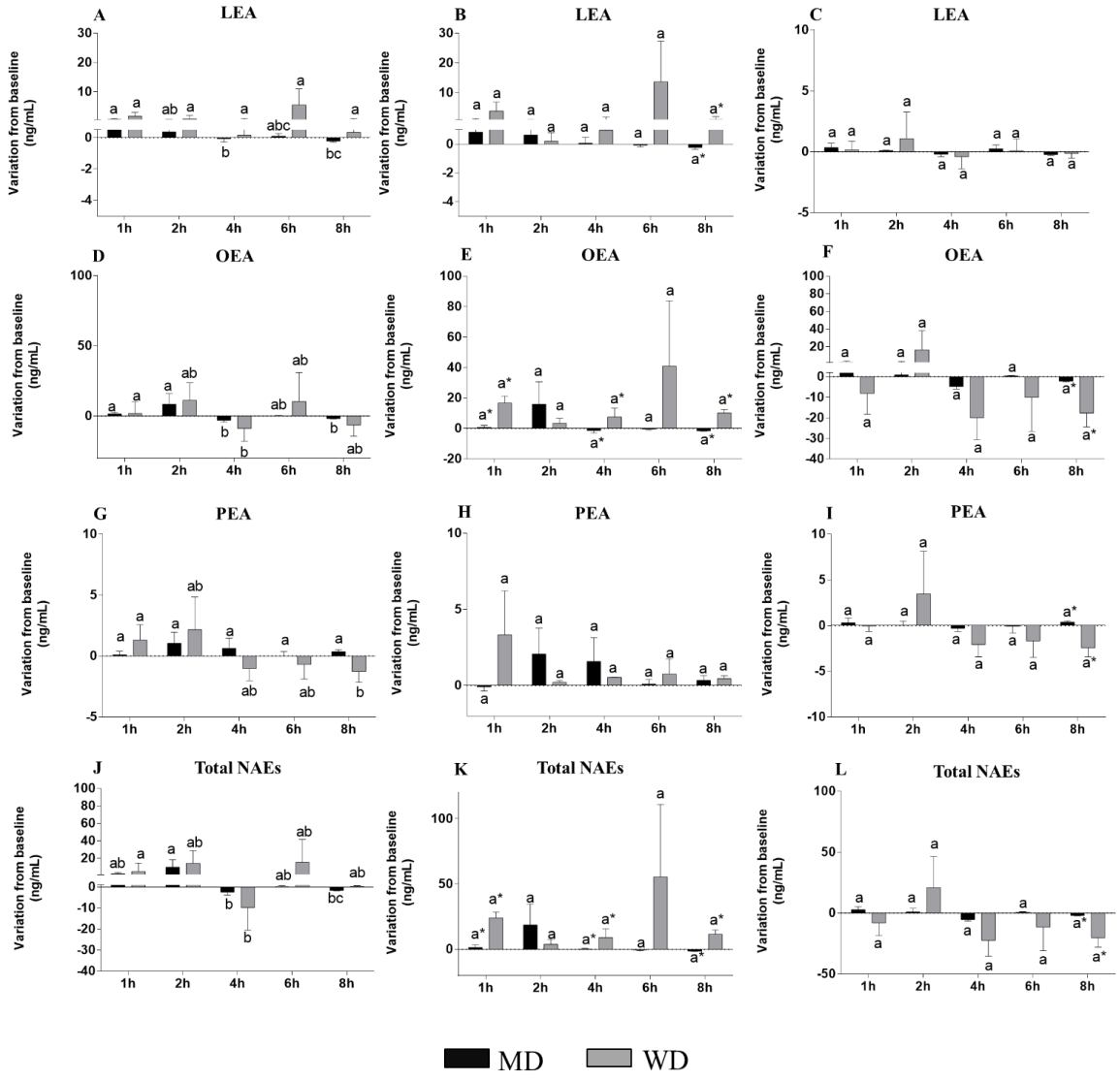

Figure 5.6. Variation from baseline of LEA (A) in DC, LEA in DC1 (B) and LEA in DC2 (C), OEA in DC (D), OEA in DC1 (E) and OEA in DC2 (F), PEA in DC (G), PEA in DC1 (H), PEA in DC2 (I), total NAEs in DC (J), total NAEs in DC1 (K), total NAEs in DC2 (L). Panels A, D, G and J refer to mean concentrations of the two donors; panels B, E, H, K refer to concentrations in donor 1; panels C, F, I, L refer to to concentrations in donor 2. The statistical tests used for analysis were Mann-Whitney and Wilcoxon tests.Different letters indicate significant differences ($p<0.05$) within the same diet. Asterisks indicate significant differences ($p<0.05$) between MD and WD (number of replicates=3).

No difference between the diets for LEA bioaccessibility in DC was found (**Figure 5.6A**).

In donor 1 a higher bioaccessibility from WD than from MD at 8h (p=0.046) was observed. Furthermore a higher bioaccessibility in donor 1 than in donor 2 when the system was fed with MD after 2h (p=0.049) was found.

Compared to the concentration in SI, the concentration of LEA in DC was reduced by a minimum of 97% after 1h to a maximum of 101% after 8h of fermentation with MD, whereas the reduction ranged between 72% (after 6h) to 96% (after 2h) upon WD.

No difference between diets for the bioaccessibility of OEA in DC (**Figure 5.6D**) was found, whereas a reduction of mean concentration with MD at 4h *vs* 1h (p=0.046), at 8h *vs* 1h (p=0.046), at 4h *vs* 2h (p=0.043) and at 8h *vs* 1h (p=0.046) was found.

Donor 1 showed a higher bioaccessibility than donor 2 that was significant after 1h (p=0.049), after 4h (p=0.049), after 6h (p=0.049) and after 8h (p=0.049) of feeding system with WD.

In donor 1 the bioaccessibility was higher from WD than from MD after 1h (p=0.049), 4h (p=0.049) and 8h (p=0.049), whereas in donor 2 the bioaccessibility was higher from MD than from WD after 8h (p=0.049).

