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In vivo food digestibility: a physiological approach for new product development

Tutor:
Ch.ma Prof.ssa Paola Vitaglione

Candidato:
Silvia Tagliamonte

Coordinatore: Ch.ma Prof.ssa Amalia Barone

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Table of contents

Chapter 1	General introduction	1
Chapter 2	Melanoidins from coffee and bread differently influence energy intake: A randomized controlled trial of food intake and gut-brain axis response	27
Chapter 3	Digestion of milk proteins affects the gastro-intestinal discomforts after milk consumption in healthy subjects: implications of the gut microbiome	55
Chapter 4	Mediterranean diet consumption affects the endocannabinoid system in overweight and obese subjects: possible links with gut microbiome, insulin resistance and inflammation	86
Chapter 5	Endocannabinoids, endocannabinoid-like molecules and their precursors in human small intestinal lumen and plasma: does diet affect them?	113
Chapter 6	General discussion	138

Abstract

In the last decades a worrisome increase in overweight/obesity, related chronic diseases and gastrointestinal discomforts (GID) prevalence has been observed. Overwhelming evidence showed that diet and lifestyle are the major drivers of these conditions underlying the lack of suitability of a 'one size fits all' approach. Inter-individual differences in genetics, body system functioning, gut microbiome and lifestyle drive personalized postprandial response to the same food/diet affecting health and wellbeing in the long-term. Therefore, developing personalised dietary advice or functional foods tailored to consumers is a promising strategy to improve human health. To do that, medicine-based evidence obtained by a **multi-system approach in well characterized people** are mandatory in order to identify the physiological markers/mechanisms of health and wellbeing in body systems that can be modulated by specific nutrients/foods/diets.

This dissertation takes up that challenge by focusing on **microbiome-gut-brain axis** and several molecules **known mediators of human health, digestion process and sensations** such as gastrointestinal hormones, neuropeptides, endocannabinoids and endocannabinoid like molecules and microbial metabolites. The specific objective was to unravel the physiological mechanisms underpinning satiety and post-prandial/long-term wellbeing in healthy, overweight/obese, ileostomy subjects by using a **multi-system approach** to design new tailored food products. To this purpose three randomized clinical trials (RCTs) and one observational study were performed. Two RCTs focused on **the effect of nutrient digestion on satiety and wellbeing after consumption** of **coffee and bread melanoidins** enriched-bread compared to a conventional bread in healthy subjects, and **cow's milk** in healthy and lactose tolerant subjects suffering with milk-related GID. The other two studies focused on **the long-term effect of diet on gut microbiome and/or endocannabinoid system**: one RCT addressed the effect of a **Mediterranean diet** consumption on gut microbiome and the circulating levels of endocannabinoids and endocannabinoid like molecules in overweight/obese subjects, whereas the observational study investigated the effect **of individual habitual diet** on ileal fluids levels of endocannabinoids and endocannabinoid like molecules in subjects with ileostomy.

ECS mediators have a pleiotropic activity in the body being tightly involved with appetite sensations and obesity development. Indeed, one of the leading causes of obesity is the overeating which may be attenuated by providing satiating foods high in fiber. Coffee and bread melanoidins, are formed at the last step of Maillard rection during food processing and share physiochemical, biochemical and biological properties with dietary fiber.

Moreover, they are well known to enhance flavour of food, which is an important factor for personalisation of food. Thus, the first RCT (**Chapter 2**) was performed to answer to the question of whether melanoidins affect post-prandial appetite sensations and energy intake and how. A meal study with a three-arms crossover design was performed in 14 healthy subjects to investigate the satiety effect of coffee melanoidins (CM) or bread melanoidins (BM) and the postprandial responses of 21 gut-brain appetite modulators. CM significantly reduced daily energy intake by 26% and lowered blood glucose peak, insulin, α-melanocyte stimulating hormone, orexin-A, β-endorphin, and blunted the response of three N-acylethanolamines (NAEs) versus BM. Findings of this study suggest the use of CM as food ingredient for the management of body weight and insulin resistance.

Besides dietary fibers, proteins and -bioactive peptides (BAPs) may be promising functional ingredients. However the relationships between protein digestion, hormonal, metabolic and endocannabinoid responses and individual post-prandial sensations was underexplored. Therefore in another *in vivo* study (**Chapter 3**) the question of whether cow's milk protein digestion affect postprandial discomforts and, eventually, the underpinning mechanisms in healthy and lactose tolerant subjects was investigated. A meal study was performed with, 20 habitual milk consumers (HMC) and 19 non-habitual milk consumers (NHMC) who were asked to consume 250 mL milk and collect blood, urine and feces. Data showed in NHMC a lower and slower appearance of milk-derived peptides in plasma (independently of intestinal permeability) and higher scores of GID. Compared to HMC, NHMC showed a lower proteolytic capacity of gut microbiome that was likely shaped by the habitual diet and contributed to the slower protein digestion later after milk consumption.

Since the ECS has been shown to be at the crossroad between gut microbiome and host metabolism, an 8-week RCT (**Chapter 4**) was performed to answer to the question of whether a switch from a western diet (WD) to a mediterranean diet (MD) was effective in modulating plasma concentration of ECs, NAEs and gut microbiome. To test this hypothesis 82 overweight and obese subjects were randomised to consume an MD tailored to their habitual energy and macronutrient intake (n=43) or to maintain their habitual diet (n=39). ECS and NAEs, metabolic and inflammatory markers and gut microbiome were monitored over the study period. The results showed that MD affected circulating ECS mediators and increased *Akkermansia muciniphila* gut levels. The EC tone at baseline drove an individualized response to an MD towards an amelioration of insulin sensitivity and inflammation. These findings reveal opportunities in the context of personalized nutrition laying the foundation for RCT specifically designed to build and validate integrative ECS-model to shape gut microbiome.

Noteworthy, a recent study showed that ECs and NAEs are also present in food at different extent, and it has been estimated they may be sufficient to elicit a physiological response along the GIT. In this scenario an observational study (**Chapter 5**) was conducted to answer to the last question of whether the habitual diet affect ECS mediators in the human intestinal lumen. Ileal fluids and blood samples from 35 subjects with ileostomy were analysed for their content in ECs and NAEs. Data showed that diet affects ECS mediators in plasma and intestinal lumen. Moreover, NAEs present in the intestinal lumen at concentrations that may be sufficient to activate the receptors lining on the gastro-intestinal mucosa and modulating appetite. These findings are preliminary for application in food innovation.

The last chapter (**Chapter 6**) is a general discussion of the overall findings as well as future perspectives based on the generated knowledge.

In conclusion, this thesis provided new evidence in the frame of food innovation and personalized nutrition by using a multi-system approach monitoring postprandial and long-term biological responses to meals or diets orchestrated by gut-brain axis and gut microbiome markers in different target populations.

Abstract

Negli ultimi decenni è stato osservato un preoccupante aumento del sovrappeso/obesità, delle annesse malattie croniche e dei disturbi gastrointestinali (GID). Evidenze schiaccianti hanno dimostrato che la dieta e lo stile di vita sono i principali fattori determinanti di queste condizioni, sottolineando la mancata efficacia di un approccio "universale". Le differenze interindividuali nella genetica, nel funzionamento del sistema corporeo, nel microbioma intestinale e nello stile di vita determinano una risposta postprandiale personalizzata allo stesso cibo/dieta che incide sulla salute e sul benessere a lungo termine. Pertanto, sviluppare consigli alimentari personalizzati o alimenti funzionali su misura per i consumatori è una strategia promettente per migliorare la salute umana. Per fare ciò, le evidenze mediche ottenute con un approccio multi-sistema in persone ben caratterizzate sono fondamentali per identificare i marcatori/meccanismi fisiologici di salute e benessere nei sistemi corporei che possono essere modulati da specifici nutrienti/alimenti/diete.

Questa tesi raccoglie questa sfida focalizzandosi sull'asse microbioma-intestino-cervello e su diverse molecole note come mediatori della salute umana, del processo di digestione e delle sensazioni come gli ormoni gastrointestinali, i neuropeptidi, gli endocannabinoidi e le molecole endocannabinoidi-simili e i metaboliti microbici. L'obiettivo specifico è stato quello di svelare i meccanismi fisiologici alla base della sazietà e del benessere post-prandiale/lungo termine in

soggetti sani, sovrappeso/obesi, ileostomizzati, utilizzando un approccio multi-sistema per progettare nuovi prodotti alimentari su misura. A questo scopo sono stati eseguiti tre studi clinici randomizzati (RCT) e uno studio osservazionale. Due RCT si sono concentrati sull'effetto della digestione dei nutrienti sulla sazietà e il benessere dopo il consumo di caffè e pane arricchito di melanoidine rispetto a un pane convenzionale in soggetti sani, e di latte vaccino in soggetti sani e tolleranti al lattosio affetti da GID legata al consumo di latte. Gli altri due studi si sono concentrati sull'effetto a lungo termine della dieta sul microbioma intestinale e/o sul sistema endocannabinoide: un RCT è stato eseguito per valutare l'effetto del consumo di una dieta mediterranea sul microbioma intestinale e sui livelli circolanti di endocannabinoidi e di molecole endocannabinoidi-simili in soggetti sovrappeso/obesi, mentre lo studio osservazionale ha studiato l'effetto della dieta abituale individuale sui livelli di fluidi ileali di endocannabinoidi (ECs) e molecole endocannabinoidi-simili (NAEs) in soggetti con ileostomia.

I mediatori del sistema endocannabinoide (ECS) svolgono un'attività pleiotropica nell'organismo, essendo strettamente coinvolti nelle sensazioni di appetito e nello sviluppo dell'obesità. Infatti, una delle cause principali dell'obesità è la sovralimentazione che può essere attenuata fornendo alimenti sazianti ad alto contenuto di fibre. Le melanoidine del caffè e del pane si formano nell'ultima fase della reazione di Maillard durante la lavorazione degli alimenti e condividono proprietà fisiochimiche, biochimiche e biologiche con la fibra alimentare. Inoltre, sono ben noti per migliorare il sapore del cibo, che è un fattore importante per la personalizzazione degli alimenti. Così, il primo RCT (**Capitolo 2**) è stato eseguito per rispondere alla domanda se le melanoidine influenzano le sensazioni di appetito post-prandiale e l'introito alimentare e come. Un "pasto test" con un disegno crossover a tre bracci è stato consumato da 14 soggetti sani per indagare l'effetto saziante di pane con melanoidine del caffè (CM) o melanoidine del pane (BM) e le risposte postprandiali di 21 modulatori intestinali dell'appetito e neuropeptidi. Il CM ha ridotto significativamente l'assunzione giornaliera di energia del 26% e ha abbassato il picco di glucosio nel sangue, l'insulina, l'ormone melanotropo-α, l'orexina-A, la β-endorfina e la risposta di tre N-acetilanolamine (NAE) rispetto al BM. I risultati di questo studio suggeriscono l'uso del CM come ingrediente alimentare per il controllo del peso corporeo e dell'insulino-resistenza.

Oltre alle fibre alimentari, le proteine e i peptidi bioattivi (BAP) sembrano essere ingredienti promettenti per lo sviluppo di alimenti funzionali. Tuttavia le relazioni tra la digestione delle proteine, le risposte ormonali, metaboliche, degli endocannabinoidi e le sensazioni post-prandiali individuali sono state poco esplorate. Pertanto, un altro studio *in vivo* (**Capitolo 3**) è

stato eseguito per rispondere alla domanda se la digestione delle proteine del latte influisce sui GID postprandiali in soggetti sani e tolleranti al lattosio e, eventualmente, investigarne i meccanismi sottostanti. È stato eseguito un pasto test su 20 consumatori abituali di latte (HMC) e 19 consumatori non abituali di latte (NHMC) ai quali è stato chiesto di consumare 50 mL di latte vaccino, sono stati raccolti campioni di sangue, urine e feci. I dati hanno mostrato una più bassa e lenta comparsa di peptidi derivati dal latte nel plasma di soggetti NHMC (indipendentemente dalla permeabilità intestinale) in concomitanza con più alti punteggi di GID. Rispetto a HMC, NHMC hanno mostrato una minore capacità proteolitica del microbioma intestinale probabilmente influenzato dalla dieta abituale e ha contribuito alla digestione proteica più lenta dopo il consumo di latte.

Poiché l'ECS ha dimostrato di essere al crocevia tra il microbioma intestinale e il metabolismo dell'ospitante, un RCT di 8 settimane (**Capitolo 4**) è stato eseguito per rispondere alla domanda se un passaggio da una dieta occidentale (WD) a una dieta mediterranea (MD) fosse efficace nel modulare la concentrazione plasmatica di ECs, NAEs e microbioma intestinale. Per testare questa ipotesi, 82 soggetti sovrappeso e obesi sono stati randomizzati per il consumo di una MD adattata alla loro abituale assunzione di energia e macronutrienti (n=43) o a mantenere la loro dieta abituale (n=39). ECs e NAEs, marcatori metabolici, infiammatori e microbioma intestinale sono stati monitorati durante il periodo di studio. I risultati hanno mostrato che MD ha influenzato i mediatori circolanti del ECS e ha aumentato i livelli di *Akkermansia muciniphila* nell'intestino. Il tono endocannabinoide al basale ha inoltre guidato una risposta individualizzata a una dieta mediterranea verso un miglioramento dell'insulino-resistenza e dell'infiammazione. Questi risultati rivelano opportunità nel contesto della nutrizione personalizzata che pone le basi per RCT specificamente progettati per costruire e convalidare un modello che integri ECS e risposta del microbioma intestinale.

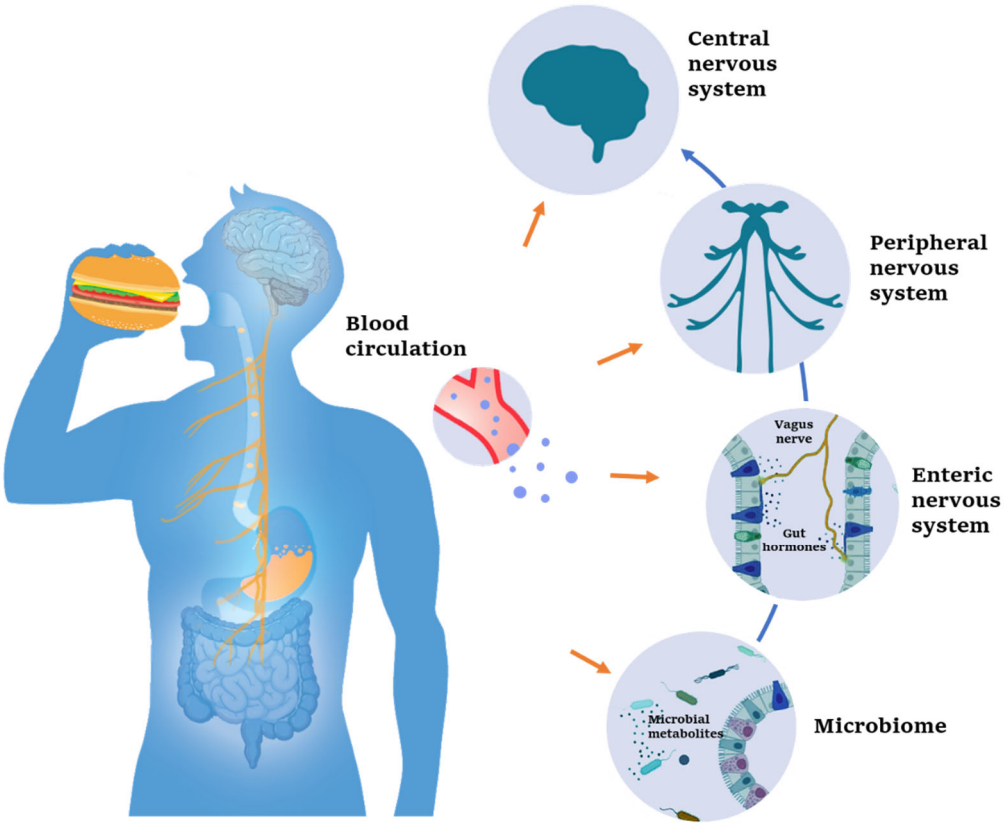
Un recente studio ha dimostrato che ECs e NAEs sono presenti in misura diversa negli alimenti, ed è stato stimato che potrebbero essere sufficienti per suscitare una risposta fisiologica lungo il GIT. In questo scenario è stato condotto uno studio osservazionale (**Capitolo 5**) per rispondere all'ultima domanda se la dieta abituale influenza i mediatori di ECS nel lume intestinale umano. I fluidi ileali e i campioni di sangue di 35 soggetti con ileostomia sono stati analizzati per il loro contenuto in ECs e NAEs. I dati hanno mostrato che la dieta influenza i mediatori di ECS nel plasma e nel lume intestinale. Inoltre, i NAEs sono presenti nel lume intestinale a concentrazioni che possono essere sufficienti per attivare i recettori localizzati sulla mucosa gastrointestinale e modulare l'appetito. Questi risultati sono ancora preliminari per l'applicazione nell'innovazione alimentare.

L'ultimo capitolo (**Capitolo 6**) è una discussione generale dei risultati complessivi così come le prospettive future basate sulla conoscenza generata.

In conclusione, questa tesi ha fornito nuove prove nel quadro di innovazione alimentare e nutrizione personalizzata utilizzando un approccio multi-sistema monitorando le risposte postprandiali e biologiche di lungo termine ai pasti o diete orchestrate da marker dell'asse intestino-cervello e microbioma intestinale in diverse popolazioni target.

Chapter 1

General introduction



1.1 Systems' biology: Personalized nutrition for the future

In 2019, according to the obesity report of the Organisation for Economic Co-operation and Development (OECD) almost one in four people in OECD countries is currently obese. The obesity epidemic has far-reaching consequences for individuals and will claim more than 90 million lives in the next 30 years (OECD, 2019). Nutrition plays a pivotal role in the development of chronic diseases. Indeed, nutritionally unbalanced diets are positively associated with the risk of coronary heart disease, stroke, type 2 diabetes and obesity (Jayedi et al., 2020). Noteworthy, the majority of the nutritional research and the corresponding guidelines reached so far, focus on population averages (EFSA, 2017; Karpyn, 2018). However, 'one-size-fits-all' nutritional approach is inappropriate where the metabolic inter-individual variability is greater than the variability induced by diet interventions (Heinzmann et al., 2012). This observation paved the way to personalized approaches by considering the high inter-person variability in response to foods and weight-loss diets (Zeevi et al., 2015). For instance, some individuals can have exaggerated glycemic response to a banana but not to a biscuit, whereas some others may have an opposite response (Zeevi et al., 2015). Therefore, individual meal-specific responses are generally much more effective than scaling in achieving a weight loss (Berry et al., 2020).

Comprehensively, tailoring dietary recommendations to specific biological requirements based on a person's health status and goals is the key objective for personalized nutrition (Van Ommen et al., 2017). Since the evolution of biological complexity is an important outcome of the evolution process the biology behind these recommendations is complex (Werner et al., 2015) and any recommendation should account for multiple biological processes occurring simultaneously in various body districts (van Ommen et al., 2017). Therefore, a systems biology–based approach which cross-examine complex biological systems by combining several '-omics' disciplines is necessary to understand how diet may influence health and disease (Van Ommen et al., 2017; Badimon et al., 2017).

In this scenario, a systems biology–based approach can study how food impacts health status by differentiating responders from non-responders to nutritional intervention and identifying the dietary bioactive compounds responsible for the health outcomes (Odriozola et al., 2015; Kussmann et al., 2006).

1.2 Foodomics for personalized nutrition: how far are we?

In the past decades, human nutrition science has developed greatly by recognizing food not

only as a simple energy source, but also as a key player in maintaining health and in reducing the risk of diseases (Capozzi and Bordoni, 2013). In this scenario, food science has extended its boundaries to various "omics" techniques, including nutrigenetics, nutrimetabolomics, nutritranscriptomics, nutriproteomics and metagenomics (Chaudhary et al., 2021), which allowed the birth of foodomics. Foodomics is an inter-disciplinary approach accross food science, human nutrition, the individual, human health, and diseases aiming to optimize human health and wellbeing (Capozzi and Bordoni, 2013), which has emerged as a key tool to achieve efficient prevention of non-communicable diseases (NCDs) (Stewart-Knox et al., 2016). Besides diet, human gut microbiome is a key player in host's metabolic balance and have to be considered as an extra-genetic factor in the context of obesity, malnutrition and many chronic diseases (Cox et al., 2013; Claus et al., 2013). Indeed, gut microbiome is increasingly recognized to potentially impact human physiology by participating in digestion, the absorption of nutrients, shaping of the mucosal immune response and the synthesis or modulation of a plethora of potentially bioactive compounds (Kolodziejczyk et al., 2019). Dietary habits constitute a major factor shaping human gut microbiome (Cuevas-Sierra et al., 2019). Therefore, diet-induced microbiota alterations may be harnessed in order to induce changes in host physiology, including disease development and progression, and have a pivotal role in the development of personalized nutritional and precision medicine approaches (Kolodziejczyk et al., 2019; De Filippis et al, 2018).

Diet macronutrient composition, fiber content, the presence of phytochemicals and other bioactive compounds (i.e., polyphenols) can all affect gut microbiome that, in turn, modulates one or more physiological pathways and body systems (**Table 1**).

Hence, there is an increasing interest on functional foods which act on specifics metabolic pathways, such as polyunsaturated fatty acids (PUFAs) to reduce hypertriglyceridemia, bioactive peptides as anti-inflammatory, anti-hypertensive and antioxidants, antimicrobial source, or even probiotics and prebiotics which help to maintain a healthy gut ecosystem (Bersi et al., 2018; Claus et al., 2014). Thus, there is now increasing interest in the concept of personalized functional foods, which are formulated to fit to an individual's 'hyperbolome' harnessing the diet-microbiome interaction (Claus et al., 2014; Vimaleswaran et al., 2014).

Table 1. Effects of different diets and fibers on gut microbiota and their impact on host health (adatapted by Cuevas-Sierra et al., 2019)

Type of diet, diet composition and supplements	Effect on gut microbiota	Effect on host health	Reference
High-fiber			
Normal diet supplemented with 25 g nonstarch polysaccharide and 22 g resistant starch	↑ Abundance of *Rominococcus bromii*	High production of SCFAs with high-resistant-starch diet Increase in butyrate production (22%) *R. bromii* facilitates fermentation of carbohydrates (cellulose, pectin, and starch) and increases energy availability	(Abell et al., 2008)
High-fat diet supplemented with 10% inulin	↑ Production of SCFAs and ↑ bacterial proliferation	No increase in body fat/weight Increased expression of genes involved in hepatic lipogenesis (*Fasn*, *Gpam*) and improvement in the ω-6-to-ω-3 ratio	(Weitkunat et al., 2015)
500 g oat products/kg and 130 g wheat starch/kg	↑ *Bifidobacteria* ↑ Production of SCFAs	Increase in acetate production High excretion of bile acids Decrease in LDL cholesterol	(Drzikova et al., 2005)
10 g fiber/d (2.26%)	↑ *Bacteroides* and ↓ Firmicutes ↓ Enterobacteriaceae ↑ Production of SCFAs ↓ Abundance of *Shigella* and *Escherichia*	Prevention of some potentially pathogenic intestinal microbes causing diarrhea (i.e., a decrease in Enterobacteriaceae such as *Shigella* and *Escherichia*)	(De Filippo et al., 2010)
High-fat			
72% fat	↓ *Lactobacillus*, *Bifidobacterium*, and *Prevotella*	Increased intestinal permeability and association with inflammatory biomarkers (IL-1, TNF-α)	(Cani et al., 2008)
60% fat	↑ Firmicutes ↓ *Bacteroides* ↑ *Ruminococcus* and Rickenellaceae and ↓ Prevotellaceae	Increased intestinal permeability and inflammatory biomarkers (TNF-α, IL-β, and IL-6)	(Kim et al., 2012)
Western diet (13.1% protein, 60.6% fat, 26.3% carbohydrates, mainly saccharose)	↑ *Escherichia coli*, *Bacteroides*, and *Prevotella*	Increase of gut permeability and alteration in mucus layer	(Martinez-Medina et al., 2014)
High-protein diet			
53% protein	↓ *Clostridium coccoides*, *Clostridium leptum*, and *Faecalibacterium prausnitzii*	Increased production of butyrate Increased substrate availability	(Liu et al., 2014)
29% protein, 66% fat, 5% carbohydrates	↓ *Roseburia*	Decrease in total fecal SCFAs Increase in hazardous metabolites (*N*-nitroso compounds)	(Russell et al., 2011)
Artificial sweetener			
Chocolate with 22.8–45.6 g MTL, MTL and PDX, or MTL and resistant starch	↑ Fecal *Bifidobacteria*, *Lactobacilli*, propionate and butyrate after PDX treatment	No significant change in bowel habit or intestinal symptoms	(Beards et al., 2010)
Probiotics			
B. longum, *B. infantis*, *and B. breve, L. acidophilus*, *L. paracasei*, *L. delbrueckii* subsp. *bulgaricus*, and *L. plantarum*	↑ Fecal *Bifidobacteria*, *Lactobacilli*, *Streptococcus*, ↓ total coliforms, *Helicobacter pylori*, *Escherichia coli*	Decrese in fasting glucose, total cholesterol, tryglicerides, insulin resistance, and inflammatory biomarker CRP	Rajkumar et al., 2014
Polyphenols			
Cocoa flavanol	↑ Fecal *Bifidobacteria*, *Lactobacilli*	Decrese in total cholesterol, tryglicerides, and inflammatory biomarker CRP	Tzounis et al., 2011

[1]FASN, Fatty Acid Synthase; GPAM, Glycerol-3-Phosphate Acyltransferase; MTL, maltitol; PDX, polydextrose; TLR, Toll-like receptor; ↑, increased; ↓, decreased.

1.3 Gastrointestinal nutrient sensing: the "gut feeling" for food

Gastrointestinal tract (GIT) is the main endocrine organ in the human body. Various subtle chemosensory mechanisms along the GIT are constantly monitoring and conveying information about the concentration of nutrients, digestion products and microbial metabolites (Steensels and Depoortere, 2008). Chemosensory receptors are expressed in several cell types of the intestinal epithelium such as Paneth, goblet, tuft and enteroendocrine cells (EECs). Upon the activation of chemosensory signaling molecules by ligand binding, might initiate a cascade of events including the release of more than 20 different peptides modulating physiological pathways underpinning nutrient homeostasis and energy balance including satiety, mechanical and chemical digestion, as well as nutrient absorption, storage and utilization (McCauley et al., 2020). This process is known as "nutrient sensing".