Compared to the concentration in SI, the concentration of OEA in DC was reduced by a minimum of 79% after 2h to a maximum of 101% after 8h using the MD, whereas the reduction ranged between 69% (after 6h) to 98% (after 1h) upon WD.

No difference between the diets for PEA bioaccessibility in DC was found (**Figure 5.6G**) and a reduction after 8h *vs* 1h (p=0.027) with WD was found.

The bioaccessibility of PEA in donor 2 was higher from MD than from WD after 8h (p=0.049).

Compared to the concentration in SI, the concentration of PEA in DC was reduced by a minimum of 84% after 2h to a maximum of 101% after 6h during the fermentation with MD, whereas the reduction ranged between 75% (after 1h) to 102% (after 6h) upon WD.

The bioaccessibility of total NAEs was not different between the two diets (**Figure 5.6J**), but a reduction after 8h *vs* 1h, 2h and 6h (p=0.028; p=0.046; p=0.046) and after 4h *vs* 2h (p=0.028) from MD was found, while a reduction after 4h *vs* 1h (p=0.046) from WD was found.

In donor 1 the bioaccessibility was higher from WD than from MD after 1h (p=0.049), after 4h (p=0.049) and after 8h (p=0.049) (**Figure 5.6 K**).

A higher bioaccessibility of total NAEs in donor 1 than in donor 2 after 4h (p=0.046) and 8h (p=0.049) from MD and after 1h (p=0.049) and 8h (p=.0.049) from WD was found.

Compared to the concentration in SI, the concentration of total NAEs in DC was reduced by a minimum of 84% after 2h to a maximum 106% after 4h using the MD, whereas the reduction ranged between 76 (after 6h) to 93% (after 1h) upon WD.

5.3.1 Assay FAAH inhibition

Figure 5.7 shows the results of the FAAH inhibition assay**.** No difference between the two diets was found. The highest percentage of inhibition was found in small intestine before the fermentation of both diets.