Enterocytes represent 80% of small intestinal epithelial cells and their main function is to select and absorb nutrients on their apical side that express several transporters and export them basally (Steensels and Depoortere, 2008). **Figure 1a** depicts several transporters implicated in the uptake of nutrient metabolites such as sugars, amino acids, and fatty acids.

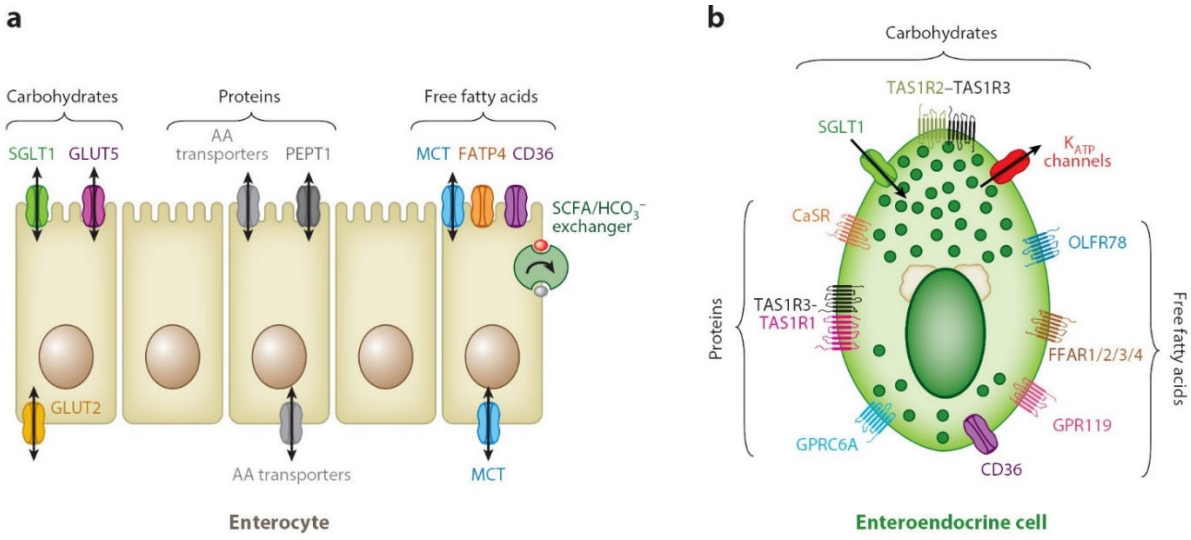


Figure 1: Chemosensing of different nutrients in enterocytes (a) and enteroendocrine cells (b) lining the gastrointestinal epithelium. Abbreviations: AA, amino acid; CaSR, calcium-sensing receptor; CD36, cluster of differentiation 36; FATP4, fatty acid transport protein 4; FFAR1/2/3/4, free fatty acid receptor 1/2/3/4; GLUT2/5, glucose transporter 2/5; GPR119, G protein–coupled receptor 119; GPRC6A: G protein–coupled receptor family C group 6 member A; KATP, ATP-sensitive potassium; MCT, monocarboxylate transporter; OLFR78, olfactory receptor 78; PEPT1/2, peptide transporter 1/2; $SCFA/HCO_3$ –, short-chain fatty acid bicarbonate exchanger; SGLT1/3, sodium-dependent glucose cotransporter 1/3; TAS1R1/2/3, taste 1 receptor family member 1/2/3. (Steensel & Depoortere 2018)

Enteroendocrine cells (EECs) are specialized cells found within the GIT which secrete >20 different peptides by sensing nutrients and other molecules while they pass through the gut (McCauley et al., 2020). At least, 12 subtypes of EECs have been described which are morphologically and biochemically similar to taste cells of the tongue taste bud cells, expressing similar hormones and receptors. Moreover, they are innervated with gustatory and vagal afferent nerve fibers and playi a key role in appetite, nutrient metabolism, gastrointestinal diseases, obesity, diabetes, immune-related diseases as well as food preferences (Depoortere, 2014; Steensel & Depoortere 2018; Berthoud et al., 2021a,b).

Figure 1b illustrates the processes underpinning the GI hormones response induced by the detection of carbohydrates, proteins and lipids in the GIT through the G-protein coupled receptors (GPCRs), other receptors like PPARs, and transporters.

Specifically, the ingested **carbohydrates** are broken down along the GIT into the monosaccharides, glucose, fructose and galactose thanks to a suite of enzymes. Glucose can be absorbed via sodium-glucose cotransporter (SGLT-1) present on the apical cell surface which uses the energy from a downhill sodium gradient to transport one glucose (or galactose) molecule with two Na^+ from the lumen into the enterocyte (McCauley et al., 2020). Besides SGLT-1, ~~sugars~~ non-caloric sweeteners are detected by the heterodimeric T1R2+T1R3 sweet receptor expressed throughout the GIT from the tongue taste cells to the large intestine (Mace et al., 2015; Nuemket et al., 2017), which are also known as the umami receptor or the metabotropic glutamate receptor. Moreover, glucose can also induce hormone secretion in a metabolism-dependent way as a result of higher ATP levels upon glucose metabolism in the EECs which in turn can affect ATP-sensitive potassium channels (KATP). Glucose sensing by the T1R2-T1R3, the glucose transporter SGLT1, and KATP channels mediate GLP-1 and GIP secretion (Mace et al., 2015; Steensel & Depoortere 2018).

On the other hand, the ingested **proteins** are broken down along the upper GIT into polypeptides, di- and tri-peptides, and amino acids (AA) by several proteases and can be sensed by several receptors depending on their size and AA composition (Mace et al., 2015; Steensel & Depoortere 2018). Upon digestion also bioactive peptides (BAPs) are formed. They are protein fragments (from two- up to twenty amino acid long) that exert a measurable biological effect on body functions and health (Tulipano et al., 2020). The trans-epithelial transport routes of peptides vary for different peptides. Four major mechanisms for peptide transport have been identified in the intestine: 1) peptide transporter-1 (PepT1), mediating the transport for di- and

tri-peptides, 2) sodium-coupled oligopeptide transporters 1 and 2 (SOPT1 and SOPT2), 3) paracellular passive transport, via intercellular junctions and 4) transcytosis (Daniel and Zietek, 2015; Chothe et al., 2011). Thereafter, di- and tri-peptides are sensed via the lysophosphatidic acid receptor 5 (LPA5R or GPR92/93), whereas AA are sensed by several taste GPCRs: the calcium-sensing receptor (CaSR), GPCR family C group 6 member A (GPRC6A), the heterodimer TS1R1+TS1R3 (Steensel & Depoortere 2018). It has been reported that protein hydrolysates (peptone), induce CCK release via GPR93 (GPR92 or LPAR5) (Choi et al., 2007). Conversely, di- and tri-peptides induce CCK secretion via CaSR while most of the AA stimulate GLP-1 secretion through GPRC6A and CCK through T1R1 + T1R3 and CaSR (Mace et al., 2015).

Dietary **triglycerides** are broken down to mono- or di-glycerides and free fatty acids (FFAs) by lipases. Similarly to peptides, they can be sensed by different receptors depending on their chain-length. For instance, short-chain FFAs (2-6 C) can be sensed by GPR41 (FFAR3) and GPR43 (FFAR2) while medium-chain FFAs (6-12 C) and long chain FFAs (>12 C) can be detected by GPR120 (FFAR4) and GPR40 (FFAR1) (Witkmap et al., 2018). Although all these receptors have been implicated in gut hormone release, GPR120 is the most extensively investigated one (Hirasawa et al., 2005; Witkamp, 2018; Iwasaki et al., 2014; Quesada-López et al., 2016). Monoglycerides and fatty acid metabolites, like 2-oleoyl glycerol, 2-palmitoylglycerol and 2-linoleoylglycerol, and oleoylethanolamide, are also known to bind GPR119 resulting in incretins-release such as GLP-1 and GIP (Cheng et al., 2015; Hansen et al., 2011; Moss et al., 2016). The physiological relevance of these fatty-acid amides will be further discussed in the next paragraph. Aside from the dietary fatty acids, short-chain FFAs produced by the gut microbiota can bind GPR41 and GPR43, thereby inducing the GLP-1 and PYY secretion (Hand et al., 2012; Tolhurst et al., 2012).

Along with macronutrients, chemosensors on EECs can also sense other compounds in the GIT eliciting gut hormone secretion. For instance, dietary bitter compounds can bind TAS2R bitter receptors (Steensel & Depoortere 2018). They are GPCRs comprising 25 functional members which exist as homo- or heterodimers. A large array of bitter compounds can induce GLP-1 and CCK and ghrelin secretion via TAS2R receptors (Mace et al., 2015). EECs can also sense phytochemicals derived from herbs and spices such as capsaicin found in chili peppers or cinnamaldehyde (cinnamon) (Holzer et al., 2011). These compounds can bind transient receptor potassium (TRP) channel consisting of 28 members in mammals (Holzer et al., 2011).

Thus, capsaicin can induce GLP-1 and PYY release through TRPV1 receptor (Smeets and Westerterp-Plantenga, 2009).

1.4 Microbiota-gut-brain axis: feeding behavior and gastrointestinal diseases

A complex crosstalk exists between intestinal bacteria, the gut and the central nervous system (CNS), referred to as the microbiota-gut–brain axis. This network includes the bidirectional communication among CNS, the enteric nervous system (ENS) which also contains intrinsic sensory neurons (referred to as a "second brain"), and neuroendocrine and neuroimmune pathways, as well as host metabolism (Gonzalez-Santana and Heijtz, 2020). Sensory neurons which are intrinsic primary afferent neurons innervate the ENS responding to mechanical and chemical stimuli via the vagus nerve (Steensel & Depoortere 2018). Moreover, EECs contains peptide-secreting vesicles within an axon-like basal process, called neuropod, which guide the secretion of hormones to those neurons innervating the small intestine and colon (Steensel & Depoortere 2018). **Figure 2** depicts the complex crosstalk network between microbiota-gut-brain axis. (). The working pathways use signals through neuronal circuits (e.g., bidirectional vagus nerve-to-brain communication, the ENS and neuropods), activation of immune responses (e.g., cytokine and chemokine release within the gut or elsewhere that subsequently influence the brain directly throughout the blood–brain barrier or indirectly via the vagus nerve), ECCs hormone signaling, and the production of microbial metabolites. In this respect, very relevant are bacterial fermentations leading to formation in the gut of short-chain fatty acids (SCFAs; propionate, butyrate, and acetate) and tryptophan-derived metabolites (Gonzalez-Santana and Heijtz, 2020).

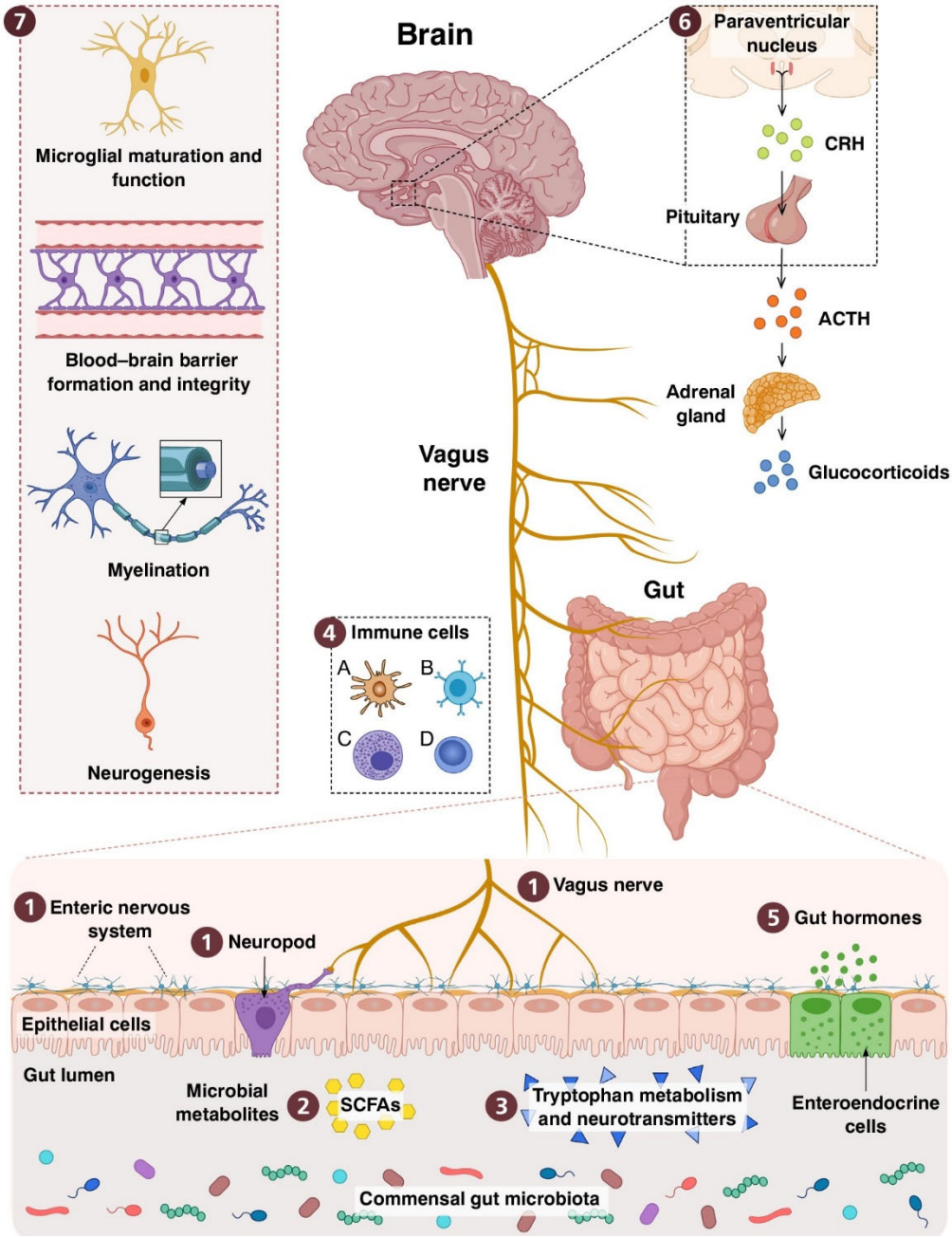


Figure 2: Overview of the potential pathways and molecules involved in the Microbiota–Gut–Brain Axis. There are several direct and indirect pathways involved in the bidirectional communication among microbiota, gut and brain, including signals carried by neuronal circuits [e.g., bidirectional vagus nerve-to-brain communication, the ENS, and neuropods (**1**)], the production of bacterial metabolites such as the SCFAs propionate, butyrate, and acetate (**2**), tryptophan metabolites and neurotransmitters (**3**), cytokines release by immune cells such as (A) dendritic cell, (B) B cell, (C) mast cell, and (D) T cell (**4**), and hormones secreted by EECs (**5**). Some of these molecules can activate the vagus nerve or reach the brain via the blood brain barrier and directly affect brain functions. Activation of the hypothalamic–pituitary–adrenal (HPA) axis is characterized by the release the adrenocorticotrophic hormone (CRH) in the pituitary gland which in turn stimulates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. Subsequently, ACTH stimulate the production and release of glucocorticoids which is corticosterone in mice and cortisol in humans, which play a key role in affecting gut physiology (e.g., modulating the intestinal epithelial barrier and immune responses) and gut microbiota composition (**6**). The gut microbiota has also been implicated in microglial maturation and function, blood–brain barrier formation and integrity,

myelination, and neurogenesis (**7**) (Gonzalez-Santana and Heijtz, 2020).

Microbiota-gut-brain axis is gaining growing attention due its involvement in eating behavior (homeostatic and hedonic), gastrointestinal motility, and inflammatory processes (van Son et al., 2021). Central regulation of food intake is crucial for energy homeostasis. Several neural circuits orchestrating feeding behavior overlap with the brain's reward system both anatomically and functionally (Rossi & Stuber, 2017). In vertebrates, food intake is regulated within two systems: the homeostatic and hedonic.

Homeostatic system: Homeostatic feeding is necessary for basic metabolic processes and modulate energy balance and energy metabolism by mediators such as leptin and ghrelin, on the other hand, hedonic feeding is driven by sensory perception or pleasure involving the motivational aspects of food (i.e., pleasure and reward) (Rossi & Stuber, 2017; van Son et al., 2021). It has been reported an interaction among these pathways as well as the interaction of the two with the gut microbiota (Lutter and Nestler, 2009; Berthoud et al., 2017). Ghrelin, an orexigenic hormone plays a key role in modulating the homeostatic eating. It is mainly produced in the stomach by the EECs, rises before meals and is suppressed after food ingestion (Williams et al., 2003). Ghrelin exerts physiological effects increasing gastric motility, gastric acid secretion, simulating food intake, however it is also involved in glucose metabolism, taste, and reward (Muller et al. 2015). Moreover, ghrelin passes the blood-brain barrier and activates orexigenic GABAergic neurons that express neuropeptide Y (NPY) and agouti-related protein (AgRP), which increase food intake and reduce energy expenditure during acute activation (van Son et al., 2021). Conversely, leptin is an anorexigenic hormone, which is produced by white adipose tissue and can cross the blood-brain barrier affecting the long-term energy balance (Jequier et al., 2002). Leptin suppresses hunger signals by inhibiting NPY/AgRP neurons (Elias et al., 1999). A decrease in plasma leptin concentrations has also been described as a hunger signal (Mars et al., 2006). Other important anorexigenic peptides which are mainly produced by the EECs postprandially are peptide YY (PYY), cholecystokinin (CCK), and glucagon-like peptide 1 (GLP-1). It has been reported a postprandial increase in plasma PYY which inhibits NPY/AgRP via neuropeptide Y receptor type 2 (Y2R) increasing satiety (van Son et al., 2021). GLP-1 plays a key role in modulating glucose by stimulating insulin secretion and lowering glucagon. Moreover, by binding GLP-1 receptor agonists (GLP-1RAs) GLP-1 can inhibit orexigenic GABAergic neurons reducing food intake and gastric emptying (van Son et al., 2021). Moreover, GLP-2 modulate CCK secretion from gallbladder, which reduces food intake activating the vagus nerve by binding the vagal CCK-A receptors (Corp et al., 1993).

Gut microbiome has been also associated to intestinal hormone secretion thus modulating the host appetite and metabolism. Indeed, the gut abundances of *Bifidobacterium* and *Lactobacillus* are negatively associated with plasma ghrelin levels and positively associated with plasma leptin concentrations (Queipo-Ortuno et al., 2013). Moreover, oral administration of microbial metabolites SCFAs were found to stimulate leptin production in cultured adipocyte cells via GPR41 receptors also increasing the circulating leptin concentrations (Xiong et al., 2004). The imidazole propionate, a gut-microbiota metabolite deriving from dietary histidine impairs glucose metabolism and contribute to type 2 diabetes (Molinaro et al., 2020; Koh et al., 2018).

Hedonic system: Dysfunction of the reward system may lead to hedonic over-eating in susceptible individuals being one of the obesity leading cause. The mesolimbic pathway referred to as the reward pathway, is a dopaminergic pathway which involve dopamine and other neurotransmitters (van Son et al., 2021). Palatable food stimulates dopamine release, activating the dopamine-reward system that promote feeding behavior (Lenoir et al., 2007). However, dopamine system modulates feeding behavior not only by the reward-related circuit, but also by contributing to the homeostatic circuit of food intake (Baik, 2021). Mounting evidence suggest the central role of the endocannabinoid system (ECS) in the modulation of the overlapping homeostatic and hedonic mechanisms of appetite and food intake (Jager and Witkamp, 2014). The physiological relevance of ECS will be further discussed in the next paragraph. Furthermore, mesolimbic dopaminergic activity can be affected by leptin and ghrelin (Leinninger et al., 2009; Skibicka et al., 2011). A considerable amount of circulating dopamine is produced in the gastrointestinal tract, however, whether peripherally produced dopamine can affect dopaminergic pathways in the brain is still unknown (Eisenhofer et al., 1997). Interestingly, altered gut microbiota composition has been found in several central disorders with dysregulated dopaminergic transmission, such as anxiety, depression, and Parkinson's disease (Simpson et al., 2020; Yang et al., 2019), hypothesizing a role of pro-inflammatory processes associated to microbial dysbiosis in contributing to these disorders (Simpson et al. 2020).

1.5 The Endocannabinoid system

The Endocannabinoid system (ECS) refers to a signally system which has a pleiotropic role in modulating appetite, food intake, macronutrient metabolism, pain sensation, blood pressure, mood, cognition, immunity, and gastrointestinal discomforts (Witkamp, 2018; Storr et al.,

2008; Hillard, 2017). The ECS includes endocannabinoids (ECs), their receptors and enzymes involved in their synthesis and degradation. The two best-characterized ECs are N-arachidonoylethanolamide (AEA) and 2-arachidonoylglycerol (2-AG) which can exert their physiological role in the body by binding CB1 and CB2 receptors located in the CNS, in the ENS, in the liver and in the adipose tissue. Besides ECs, several structural congeners referred to as N-acylethanolamines (NAEs) have been characterized. They include oleoylethanolamine (OEA), linoleyethanolamine (LEA), and palmitoyletahanolamine (PEA), and show similar mechanisms of action, tissue distribution as well as pathways of formation and breakdown with ECs. However, differently from ECs, these "endocannabinoid-like molecules" can act through GPR119, GPR55, peroxisome proliferator-activated receptors (PPARs) and the transient potential vanilloid subtype 1 (TRPV1). Structurally, also AEA is a fatty acid amide which can bind CB1 and CB2 (Witkamp, 2018; Di Marzo 2018).

Regarding NAEs and AEA synthesis, it takes place as a multi-step process. First, N-acylphosphatidylethanolamines (NAPEs) are formed through the action of calcium-dependent N-acyltransferase (NAT). Thereafter, NAPEs are cleaved by NAPE-specific phospholipase D (NAPE-PLD) to yield NAEs (**Figure 3**). Conversely, the synthesis of 2-AG is catalysed by two different enzymes: phospholipase C (PLC) and diacylglycerol lipase (DAGL) starting from Diacylglycerol (DAG) or 2-arachidonoyl-lysophospolipid (lysoPI) (**Figure 3**). Further, NAEs can be hydrolyzed by fatty acid amide hydrolase (FAAH) and N-acyl ethanolamine-hydrolysing acid amidase (NAAA), whereas 2-AG is degraded by monoacylglycerol lipase (MGL) (Witkamp, 2018; Simon and Cota, 2017).

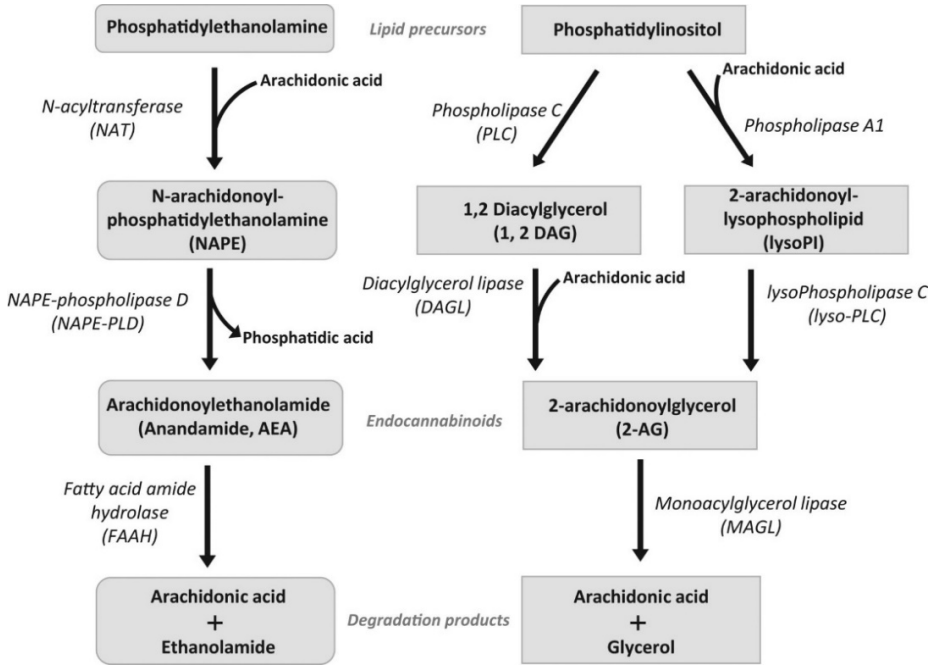


Figure 3: General overview of the pathways for N-acylethanolamine (NAEs) and 2-arachidonoyls synthesis and metabolism (Simon and Cota, 2017).

1.5.1 The endocannabinoid system and the gut-brain axis

Homeostatic and hedonic hunger: ECs and NAEs play a pivotal role in modulating the homeostatic and hedonic feeding via different signaling networks in the ENS and CNS. The activation of CB1 receptor in the hypothalamus, the corticolimbic circuits, including the nucleus accumbens (NAc) and the ventral tegmental area, and the brainstem modulate the homeostatic and hedonic feeding (Gatta-Cherifi & Cota, 2015). 2-AG is involved in this reward-driven feeding through activating the dopaminergic pathway of pleasure playing a key role in the palatable food "liking" and "wanting" system. Differently, AEA is another orexigenic compound which is likely involved in homeostatic feeding processes (Hillard, 2017). The activation of CB1 by ECs increases the consumption of foods by decreasing levels of the anorectic hormone leptin and CCK (Di Marzo et al., 2001; Di Patrizio et al., 2015). Conversely, NAEs act as anorectic mediators by binding PPAR-α, GPR119, and TRPV1 (Witkmap, 2018). For instance, PEA may indirectly impact appetite through leptin signalling in the hypothalamus (Raso et al., 2014). OEA is the best-characterized NAE known to increase satiety both centrally and locally mediated, especially via PPAR-α and GPR119 stimulating

GLP-1 and GIP secretion (Cheng et al., 2015). SEA may inhibit food intake through the downregulation of the specific liver enzyme, stearoyl-coenzyme A desaturase-1 (SCD-1), which may decrease the leptin levels (Terrazzino et al., 2004). Dysregulation of this system in hunger signalling can have multiple implications as was shown in people with anorexia and obesity (Monteleone et al., 2015; Hillard, 2017).

Endocannabinoids and gut homeostasis: Endocannabinoid receptors are widely distributed along the GIT being a key modulator of gastrointestinal physiology, influencing satiety, emesis, gastric emptying, immune function, mucosal integrity, motility, secretion, and visceral sensation (Lee et al., 2016). Indeed, it has been shown the efficacy of CB_1 receptor antagonists in increasing motility in mice (Izoo et al., 2001), whereas the use of FAAH blockers resulted in a slower gastrointestinal motility (Capasso et al., 2005). Further, blocking the degradation of 2-AG by inhibiting MGL results in reduced gut transit in mice (Duncan et al., 2008). These results were further supported data from clinical trials involving patients with metabolic disorders and obesity (Ruilope et al., 2008; Depres et al., 2005; Addy et al., 2008). The use of CB_1 antagonists rimonabant and taranabant resulted in higher frequency of diarrhoea and other gastrointestinal motor side effects like nausea and vomiting (Ruilope et al., 2008; Depres et al., 2005; Addy et al., 2008). Moreover, the activation of TRPV1 receptors by AEA can exacerbate pain and nociception (Di Marzo, 2018). In view of this, recent evidence suggested the role of these compound in pathogenesis of irritable bowel disorders (Di Marzo, 2018).

Endocannabinoid and gut microbiota: The endocannabinoid system modulate gut-barrier function, gut permeability and metabolic endotoxaemia through CB1 receptors (Cani et al., 2016). Activating CB1 receptors in mice resulted in higher lipopolysaccharide (LPS) levels released from gut bacteria, causing increased gut permeability and adipogenesis (Muccioli et al., 2010). Moreover, *Akkermansia muciniphila* administration to high-fat diet (HFD)-fed mice led to an increase in intestinal levels of 2-AG and decreased circulating LPS (Cani et al., 2016). Intestinal AEA is negatively associated to gut-barrier function, while intestinal 2-AG, OEA and PEA are considered "gate keepers" due their contribution to lock the barrier and reduce intestinal inflammation. Indeed, OEA by GPR119 receptors on EECs may elicit the secretion of GLP-1 and GLP-2 (involved in glucose homeostasis and gut-barrier function, respectively) (Cani et al., 2016). Further, PEA exhibits anti-inflammatory effects by binding PPAR-α on EECs (Borrelli et al., 2015).

1.5.2 Effect of food intake in modulating ECs and NAEs

Diet affects tissue and circulating levels of ECs and NAEs both postprandially and in the long-term. **Figure 4** shows feeding-or fasting-induced changes in endocannabinoid levels in gut, brain and other tissues. It has been reported a post-prandial increase in circulating 2-AG levels when individuals are presented with a palatable food compared to a non-palatable food through the rewarding mediator ghrelin; conversely, AEA and other NAEs levels decline (Monteleone et al., 2012). NAEs and AEA are acutely suppressed by insulin, whereas low insulin sensitivity results in glucose mediated NAE elevation (Di Marzo et al., 2009; Gatta-Cherifi et al., 2012; Fanelli et al., 2018). Furthermore, plasma AEA and NAEs concentrations in blood tend to mirror those of free fatty acids, both in fasting and in non-fasting condition (Joosten et al., 2010).

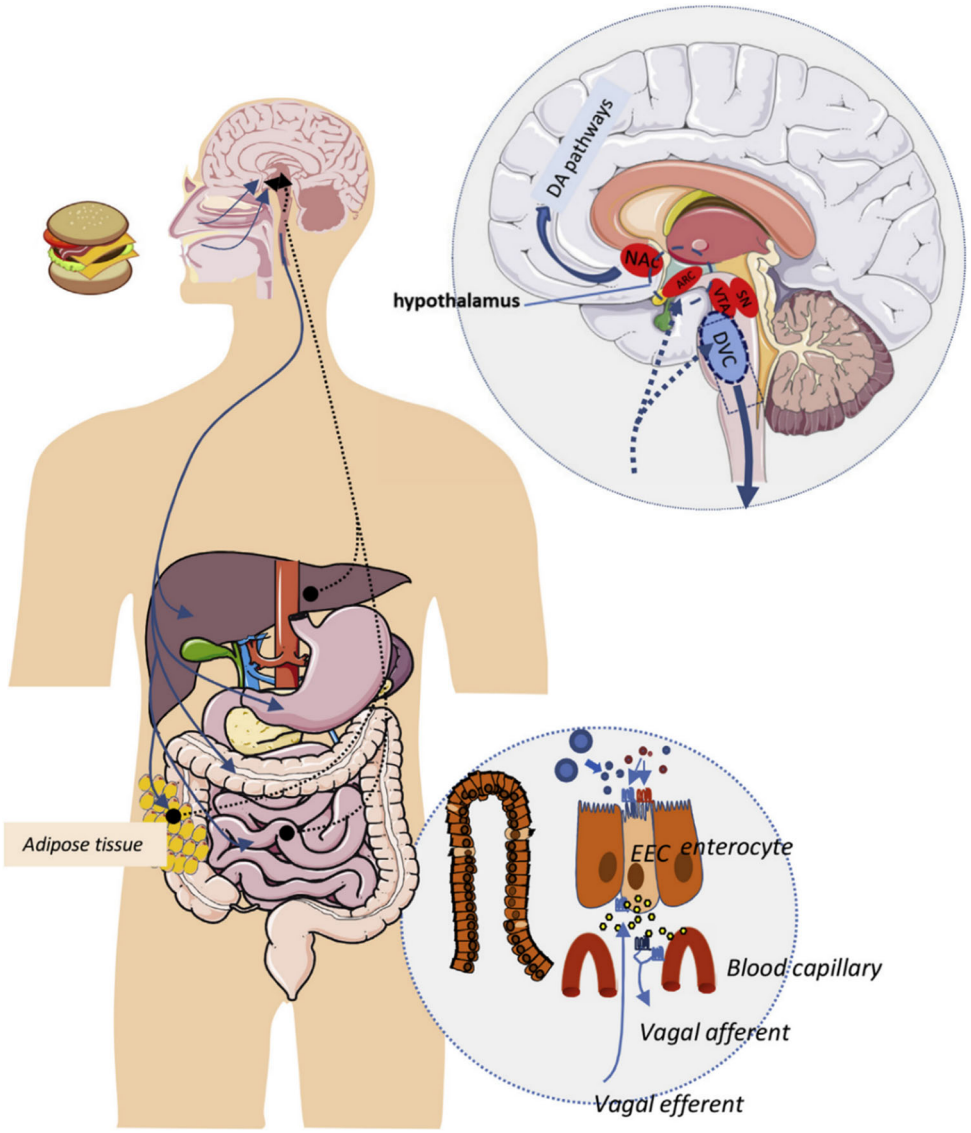


Figure 4: Overview of the short-term effects of oro-sensory food exposure and consumption on endocannabinoids (ECs) and N-acylethanolamines (NAEs) release. During the cephalic phase, oro-nasal sensing is signaled to the brain, eliciting changes

in ECs levels. Signaling via Vagal efferent induce ECs and NAEs release in gut tissues. These molecules in turn elicit hormonal and neuronal signaling to the brain and periphery by binding several receptors along the GIT. Nutrient arriving in the GIT upon food intake reduce local ECs and NAEs release (Witkamp, 2018).

With regard to NAEs, Mennella and colleagues showed that the oleic acid content in a meal increased OEA post-prandial levels, while causing a reduction in appetite sensations and energy intake at subsequent meal (Mennella et al., 2014).

Few evidence showed also the effect of the long-term diet in modulating ECs and NAEs. This could depend on the local availability of fatty acid precursors stored in the cells (which reflect the habitual diet). For instance, increasing the intake of n-3 long-chain PUFA lead to a decrease of AEA and 2-AG both in humans and in mice. Moreover, 24-week dietary supplementation with krill powder reduced AEA plasma levels in obese subjects. Finally, a 5-day high-fat diet can lead to an increase of N-acylphosphatidyletholamines (NAPEs), which are known to be NAEs precursors, decreasing food intake in rats (Gillum et al., 2018). Interestingly we recently found that ECs and NAEs are present in food at different extent, and it has been estimated that a Mediterranean diet could provide a higher amount of NAEs compared to a Western diet (De Luca et al., 2019). Notwithstanding, no information exists regarding how these compounds within foods are absorbed and/or metabolized *in vivo.*

References

1. Abell, G. C., Cooke, C. M., Bennett, C. N., Conlon, M. A., & McOrist, A. L. (2008). Phylotypes related to Ruminococcus bromii are abundant in the large bowel of humans and increase in response to a diet high in resistant starch. *FEMS microbiology ecology*, *66*(3), 505-515.
2. Addy C, Rothenberg P, Li S *et al.* Multiple-dose pharmacokinetics, pharmacodynamics, and safety of taranabant, a novel selective cannabinoid-1 receptor inverse agonist, in healthy male volunteers. *J Clin Pharmacol* 2008; **48**: 734– 44.
3. Badimon, L., Vilahur, G., & Padro, T. (2017). Systems biology approaches to understand the effects of nutrition and promote health. *British Journal of Clinical Pharmacology*, *83*(1), 38-45.
4. Baik, J. H. (2021). Dopaminergic Control of the Feeding Circuit. *Endocrinology and Metabolism*, *36*(2), 229.
5. Beards, E., Tuohy, K., & Gibson, G. (2010). A human volunteer study to assess the impact of confectionery sweeteners on the gut microbiota composition. *British journal of nutrition*, *104*(5), 701-708.
6. Berry, S. E., Valdes, A. M., Drew, D. A., Asnicar, F., Mazidi, M., Wolf, J., ... & Spector, T. D. (2020). Human postprandial responses to food and potential for precision nutrition. *Nature medicine*, *26*(6), 964-973.
7. Bersi, G., Barberis, S. E., Origone, A. L., & Adaro, M. O. (2018). Bioactive peptides as functional food ingredients. Role of materials science in food bioengineering, 147-186.
8. Berthoud, H. R., Albaugh, V. L., & Neuhuber, W. L. (2021). Gut-brain communication and obesity: understanding functions of the vagus nerve. *Journal of Clinical Investigation*, *131*(10), e143770.
9. Berthoud, H. R., Morrison, C. D., Ackroff, K., & Sclafani, A. (2021). Learning of food preferences: Mechanisms and implications for obesity & metabolic diseases. *International Journal of Obesity*, *45*(10), 2156-2168.
10. Berthoud, H. R., Münzberg, H., & Morrison, C. D. (2017). Blaming the brain for obesity: integration of hedonic and homeostatic mechanisms. *Gastroenterology*, *152*(7), 1728-1738.
11. Borrelli, F. et al. Palmitoylethanolamide, a naturally occurring lipid, is an orally effective intestinal antiinflammatory agent. Br. J. Pharmacol. 172, 142–158 (2015).
12. Cani, P. D., Bibiloni, R., Knauf, C., Waget, A., Neyrinck, A. M., Delzenne, N. M., & Burcelin, R. (2008). Changes in gut microbiota control metabolic endotoxemia-induced

inflammation in high-fat diet–induced obesity and diabetes in mice. *Diabetes*, *57*(6), 1470-1481.

13. Cani, P. D., Plovier, H., Van Hul, M., Geurts, L., Delzenne, N. M., Druart, C., & Everard, A. (2016). Endocannabinoids—at the crossroads between the gut microbiota and host metabolism. *Nature Reviews Endocrinology*, *12*(3), 133-143.
14. Capasso R, Matias I, Lutz B *et al.* Fatty Acid amide hydrolase controls mouse intestinal motility *in vivo*. *Gastroenterology* 2005; **129**: 941– 51.
15. Capozzi, F., & Bordoni, A. (2013). Foodomics: a new comprehensive approach to food and nutrition. *Genes & nutrition*, *8*(1), 1-4.
16. Chaudhary, N., Kumar, V., Sangwan, P., Pant, N. C., Saxena, A., Joshi, S., & Yadav, A. N. (2021). Personalized nutrition and-omics. *Comprehensive Foodomics*, 495.
17. Cheng, Y. H., Ho, M. S., Huang, W. T., Chou, Y. T., & King, K. (2015). Modulation of glucagon-like peptide-1 (GLP-1) potency by endocannabinoid-like lipids represents a novel mode of regulating GLP-1 receptor signaling. *Journal of Biological Chemistry*, *290*(23), 14302-14313.
18. Cheng, Y. H., Ho, M. S., Huang, W. T., Chou, Y. T., & King, K. (2015). Modulation of glucagon-like peptide-1 (GLP-1) potency by endocannabinoid-like lipids represents a novel mode of regulating GLP-1 receptor signaling. *Journal of Biological Chemistry*, *290*(23), 14302-14313.
19. Choi S, Lee M, Shiu AL, Yo SJ, Hallden G, and Aponte GW. GPR93 activation by protein hydrolysate induces CCK transcription and secretion in STC-1 cells. Am J Physiol Gastrointest Liver Physiol. 2007;292(5):G1366-75.
20. Chothe, P., Singh, N., & Ganapathy, V. (2011). Evidence for two different broad-specificity oligopeptide transporters in intestinal cell line Caco-2 and colonic cell line CCD841. American Journal of Physiology-Cell Physiology, 300(6), C1260-C1269.
21. Claus, S. P. (2013). Fighting undernutrition: Don't forget the bugs. *Cell host & microbe*, *13*(3), 239-240.
22. Claus, S. P. (2014). Development of personalized functional foods needs metabolic profiling. *Current Opinion in Clinical Nutrition & Metabolic Care*, *17*(6), 567-573.
23. Corp, E.S.; McQuade, J.; Moran, T.H.; Smith, G.P. Characterization of type A and type B CCK receptor binding sites in rat vagus nerve. *Brain Res.* **1993**, *623*, 161–166.
24. Cox, L. M., & Blaser, M. J. (2013). Pathways in microbe-induced obesity. *Cell metabolism*, *17*(6), 883-894.

25. Cuevas-Sierra, A., Ramos-Lopez, O., Riezu-Boj, J. I., Milagro, F. I., & Martinez, J. A. (2019). Diet, gut microbiota, and obesity: links with host genetics and epigenetics and potential applications. *Advances in Nutrition*, *10*(suppl_1), S17-S30.
26. Daniel, H., & Zietek, T. (2015). Taste and move: glucose and peptide transporters in the gastrointestinal tract. *Experimental Physiology*, *100*(12), 1441-1450.
27. De Filippis, F., Vitaglione, P., Cuomo, R., Berni Canani, R., Ercolini, D. (2018). Dietary interventions to modulate the gut microbiome—how far away are we from precision medicine. Inflammatory bowel diseases, 24(10), 2142-2154.
28. De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J. B., Massart, S., ... & Lionetti, P. (2010). Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proceedings of the National Academy of Sciences*, *107*(33), 14691-14696.
29. De Luca, L., Ferracane, R., & Vitaglione, P. (2019). Food database of N-acyl-phosphatidylethanolamines, N-acylethanolamines and endocannabinoids and daily intake from a Western, a Mediterranean and a vegetarian diet. *Food chemistry*, *300*, 125218.
30. Depoortere, I. (2014). Taste receptors of the gut: emerging roles in health and disease. *Gut*, *63*(1), 179-190.
31. Despres JP, Golay A, Sjostrom L. Effects of rimonabant on metabolic risk factors in overweight patients with dyslipidemia. *N Engl J Med* 2005; **353**: 2121– 34.
32. Di Marzo V., Verrijken A., Hakkarainen A., Petrosino S., Mertens I., Lundbom N. Role of insulin as a negative regulator of plasma endocannabinoid levels in obese and nonobese subjects. *European Journal of Endocrinology.* 2009;161:715–722.
33. Di Marzo, V., Goparaju, S. K., Wang, L., Liu, J., Bátkai, S., Járai, Z., ... & Kunos, G. (2001). Leptin-regulated endocannabinoids are involved in maintaining food intake. *Nature*, *410*(6830), 822-825.
34. DiPatrizio, N. V., Igarashi, M., Narayanaswami, V., Murray, C., Gancayco, J., Russell, A., ... & Piomelli, D. (2015). Fasting stimulates 2-AG biosynthesis in the small intestine: role of cholinergic pathways. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *309*(8), R805-R813.
35. Drzikova, B., Dongowski, G., & Gebhardt, E. (2005). Dietary fibre-rich oat-based products affect serum lipids, microbiota, formation of short-chain fatty acids and steroids in rats. *British journal of nutrition*, *94*(6), 1012-1025.
36. Duncan, M., Mouihate, A., Mackie, K., Keenan, C. M., Buckley, N. E., Davison, J. S., ... & Sharkey, K. A. (2008). Cannabinoid CB2 receptors in the enteric nervous system

modulate gastrointestinal contractility in lipopolysaccharide-treated rats. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *295*(1), G78-G87.

37. Eisenhofer, G.; Aneman, A.; Friberg, P.; Hooper, D.; Fandriks, L.; Lonroth, H.; Hunyady, B.; Mezey, E. Substantial production of dopamine in the human gastrointestinal tract. *J. Clin. Endocrinol. Metab.* **1997**, *82*, 3864–3871.
38. Elias, C.F.; Aschkenasi, C.; Lee, C.; Kelly, J.; Ahima, R.S.; Bjorbæk, C.; Flier, J.S.; Saper, C.B.; Elmquist, J.K. Leptin differentially regulates NPY and POMC neurons projecting to the lateral hypothalamic area. *Neuron* **1999**, *23*, 775–786.
39. European Food Safety Authority. Dietary reference values for nutrients summary report. EFSA Supporting Publications 14, e15121E (2017).
40. Fanelli, F., Mezzullo, M., Repaci, A., Belluomo, I., Gasparini, D. I., Di Dalmazi, G., ... & Pagotto, U. (2018). Profiling plasma N-Acylethanolamine levels and their ratios as a biomarker of obesity and dysmetabolism. *Molecular metabolism*, *14*, 82-94.
41. Gatta-Cherifi B., Matias I., Vallée M., Tabarin A., Marsicano G., Piazza P.V. Simultaneous postprandial deregulation of the orexigenic endocannabinoid anandamide and the anorexigenic peptide YY in obesity. *International Journal of Obesity (London)* 2012;36:880–885.
42. Gillum, M. P., Zhang, D., Zhang, X. M., Erion, D. M., Jamison, R. A., Choi, C., ... & Shulman, G. I. (2008). N-acylphosphatidylethanolamine, a gut-derived circulating factor induced by fat ingestion, inhibits food intake. *Cell*, *135*(5), 813-824.
43. Gonzalez-Santana, A., & Heijtz, R. D. (2020). Bacterial peptidoglycans from microbiota in neurodevelopment and behavior. *Trends in molecular medicine*, *26*(8), 729-743.
44. Hand KV, Bruen CM, O'Halloran F, Panwar H, Calderwood D, Giblin L, and Green BD. Examining acute and chronic effects of short- and long-chain fatty acids on peptide YY (PYY) gene expression, cellular storage and secretion in STC-1 cells. European journal of nutrition. 2012.
45. Hansen, K. B., Rosenkilde, M. M., Knop, F. K., Wellner, N., Diep, T. A., Rehfeld, J. F., ... & Hansen, H. S. (2011). 2-Oleoyl glycerol is a GPR119 agonist and signals GLP-1 release in humans. The Journal of Clinical Endocrinology & Metabolism, 96(9), E1409-E1417.
46. Heinzmann, S. S., Merrifield, C. A., Rezzi, S., Kochhar, S., Lindon, J. C., Holmes, E., & Nicholson, J. K. (2012). Stability and robustness of human metabolic phenotypes in response to sequential food challenges. *Journal of proteome research*, *11*(2), 643-655.
47. Hillard, C. J. (2018). Circulating endocannabinoids: from whence do they come and where are they going?. *Neuropsychopharmacology*, *43*(1), 155-172.

48. Hirasawa A, Tsumaya K, Awaji T, Katsuma S, Adachi T, Yamada M, Sugimoto Y, Miyazaki S, and Tsujimoto G. Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120. Nat Med. 2005;11(1):90-4.
49. Holzer P. 2011. TRP channels in the digestive system. Curr. Pharm. Biotechnol. 12:24–34.
50. Iwasaki K, Harada N, Sasaki K, Yamane S, Iida K, Suzuki K, Hamasaki A, Nasteska D, Shibue K, Joo E, et al. Free fatty acid receptor GPR120 is highly expressed in enteroendocrine K-cells of the upper small intestine and has a critical role in GIP secretion after fat ingestion. Endocrinology. 2014:en20141653.
51. Izzo AA, Fezza F, Capasso R *et al.* Cannabinoid CB1-receptor mediated regulation of gastrointestinal motility in mice in a model of intestinal inflammation. *Br J Pharmacol* 2001; **134**: 563– 70.
52. Jager, G., & Witkamp, R. F. (2014). The endocannabinoid system and appetite: relevance for food reward. *Nutrition research reviews*, *27*(1), 172-185.
53. Jayedi A, Soltani S, Abdolshahi A, Shab-Bidar S (2020). Healthy and unhealthy dietary patterns and the risk of chronic disease: an umbrella review of meta-analyses of prospective cohort studies. *British Journal of Nutrition*, *124*(11), 1133-1144.
54. Jequier, E. Leptin signaling, adiposity, and energy balance. *Ann. N. Y. Acad. Sci.* **2002**, *967*, 379–388.
55. Joosten, M. M., Balvers, M. G., Verhoeckx, K. C., Hendriks, H. F., & Witkamp, R. F. (2010). Plasma anandamide and other N-acylethanolamines are correlated with their corresponding free fatty acid levels under both fasting and non-fasting conditions in women. *Nutrition & Metabolism*, *7*(1), 1-6.
56. Karpyn, A. *Food and Public Health: A Practical Introduction* (Oxford University Press, 2018).
57. Kim, K. A., Gu, W., Lee, I. A., Joh, E. H., & Kim, D. H. (2012). High fat diet-induced gut microbiota exacerbates inflammation and obesity in mice via the TLR4 signaling pathway.
58. Koh, A.; Molinaro, A.; Stahlman, M.; Khan, M.T.; Schmidt, C.; Manneras-Holm, L.; Wu, H.; Carreras, A.; Jeong, H.; Olofsson, L.E.; et al. Microbially Produced Imidazole Propionate Impairs Insulin Signaling through mTORC1. *Cell* **2018**, *175*, 947–961.
59. Kolodziejczyk, A. A., Zheng, D., Elinav, E. (2019). Diet–microbiota interactions and personalized nutrition. Nature Reviews Microbiology, 1-12.
60. Kussmann M, Raymond F, Affolter M. OMICS-driven biomarkerdiscovery in nutrition and health. J Biotechnol 2006; 124: 758–87.

61. Lee, Y., Jo, J., Chung, H. Y., Pothoulakis, C., & Im, E. (2016). Endocannabinoids in the gastrointestinal tract. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *311*(4), G655-G666.
62. Leinninger, G.M.; Jo, Y.H.; Leshan, R.L.; Louis, G.W.; Yang, H.; Barrera, J.G.; Wilson, H.; Opland, D.M.; Faouzi, M.A.; Gong, Y.; et al. Leptin acts via leptin receptor-expressing lateral hypothalamic neurons to modulate the mesolimbic dopamine system and suppress feeding. Cell Metab. 2009, 10, 89–98.
63. Lenoir, M.; Serre, F.; Cantin, L.; Ahmed, S.H. Intense sweetness surpasses cocaine reward. *PLoS ONE* **2007**, *2*, e698.
64. Liu, X., Blouin, J. M., Santacruz, A., Lan, A., Andriamihaja, M., Wilkanowicz, S., ... & Davila, A. M. (2014). High-protein diet modifies colonic microbiota and luminal environment but not colonocyte metabolism in the rat model: the increased luminal bulk connection. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *307*(4), G459-G470.
65. Lutter, M., & Nestler, E. J. (2009). Homeostatic and hedonic signals interact in the regulation of food intake. *The Journal of nutrition*, *139*(3), 629-632.
66. Mace, O. J., Tehan, B., & Marshall, F. (2015). Pharmacology and physiology of gastrointestinal enteroendocrine cells. *Pharmacology research & perspectives*, *3*(4), e00155.
67. Mace, O. J., Tehan, B., & Marshall, F. (2015). Pharmacology and physiology of gastrointestinal enteroendocrine cells. *Pharmacology research & perspectives*, *3*(4), e00155.
68. Mars, M., De Graaf, C., De Groot, C. P. G. M., Van Rossum, C. T. M., & Kok, F. J. (2006). Fasting leptin and appetite responses induced by a 4-day 65%-energy-restricted diet. *International journal of obesity*, *30*(1), 122-128.
69. Martinez-Medina, M., Denizot, J., Dreux, N., Robin, F., Billard, E., Bonnet, R., ... & Barnich, N. (2014). Western diet induces dysbiosis with increased E coli in CEABAC10 mice, alters host barrier function favouring AIEC colonisation. *Gut*, *63*(1), 116-124.
70. Mattace Raso, G., Santoro, A., Russo, R., Simeoli, R., Paciello, O., Di Carlo, C., ... & Meli, R. (2014). Palmitoylethanolamide prevents metabolic alterations and restores leptin sensitivity in ovariectomized rats. *Endocrinology*, *155*(4), 1291-1301.
71. McCauley, H. A., Matthis, A. L., Enriquez, J. R., Nichol, J. T., Sanchez, J. G., Stone, W. J., ... & Wells, J. M. (2020). Enteroendocrine cells couple nutrient sensing to nutrient absorption by regulating ion transport. *Nature communications*, *11*(1), 1-10.