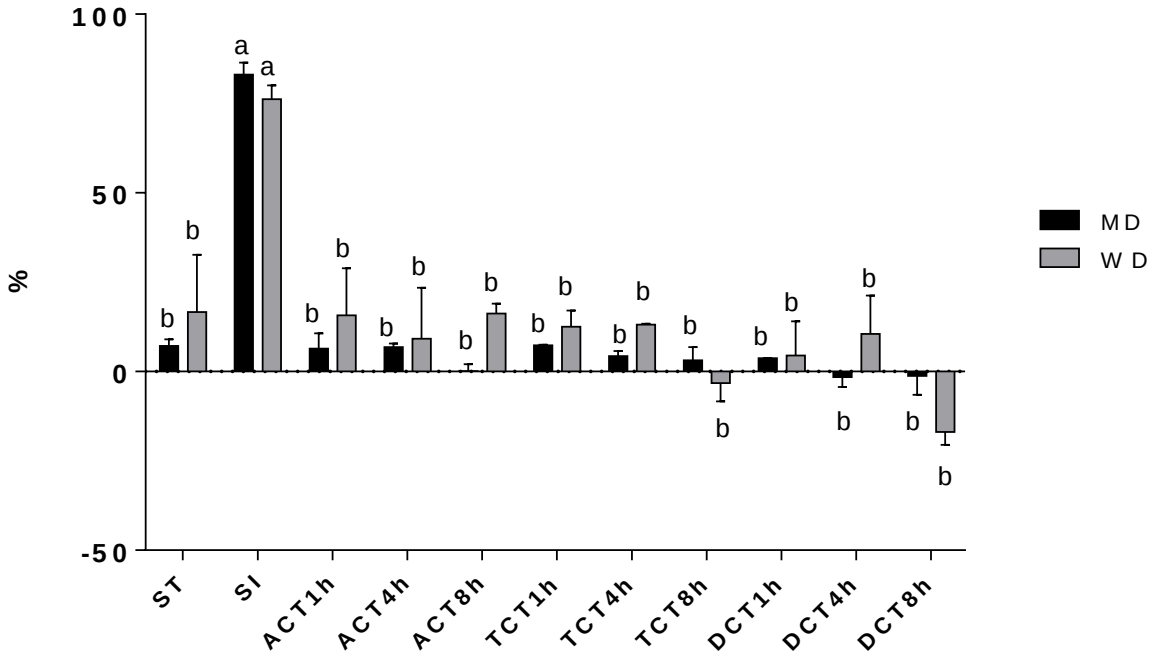

Figure 5.7. Percentage of inhibiton in supernatants of MD and WD deriving from SHIME®. The statistical tests used for analysis were Mann-Whitney test. Different letters indicate significant differences ($p<0.05$) between MD and WD (number of replicates=3).

5.4 Discussion

In the present study the bioaccessibility of NAEs and ECs deriving from MD and WD along GIT *in vitro* using SHIME® was assessed.

Data showed a low bioaccessibility of NAEs and ECs from both the diets. Moreover, considering the amount of NAPEs (24 mg with MD and 9 mg with WD) added with the two diets and the NAEs retrieved in the system, a weak activity of microbial NAPE-PLD and/or an over-activity of FAAH could be hypothesised.

Previous studies showed the link between diet and ECs system and between ECs system and gut microbiota, but the mechanisms of this link is not yet clarified.

Data showed a lack of influence of diets on the concentrations of NAEs and ECs in the colon but an influence of the fecal donors. Indeed, the donor 2 was always associated with higher variation from baseline of total NAEs and particularly OEA in AC with MD and with WD.

In TC the variations from baseline of LEA, OEA and total NAEs was higher in donor 2 than in donor 1 upon WD, while the variation from baseline of PEA was higher in donor 1 than in donor 2. No difference was found upon MD.

This behavior changed in DC where the higher variation from baseline of LEA upon MD, OEA and total NAEs upon WD was higher in donor 1 than in donor 2.