72. Mennella, I., Savarese, M., Ferracane, R., Sacchi, R., & Vitaglione, P. (2015). Oleic acid content of a meal promotes oleoylethanolamide response and reduces subsequent energy intake in humans. *Food & function*, *6*(1), 203-209.
73. Molinaro, A.; Bel Lassen, P.; Henricsson, M.; Wu, H.; Adriouch, S.; Belda, E.; Chakaroun, R.; Nielsen, T.; Bergh, P.O.; Rouault, C.; et al. Imidazole propionate is increased in diabetes and associated with dietary patterns and altered microbial ecology. *Nat. Commun.* **2020**, *11*, 5881.
74. Moss, C. E., Glass, L. L., Diakogiannaki, E., Pais, R., Lenaghan, C., Smith, D. M., ... & Reimann, F. (2016). Lipid derivatives activate GPR119 and trigger GLP-1 secretion in primary murine L-cells. *Peptides*, *77*, 16-20.
75. Muccioli, G. G., Naslain, D., Bäckhed, F., Reigstad, C. S., Lambert, D. M., Delzenne, N. M., & Cani, P. D. (2010). The endocannabinoid system links gut microbiota to adipogenesis. *Molecular systems biology*, *6*(1), 392.
76. Muller, T.D.; Nogueiras, R.; Andermann, M.L.; Andrews, Z.B.; Anker, S.D.; Argente, J.; Batterham, R.L.; Benoit, S.C.; Bowers, C.Y.; Broglio, F.; et al. Ghrelin. *Mol. Metab.* **2015**, *4*, 437–460.
77. Nuemket, N., Yasui, N., Kusakabe, Y., Nomura, Y., Atsumi, N., Akiyama, S., ... & Yamashita, A. (2017). Structural basis for perception of diverse chemical substances by T1r taste receptors. *Nature communications*, *8*(1), 1-10.
78. OCSE – The Heavy Burden of Obesity. The Economics of Prevention – (2019).
79. Odriozola L, Corrales FJ. Discovery of nutritional biomarkers:future directions based on omics technologies. Int J Food Sci Nutr2015; 66: S31–40.
80. Queipo-Ortuno, M.I.; Seoane, L.M.; Murri, M.; Pardo, M.; Gomez-Zumaquero, J.M.; Cardona, F.; Casanueva, F.; Tinahones, F.J. Gut microbiota composition in male rat models under different nutritional status and physical activity and its association with serum leptin and ghrelin levels. *PLoS ONE* **2013**, *8*, e65465.
81. Quesada-López, T., Cereijo, R., Turatsinze, J. V., Planavila, A., Cairó, M., Gavaldà-Navarro, A., ... & Villarroya, F. (2016). The lipid sensor GPR120 promotes brown fat activation and FGF21 release from adipocytes. *Nature communications*, *7*(1), 1-17.
82. Rajkumar, H., Mahmood, N., Kumar, M., Varikuti, S. R., Challa, H. R., & Myakala, S. P. (2014). Effect of probiotic (VSL# 3) and omega-3 on lipid profile, insulin sensitivity, inflammatory markers, and gut colonization in overweight adults: a randomized, controlled trial. *Mediators of inflammation*, *2014*.

83. Rossi, M. A., & Stuber, G. D. (2018). Overlapping brain circuits for homeostatic and hedonic feeding. *Cell metabolism*, *27*(1), 42-56.
84. Rossi, M. A., & Stuber, G. D. (2018). Overlapping brain circuits for homeostatic and hedonic feeding. *Cell metabolism*, *27*(1), 42-56.
85. Ruilope LM, Despres JP, Scheen A *et al.* Effect of rimonabant on blood pressure in overweight/obese patients with/without co-morbidities: analysis of pooled RIO study results. *J Hypertens* 2008; **26**: 357– 67.
86. Russell, W. R., Gratz, S. W., Duncan, S. H., Holtrop, G., Ince, J., Scobbie, L., ... & Flint, H. J. (2011). High-protein, reduced-carbohydrate weight-loss diets promote metabolite profiles likely to be detrimental to colonic health. *The American journal of clinical nutrition*, *93*(5), 1062-1072.
87. Simon, V., & Cota, D. (2017). Mechanisms in endocrinology: endocannabinoids and metabolism: past, present and future. *European journal of endocrinology*, *176*(6), R309-R324.
88. Simpson, C.A.; Diaz-Arteche, C.; Eliby, D.; Schwartz, O.S.; Simmons, J.G.; Cowan, C.S.M. The gut microbiota in anxiety and depression—A systematic review. *Clin. Psychol. Rev.* **2020**, *83*, 101943.
89. Skibicka, K.P.; Hansson, C.; Alvarez-Crespo, M.; Friberg, P.A.; Dickson, S.L. Ghrelin directly targets the ventral tegmental area to increase food motivation. *Neuroscience* **2011**, *180*, 129–137.
90. Smeets AJ, Westerterp-Plantenga MS. 2009. The acute effects of a lunch containing capsaicin on energy and substrate utilisation, hormones, and satiety. Eur. J. Nutr. 48:229–34.
91. Steensels, S., & Depoortere, I. (2018). Chemoreceptors in the Gut. *Annual review of physiology*, *80*, 117-141.
92. Stewart-Knox, B. J., Markovina, J., Rankin, A., Bunting, B. P., Kuznesof, S., Fischer, A. R. H., ... & Frewer, L. J. (2016). Making personalised nutrition the easy choice: creating policies to break down the barriers and reap the benefits. *Food Policy*, *63*, 134-144.
93. Storr, M. A., Yüce, B., Andrews, C. N., & Sharkey, K. A. (2008). The role of the endocannabinoid system in the pathophysiology and treatment of irritable bowel syndrome. *Neurogastroenterology & Motility*, *20*(8), 857-868.
94. Terrazzino, S., Berto, F., Carbonare, M. D., Fabris, M., Guiotto, A., Bernardini, D., & Leon, A. (2004). Stearoylethanolamide exerts anorexic effects in mice via downregulation of liver

stearoyl-coenzyme A desaturase-1 mRNA expression. *The FASEB Journal*, *18*(13), 1580-1582.

95. Tolhurst G, Heffron H, Lam YS, Parker HE, Habib AM, Diakogiannaki E, Cameron J, Grosse J, Reimann F, and Gribble FM. Short-chain fatty acids stimulate glucagonlike peptide-1 secretion via the G-protein-coupled receptor FFAR2. Diabetes. 2012;61(2):364-71.
96. Tzounis, X., Rodriguez-Mateos, A., Vulevic, J., Gibson, G. R., Kwik-Uribe, C., & Spencer, J. P. (2011). Prebiotic evaluation of cocoa-derived flavanols in healthy humans by using a randomized, controlled, double-blind, crossover intervention study. *The American journal of clinical nutrition*, *93*(1), 62-72.
97. van Ommen, B., van den Broek, T., de Hoogh, I., van Erk, M., van Someren, E., Rouhani-Rankouhi, T., ... & Wopereis, S. (2017). Systems biology of personalized nutrition. *Nutrition reviews*, *75*(8), 579-599.
98. van Son, J., Koekkoek, L. L., La Fleur, S. E., Serlie, M. J., & Nieuwdorp, M. (2021). The Role of the Gut Microbiota in the Gut–Brain Axis in Obesity: Mechanisms and Future Implications. *International Journal of Molecular Sciences*, *22*(6), 2993.
99. Vimaleswaran, K. S., Le Roy, C. I., & Claus, S. P. (2015). Foodomics for personalized nutrition: how far are we?. *Current Opinion in Food Science*, *4*, 129-135.
100. Weitkunat, K., Schumann, S., Petzke, K. J., Blaut, M., Loh, G., & Klaus, S. (2015). Effects of dietary inulin on bacterial growth, short-chain fatty acid production and hepatic lipid metabolism in gnotobiotic mice. The Journal of nutritional biochemistry, 26(9), 929-937.
101. Williams, D.L.; Cummings, D.E.; Grill, H.J.; Kaplan, J.M. Meal-related ghrelin suppression requires postgastric feedback. *Endocrinology* **2003**, *144*, 2765–2767.
102. Witkamp, R. F. (2018). The role of fatty acids and their endocannabinoid-like derivatives in the molecular regulation of appetite. *Molecular aspects of medicine*, *64*, 45-67.
103. Xiong, Y.; Miyamoto, N.; Shibata, K.; Valasek, M.A.; Motoike, T.; Kedzierski, R.M.; Yanagisawa, M. Short-chain fatty acids stimulate leptin production in adipocytes through the G protein-coupled receptor GPR41. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 1045–1050.
104. Yang, D.; Zhao, D.; Ali Shah, S.Z.; Wu, W.; Lai, M.; Zhang, X.; Li, J.; Guan, Z.; Zhao, H.; Li, W.; et al. The Role of the Gut Microbiota in the Pathogenesis of Parkinson's Disease. *Front. Neurol.* **2019**, *10*, 1155.

105. Zeevi, D. et al. Personalized nutrition by prediction of glycemic responses. *Cell* **163**, 1079–1094 (2015).

Chapter 2

Melanoidins from coffee and bread differently influence energy intake: A randomized controlled trial of food intake and gut-brain axis response

Joel M. Walker, Ilario Mennella, Rosalia Ferracane, Silvia Tagliamonte, Ann-Katrin Holik, Kathrin Hölz, Mark M. Somoza, Veronika Somoza, Vincenzo Fogliano, Paola Vitaglione

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Abstract

Melanoidins are Maillard reaction products similar to dietary fiber but their effect on food intake was under-investigated. A randomized, crossover study of 14 healthy subjects investigated whether melanoidins modulated energy intake and affected the postprandial dynamics of 21 gut-brain modulators of appetite including gastrointestinal peptides, endocannabinoids, *N*-acylethanolamines and neuropeptides. At breakfast the subjects consumed a bread enriched with either 3 g coffee melanoidins (CM), 3 g bread melanoidins (BM), or a conventional bread (CT). Energy intakes at lunch and throughout the day were measured, while appetite sensations and blood samples were collected before and 30, 60, 120, and 180 min after breakfast. Compared to CT, CM significantly reduced daily energy intake by 26%. CM lowered blood glucose peak, insulin, α-melanocyte stimulating hormone, orexin-A, β-endorphin, and blunted the response of three *N*-acylethanolamines versus BM. Coffee melanoidins at breakfast reduces daily energy intake and modulates postprandial glycemia and other biomarkers.

Keywords: obesity; dietary fibre; diabetes; processed foods; clinical trial; Maillard reaction

1. Introduction

Heat-treated and highly-processed foods including bakery products, coffee, milk, beer, cocoa, soy sauce and vinegar are dietary sources of high molecular weight compounds named melanoidins (Borrelli et al., 2003; Borrelli, Visconti, Mennella, Anese, & Fogliano, 2002; Fogliano & Morales, 2011; Moreira, Nunes, Domingues, & Coimbra, 2012). They are the final heterogeneous polymers formed by the Maillard reaction between amino acids and reducing carbohydrates in food. Maillard reaction products have been attributed a nutritional quality lowering effect due to reduced protein digestibility and, particularly, the advanced glycation end-products, which have been linked with health conditions and diseases such as aging, diabetes and atherosclerosis (ALjahdali & Carbonero, 2019). In contrast, Maillard reaction products have also been the subject of many research studies because of the potential health benefits mediated by the interplay between these non-digestible dietary elements and the gut microbiota. Since a common diet can provide around 10 g/day of melanoidins (Fogliano & Morales, 2011; Pastoriza & Rufian-Henares, 2014), melanoidins are good contributors to the digestion-resistant polymers that reach the colon and are fermented by the local microbiota (Fogliano & Morales, 2011; Morales, Somoza, & Fogliano, 2012). In addition, melanoidins possess antioxidative (Borrelli et al., 2002) and anti-inflammatory properties (De Marco, Fischer, & Henle, 2011) shown in *vitro* and in mice (Choi, Jung, & Ko, 2018) as well as a hepatoprotective effect shown in a rat model of liver disease (Vitaglione et al., 2010). Since these molecules are formed in food products upon heating, their structure and composition vary depending on the availability of precursors and the presence of other food components that may be involved in the reaction. Therefore, melanoidins from coffee and bread, which are the most abundant melanoidins in the Western diet (Fogliano & Morales, 2011), are chemically different. In coffee, melanoidins are formed mainly by polysaccharides and polyphenols due to the presence of arabinogalactans and chlorogenic acids in green coffee beans (Morales et al., 2012) and have a limited ability to form gel structures in the stomach and the intestine (Nunes & Coimbra, 2007). In contrast, bread melanoidins derive mainly from gluten proteins and starch (Morales et al., 2012). Bread melanoidins are only partially digested by digestive enzymes in the stomach and remain insoluble throughout the gastrointestinal tract (Helou et al., 2017; Helou et al., 2015). Although melanoidins share physicochemical, biochemical and biological properties with dietary fibers (Fogliano & Morales, 2011), the effect of melanoidins on satiety and biochemical pathways underpinning appetite sensations in humans is unknown.

Humans have an innate tendency to overeat, which is an important component of the current

obesity epidemic. An elegant study recently demonstrated that ultra-processed diets cause excess calorie intake by affecting appetite cues (Hall et al., 2019). Food intake results in a systemic modulation of hormones, peptides, signaling molecules, and hedonic feelings leading to satiety (Berthoud, 2011; Keesey & Powley, 2008) through feedback mechanisms that enable the gut-brain axis to monitor changes in the nutrient status within the body (Camilleri, 2015). The body's response to food ingestion results in an increase of plasma glucose, insulin, PYY, and GLP-1, which elicits satiety and limits food intake through the down-regulation of activity in the hypothalamus (Zanchi et al., 2017), while the orexigenic peptides ghrelin and orexin-A (So et al., 2018) along with the endocannabinoids (ECs), anandamide (AEA) and 2-arachidonoylglycerol (2-AG), can stimulate hypothalamus activity (Di Marzo, Ligresti, & Cristino, 2009; Zanchi et al., 2017). In addition, endocannabinoid-like molecules, including *N*-acylethanolamines (NAEs) such as oleoylethanolamide (OEA), linoleoylethanolamide (LEA), and palmitoylethanolamide (PEA), have a pleiotropic activity in the body and affect energy metabolism leading to appetite reduction through a wide range of receptors located in the central nervous system and along the gastrointestinal tract (Mennella et al., 2016; Mennella, Savarese, Ferracane, Sacchi, & Vitaglione, 2015; Witkamp, 2018).

The primary aim of this study was to evaluate whether coffee and bread melanoidins influence appetite sensations and energy intake in humans. Secondarily, we aimed to assess the postprandial responses of blood glucose and 21 biomarkers of the gut-brain axis involved in appetite cues and to display relationships between appetite sensations, metabolic biomarkers and energy intake.

2. Subjects and Methods

2.1. Isolation of coffee melanoidins

Prepackaged Lavazza Suerte ground coffee (Lavazza), a medium roast coffee, was purchased from a local market. The coffee powder was diluted with water (1:4 wt:vol) and cooked at 90 °C with a stirring rate of 2,000 rpm for 30 min in a BIMBY cooker (Vorwerk). The coffee mix was allowed to settle for 10 min and was poured into a French press (Brazil model, Bodum). The French press coffee was filtered through unbleached filter paper (Melitta), collected, and transferred to dialysis tubing with a molecular weight cut-off of 12-14 kDa (Spectra/Por, Spectrum Laboratories). The filtered coffee was dialyzed against tap water at 4 °C until the water remained clear. The dialyzed coffee was collected and the weight of the coffee

melanoidins was determined by freeze-drying the dialyzed coffee melanoidins solution. The freeze-dried powder was stored at -20 °C until use in the preparation of the coffee melanoidins-enriched bread.

2.2. Isolation of bread melanoidins

Bread melanoidins were prepared following the protocol of Fogliano and Morales with a few modifications (Fogliano & Morales, 2011). Bread crisps dough was prepared with wheat flour (*tipo* 00) and water at a ratio of 1.8:1 (wt:vol). The dough was spread to a thickness of 0.3 cm, cut into circular disks with a diameter of 6 cm, and holes were made using a fork to minimize the formation of air pockets during baking. Crisps were baked for 60 min at 200 °C in a convective oven (Electrolux Rex). The baked crisps were finely ground at 4,000 rpm for 30 sec followed by 10,000 rpm for 5 sec using the BIMBY mixer. The ground crisps were sifted through a 300 μm sieve and stored at -20 °C until needed.

The melanoidins content in the bread crisps powder were determined (Fogliano & Morales, 2011). A 500 mg sample of bread crisp powder was incubated in 6 mL of 20 mM Tris pH 8.0 along with 0.1 mg/mL protease Pronase E from *Streptomyces griseus* (Sigma-Aldrich) for 40 h at 37 °C. The supernatant was cleared at 2,800 *g* for 10 min. Three mL of supernatant were incubated with 3 mL of 40% trichloroacetic acid for two h at room temperature. The precipitated bread crust was concentrated in a Centricon YM-10 (Merck-Millipore). The retentate was freeze-dried and the melanoidins present in the bread crisps were calculated.

2.3. Preparation of the foods for the randomized, controlled, crossover study

Three breads were formulated and prepared. They were a conventional 100% refined wheat flour bread (control bread, CT) and two breads enriched with 3% coffee-melanoidins (CM) or bread melanoidins (BM). To design the melanoidins-enriched breads we took into account the reported daily intakes of coffee and bread melanoidins (which are 0.5-2 g/d and 1.8-15 g/d, respectively) (Fogliano & Morales, 2011) and we estimated that the addition of an extra 3 g of coffee or bread melanoidins would represent a large intake for both types of melanoidins at one meal. Coffee melanoidins or bread melanoidins were incorporated into the dough by replacing wheat flour in the recipe of the conventional bread in order to have a final amount of 3 g melanoidins per 100 g bread. The doughs were colored with a 0.3% (w:w) brown food coloring (Wilton Industries) to mask the color differences arising from melanoidins enrichment. The composition of the three breads is summarized in **Table S1**. The doughs were baked for 25 min

at 200 ºC in a convective oven (Electrolux Rex). The prepared breads were all weighed, placed in freezer bags, and frozen until the study session.

A study day breakfast consisted of 100 g of one of the breads and 125 mL of 1% lactose-free milk (Parmalat). The nutritional composition of the three breakfasts is reported in **Table S2**. The experimental day *ad libitum* lunch provided pre-weighed portions of typical Italian dishes in a buffet style including pasta with tomato sauce, meat, fish, green salad, French fries, bread, and fruit. The food was prepared by a local restaurant following fixed recipes on each experimental day. The selected dishes were based on the subjects' preferences as determined from their written responses in a food preference questionnaire that each subject completed during the enrollment visit. After lunch, the dishes were weighed again and the food consumed was measured to the nearest 1 g. The energy intake and nutrient intake were then calculated based on the food analysis from the Italian research center for food and nutrition (CREA - Centro di ricerca per gli alimenti e la nutrizione, Italy).

2.4. Subjects

Recruitment was conducted at the University of Naples among people working or studying in the Department of Agricultural Sciences and informed of the study by public announcements on social media. The participant flow and their general characteristics were reported in **Figure 1** and **Table 1,** respectively. Twenty subjects were screened and fourteen subjects were selected based on the data obtained from a questionnaire of their medical status, their food preferences and their subjective eating habits. The selected subjects were healthy. They were not taking any medications (birth control pill was allowed) or drugs. The subjects had to be habitual consumers of breakfast and could not be in a restrictive diet regimen. The subjects signed an informed consent form to be enrolled in the study.

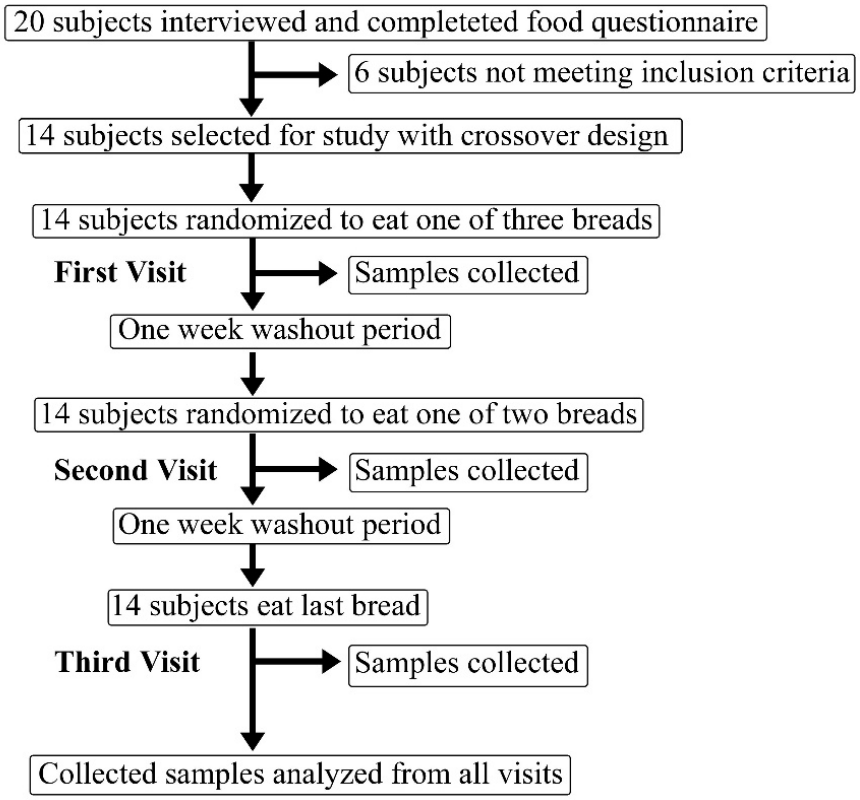

Figure 1: Participant flow diagram. The participant flow through the entire study is shown.

Table 1: Characteristics of the study participants. Data shown as means ± SE.

	Units	Values	Range
Subjects		**14**	
Female/Male	***n/n***	**7/7**	
Age	**y**	**26.4 ± 0.57**	**19-49**
Body mass index	**kg/m^2**	**22.2 ± 0.14**	**19-25**

2.5. Study design

This study was registered at clinicaltrials.gov as NCT01851304. It was conducted following the guidelines laid down by the Code of Ethics of the World Medical Association (Declaration

of Helsinki) and all procedures involving human subjects were approved by the Ethics Committee of the University of Naples (Protocol number: 235/13). Written informed consent was obtained from all subjects and the privacy rights of all subjects were observed. The study started in September 2014 and finished in July 2015.

A randomized, controlled, crossover, and single blind designed study was performed. The sample size was calculated on the study primary outcome: appetite feelings and energy intake at the subsequent lunch. It was calculated that an $\alpha = 0.05$ with a power of 80%, 12 subjects would be needed to detect a 19% difference in energy intake at the *ad libitum* lunch. Considering the postprandial hormonal responses as secondary outcomes, it was also calculated that 8 subjects would allow for the detection of a 23% and 16% difference in $AUC_{0\text{-}180}$ of ghrelin and PYY, respectively. Each blinded subject participated in three study days that were separated by a 1-week washout period. The order of treatments was randomized for each subject via computer-generated randomization tables (Microsoft Excel) that were provided by an experimenter to the nutritionist in charge of the meal planning over the study days. The experimental design for each study day is depicted in **Figure S1**.

The consumption of either the CM or the BM was well tolerated and there were no reported side effects during the study.

The subjects were advised to eat a dinner in the evening prior to each study day, which minimized the consumption of dietary fiber and avoided the second-meal effect (Wolever, Jenkins, Ocana, Rao, & Collier, 1988). They were also instructed to consume white rice and animal protein-containing foods; to avoid alcoholic and soft drinks; to always consume the same foods at dinner before each study day; to finish eating by 22:00 h; and to fast overnight with *ad libitum* access to water. Before beginning a study day, subjects completed a questionnaire about their current health. Subjects experiencing any physiological or psychological discomfort were excluded from the current visit and asked to come to the next experimental day.

The fasted subjects arrived at 08:30 h at the laboratory of Food and Health of the Department of Agricultural Sciences of the University of Naples. The appetite sensations of hunger, desire to eat, fullness, and satiety were measured at time points 0 (just prior to breakfast), 30 min, 60 min, 120 min and 180 min after breakfast using a 100 mm Visual Analog Scale (VAS) anchored by specific questions (Green, Delargy, Joanes, & Blundell, 1997). The questions were ‘How

hungry do you feel?' 'How much could you eat at this moment?' 'How full do you feel?' 'How satiated do you feel?' and the recorded mark indicated the strength of each sensation (Green et al., 1997).

Immediately after the recording of baseline appetite sensations, blood glucose was measured with a skin prick test using a One Touch meter (LifeScan). Then a cannula was placed into an arm vein of each subject and blood was collected at that time and at 30 min, 60 min, 120 min and 180 min after breakfast. Fifteen milliliters of blood were drawn into three EDTA-coated vacutainer tubes (Becton Dickinson). The protease inhibitors dipeptidyl peptidase IV (DPPIV, EMD Millipore) and phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich) were added immediately after blood collection to inhibit the degradation of acylated ghrelin and PYY. The tubes were centrifuged at 2,400 *g* for 10 min at 4 °C. The supernatant was collected and stored at -40 °C until sample measurement.

After the collection of the last blood sample (180 min) subjects were offered an *ad libitum* lunch as described above (*Par. 2.3.*). Before leaving the laboratory, subjects were instructed on how to compile a weighted food diary throughout the rest of the day until the following morning. They received a food diary entry scheme with four columns: one to report the time, one for the food or the beverage consumed, one for the weight/volume of the food, and one for the notes. Subjects were instructed to write everything they consumed in the food diary. When subjects consumed a commercial food, they were required to report the commercial product and brand into the notes. For food prepared at home, they were required to report in the notes the weight of added fats or sugars, as well as indicate the percentage of ingredients in the dish in order to allow a proper calculation of the nutritional composition of the related food item. On the day after an experimental day, the weighed food diary was sent by e-mail from each subject to the nutritionist collaborating with the study. The nutritionist called each subject by phone to confirm the contents of their food diary and to avoid any mistakes possibly occurring due to the subject's forgetfulness. The weighed food diaries were then analysed by the nutritionist to calculate the energy intake using the same food nutritional composition database that was used for assessing the energy intake at the *ad libitum* lunch (*Par. 2.3.*).

2.6. Gastrointestinal hormones

Insulin, ghrelin, leptin, peptide YY (PYY), glucagon peptide 1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP), pancreatic polypeptide (PP) and glucagon were measured in

plasma samples prepared with protease inhibitors using the EMD Millipore human metabolic hormone magnetic bead panel following the manufacturer's instructions. The plate was read in a Bio-Plex 200 System with Luminex Technology (Bio-Rad) and the values calculated with a logistic 5-parameter or 4-parameter regression line equation using the Bio-Plex Manager 5.0 software (Bio-Rad).

2.7. Postprandial insulin sensitivity

Postprandial oral glucose insulin sensitivity (OGIS) was calculated by the meal glucose tolerance test (MGTT) over 3 h according to Mari *et al.* using an Excel spreadsheet for 3 h OGTT (Mari, Schmitz, et al., 2002; Mari, Tura, Gastaldelli, & Ferrannini, 2002).