Such differences between donors were likely due to a different microbiota composition. Indeed, the ratio of *Firmicutes/Bacteroidetes* was 1.55 in AC1 and 2.43 in AC2, 0.85 in TC1 and 0.70 in TC2, as well as 1.12 in DC1and 0.85 in DC2 (*data not shown*).

A recent study showed that the treatment with OEA (10 mg/kg) for 11 days changed the composition of gut microbiota in mice. OEA treatment shifted the *Firmicutes/Bacteroidetes* in favour of *Bacteroidetes* and reduced *Firmicutes*, the ratio was similar to the ratio of TC1 and DC2 (Di Paola *et al.*, 2018).

In contrast, another study showed that a treatment with PEA (30 mg/kg) for 10 days changed the gut microbiota with an increase of *Firmicutes* and a reduction of *Bacteroidetes* determining a ratio that was similar to AC1 (Cristiano *et al.*, 2018).

In humans, an alteration of nutrient load of the diet changed the gut microbiota. Jumpertz & coworkers (2011) observed that in normal weight subjects a 20% increase in *Firmicutes* and a corresponding decrease in *Bacteroidetes* were associated with an increased energy harvest of 150 kcal (Jumpertz *et al.*, 2011). De Filippo *et al*. (2010) showed that the *Firmicutes* are most abundant in European children compared to Burkina Faso children, and the ratio *Firmicutes/Bacteroidetes* was higher in European children than in Burkina Faso children. In humans, the ratio *Firmicutes/Bacteroidetes* differs in obese and lean humans and this ratio decreased with weight loss after a low calories diet (Ley *et al.*, 2006).

A study conducted in patients with ulcerative colitis (UC) and Crohn's disease following a MD showed that the subjects with UC increased the levels of NAPEs in feces suggesting a modification of the ECs system and demonstrating the link of the ECs system with gastrointestinal functions (Santoru *et al.*, 2017).

Another study showed that the administration of engineered NAPE-expressing *E.coli* Nissle 1917 for 8 weeks reduced obesity in mice fed with a high fat diet. Furthermore, it was shown an increase of hepatic NAEs after the administration of P-NAPE-ECn suggesting the possibility that NAPEs were absorbed by colon and transformed in NAEs to entry in portal circulation (Chen *et al.*, 2014).

Furthermore the synthesis and the degradation of NAEs and ECs in intestine was shown to be modulated by gut microbiota, where high-fat diet increased NAPE-PLD activity and reduced FAAH in intestine and in adipose tissue of mice, whereas the treatment with prebiotics reduced these effects. Anyway, the mechanisms by which the gut microbiota participate in this regulation is not yet understood (Cani, 2012).

PEA is an anti-inflammatory compound that has beneficial effects on colon inflammation. The action of this compound is terminated by hydrolysis of FAAH and NAAA. Alhouayek, *et al.* (2015) showed that in murine model of inflammatory bowel diseases (IBDs) the NAAA inhibition increased the levels of PEA in the colon and reduced the colon inflammation similar to the action of PEA, while FAAH inhibition did not increase PEA levels in the colon. So NAAA is the enzyme responsible for the control of PEA in the colon.

Regarding the FAAH inhibition, it was highest in SI for both the diets. It was hypothesised that the presence of bile salts and pancreatin in the system might increase the release of compounds having an inhibitory activity on FAAH.

Indeed, many *in vitro* studies showed the presence of natural inhibitor of FAAH present in plants (Thors *et al.,* 2007; Thors *et al.,* 2008).

New compounds such as *N*-arachidonoyl-amino acids are able to inhibit FAAH *in vitro* (Cascio *et al.,* 2004). Recently, Donvito & coworkers (2018) showed that the N-oleoyl-glycine (O-Gly) is able to inhibit FAAH *in vitro* with a IC_{50} 8.65 μM and the FAAH inhibition produced by maximum dose tested (50 μM) was 89.4%. However, after the treatment with the two diets O-Gly was present at same concentration in the supernatants deriving from AC, TC and DC (data not shown), thus its influence in our results could be excluded.