2.8. Neuropeptides

Cortisol, β-endorphin, α-melanocyte stimulating hormone (α-MSH), oxytocin, orexin-A, neurotensin, and substance P were simultaneously measured using the EMD Millipore human neuropeptide magnetic bead panel; read with a Bio-Plex 200 system using Luminex Technologies; and the measurements calculated with the Bio-Plex Manager 5.0 software. Melatonin was measured by ELISA following the manufacturer's protocol (IBL International). Each plate was scanned on a dual-wavelength plate reader (Thermo Scientific Multiscan FC, Thermo Scientific) at 405 nm with reference wavelength of 620 nm and data collected with the Scanit Software version 2.5.1 (Thermo Scientific). The melatonin concentration was determined using a logistic 4-parameter regression line.

2.9. Plasma endocannabinoids (ECs) and N-acylethanolamines (NAEs)

Plasma samples were spiked with an internal standard and two ECs, anandamide (AEA) and 2-arachidonoylglycerol (2-AG), along with three NAEs, linoleoylethanolamide (LEA), palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) were extracted from the plasma samples and analyzed by LC/MS/MS (Mennella, Ferracane, Zucco, Fogliano, & Vitaglione, 2015).

2.10. Statistical analysis

The statistical differences between time points and bread treatments were calculated using SPSS 16.0 and SigmaPlot 11.0. For all the measured markers, the mean ± standard error mean (SE) for each time point was determined. The area under the curve (AUC) for each marker was

calculated using the linear trapezoidal rule. All measured markers including the energy intake data and the area under the curves were normalized using LN transformation. The transformed data were subjected to repeated measures analysis of variance (ANOVA) over time for each bread and to one-way ANOVA between breads at each time point and AUC interval. Statistically significant differences ($p < 0.05$) were further investigated by the Bonferroni *post-hoc* test. Paired *t*-tests with a significant difference of $p < 0.05$ were done between CM and BM at all time points and AUC intervals for each marker.

The AUC of each variable calculated over all treatments by all participants was subjected to a pairwise Pearson's correlation test. The heatmap correlation matrix was visualized using the Hmisc package and the function heatmap.2 of R software, version 3.6.2 (https://www.r-project.org).

3. Results

3.1. Short-term appetite sensations

Participants perceived similar hunger, fullness, satiety and prospective food consumption over the three hours after breakfast with all three types of bread (**Table S3**). Compared to baseline, however, both CM and CT consumption reduced hunger after 30 min and it remained low at 60 min after CT (**Figure 2A**). That profile paralleled a decreased prospective consumption at 30 min (**Figure 2B**) and an increased satiety and fullness at 30, 60 and 120 min vs baseline only after CT ($p < 0.05$) (**Figure 2C-D**). Participant sensations did not vary over time when BM was consumed.

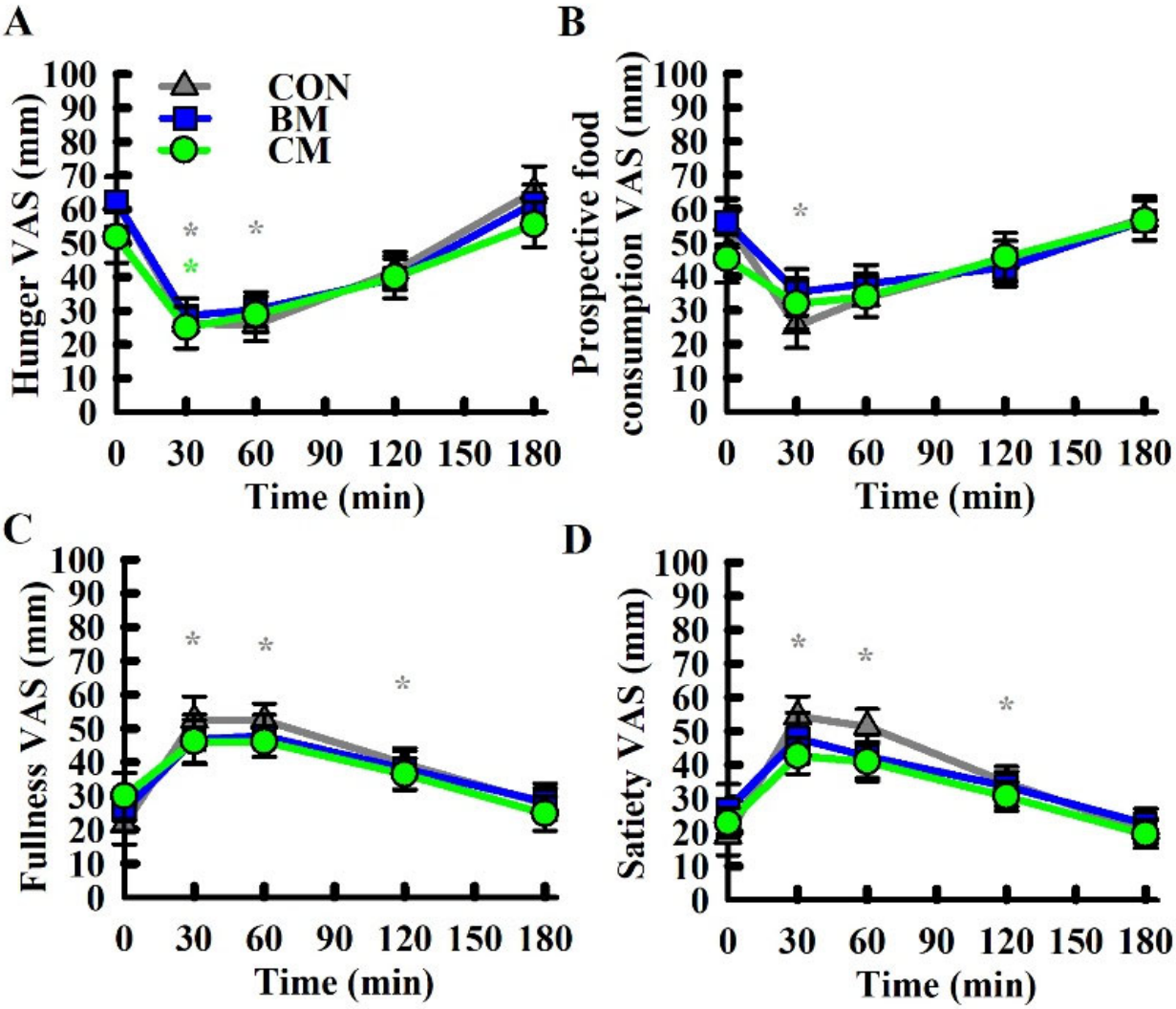

Figure 2: Subjective appetite response (A-D). The subjective appetite response measurements are shown as means ± SE, n=14. *$p < 0.05$ for the measured time point compared with baseline within each bread treatment (repeated-measures ANOVA, Bonferroni *post-hoc* test).

3.2. Daily energy intake

In accordance with the similar appetite sensations recorded over the three hours after the three breakfasts, participants did not vary their energy intake at the subsequent lunch offered 180 min after breakfast (**Figure 3**). However, energy intake over the period following lunch was significantly reduced by 26% when participants consumed CM bread at breakfast compared with CT bread (901 ± 102 kcal, 3,770 ± 427 kJ, vs 1,216 ± 97.1 kcal, 5,088 ± 406 kJ; $p = 0.04$).

A similar trend was observed with BM but the 18% decrease was not enough to achieve a statistically significant difference (967 ± 114 kcal (4,046 ± 477 kJ) vs 1,216 ± 97.1 kcal (5,088 ± 406 kJ); $p = 0.08$).

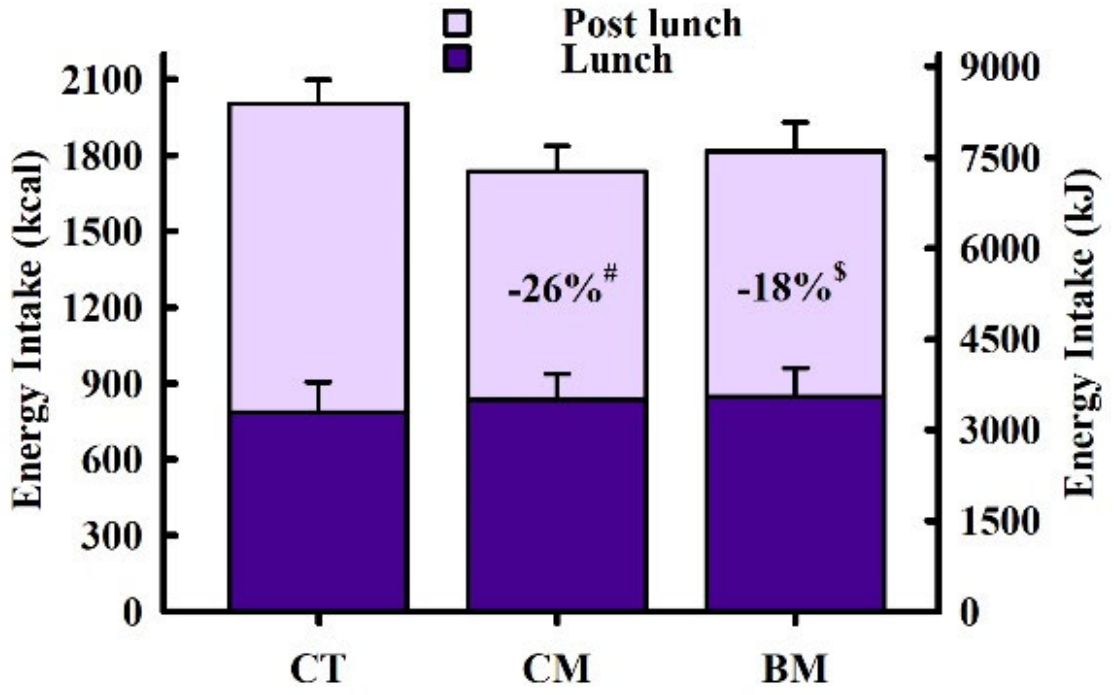

Figure 3: Energy intake. Energy intakes at lunch and during the 24 h post lunch period are expressed as means ± SE, n=14. $^{\#}p = 0.04$ for CM versus CT bread treatments in the post lunch period, $^{\$}p = 0.08$ for BM versus CT bread treatments in the post lunch period (ANOVA and Bonferroni *post-hoc* test).

3.3. Influence on glucose and gastrointestinal markers

The blood glucose response was similar over the three hours after all of the breakfasts (**Table S3**). Participants experienced blood glucose responses with a peak between 30 min and 60 min followed by a rebound to baseline values at 180 min (**Figure 4A**). Interestingly, the blood glucose peak at 60 min after breakfast was significantly lower with CM than after BM (1.31 ± 0.24 mmol/L vs 1.85 ± 0.17 mmol/L, $p = 0.04$). Moreover, CM elicited a lower late postprandial response of insulin than CT (**Figure 4B** and **Table S4**). After CM ingestion, plasma insulin

concentration peaked at 30 min, and at 120 min had returned to a value that was similar to baseline but significantly lower than after CT (0.877 ± 0.178 µg/L vs 1.50 ± 0.220 µg/L, p = 0.024); while at 180 min insulin concentration after CM was lower than after BM (0.456 ± 0.062 µg/L vs. 0.619 ± 0.134 µg/L, p = 0.017). The OGIS index did not show any difference in the individual insulin sensitivity over 180 min after breakfasts (**Figure S2**). On the other hand, data showed that ghrelin concentration within 60 min after breakfast with CM decreased faster and deeper than after either BM or CT (**Figure 4C**). This fall was followed by a rapid increase within 180 min after CM consumption that resulted in a significantly higher late response of ghrelin after CM than BM (**Table S4**).

The counteraction of anorexigenic peptides explained the lack of effect on energy intake at lunch despite the stronger response of the orexigenic ghrelin following CM consumption (**Figure 4D-I**). Although ghrelin, leptin, PP, PYY, glucagon, GIP, and GLP-1 showed similar responses after all bread consumption, we found differences in time-concentration profiles of some of them between breads. Particularly, plasma GIP strongly increased in the first hour after both CM and BM bread ingestion followed by a decrease towards baseline at 180 min after breakfast. Pancreatic polypeptide showed a strong response to CM bread ingestion 180 min after breakfast while BM and CT displayed a broader peak over the entire 180 min after breakfast. Plasma glucagon and leptin levels were affected only after CT ingestion.

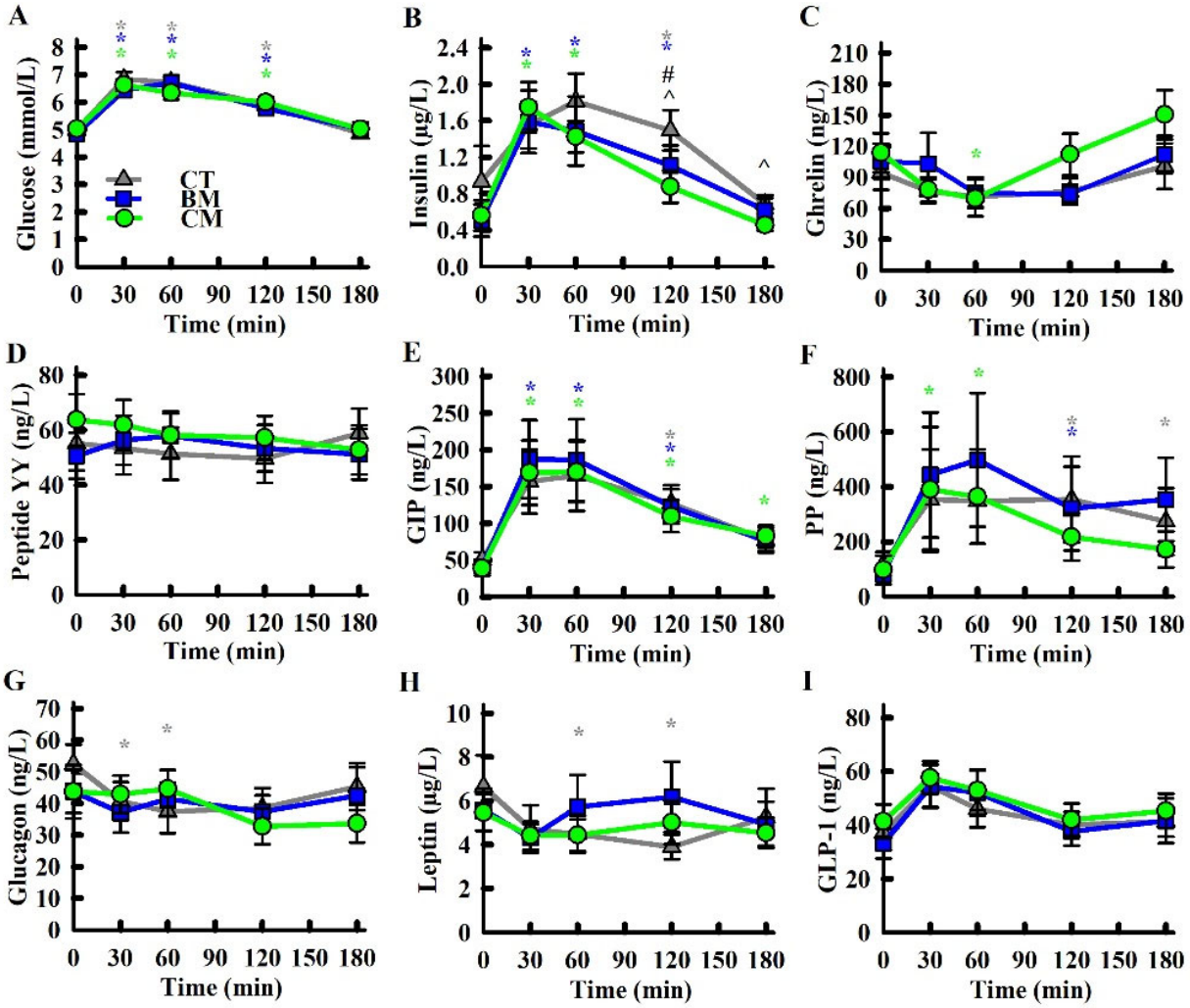

Figure 4: Gastrointestinal markers response (A-I). Measurement of glucose and the gastrointestinal markers are shown as means ± SE, n=14. $^{\#}p < 0.05$ between CM and CT breads at this time point (one-way ANOVA with Bonferroni *post-hoc* test). $*p < 0.05$ for the measured time point compared with baseline within each bread treatment (repeated-measures ANOVA, Bonferroni *post-hoc* test). $^{\wedge}p < 0.05$ between CM and BM at this time point (paired *t*-test).

3.4. Influence on endocannabinoids and N-acylethanolamines

We did not find any difference between the entire 180 min postprandial ECs and NAEs response with any of the breads (**Table S3**; **Figure 5**).

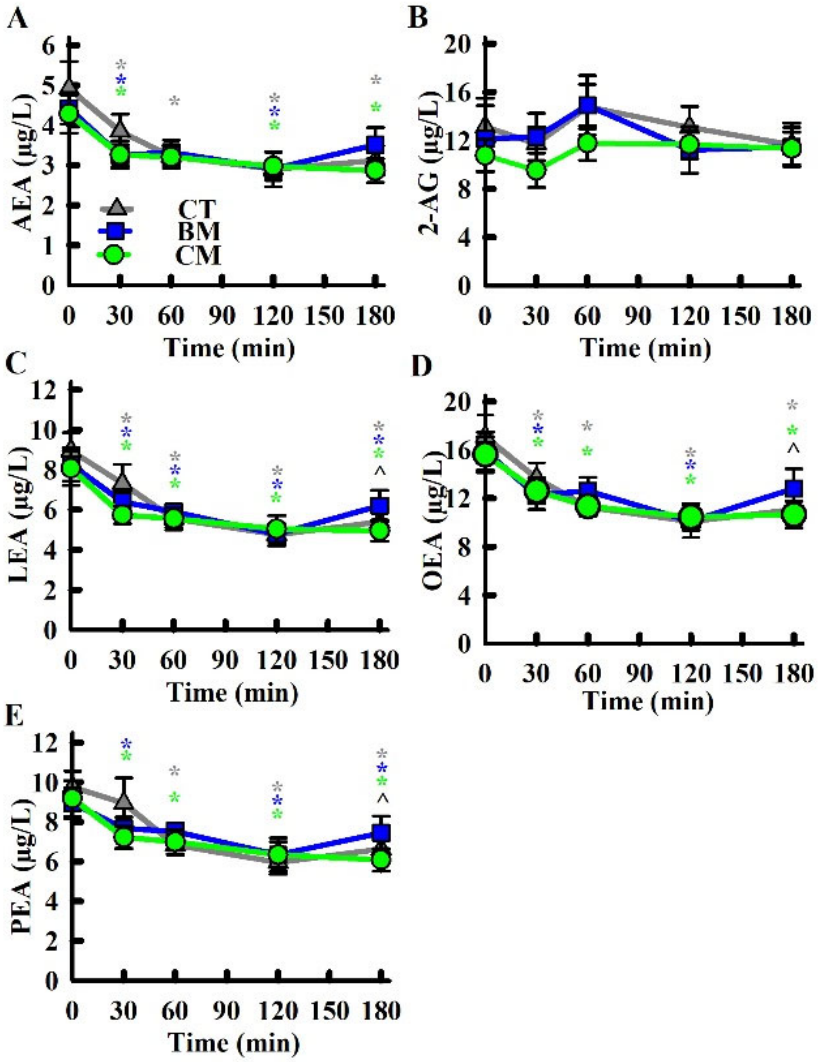

Figure 5: Endocannabinoids and *N*-acylethanolamines response (A-E). Measurement of the endocannabinoids and *N*-acylethanolamines are shown as means ± SE, n=14. *$p < 0.05$ for the measured time point compared with baseline within each bread treatment (repeated-measures ANOVA, Bonferroni *post-hoc* test). ^$p < 0.05$ between CM and BM at this time point (paired *t*-test).

In general, 2-AG concentration did not change postprandially after bread ingestion. AEA and the NAEs, on the other hand, decreased within 120 min and showed some variability at 180 min. At the 180 min time point, BM consumption resulted in AEA and OEA returning to baseline ($p > 0.05$), while CM and CT remained significantly lower than baseline. All three breads maintained a significant reduction in plasmatic LEA and PEA at 180 min. Lower concentrations of all of the NAEs at 180 min were always observed after CM vs BM (LEA was

4.96 ± 0.527 µg/L vs 6.20 ± 0.785 µg/L, OEA was 10.6 ± 1.10 µg/L vs 12.8 ± 1.65 µg/L, PEA was 6.08 ± 0.570 µg/L vs 7.45 ± 0.846 µg/L, $p < 0.05$, respectively).

3.5. Influence on neuropeptides

For 5 out of 8 neuropeptides monitored, we did not observe any difference in the postprandial plasma response between breads (**Figure 6A-D and Figure S3A-D**) while three neuropeptides showed significant variations. Compared to BM, CM showed a significantly lower plasma response of α-MSH over 180 min (**Table S3**), orexin-A over 30-180 min ($AUC_{30\text{-}180min}$ were 44.9 ± 5.82 µg*min/L vs 52.0 ± 5.72 µg*min/L, $p = 0.042$, respectively) and β-endorphin over 60-180 min (**Table S4**). Moreover, we found a decrease in cortisol concentration 120 min after CM bread consumption compared to baseline and a lower plasma β-endorphin concentration 120 min after CM than BM.

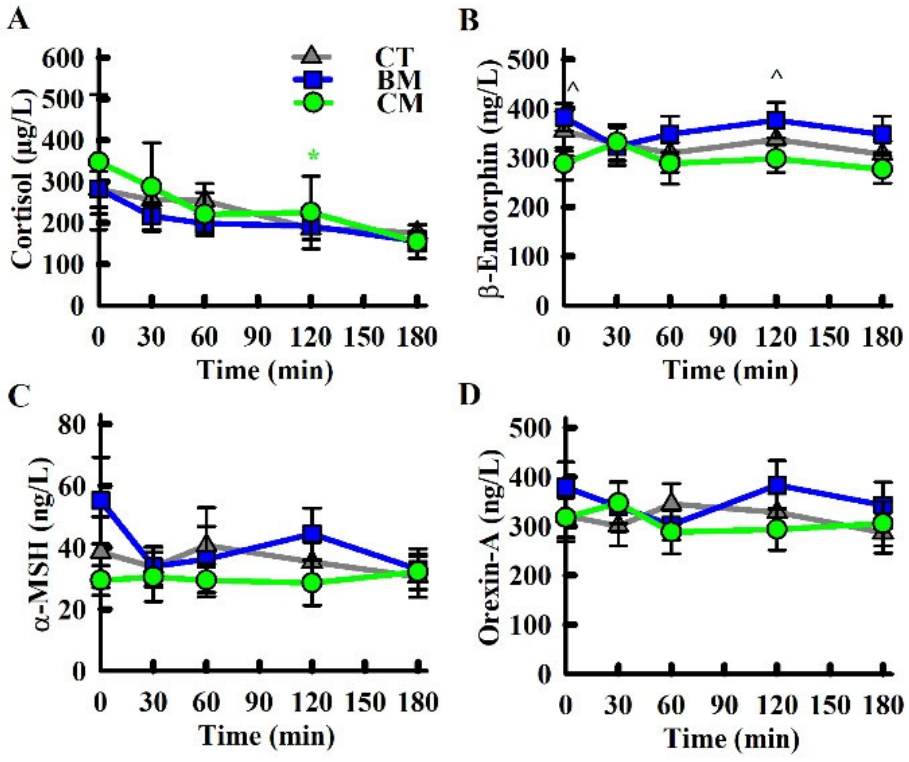

Figure 6: Neuropeptides response (A-D). Measurement the neuropeptides are shown as means ± SE, n=14. *$p < 0.05$ for the measured time point compared with baseline within each bread treatment (repeated-measures ANOVA, Bonferroni *post-hoc* test). ^$p < 0.05$ between CM

and BM at this time point (paired *t*-test).

3.6. Postprandial interplay between gut and brain biomarkers

In order to evaluate the postprandial interplay between responses of gut and brain biomarkers with evolution of appetite sensations and the subsequent energy intake, we performed a correlation analysis between variables monitored within 180 min postprandially. **Figure 7** illustrates the strength of the Pearson's correlations between the postprandial responses for each pair of biomarkers. We confirmed some relationships between specific appetite sensations and the response of GLP-1, PP, insulin, leptin, β-endorphin, substance-P and orexin-A. Moreover, we unraveled novel relationships between α-MSH and neurotensin with hunger, all NAEs and AEA with satiety neurohormonal response, and 2-AG with energy intake.