5.5 Conclusions

This study showed that both the concentration of NAEs and FAAH inhibition upon a MD and a WD were not influenced by diets but mainly by fecal donor. This was associated with the different composition of the gut microbiota of the two donors.

Considering the amount of NAPEs (precursors of NAEs) added to the diet and the amount of NAEs retrieved, a weak activity of NAPE-PLD was suggested.

Further *in vivo* studies are necessary to confirm the influence of gut microbiota on metabolic fate of NAEs and ECs along gastrointestinal tract.

5.6 Abbreviations

NAEs: N-acylethanolamines; ECs: endocannabinoids, NAPEs: N-acylphosphatidylethanolamine; NAT: N-acytransferase; FFAs: free fatty acids; NAPE-PLD: N-acyl phosphatidylethanolamine-specific phospholipase D; FAAH: fatty acid amide hydrolase; NAAA: N-acyethanolamine-hydrolyzing acid amidase; ECs: endocannabinoids; AEA: anandamide; 2-AG: 2-arachidonoylglycerol: CB: cannabinoid receptor; PPAR-α: peroxisome proliferator-activated-α; GPR: G- protein-coupled receptors; GIT: gastrointestinal tract; GLP-1: glucagone like peptide-1; GIP: glucose dependent insulinotropic peptide; PYY; peptide YY; LPS: lipopolysaccharide; GIT: gastrointestinal tract; SHIME®: Simulator of the Human Intestinal Microbial; AC: ascending colon; TC: transverse colon; DC: descending colon; SI: small intestine; MD: Mediterranean diet; WD: Western diet; LEA: Linoleoylethanolamide; PEA: Palmitoylethanolamide; OEA: Oleoylethanolamide. SEM: standard error of means; O-Gly: N-oleoyl-glycine.

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Chapter 6. Summary, conclusions and future perspectives

NAPEs, NAEs and ECs are bioactive molecules that participate to the hedonic and homeostatic phenomena underpinning food intake.

NAEs have a wide range of biochemical effects and derive from hydrolysis of NAPEs. This reaction is catalysed by the enzyme NAPE-PLD present in many plant and animal tissues. Subsequently, NAEs are hydrolysed in mammal tissues and in plants by FAAH which leads to the formation of free fatty acids and ethanolamines (Coulon *et al.,* 2012).

Animal studies showed that NAEs and ECs are involved in different biological pathways since they are agonists of cannabinoid receptor-1 (CB1), transient receptor potential vanilloid 1 (TRPV1) and the peroxisome proliferator-activated receptor-α (PPAR-α). Moreover, NAEs are also able to link GPR119 which is expressed on cells in the small intestine, colon and stomach (Hansen & Diep, 2009).

The receptors are involved in food intake and in blood glucose control due to the regulation from the gastrointestinal tract (GIT) of GLP-1 secretion (Izzo & Sharkey, 2010).

Although NAPEs, NAEs and ECs are compounds widespread in nature, only few studies reported the amount of ECs and NAEs in some food products (Di Marzo *et al.,* 1998; Chapman *et al.,* 1999; Venables *et al.,* 2005). Anyway, the possibility that some of these food-derived compounds act through the receptors present along the GI mucosa depends from their survival to the degrading enzymes (Izzo & Sharkey, 2010).

The gut microbiota is able to control levels of ECs in both the gut and the adipose tissue by regulating NAPE-PLD, CB1 and FAAH expression in obese mice (Muccioli *et al.,* 2010). In humans, a relationship between the changes in the gut microbiota and in the ECs system tone have not yet demonstrated.

The studies described in this PhD thesis investigated the presence of NAPEs, NAEs and ECs in foods and tested their bioaccessibility in the oral cavity during food mastication *in vivo* and

in the GIT *in vitro* to evaluate their potential availability to activate the receptors present on the alimentary canal mucosa.