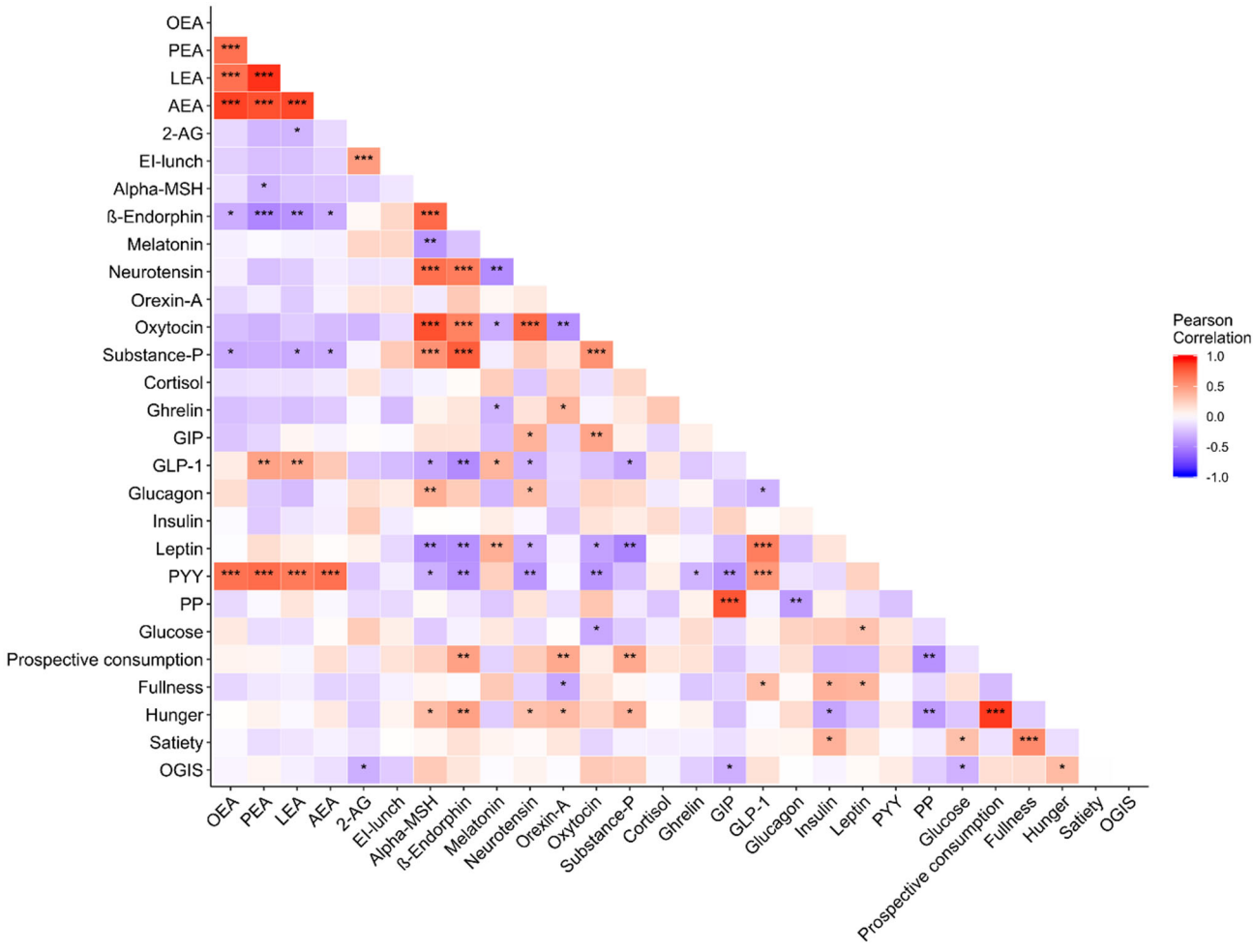

Figure 7: Correlation matrix between markers. Heatmap showing the correlation matrix

between all tested markers. The color scale represents the magnitude of Pearson's correlations between changes in each pair of variables AUC, with red indicating positive and blue indicating negative correlation. *p < 0.05, **p<0.01, ***p<0.001 indicate significant Pearson's correlations.

4. Discussion

In this study, we aimed to assess whether coffee and bread melanoidins modulate energy intake and how they affect postprandial dynamics of 21 gut-brain modulators of appetite, including 8 gastrointestinal peptides, 2 endocannabinoids, 3 *N*-acylethanolamines, and 8 neuropeptides in humans. Participants reported similar appetite sensations over 180 min after the three breakfasts and energy intake at the subsequent lunch. However, CM significantly decreased energy intake by 26% over the post lunch period compared to CT, while the 18% reduction recorded with BM indicated a clear trend although it did not reach statistical significance.

The reduction of energy intake over the day after lunch suggested that melanoidin-enriched breads mainly targeted physiological mechanisms taking place in the distal gastrointestinal tract. This is a typical effect of low processed and whole food because of a delayed digestion compared with highly processed and finely grounded food, as previously demonstrated with a rye-based product (Isaksson et al., 2011). A similar effect was also observed with microencapsulated bitter compounds, which were specifically designed to release the bioactive compounds into the duodenum in order to target the chemosensory receptors on intestinal mucosa (Mennella et al., 2016). Recalling the evidence that coffee melanoidins lead to intestinal bacterial growth (Morales et al., 2012), we can hypothesize in this study that coffee melanoidins are metabolized by the gut microbiota in the colon, thus releasing metabolites that can elicit the secretion of anorexigenic hormones by enteroendocrine cells (Fetissov, 2017). Therefore, a concomitant effect of the food structure delaying digestion of macronutrients and delivering polyphenols from CM or proteins from BM, as well as a satiety response induced by microbial metabolites likely contributed to the delayed satiety observed in our study.

Looking at postprandial metabolic responses, results showed a generally lower glucose and insulin concentration following CM consumption. This effect was independent from postprandial insulin sensitivity as assessed by OGIS and a delayed gastric emptying. Indeed, we found a stronger response of ghrelin that might indicate an even faster gastric emptying following CM (Levin et al., 2006). A similar effect on postprandial insulin response in humans

was also observed in previous studies with a lupin-based bread and a wholegrain bread (Keogh, Atkinson, Eisenhauer, Inamdar, & Brand-Miller, 2011) as well as with a β-glucan-enriched bread (Vitaglione, Lumaga, Stanzione, Scalfi, & Fogliano, 2009). Interestingly, rye products also displayed an improved postprandial glycemic response when served at breakfast (Rosen, Ostman, & Bjorck, 2011) that was likely due to their content of bioactive phenolic compounds inhibiting activity of carbohydrate digestive enzymes (Rosen, Ostman, Shewry, et al., 2011). We hypothesize that high molecular weight coffee melanoidins having chlorogenic acids incorporated into their structures (Moreira et al., 2012) could elicit a metabolic effect similar to polyphenol-bound dietary fiber present in cereals (Vitaglione et al., 2015). This idea that the polyphenol-based and antioxidant structure of CM (Borrelli et al., 2002) might have affected the postprandial metabolic response was also supported by the observation of a lower α-MSH response after CM vs BM. That result suggested a lower impact of CM on postprandial oxidative and inflammatory phenomena along the intestine because α-MSH has been indicated as an anti-inflammatory peptide secreted by many tissues, including intestinal cells, to counteract inflammation (Hoggard, Hunter, Duncan, & Rayner, 2004; Singh & Mukhopadhyay, 2014).

Regarding the response of ECs, that 2-AG did not respond to food intake was already found in previous studies in normal weight subjects (Gatta-Cherifi et al., 2012; Mennella, Savarese, et al., 2015) while it was in disagreement with one study reporting an increase of 2-AG 60 min after a meal in overweight subjects (Tischmann et al., 2019).

A previous study demonstrated that plasma OEA could reflect the fatty acid intake (Fu et al., 2011). It could then be hypothesized that the lower concentration of NAEs at 180 min after CM vs BM could be caused by a slower digestion of the breakfast containing CM that provided fewer precursors for LEA, OEA, and PEA synthesis in the intestine and/or a slower spill-over of NAEs from the gut into the bloodstream (DiPatrizio, Astarita, Schwartz, Li, & Piomelli, 2011; Naughton, Mathai, Hryciw, & McAinch, 2013). Moreover, since plasma NAEs concentration is dependent on the formation and degradation pathways operated by specific enzymes such as the NAPE-PLD and FAAH (Ahn, McKinney, & Cravatt, 2008; Hansen, 2014), it is also likely that the two types of melanoidins differently influenced, by direct or indirect mechanisms, the activity of these enzymes. Further studies should test these hypotheses.

The lack of a breakfast effect on individual appetite sensations and behavior at lunch was observed despite different responses after breakfast from several biomarkers including insulin,

β-endorphin, LEA, OEA and PEA. This supported the evidence that eating episodes result from the cumulative effects of a number of submaximal hormone responses that may have additive or opposing effects. In order to shed light on the biochemical modulation of appetite cues and eating behavior, we displayed the pattern of relationships between the postprandial responses of the monitored biomarkers.

Our data confirmed the results from previous studies that fullness and satiety increase is associated with increased responses of the anorexic hormones GLP-1, leptin, and insulin; hunger reduction is associated with increased insulin and the anorexic hormones PP, PYY, leptin, and GLP-1 (Murphy & Bloom, 2006; Suzuki, Simpson, Minnion, Shillito, & Bloom, 2010); and hunger increase is associated with an increased orexigenic β-endorphin (Veening & Barendregt, 2015), substance-P (Karagiannides et al., 2008) and orexin-A (Edwards et al., 1999). In disagreement with the previous literature, we provided evidence for possible involvement of the neuropeptides α-MSH and neurotensin in the evolution of hunger (Izaguirre, Catalan, & Fruhbeck, 2016; Sohn, 2015).

In parallel with expected correlations between the responses of gut peptides and neuropeptides, new correlations between those responses and the dynamics of NAEs and ECs were displayed. Three previous studies postprandially evaluated only ECs and gut peptides: one study had normal weight subjects (Monteleone et al., 2012), one study had obese subjects (Rigamonti et al., 2015), and one study had patients with Prader-Willi syndrome (Rigamonti et al., 2017). No previous study focused on NAEs, gut peptides and neuropeptides in humans.

While it is known that circulating OEA elicits satiety (Witkamp, 2018), our data showed that all three NAEs may contribute to the anorexic effect of gut peptides and neuropeptides. In contrast with the previous evidence that AEA increases appetite and energy intake (Di Marzo & Matias, 2005), we found that this endocannabinoid correlates with some gut peptides and neuropeptides likely eliciting an anorexic effect. We hypothesized that the longer postprandial period evaluated by us (180 min) compared to the study by Gatta-Cherifi *et.al.* (60 min) might account for this difference (Gatta-Cherifi et al., 2012). Nonetheless, the positive and strong correlation we found between 2-AG and energy intake is in agreement with all previous literature indicating the implication of this endocannabinoid in the regulation of food intake (Witkamp, 2018).

One study limitation involves the consumption of milk at breakfast, as we cannot exclude the

possibility that milk proteins influenced CM and BM effects. However, providing milk at breakfast aligned the experimental meal with the usual dietary habits of the participants thus avoiding a possible bias of laboratory experimental procedures.

5. Conclusions

For the first time, we show in this study that melanoidins, particularly from coffee, affect daily energy intake in humans by targeting physiological mechanisms taking place in the distal intestinal tract. Although both types of melanoidins similarly influence appetite and subsequent energy intake, coffee melanoidins lower the blood glucose peak and insulin response enhancing ghrelin, and blunt the response of all NAEs, α-MSH, orexin-A and β-endorphin. These effects are likely due to the chemical structure of coffee melanoidins that incorporate chlorogenic acids and possibly elicit metabolic effects similar to polyphenol-bound dietary fiber of cereals, such as modulation of digestive enzyme activity, control of oxidative and inflammatory processes in the gastrointestinal tract and modulation of the gut microbiota composition.

Additionally, we shed light on the interplay between postprandial appetite sensations and neurohormonal responses in humans. We confirm some relationships between specific appetite sensations and the response of GLP-1, PP, insulin, leptin, β-endorphin, substance-P and orexin-A. We unravel novel relationships between α-MSH and neurotensin with hunger, all NAEs and anandamide with satiety neurohormonal response, and 2-AG with energy intake.

Overall, our findings have impact in both food science and physiology. Thermal treatment of food can lead to the formation of often disregarded molecules, such as melanoidins, possessing metabolic properties that can support their isolation and utilization as novel functional ingredients to control daily energy intake. The validation of food physiological effects by a multi-system approach integrating human sensations and behaviors with homeostatic and non-homeostatic postprandial responses, despite being costly and time consuming, is mandatory to explain the mechanisms underpinning appetite cues and dietary behavior. This approach can provide a proper scientific foundation for the design of personalized food, which is the stepping stone to open the era of personalized nutrition.

Disclosure Statement:

The authors have no conflicts of interest to declare.

References

1. Ahn, K., McKinney, M. K., & Cravatt, B. F. (2008). Enzymatic pathways that regulate endocannabinoid signaling in the nervous system. *Chem Rev, 108*(5), 1687-1707. doi: 10.1021/cr0782067
2. ALjahdali, N., & Carbonero, F. (2019). Impact of Maillard reaction products on nutrition and health: Current knowledge and need to understand their fate in the human digestive system. *Crit Rev Food Sci Nutr, 59*(3), 474-487. doi: 10.1080/10408398.2017.1378865
3. Berthoud, H. R. (2011). Metabolic and hedonic drives in the neural control of appetite: who is the boss? *Curr Opin Neurobiol, 21*(6), 888-896. doi: 10.1016/j.conb.2011.09.004
4. Borrelli, R. C., Mennella, C., Barba, F., Russo, M., Russo, G. L., Krome, K., . . . Fogliano, V. (2003). Characterization of coloured compounds obtained by enzymatic extraction of bakery products. *Food Chem Toxicol, 41*(10), 1367-1374.
5. Borrelli, R. C., Visconti, A., Mennella, C., Anese, M., & Fogliano, V. (2002). Chemical characterization and antioxidant properties of coffee melanoidins. *J Agric Food Chem, 50*(22), 6527-6533.
6. Camilleri, M. (2015). Peripheral mechanisms in appetite regulation. *Gastroenterology, 148*(6), 1219-1233. doi: 10.1053/j.gastro.2014.09.016
7. Choi, S., Jung, S., & Ko, K. S. (2018). Effects of Coffee Extracts with Different Roasting Degrees on Antioxidant and Anti-Inflammatory Systems in Mice. *Nutrients, 10*(3). doi: 10.3390/nu10030363
8. De Marco, L. M., Fischer, S., & Henle, T. (2011). High molecular weight coffee melanoidins are inhibitors for matrix metalloproteases. *J Agric Food Chem, 59*(21), 11417-11423. doi: 10.1021/jf202778w
9. Di Marzo, V., Ligresti, A., & Cristino, L. (2009). The endocannabinoid system as a link between homoeostatic and hedonic pathways involved in energy balance regulation. *Int J Obes (Lond), 33 Suppl 2*, S18-24. doi: 10.1038/ijo.2009.67
10. Di Marzo, V., & Matias, I. (2005). Endocannabinoid control of food intake and energy balance. *Nat Neurosci, 8*(5), 585-589. doi: 10.1038/nn1457
11. DiPatrizio, N. V., Astarita, G., Schwartz, G., Li, X., & Piomelli, D. (2011). Endocannabinoid signal in the gut controls dietary fat intake. *Proc Natl Acad Sci U S A, 108*(31), 12904-12908. doi: 10.1073/pnas.1104675108
12. Edwards, C. M., Abusnana, S., Sunter, D., Murphy, K. G., Ghatei, M. A., & Bloom, S. R. (1999). The effect of the orexins on food intake: comparison with neuropeptide Y, melanin-

concentrating hormone and galanin. *J Endocrinol, 160*(3), R7-12. doi: 10.1677/joe.0.160r007

13. Fetissov, S. O. (2017). Role of the gut microbiota in host appetite control: bacterial growth to animal feeding behaviour. *Nat Rev Endocrinol, 13*(1), 11-25. doi: 10.1038/nrendo.2016.150
14. Fogliano, V., & Morales, F. J. (2011). Estimation of dietary intake of melanoidins from coffee and bread. *Food Funct, 2*(2), 117-123. doi: 10.1039/c0fo00156b
15. Fu, J., Dipatrizio, N. V., Guijarro, A., Schwartz, G. J., Li, X., Gaetani, S., . . . Piomelli, D. (2011). Sympathetic activity controls fat-induced oleoylethanolamide signaling in small intestine. *J Neurosci, 31*(15), 5730-5736. doi: 10.1523/JNEUROSCI.5668-10.2011
16. Gatta-Cherifi, B., Matias, I., Vallee, M., Tabarin, A., Marsicano, G., Piazza, P. V., & Cota, D. (2012). Simultaneous postprandial deregulation of the orexigenic endocannabinoid anandamide and the anorexigenic peptide YY in obesity. *Int J Obes (Lond), 36*(6), 880-885. doi: 10.1038/ijo.2011.165
17. Green, S. M., Delargy, H. J., Joanes, D., & Blundell, J. E. (1997). A satiety quotient: a formulation to assess the satiating effect of food. *Appetite, 29*(3), 291-304.
18. Hall, K. D., Ayuketah, A., Brychta, R., Cai, H., Cassimatis, T., Chen, K. Y., . . . Zhou, M. (2019). Ultra-Processed Diets Cause Excess Calorie Intake and Weight Gain: An Inpatient Randomized Controlled Trial of Ad Libitum Food Intake. *Cell Metab, 30*(1), 67-77 e63. doi: 10.1016/j.cmet.2019.05.008
19. Hansen, H. S. (2014). Role of anorectic N-acylethanolamines in intestinal physiology and satiety control with respect to dietary fat. *Pharmacol Res, 86*, 18-25. doi: 10.1016/j.phrs.2014.03.006
20. Helou, C., Anton, P. M., Niquet-Leridon, C., Spatz, M., Tessier, F. J., & Gadonna-Widehem, P. (2017). Fecal excretion of Maillard reaction products and the gut microbiota composition of rats fed with bread crust or bread crumb. *Food Funct, 8*(8), 2722-2730. doi: 10.1039/c7fo00430c
21. Helou, C., Denis, S., Spatz, M., Marier, D., Rame, V., Alric, M., . . . Gadonna-Widehem, P. (2015). Insights into bread melanoidins: fate in the upper digestive tract and impact on the gut microbiota using in vitro systems. *Food Funct, 6*(12), 3737-3745. doi: 10.1039/c5fo00836k
22. Hoggard, N., Hunter, L., Duncan, J. S., & Rayner, D. V. (2004). Regulation of adipose tissue leptin secretion by alpha-melanocyte-stimulating hormone and agouti-related protein: further evidence of an interaction between leptin and the melanocortin signalling

system. *J Mol Endocrinol, 32*(1), 145-153. doi: 10.1677/jme.0.0320145

23. Isaksson, H., Rakha, A., Andersson, R., Fredriksson, H., Olsson, J., & Aman, P. (2011). Rye kernel breakfast increases satiety in the afternoon - an effect of food structure. *Nutr J, 10*, 31. doi: 10.1186/1475-2891-10-31
24. Izaguirre, M., Catalan, V., & Fruhbeck, G. (2016). Elucidating the Role of Peripheral Neurotensin in Appetite Control. *Endocrinology, 157*(9), 3391-3393. doi: 10.1210/en.2016-1542
25. Karagiannides, I., Torres, D., Tseng, Y. H., Bowe, C., Carvalho, E., Espinoza, D., . . . Kokkotou, E. (2008). Substance P as a novel anti-obesity target. *Gastroenterology, 134*(3), 747-755. doi: 10.1053/j.gastro.2007.12.032
26. Keesey, R. E., & Powley, T. L. (2008). Body energy homeostasis. *Appetite, 51*(3), 442-445. doi: 10.1016/j.appet.2008.06.009
27. Keogh, J., Atkinson, F., Eisenhauer, B., Inamdar, A., & Brand-Miller, J. (2011). Food intake, postprandial glucose, insulin and subjective satiety responses to three different bread-based test meals. *Appetite, 57*(3), 707-710. doi: 10.1016/j.appet.2011.08.015
28. Levin, F., Edholm, T., Schmidt, P. T., Gryback, P., Jacobsson, H., Degerblad, M., . . . Naslund, E. (2006). Ghrelin stimulates gastric emptying and hunger in normal-weight humans. *J Clin Endocrinol Metab, 91*(9), 3296-3302. doi: 10.1210/jc.2005-2638
29. Mari, A., Schmitz, O., Gastaldelli, A., Oestergaard, T., Nyholm, B., & Ferrannini, E. (2002). Meal and oral glucose tests for assessment of beta -cell function: modeling analysis in normal subjects. *Am J Physiol Endocrinol Metab, 283*(6), E1159-1166. doi: 10.1152/ajpendo.00093.2002
30. Mari, A., Tura, A., Gastaldelli, A., & Ferrannini, E. (2002). Assessing insulin secretion by modeling in multiple-meal tests: role of potentiation. *Diabetes, 51 Suppl 1*, S221-226. doi: 10.2337/diabetes.51.2007.s221
31. Mennella, I., Ferracane, R., Zucco, F., Fogliano, V., & Vitaglione, P. (2015). Food Liking Enhances the Plasma Response of 2-Arachidonoylglycerol and of Pancreatic Polypeptide upon Modified Sham Feeding in Humans. *J Nutr, 145*(9), 2169-2175. doi: 10.3945/jn.114.207704
32. Mennella, I., Fogliano, V., Ferracane, R., Arlorio, M., Pattarino, F., & Vitaglione, P. (2016). Microencapsulated bitter compounds (from Gentiana lutea) reduce daily energy intakes in humans. *Br J Nutr*, 1-10. doi: 10.1017/S0007114516003858
33. Mennella, I., Savarese, M., Ferracane, R., Sacchi, R., & Vitaglione, P. (2015). Oleic acid content of a meal promotes oleoylethanolamide response and reduces subsequent energy

intake in humans. *Food Funct, 6*(1), 204-210. doi: 10.1039/c4fo00697f

34. Monteleone, P., Piscitelli, F., Scognamiglio, P., Monteleone, A. M., Canestrelli, B., Di Marzo, V., & Maj, M. (2012). Hedonic eating is associated with increased peripheral levels of ghrelin and the endocannabinoid 2-arachidonoyl-glycerol in healthy humans: a pilot study. *J Clin Endocrinol Metab, 97*(6), E917-924. doi: 10.1210/jc.2011-3018
35. Morales, F. J., Somoza, V., & Fogliano, V. (2012). Physiological relevance of dietary melanoidins. *Amino Acids, 42*(4), 1097-1109. doi: 10.1007/s00726-010-0774-1
36. Moreira, A. S., Nunes, F. M., Domingues, M. R., & Coimbra, M. A. (2012). Coffee melanoidins: structures, mechanisms of formation and potential health impacts. *Food Funct, 3*(9), 903-915. doi: 10.1039/c2fo30048f
37. Murphy, K. G., & Bloom, S. R. (2006). Gut hormones and the regulation of energy homeostasis. *Nature, 444*(7121), 854-859. doi: 10.1038/nature05484
38. Naughton, S. S., Mathai, M. L., Hryciw, D. H., & McAinch, A. J. (2013). Fatty Acid modulation of the endocannabinoid system and the effect on food intake and metabolism. *Int J Endocrinol, 2013*, 361895. doi: 10.1155/2013/361895
39. Nunes, F. M., & Coimbra, M. A. (2007). Melanoidins from coffee infusions. Fractionation, chemical characterization, and effect of the degree of roast. *J Agric Food Chem, 55*(10), 3967-3977. doi: 10.1021/jf063735h
40. Pastoriza, S., & Rufian-Henares, J. A. (2014). Contribution of melanoidins to the antioxidant capacity of the Spanish diet. *Food Chem, 164*, 438-445. doi: 10.1016/j.foodchem.2014.04.118
41. Rigamonti, A. E., Bini, S., Piscitelli, F., Lauritano, A., Di Marzo, V., Vanetti, C., . . . Sartorio, A. (2017). Hedonic eating in Prader-Willi syndrome is associated with blunted PYY secretion. *Food Nutr Res, 61*(1), 1297553. doi: 10.1080/16546628.2017.1297553
42. Rigamonti, A. E., Piscitelli, F., Aveta, T., Agosti, F., De Col, A., Bini, S., . . . Sartorio, A. (2015). Anticipatory and consummatory effects of (hedonic) chocolate intake are associated with increased circulating levels of the orexigenic peptide ghrelin and endocannabinoids in obese adults. *Food Nutr Res, 59*, 29678. doi: 10.3402/fnr.v59.29678
43. Rosen, L. A., Ostman, E. M., & Bjorck, I. M. (2011). Effects of cereal breakfasts on postprandial glucose, appetite regulation and voluntary energy intake at a subsequent standardized lunch; focusing on rye products. *Nutr J, 10*, 7. doi: 10.1186/1475-2891-10-7
44. Rosen, L. A., Ostman, E. M., Shewry, P. R., Ward, J. L., Andersson, A. A., Piironen, V., . . . Bjorck, I. M. (2011). Postprandial glycemia, insulinemia, and satiety responses in healthy subjects after whole grain rye bread made from different rye varieties. 1. *J Agric Food*

Chem, 59(22), 12139-12148. doi: 10.1021/jf2019825

45. Singh, M., & Mukhopadhyay, K. (2014). Alpha-melanocyte stimulating hormone: an emerging anti-inflammatory antimicrobial peptide. *Biomed Res Int, 2014*, 874610. doi: 10.1155/2014/874610
46. So, M., Hashimoto, H., Saito, R., Yamamoto, Y., Motojima, Y., Ueno, H., . . . Ueta, Y. (2018). Inhibition of ghrelin-induced feeding in rats by pretreatment with a novel dual orexin receptor antagonist. *J Physiol Sci, 68*(2), 129-136. doi: 10.1007/s12576-016-0517-5
47. Sohn, J. W. (2015). Network of hypothalamic neurons that control appetite. *BMB Rep, 48*(4), 229-233. doi: 10.5483/bmbrep.2015.48.4.272
48. Suzuki, K., Simpson, K. A., Minnion, J. S., Shillito, J. C., & Bloom, S. R. (2010). The role of gut hormones and the hypothalamus in appetite regulation. *Endocr J, 57*(5), 359-372. doi: 10.1507/endocrj.k10e-077
49. Tischmann, L., Drummen, M., Gatta-Cherifi, B., Raben, A., Fogelholm, M., Hartmann, B., . . . Adam, T. C. (2019). Effects of a High-Protein/Moderate-Carbohydrate Diet on Appetite, Gut Peptides, and Endocannabinoids-A Preview Study. *Nutrients, 11*(10). doi: 10.3390/nu11102269
50. Veening, J. G., & Barendregt, H. P. (2015). The effects of beta-endorphin: state change modification. *Fluids Barriers CNS, 12*, 3. doi: 10.1186/2045-8118-12-3
51. Vitaglione, P., Lumaga, R. B., Stanzione, A., Scalfi, L., & Fogliano, V. (2009). beta-Glucan-enriched bread reduces energy intake and modifies plasma ghrelin and peptide YY concentrations in the short term. *Appetite, 53*(3), 338-344. doi: 10.1016/j.appet.2009.07.013
52. Vitaglione, P., Mennella, I., Ferracane, R., Rivellese, A. A., Giacco, R., Ercolini, D., . . . Fogliano, V. (2015). Whole-grain wheat consumption reduces inflammation in a randomized controlled trial on overweight and obese subjects with unhealthy dietary and lifestyle behaviors: role of polyphenols bound to cereal dietary fiber. *Am J Clin Nutr, 101*(2), 251-261. doi: 10.3945/ajcn.114.088120
53. Vitaglione, P., Morisco, F., Mazzone, G., Amoruso, D. C., Ribecco, M. T., Romano, A., . . . D'Argenio, G. (2010). Coffee reduces liver damage in a rat model of steatohepatitis: the underlying mechanisms and the role of polyphenols and melanoidins. *Hepatology, 52*(5), 1652-1661. doi: 10.1002/hep.23902
54. Witkamp, R. F. (2018). The role of fatty acids and their endocannabinoid-like derivatives in the molecular regulation of appetite. *Mol Aspects Med, 64*, 45-67. doi:

10.1016/j.mam.2018.01.002

55. Wolever, T. M., Jenkins, D. J., Ocana, A. M., Rao, V. A., & Collier, G. R. (1988). Second-meal effect: low-glycemic-index foods eaten at dinner improve subsequent breakfast glycemic response. *Am J Clin Nutr, 48*(4), 1041-1047. doi: 10.1093/ajcn/48.4.1041
56. Zanchi, D., Depoorter, A., Egloff, L., Haller, S., Mahlmann, L., Lang, U. E., . . . Borgwardt, S. (2017). The impact of gut hormones on the neural circuit of appetite and satiety: A systematic review. *Neurosci Biobehav Rev, 80*, 457-475. doi: 10.1016/j.neubiorev.2017.06.013

Chapter 3

Digestion of milk proteins affects the gastro-intestinal discomforts after milk consumption in healthy subjects: implications of the gut microbiome

Silvia Tagliamonte, …… and Paola Vitaglione

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Abstract

Objective: To clarify the implication of milk protein digestion and related physiological responses (primary outcome), gut microbiome, and gut permeability in the occurrence of gastrointestinal discomforts (GID) after cow's milk consumption in healthy people.

Design: Thirty-nine lactose tolerant healthy subjects, 19 non-habitual milk consumers with GID (NHMC) and 20 habitual milk consumers without GID (HMC), participated in a milk load (250 mL) and a gut permeability test, and provided a faecal sample for gut microbiome analysis. We measured plasma concentration of milk-derived peptides and amino acids, hormones and endocannabinoids and glucose and serum dipeptidyl peptidase-IV (DPPIV) activity over 6h along with indoxyl sulfate urinary excretions and GID over 24h after milk consumption.

Results: Compared to HMC, NHMC showed blunted plasma profiles of 31 peptides and higher scores of GID, along with unchanged plasma ghrelin and anandamide levels, lower insulin, and higher blood glucose peak and serum DPPIV activity after milk consumption. The intestinal permeability was similar between the groups while the habitual diet, lower in dairy products and higher in dietary fiber to protein ratio in NHMC, shaped the gut microbiome with lower level of *Bifidobacteria*, higher level of *Prevotella* and other differences explaining the lower gut proteolytic activity along with the postprandial plasma peptides and urinary indoxyl sulfate. NHMC participants were more anxious than HMC.

Conclusions: A slower and weaker digestion of milk proteins influenced by a lower gut microbiome proteolytic activity, possibly shaped by diet, explained the occurrence of GID in healthy subjects independently from the gut permeability.

1. Introduction

More than 40% of people worldwide suffer from gastrointestinal symptoms including abdominal pain, diarrhoea, constipation, bloating, fullness, nausea, and vomiting without any underlying structural abnormality, diagnosed with functional gastrointestinal disorders (FGIDs) (Sperber et al., 2021). Patients with FGIDs often report reinforced symptoms after consuming some food, such as milk, wheat, onions, garlic, chili, beans and coffee (Böhn et al., 2013; Laatikainen et al., 2020; Black et al., 2020; Fikree and Byrne, 2021). However, gastrointestinal discomforts after consuming cow's milk occur in general population even in absence of FGIDs (Pasqui et al. 2015) or lactose intolerance (Suchy et al., 2010; Laatikainen et al., 2020; Carroccio et al., 2021) leading to a prevalence of self-perception of lactose intolerance that has been estimated between 8 and 20% (Porzi et al., 2021; Nicklas et al., 2011). Following their symptoms and self-perception of lactose intolerance these people often exclude cow's milk and dairy products from their diet, may get suboptimal intakes of calcium and may increase their risk of hypertension and diabetes (Nicklas et al., 2011). The reasons why healthy people suffer from milk-induced gastrointestinal discomforts (GID) have not been clearly ascertained.

Some evidence suggested that the circulating levels of the peptide β-casomorphin-7 (BCM7), might explain the higher GID following A1 than A2 milk consumption (Jianqin et al, 2015; Deth et al., 2016; He et al., 2017; Tulipano et al. 2020). Indeed, BCM7 was believed to be exclusively produced from the genetic variant A1 β-casein at a pH between 2 and 5 (Parashar et al., 2015), and to cause GID through activation of μ-opioid receptors slowing down gastrointestinal transit time (Aslam et al., 2020). Studies that clearly demonstrate a causal role of milk containing A1 β-casein and BCM7 implications in GID are missing. Two RCTs in self-reported lactose intolerant subjects consuming for 8 weeks A1/A2 or A2 milk confirmed higher symptoms with A1/A2 milk (Jianqin et al. 2016; Ho et al., 2014). In one study the symptoms were associated with increased plasma BCM7 levels in fasting subjects but BCM7 levels were not assessed postprandially along with GID occurrence (Jianqin et al. 2016). In the other study the gastrointestinal symptoms were associated with increased gastrointestinal transit time that was hypothesized because of higher stool consistencies, self-assessed by the participants (Ho et al. 2014). However, recent *in vitro* evidence revealed that BCM7 can be formed also from A2 milk, at lower levels than A1 milk (Lambers et al. 2021). Moreover, many bioactive peptides (BAPs) are formed during digestion of milk (for a review see Nielsen et al., 2017) and, according to the activity of the brush border enzymes and gut permeability, they could cross the intestinal barrier and modulate gastrointestinal motility, digestive processes and inflammatory responses *in vivo* (Teschemacher, 2003; Pimentel et al. 2017). For instance,

dipeptidyl peptidase-IV (DPPIV) is the sole brush-border enzyme capable of breaking down BCM7, its expression is up-regulated in the small intestinal mucosa of mice fed with the genetic A1-variant of β-casein and it influences the BCM7 survival in the gut (Tulipano, 2020). Intestinal DPPIV can also hydrolyse other dietary peptides in the gut and, interestingly a soluble form of DPPIV is present in the bloodstream where it cleaves and clips many dietary and endogenous peptides such as incretins (Hasan and Hocher, 2017), neuropeptides, chemokines and other chemoattractants thus affecting metabolic, immunity and inflammatory processes in the body (Trzaskalski et al. 2020). Inhibitors of DPPIV have attracted attention as therapies of many disease conditions such as type-2 diabetes, immune and inflammatory diseases (Shao et al., 2020) and many milk-derived BAPs were reported to have DPPIV inhibitory activity (Tulipano et al., 2020); however, the relationship between DPPIV activity and milk intake is still underexplored in humans.

Mounting evidence indicates that Endocannabinoids (ECs) and N-acylethanolamines (NAEs) can be involved in physiological mechanisms underpinning GID (Witkamp, 2018). These lipid mediators have a pleiotropic activity in the body and modulate several biological pathways underpinning pain sensation and other phenomena such as appetite, macronutrient metabolism, inflammation, and immunity (Witkamp, 2018). On the other hand, it is widely accepted that gut microbiome has a crucial role in nutrient digestion, energy balance and pain regulation through the bidirectional communication in gut-brain axis (Rowland et al., 2018; Guo et al., 2019)

In this study we explored the implication of milk protein digestion and related physiological responses (primary outcome), gut permeability and gut microbiome in the occurrence of gastrointestinal discomforts (GID) after cow's milk consumption in healthy lactose tolerant subjects.

2. Materials and methods

2.1. Study design and participants

The study was conducted at the University of Naples Federico II and was approved by the related Ethics Committee (Protocol number:177/18). Each participant provided written informed consent and received no financial compensation. The trial was registered at ClinicalTrials.gov (number NCT04205045). The protocol ended when the last group of participants completed the protocol (Study Start Date: October 2018; Actual Primary Completion Date: January 2020; Study Completion Date: January 2020).

The study design and participant flow throughout the study are reported in the **Supplementary**

Figure 1. Baseline and post-prandial plasmatic concentrations of milk-derived BAPs, gastrointestinal hormones (including insulin, glucose-dependent insulinotropic peptide, ghrelin, C-peptide), ECs, and NAEs were primary outcomes of the study. Gut microbiome, urinary excretions of lactulose, mannitol and sucralose upon a gut permeability test (GPT), fasting serum DPPIV concentration, plasmatic baseline and postprandial concentration of amino acids (AA), glycaemia, and serum DPPIV activity, postprandial GID, and urinary excretions of indoxyl sulfate and lactose were secondary outcomes.

Potentially eligible adults (n=101) were 18-60 years, with a BMI 18.5-30 kg/m^2, non-pregnant or lactating females, not taking medicines, without relevant organic, systemic and metabolic diseases, a history of abdominal surgeries, food intolerance or alcohol abuse, habitual consumption of probiotics or laxatives, or antibiotics. They were further screened on the basis of weekly consumption of milk (<150 mL/week or >700 mL/week), absence or occurrence of GID after milk consumption and a lactose breath test (detailed in Supplementary methods). Participants were diagnosed as lactose intolerants and excluded from the study if H_2 concentrations in the breath were ≥20 ppm over baseline values along with presence of GID, whereas volunteers with a positive test and no GID during the test (Lactose malabsorbers) and with a negative test were included into the study. Subjects reporting no milk-related GID and habitual milk consumption >700 mL/week were recruited in HMC group, whereas those reporting milk related GID and habitual milk consumption <150 mL/week were recruited in NHMC group. Frequencies of self-reported GID after milk consumption from NHMC group at recruitment are displayed in the **Supplementary Table 1**.

Enrolled subjects participated in three visits to undergo anthropometric, lifestyle and psychological characterization, a gut permeability test and a milk-load test.

2.2. Subject characterization

Anthropometric characterization of participants consisted in measuring body weight and height. Lifestyle characterization included the record of dietary habits through a Food Frequency Questionnaire (FFQ) (Vitaglione *et al.*, 2015), dietary behaviour through the Three-Factor Eating Questionnaire (TFEQ) (Stunkard *et al*., 1985), physical activity level through the International Physical Activity Questionnaire (IPAQ) (Craig *et al.*, 2003) and frequency and consistency of faeces through the King's Stool Chart (KSG) (Whelan *et al.*, 2004; Whelan *et al.*, 2008). Psychological characterization included assessment of individual psychological profile through the Depression, Anxiety and Stress Scale (DASS) (Lovibond *et al.*, 1995) and the health-related quality-of-life questionnaire (SF-12) (Ware *et al.*, 1996).

Before leaving the laboratory, participants were instructed on how to collect a faecal sample

according to the standard operating procedure (SOP 004) of the International Human Microbiome Standards (www.microbiome-standards.org) for gut microbiome analysis (Meslier et al., 2020; Tagliamonte *et al.*, 2021).

2.4 Gut permeability test

A three probes-based gut permeability test (GPT) was performed to detect small and large intestinal permeability. A full description of the test and data analysis is reported in the **Supplementary Materials**

2.5. Milk load test

After 2-days of milk and dairy product-free diet including a standardised lunch and dinner (boiled rice and roasted chicken/fish) on the day before the test, following an overnight fast, participants underwent a blood drawing and measurement of blood glucose on capillary blood, and collected a urine sample before drinking 250 mL of ultra-high temperature processed (UHT) semi-skimmed cow's milk containing A1/A2 ß-caseins (provided by Lactalis). Blood samples were collected by venipuncture into serum separator, EDTA-containing tubes, and EDTA aprotinin-containing tubes at 0.5, 1, 2, 4, and 6 hours in parallel with measures of blood glucose, whereas urine samples were collected into urine pot at 1, 2, 4, 6, 8, 12, and 24 hours after milk consumption from volunteers who also rated the GI symptoms and appetite sensations by VAS questionnaires. Once prepared, serum and plasma samples were aliquoted and immediately frozen at −80°C until analysis. Moreover, participants were asked to avoid milk and dairy products up to 24 hours after milk ingestion and standardized lunch (bread and lactose-free raw ham) and dinner (bread and tuna) were provided to each volunteer. Compliance to the protocol was assessed by a 24-hour self-recorded food diary.

2.6 Metabolomics

Blood samples were analysed to assess: the concentration of milk-derived bioactive peptides (BAPs) and amino acids (AA) by LC-HRMS; the serum DPPIV concentration and activity by Bio-Plex Pro immunoassay kits; plasma endocannabinoids (ECs) and N-acylethanolamines (NAEs) by LC-HRMS; gastrointestinal hormones (such as Ghrelin, Insulin, C-peptide, and GIP) by Bio-Plex Pro immunoassay kits and blood glucose by finger pricking and using a bedside glucometer (OneTouch Sure Step; Life Scan Inc.). Urine samples were analysed to assess: the concentration of lactose, glucose and galactose and indoxyl sulphate by LC-MS/MS. Details are available in the **Supplementary Materials** and **Supplementary Table 2-5**.

2.7 Metagenomics

Microbial DNA extraction from faecal samples and metagenomic library preparation were carried out following the protocol reported by Meslier *et al* (2020), according to the IHMSC

SOP 07. A full description of the sequencing and data analysis procedures is reported in the **Supplementary Materials**.

2.8 Statistical analysis

The sample size was calculated considering as primary endpoints postprandial plasma BAPs, GI hormones, ECs and NAEs concentration. A sample size of 19 participants per group would be adequate to detect a 40% change in plasma BAPs, by using data reported in previous studies (Deth et al., 2016).

Concerning postprandial circulating GI hormones, ECs and NAEs, a sample size of 13 volunteers would be adequate to detect a significant postprandial change in agreement with Mennella and co-workers (2015; 2016). Therefore 19 participants for each group would be sufficient to detect significant differences on selected biomarkers with an α error of 0.05, 80% power and 2-sided testing.

Statistical analysis and visualization were carried out in R environment version 4.0.3 (https://www.r-project.org). After being checked for normality, significantly skewed variables were transformed in ln (x+k) with k values zeroing the skewness. For those that showed a normal distribution at the Shapiro-Wilk test an independent sample T test was performed to check differences between groups, whereas for those variables including potential confounding factors, an ANCOVA analysis was performed including covariates in the analysis. For those non-parametric variables, Mann-Whitney test was performed to compare between group differences. The chi-square test of independence was performed using *chisq.test* function (*stats* package) to analyse the frequency table formed by two categorical variables.

Postprandial differences over time within and between the groups, for normally distributed variables, were tested by one-way ANOVA with repeated measures and Bonferroni adjustment for multiple comparisons, whereas when normal distribution criterion was not met, Friedman test and pairwise Wilcoxon as a *post hoc* was performed.

The postprandial total area under the curves (AUCs) were estimated using the linear trapezoidal rule, and differences in AUCs between the groups were assessed by proper parametric or non-parametric analyses including as covariates the potential confounding variables measured at baseline. Two-tailed P values lower than 0.05 were considered significantly different. Data are expressed as means±SEM.

In order to explore differences in microbiome profiles, a linear discriminant analysis (LDA) effect size (LEfSE) was applied (Segata et al., 2011).

3. Results

A higher anxiety level, a lower consumption of dairy products and a higher intake of dietary fiber to protein ratio were distinctive characteristics of participants suffering from milk-induced GID, whereas gut permeability was similar between groups.

Table 1 shows the general, the anthropometric, the lifestyle and psychological characteristics of the 19 (10F/9M, average BMI 23.5±0.7 kg/m^2, age 24.2±0.9 years) NMHC and 20 (10F/10M, average BMI 25.6±0.8 kg/m^2, age 25±0.7 years) HMC participants.

The two groups were similar for all the characteristics assessed except for anxiety level that was higher in NHMC and for some aspects of the diet. Compared to HMC, NHMC consumed a significantly lower amount of conventional milk (selection criterion) but higher amount of lactose-free milk (about 37 g/day), and lower amounts of dairy products. NHMC also showed a higher intake of dietary fiber/protein ratio and a trend towards higher intake of plant protein/animal protein ratio.

Table 1: General, anthropometric, lifestyle, and psychological characteristics of habitual milk consumers (HMC) and non habitual milk consumers (NHMC).

	NHMC (n=19)	**HMC (n=20)**	**P-value**
Gender (M/F)	9/10	10/10	
Age	24.16 ± 0.90	25.00 ± 0.65	0.55
Body weight (kg)	66.53 ± 2.88	72.44 ± 3.12	0.17
BMI (kg/m^2)	23.50 ± 0.65	25.56 ± 0.82	0.06
Total METs [a]	2723.89 ± 556.86	2590.42 ± 562.52	0.77
King's stool chart score	3.74 ± 0.50	3.82 ± 0.52	0.84
TFEQ RESTRAINT	10.32 ± 1.42	9.85 ± 1.02	0.79
TFEQ DISINHIBITION	5.89 ± 0.96	7.35 ± 0.90	0.27
TFEQ HUNGER	5.05 ± 0.86	5.25 ± 0.74	0.86
QUALITY OF LIFE-PCS [b]	56.53 ± 1.06	56.92 ± 0.73	0.42
QUALITY OF LIFE-MCS [c]	41.06 ± 2.33	45.51 ± 2.12	0.08
Depression			
normal/mild, n (%)	16 (84.2%)	15 (75.0%)	0.75
moderate/severe/extremely severe, n (%)	3 (15.8%)	5 (25.0%)	
Anxiety			
normal/mild, n (%)	10 (52.6%)	19 (95%)	**0.008***
moderate/severe/extremely severe, n (%)	9 (47.4%)	1 (5%)	

Stress			
normal/mild, n (%)	14 (73.7%)	16 (80.0%)	0.93
moderate/severe/ extremely severe, n (%)	5 (26.3%)	4 (20.0%)	
Habitual diet			
Milk intake (mL/week)	80.52 ± 26.11	1197.75 ± 150.13	**<0.001***
Lactose-free milk intake (mL/week)	276.32 ± 77.74	11.17 ± 10.00	**0.012***
Dairy products intake (g/week)	1013.00 ± 149.54	1995.40 ± 136.98	**<0.001***
Aged cheese intake (g/week)	123.96 ± 34.76	234.6 ± 43.11	**0.022***
Daily energy intake (kcal/day)	1942.33 ± 194.12	2133.56 ± 140.80	0.43
% Energy from protein (%)	17.90 ± 0.52	18.63 ± 0.61	0.37
% Energy from fats (%)	37.27 ± 1.98	36.39 ± 1.02	0.73
% Energy from carbohydrates (%)	40.93 ± 1.92	41.74 ± 1.32	0.75
% Energy from fiber (%)	2.74 ± 0.27	2.21 ± 0.15	0.10
% Energy from alcohol (range/mean) (%)	1.19 ± 0.30	0.98 ± 0.23	0.52
Italian Mediterranean Index (range/mean)	5.68 ± 0.31	5.75 ± 0.33	0.97
Plant protein/animal protein intake ratio	0.55 ± 0.06	0.43 ± 0.04	0.06
Dietary Fiber/Protein intake ratio	0.31 ± 0.03	0.24 ± 0.02	**0.016***

* p<0.05 HMC *vs* NHMC assessed by Independent sample T test or Mann-Whitney test. Data are expressed as means ± SEM.
[a] Metabolic equivalent of tasks;
[b] Physical component summary;
[c] Mental component summary.

Gut permeability did not differentiate participants suffering from milk-related GID. Indeed, NHMC and HMC showed similar 24h urinary excretions of lactulose, mannitol, and sucralose after the ingestion of sugar solution (**Figure 1**).

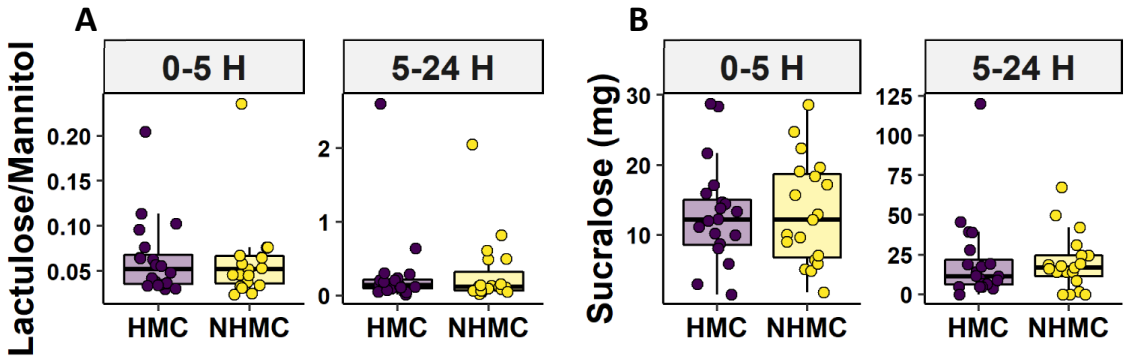

Figure 1: A) Urinary excretion of lactulose/mannitol and B) Urinary excretion of sucralose in habitual milk consumers (HMC) and non-habitual milk consumers (NHMC) within 0-5 h and 5-24 h intervals upon the gut permeability test. The box plots show the data distribution based on first quartile, median and third quartile.

A slower protein digestion in participants with GID was accompanied by weaker hormonal and endocannabinoid responses

After milk consumption, a slower and lower appearance of overall bioactive peptides (BAPs) in plasma from NHMC than HMC occurred (**Figure 2a**). The plasma profile of overall BAPs did not change until 4h when BAPs concentration weakly peaked to fall to baseline within 6h after milk consumption in NHMC. Contrarily in HMC, overall plasma BAPs peaked after 30 min, returned to baseline after 4h and peaked again 6h after milk consumption.

The smaller circulating levels of BAPs (**Figure 2b**) elicited a weaker response of ghrelin, insulin and endocannabinoids in NHMC. Indeed, NHMC participants exhibited unchanged plasma levels of ghrelin over time, while in HMC ghrelin concentration fell 30 and 60 min after milk consumption (**Figure 2c**). Moreover, NHMC showed a significantly lower circulating level of insulin 1 hour after milk consumption compared to HMC (**Figure 2c**). The plasma insulin peak after 30 min in NHMC was likely due to the insulinotropic effect of branched chain aminoacids, significantly higher in plasma from NHMC vs HMC 30 min after milk consumption (**Figure 2e**). However, the insulin response in NHMC was insufficient to control blood glucose that peaked 30 min after milk consumption (**Figure 2f**) and to lower AEA that did not change postprandially contrarily to the reduction in HMC and in accordance with ghrelin response (**Figure 2d**). No changes were found in circulating levels of 2-AG in any group; however, plasma levels of 2-AG were significantly lower 4h and 6h after milk consumption in NHMC than HMC. Contrarily to AEA a faster reduction of circulating N-acylethanolamines (NAEs) was found in NHMC than HMC (see **Supplementary Figure 2** for the profile of each NAE).

Despite differences in the profile of some aminoacids, postprandial plasma profile of total aminoacids did not differ between NHMC and HMC participants (**Supplementary Figure 3**). Similarly, the two groups showed a similar plasma response of C-peptide and GIP (**Supplementary Figure 4**).

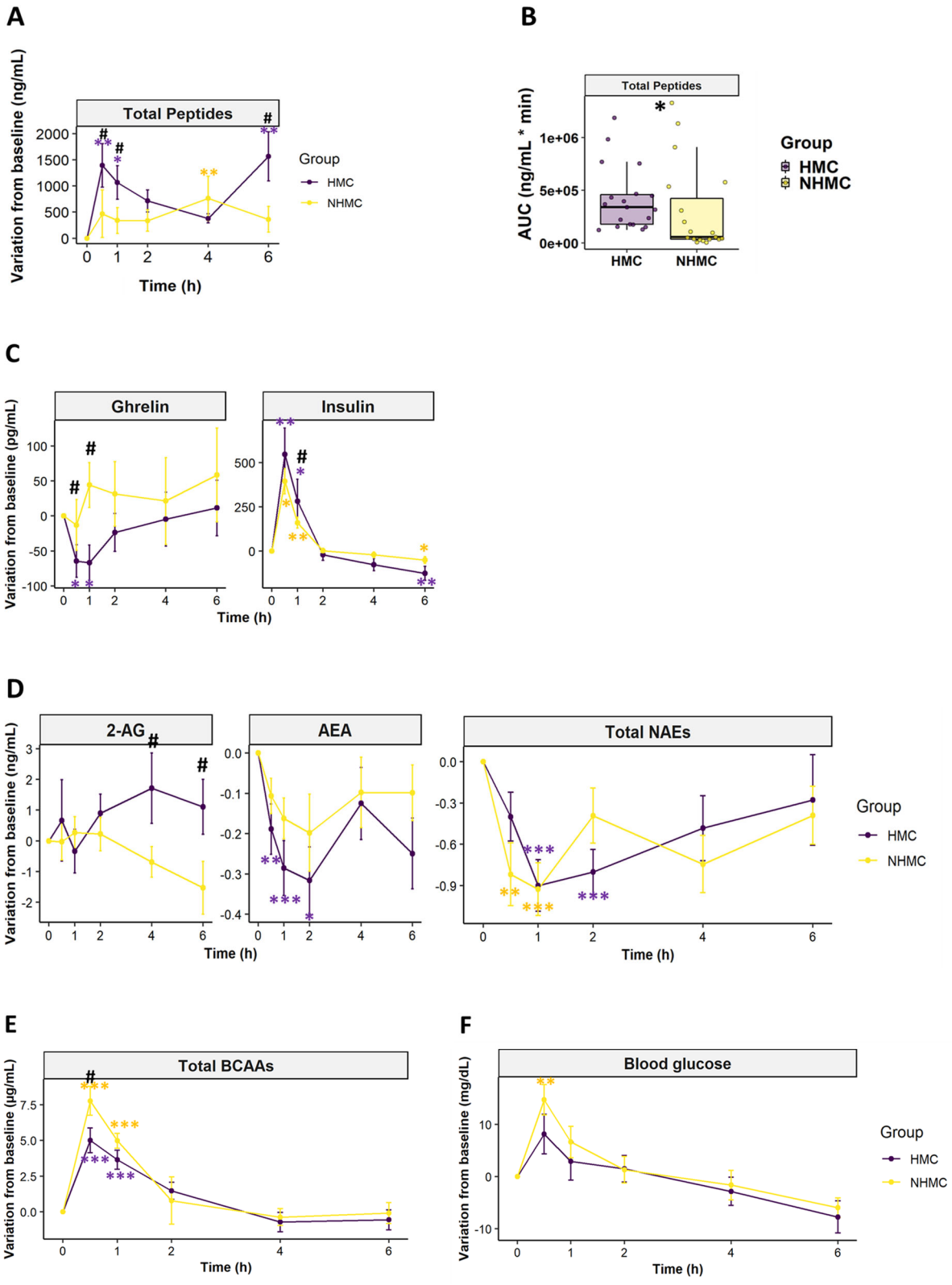

Figure 2: A) Plasma total milk-derived bioactive peptides **(**BAPs) in habitual milk consumers (HMC) and Non-habitual milk consumers (NHMC) upon 250 mL of milk consumption. Data are shown as means ± SEM. * p-value<0.05; ** p –value<0.01 within-group difference versus baseline assessed by One-way ANOVA repeated measure. # p<0.05 between-group difference

assessed by One-way ANOVA repeated measure.