In **Chapter 1** the mechanisms involved in gut-brain axis and nutrient sensing were described, by mainly focusing on the ascertained and potential role of NAPEs, NAEs and ECs in the physiological systems underpinning homeostatic and hedonic control of food intake.

In **Chapter 2** the concentration of NAPEs, NAEs and ECs in 43 foods of different origin was assessed and the daily intakes upon a Mediterranean (MD), Vegetarian (VD) and Western diet (WD) were calculated. Data showed that NAPEs and NAEs were more abundant in vegetal than in animal food products. Furthermore, NAPEs daily intake was higher in MD (263 mg/day) and VD (242 mg/day) compared to WD (163 mg/day); also NAEs daily intake was higher in MD (0.25 mg/day) and VD (0.28 mg/day) compared to WD (0.08 mg/day); conversely ECs daily intake was higher in WD and MD (0.17 mg/day) than in VD (0.01 mg/day). These amounts may be enough to activate receptors along GIT.

In fact, *in vitro* studies showed that NAEs activate GPR119 with an EC_{50} between 65 µg/L and 1.0 mg/L for OEA, 180 µg/L for LEA and 250 µg/L for PEA (Hansen *et al.*, 2011; Overton *et al.*, 2006); while Fu *et al.* (2003) also showed that OEA can activate PPAR-α with an EC_{50} of 39 µg/L influencing food intake and lipid metabolism.

Therefore, data suggested that NAPEs and NAEs travelling in the GIT upon food consumption may contribute to nutrient metabolism, to the regulation of food intake and body weight as well as to the gut permeability and GIT motility through the nutrient sensing in the gut. Further studies are necessary to define the contribution of these compounds on the benefits elicited by MD and VD *in vivo*.

An overall graphical summary of **chapter 2** was shown in **Figure 6.1**.

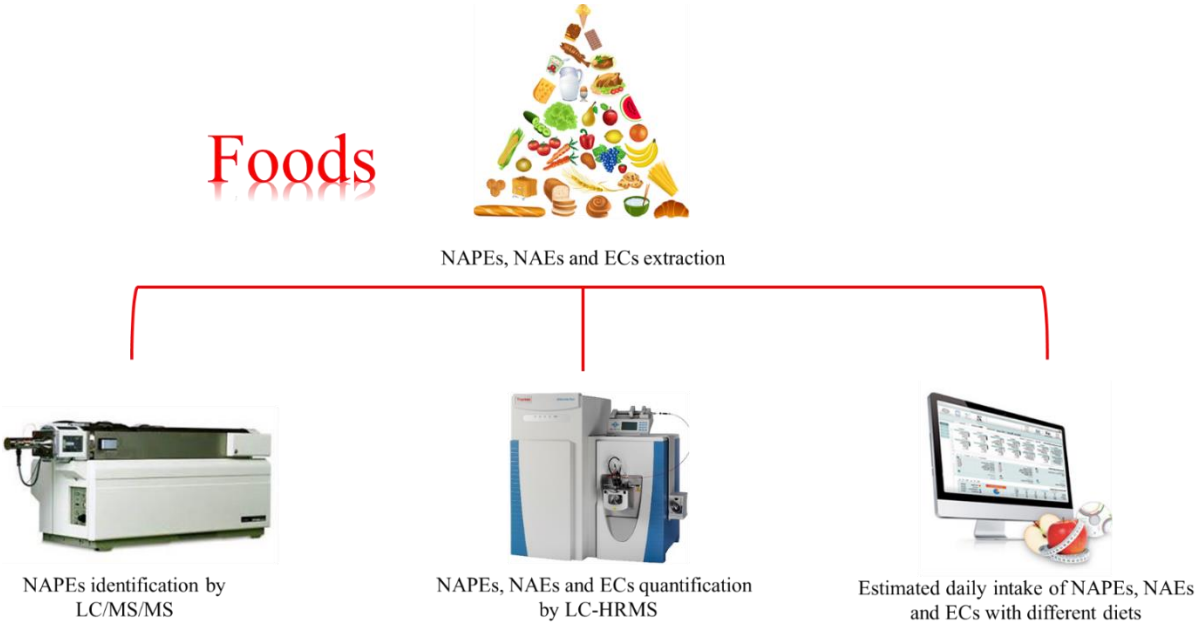

Figure 6.1. Graphical summary of Chapter 2: extraction of NAPEs, NAEs and ECs from foods and estimation of daily intakes of these compounds from different diets.

In the **Chapter 3** a sham feeding study was described. It showed the concentration of NAEs in stimulated saliva of healthy normal weight subjects. Interestingly, salivary concentration of NAEs increased upon food mastication compared to the mastication of a parafilm piece (stimulated saliva). The concentration of NAEs in the oral cavity within the first hours after food mastication was influenced by the type of dietary fiber (3% of β-glucan or whole-wheat bran) used for the preparation of biscuits.

The post-prandial concentration of salivary oleoylethanolamide (OEA) was associated with the sensations of fullness and satiety as well as the liking of the biscuits for sweetness. These links could explain the potential role of salivary OEA upon food preference and individual choice.

In **Figure 6.2** the graphical summary of this chapter was shown.

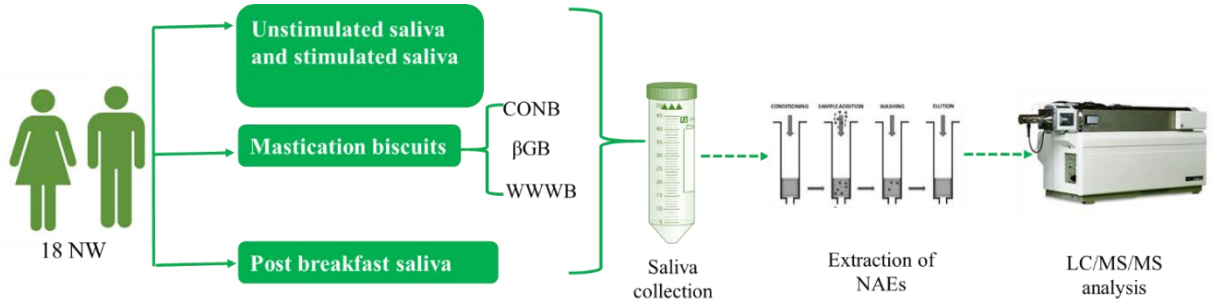

Figure 6.2. Graphical summary of Chapter 3: salivary NAEs upon mastication of different types of biscuit and in postprandial phase

In the **Chapter 4** another sham feeding study was described. It aimed at evaluating the influence of individual nutritional status on bioaccessibility (release in saliva) of NAPEs and NAEs upon mastication of three biscuits differing for amount and type of fat. To this purpose three types of biscuits were prepared with 8% of extravirgin olive oil (EVOB), 8% of palm oil (PALMB) and without added fat (CONB). Data showed that the obese (OB) subjects had a higher release of NAPEs in unstimulated saliva than normal weight (NW) subjects, while the concentration of NAEs in both unstimulated saliva and stimulated saliva was higher in OB than in NW subjects.

During the mastication of biscuits a higher concentration of salivary NAPEs and NAEs than a no food condition in both NW and OB subjects was found. An overall graphical summary of this chapter is shown in **Figure 6.3**.

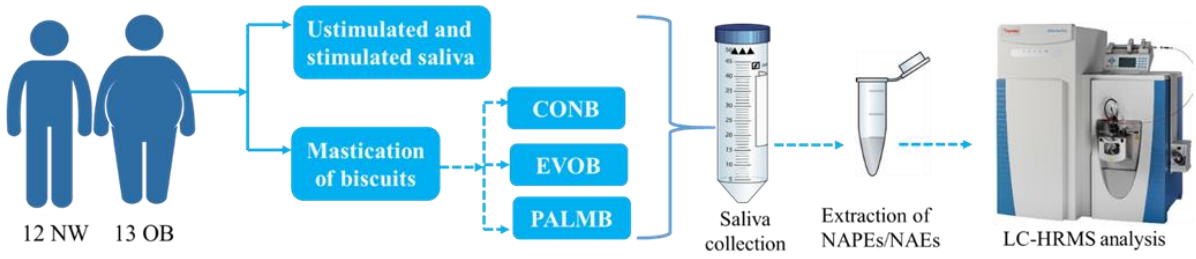

Figure 6.3. Graphical summary of Chapter 4: salivary NAPEs and NAEs upon mastication of biscuits prepared with different fats.

In a **Chapter 5** the bioaccesibility of NAEs and ECs along the gastrointestinal tract *in vitro* using Simulator of the Human Intestinal Microbial Ecosystem (SHIME®) simulating both physiological conditions and the microbial ecosystem found in the ascending (AC), transverse (TC) and descending (DC) colon was assessed. To this purpose we prepared two different diets: Mediterranean (MD) and Western diet (WD) and the supernatants deriving from the different phase of colon were analysed by HRMS analysis to measure NAEs and ECs. Data showed that the diets did not influence the concentration of these compounds, but the microbiota composition could influence the release of compounds in the colon phase. In **Figure 6.4** the graphical summary of this chapter was shown.

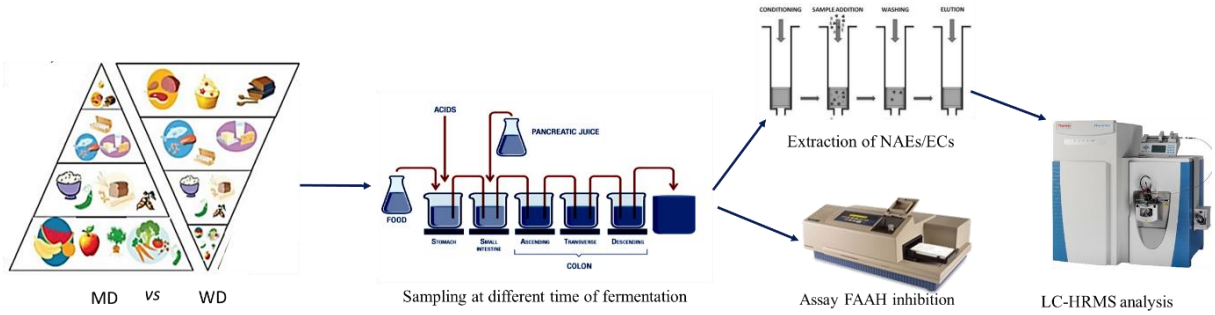

Figure 6.4. Graphical summary of Chapter 5: study of the bioaccessibility of NAEs and ECs from a Mediterranean (MD) and a Western diet (WD) along the gastrointestinal tract was assessed *in vitro* by using SHIME®.

Considering the results obtained from the experiments performed in this PhD thesis it is possible to conclude that:

- NAPEs, NAEs and ECs are present at different concentrations in many food products.
- *In vivo* studies showed that these compounds are released from food matrix during the mastication and that other food components (fibers and fats) as well as the individual nutritional status of individuals may influence both the delivery of these compounds from the food (bioaccessibility) as well as their persistence in the oral cavity after eating; this might influence liking of food and subsequent food intake.
- During *in vitro* digestion with SHIME® the concentration of NAPEs, NAEs and ECs was hypothesised to be majorly influenced by the microbiota composition inside the system acting on the biosynthesis and degradation of NAEs and ECs arriving into the colon, independently from the diet.
- Specifically designed studies are necessary to elucidate better the influence of enzymatic activity of NAPE-PLD and FAAH at different sites of the alimentary canal and in the gut microbiota to ascertain the metabolic fate of dietary NAPEs, NAEs and ECS *in vivo* and to possibly find dietary strategy to modulate the concentration of these bioactive compounds at intestinal level.

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