B) Area under the curves (AUCs) of overall circulating BAPs in HMC and NHMC upon 250 mL milk consumption. * p-value<0.05 between-group difference HMC *vs* NHMC assessed by ANCOVA adjusted for baseline values.

C) Time-concentration curves of plasma ghrelin and insulin in HMC and NHMC upon 250 mL of milk consumption. Data are shown as means ± SEM. * p –value <0.05; ** p –value<0.01; *** p-value<0.001 within-group difference versus baseline and Bonferroni adjustment for multiple comparisons; # p<0.05 between-group difference assessed by One-way ANOVA repeated measure controlling for BMI.

D) Time-concentration curves of plasma branched-chain amino acids (BCAAs) in HMC and NHMC upon 250 mL of milk consumption. Data are shown as means ± SEM. # p<0.05 between-group difference HMC *vs* NHMC assessed by One-way ANOVA repeated measure.

E) Time-concentration curves of blood glucose in HMC and NHMC upon 250 mL milk consumption. Data are shown as means ± SEM. ***p*<0.05 for the measured time point compared with baseline within each group assessed by One-way ANOVA repeated measure and Bonferroni adjustment for multiple comparisons.

The higher circulating levels of opioid agonists *vs* antagonists BAPs explained the GID early after milk consumption.

In an attempt to clarify the relationships between protein digestion and postprandial GID, the milk-derived BAPs were sub-grouped for their known bioactivity as opioid agonists, opioid antagonists, DPPIV inhibitors and peptides with other activities including antioxidant, anti-inflammatory, anti-hypertensive, and anxiolytic. The plasma profiles of the 31 BAPs are reported in **Supplementary Figure 5**.

Looking at the BAPs profiles and levels, data showed that opioid antagonist BAPs and those with other activities, were significantly lower in NHMC vs HMC, whereas the statistical significance was not reached for the differences in opioid agonists and DPPIV inhibitors BAPs (**Figure 3a** and **3b**). The lower circulating level of opioid antagonists in NHMC may support a slower postprandial GI transit time in NHMC vs HMC. Moreover, focusing on the opioid agonists and antagonists BAPs, the higher relative amount of opioid agonists vs antagonists in circulation in NHMC than HMC might explain the higher frequency and severity of uncomfortable fullness only over 1h postprandially (**Figure 3c** and **Figure 3d**). Indeed, this symptom was perceived most in NHMC early postprandially and lowered later on, between 2

and 6h, when gas, abdominal bloating, and more frequent bowel movements increased (**Figure 3e**).

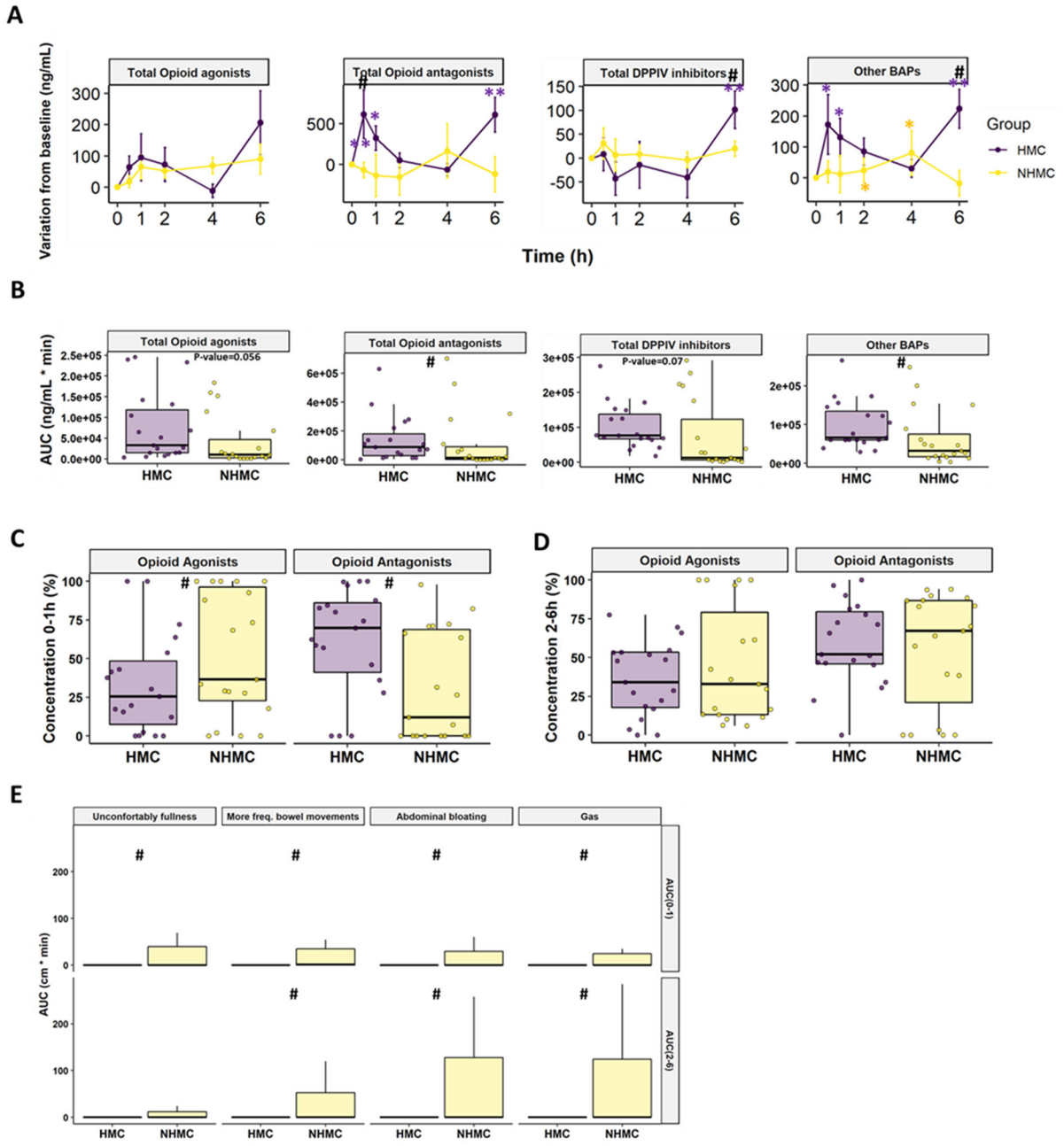

Figure 3: A) Plasma time-concentration curves of milk-derived bioactive peptides (BAPs) subgrouped according to the biological properties, in habitual milk consumers (HMC) and Non-habitual milk consumers (NHMC) upon 250 mL milk consumption. * p–value <0.05; ** p–value<0.01 within-group difference versus baseline and Bonferroni adjustment for multiple comparisons. # p-value<0.05 between-group difference HMC *vs* NHMC.

B) Areas under the curve (AUCs) of BAPs subgrouped according to the biological properties in HMC and NHMC upon 250 mL milk consumption. # p-value<0.05 between-group difference

HMC *vs* NHMC assessed by ANCOVA adjusted for baseline values.
C) Circulating relative amount of opioid agonists and antagonists BAPs over 0-1h time-period in HMC and NHMC upon 250 mL milk consumption. # p-value<0.05 between-group difference HMC *vs* NHMC assessed by ANCOVA adjusted for baseline values.
D) Circulating relative amount of opioid agonists and antagonists BAPs over 2-6h time-period in HMC and NHMC upon 250 mL milk consumption.
E) Most reported gastrointestinal discomforts over 0-1h and 2-6h time-periods in HMC and NHMC upon 250 mL milk consumption. # p-value<0.05 between-group difference HMC *vs* NHMC assessed by One-way ANOVA.

A lower proteolytic activity of the gut microbiota explained the GID and the lower hunger more later after milk consumption.

Compared to HMC, the significantly lower plasma concentration of overall BAPs 6h after milk consumption in NHMC (Figure 2a) indicated a less efficient digestion of proteins in the distal gastro-intestinal tract. The presence of undigested proteins in the large intestine was coherent with the lower urinary excretion of indoxyl sulfate (**Figure 4**) and explained the lower hunger recorded between 6-24h after the milk consumption in NHMC vs HMC (**Figure 5**).

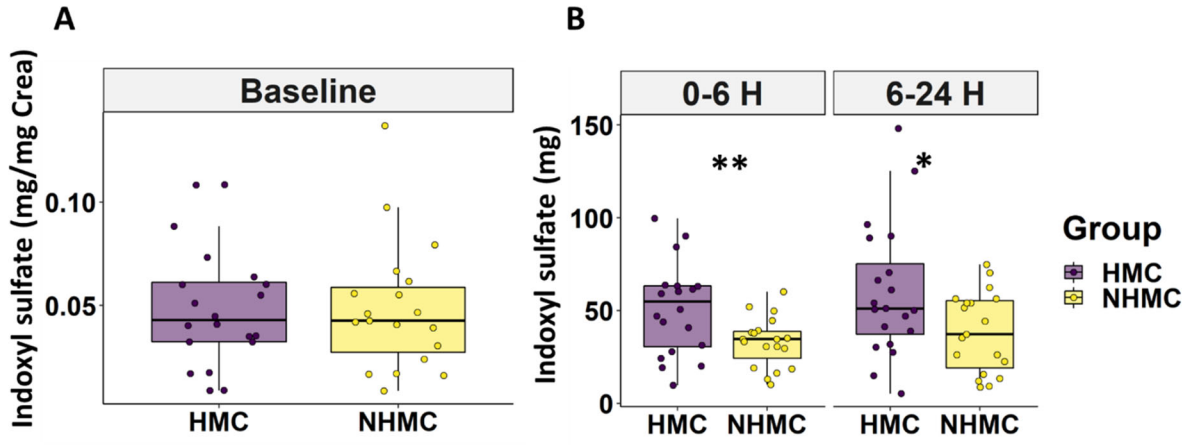

Figure 4: A) Urinary Indoxyl sulfate excretion in habitual milk consumers (HMC) and Non-habitual milk consumers (NHMC) at baseline and B) within the interval 0-6 h and 6-24 h (b) upon milk consumption. * $p<0.05$; ** $p<0.01$ HMC *vs* NHMC assessed by Independent sample T test. The box plots show the data distribution based on first quartile, median and third quartile.

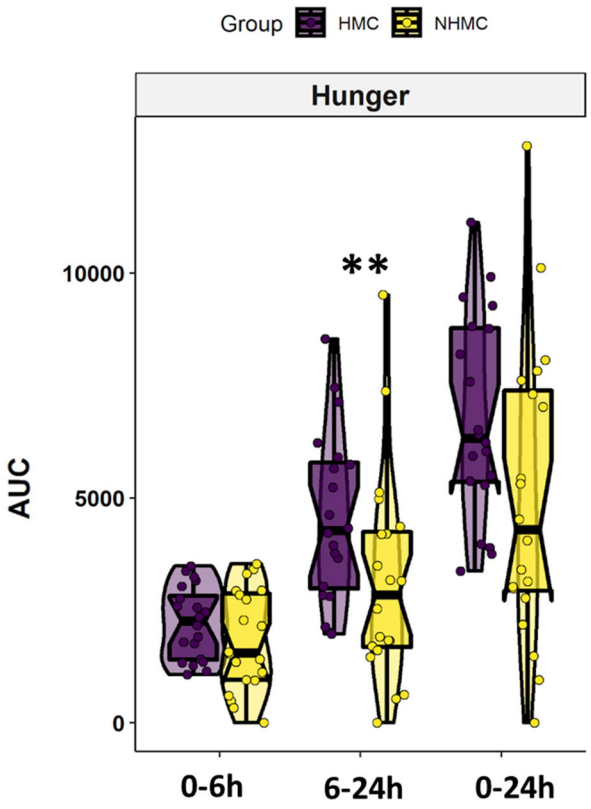

Figure 5: Violin plots representing the hunger AUC sensation monitored by visual analog scales (VAS) in participants from habitual milk consumer group (HMC) and Non-habitual milk consumer group (NHMC) upon 250 mL milk consumption. ** p–value<0.05 between-group difference HMC *vs* NHMC assessed by ANCOVA adjusted for baseline values.

This framework was explained by the lower proteolytic activity of the gut microbiota in NHMC, according to the lower abundance of proteases' genes (**Figure 6a,b**). Interestingly, gut microbiome in NHMC also showed a lower abundance of glycoside hydrolases genes (**Supplementary Figure 6a**), however this feature did not affect lactose digestion as evidenced by urinary excretion of lactose, glucose and galactose over the milk load test (**Supplementary Figure 6b**). Gut microbial diversity and gene richness were similar between the groups (**Figure 6c** and **Figure 6d**). However, differences in gut microbiome composition between NHMC and HMC were observed. Compared to HMC, NHMC showed a lower abundance of *Actinobacteria* phylum and higher abundance of *Paraprevotella* and *Prevotella*, along with higher abundance of *Bacteroides clarus*, *Coprococcus eutactus* and *Ruminococcus lactaris.* Conversely, *Bifobacterium adolescentis*, *Bifidobacterium longum*, *Dialister invisus* were higher in HMC

than NHMC (**Figure 6e**). Moreover, NHMC showed a lower abundance of KEGG metabolic pathways (**Supplementary Figure 7**).

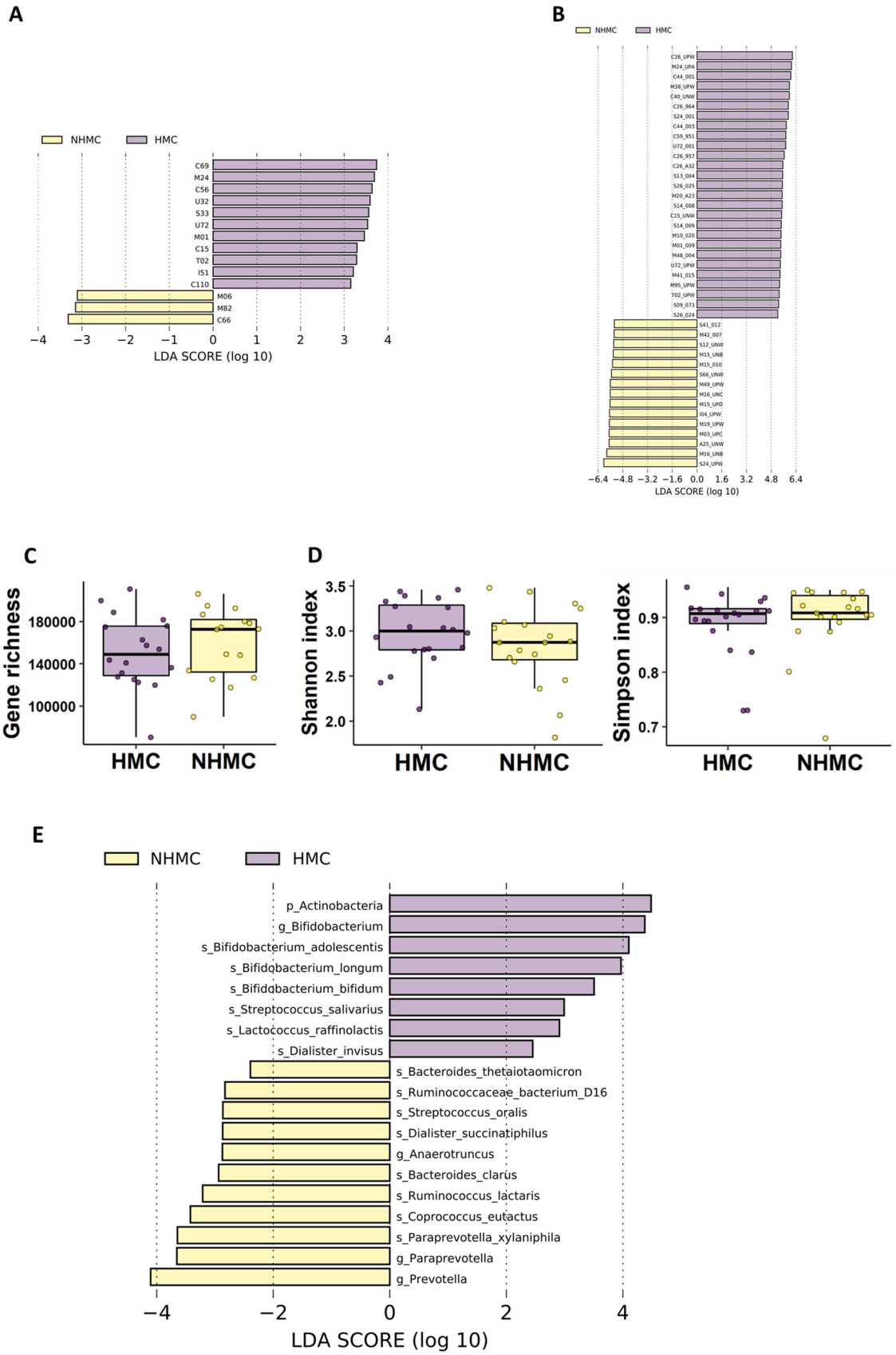

Figure 6: Linear discriminant analysis effect size (LEfSe) showing proteases families (A) and genes (B) that were differentially abundant between habitual milk consumers (HMC) in light violet and Non-habitual milk consumers (NHMC) in yellow. The proteases with statistically significant change ($p < 0.05$) and the logarithmic linear discriminant analysis (LDA) score threshold was set to 2. Proteases families: C, Cysteine; I, inhibitor; M, Metallo; S, Serine; T, Threonine; U, Unknown. C) Microbial gene richness of habitual milk consumers (HMC) and Non-habitual milk consumers (NHMC), D) Microbial Shannon and Simpson diversity indices of habitual milk consumers (HMC) and Non-habitual milk consumers (NHMC). E) Linear discriminant analysis effect size (LEfSe) showing species that were differentially abundant between habitual milk consumers (HMC) in light violet and Non-habitual milk consumers (NHMC) in yellow. The taxon of bacteria with statistically significant change ($p < 0.05$) and the logarithmic linear discriminant analysis (LDA) score threshold was set to 2. The name of the taxon level is abbreviated as p-phylum; g-genus and s-species.

Plasma concentrations of 10 milk-derived BAPs may be long-term biomarkers of intake of dairy products and DPPIV-inhibitor BAPs affect serum DPPIV activity.

In participants, fasting from 10h after 2 days of a dairy product-free diet, 10/31 BAPs were found at significantly lower concentrations in NHMC vs HMC (see Supplementary Figure 5). Among those BAPs, 3 were opioid agonists, 1 opioid antagonists, 3 DPPIV inhibitors and 3 were other BAPs.

Interestingly, the lower concentration of DPPIV-inhibitor peptides at baseline was accompanied by a trend towards higher serum DPPIV activity ($p=0.058$) in NHMC than HMC, that became significant 6 hours after milk consumption (**Figure 7a**) and over six hours postprandially (**Figure 7b**). That effect was possibly due to the increased postprandial plasma concentration of BAPs with a DPPIV inhibitor activity only in HMC, as demonstrated by the significant inverse correlation between plasma DPPIV inhibitors' concentration and serum DPPIV activity ($r= -0.413$, $p=0.011$).

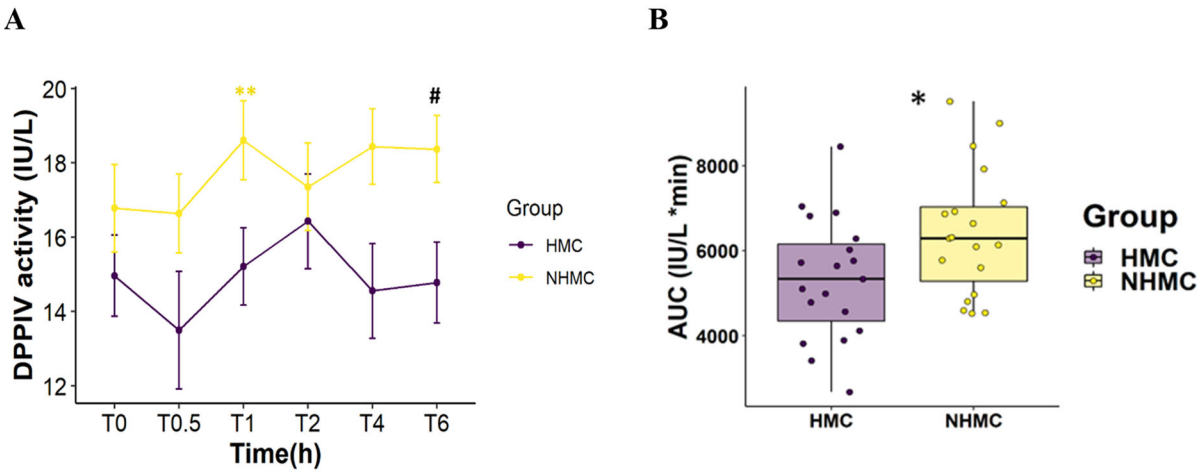

Figure 7: A) Serum Dipeptidyl peptidase IV (DPPIV) activity profile and B) areas under the curve (AUCs) B) in participants from habitual milk consumers (HMC) and Non-habitual milk consumers (NHMC) upon 250 mL of milk consumption. In A) Data are shown as means ± SEM. ***p*<0.05 for the measured time point compared with baseline within each group assessed by One-way ANOVA repeated measure and Bonferroni adjustment for multiple comparisons. # p<0.05 between-group difference assessed by One-way ANOVA repeated measure. In B) * p-value<0.05 between-group difference HMC *vs* NHMC assessed by ANCOVA adjusted for baseline values. The box plots show the data distribution based on first quartile, median and third quartile.

4. Discussion

This study showed that healthy people suffering with GID after cow's milk consumption presented a slower and weaker digestion of milk proteins that was proved by the lower plasma levels of 31 milk-derived peptides and urinary excretion of indoxyl sulfate along with the occurrence of GID.

While previous studies focused on one casein-derived opioid receptor agonist, BCM7 (Jianquin et al, 2015; Deth et al., 2016), we quantified eleven milk protein-derived peptides between opioid agonists (n=7) and opioid antagonists (n=4). By binding of opioid receptors in the enteric nervous system, opioid peptides control gastrointestinal secretion and motility (Holzer, 2009) causing inhibition of gastric emptying, constipation, abdominal distention, bloating, abdominal discomfort and gastroesophageal reflux can occur (Holzer, 2007; Kurz and Sessler, 2003). Conversely, opioid antagonist peptides contrast those effects (Holzer, 2009). Therefore, the higher postprandial contribution of opioid agonists *vs* antagonists over the first hour explained the uncomfortable fullness and the slower gastrointestinal transit time that was maintained for 6h after milk consumption by the low level of opioid antagonists BAPs in participants with GID.

The slower protein digestion explained the blunted responses of ghrelin, insulin and endocannabinoids in the participants with GID.

A prolonged sensing of aminoacids and peptones in the stomach (Vancleef et al., 2015) and/or the physiological response to anxiety possibly enhanced by milk consumption (Carlini et al., 2004) in the participants with GID explained the unchanged plasma ghrelin after milk consumption. This phenomenon, in combination with the lower insulin response, explained the blood glucose peak. Indeed, ghrelin increases hepatic glucose production and decreases the glucose disposal rate (Sun et al., 2006; Vestergaard et al., 2008). Conversely, the expected postprandial reduction of ghrelin, resulting from the protein sensing and/or increased osmolarity in the small intestinal lumen as well as protein-induced postabsorptive signals, improved glucose homeostasis in the participants without GID (Dranse et al., 2018; Overduin et al., 2014).

In accordance to previous evidence, the plasma response of anandamide mirrored plasma ghrelin and was coherent with insulin response (Hillard, 2017; Gatta-Cherifi et al., 2012, Di Marzo et al., 2009) while postprandial plasma 2-AG levels did not change after the meal (Walker et al., 2020; Gatta-Cherifi et al., 2012; Mennella et al., 2015).On the other hand, the faster reduction of circulating NAEs in the participants with GID might indicate a slower

intestinal uptake of fatty acids that are the precursors of NAEs in the enteroendocrine cells (Joosten et al., 2010).

We hypothesised that **undigested proteins in the intestinal lumen determined the GID and reduced hunger** through the wide net of chemo- and mechano-sensors lining on the gastrointestinal mucosa (Berthoud et al., 2021; Linan-Rico et al., 2016; Van Avesaat et al., 2015). Having demonstrated a similar gut permeability between the participants, **proteolytic capability of the gut microbiome likely affected the plasma levels of peptides late after milk consumption**. Although milk proteins are highly digestible proteins, a residual amount escapes human digestion and enters the colon. The extent of colonic protein metabolism results from the amount of proteins/peptides available in the intestinal lumen and the capability of the microbiome to break down/ferment them (Peled and Livney, 2021). The lower proteolytic activity in the gut of the participants with GID was supported by a lower level of protease genes in the microbiome, along with differences in the levels of species belonging to *Streptococcus*, *Ruminococcus* and *Bacteroides*, previously associated at different extent with faecal protease activity (Caroll et al., 2013). Therefore, in people with GID undigested proteins or peptones that entered the colon were not further hydrolysed by bacterial proteases and could not be absorbed (Freeman, 2015), contrary to what occurred in participants without GID experimenting the second plasma peptide peak 6h after milk consumption. The weaker colonic proteolytic activity could determine a lower availability of tryptophan, explaining the lower urinary excretion of indoxyl sulfate, the hepatic metabolite of indole, a gut microbial metabolite of tryptophan (Agus et al., 2018). However, as NHMC exhibited higher intestinal levels of *Bacteroides thetaiotaomicron*, capable to produce phenylpropanoid metabolites from tryptophan fermentation (Russell et al., 2013), we could not exclude that other tryptophan-metabolizing pathways in the intestine took over and led to the production of tryptophan metabolites that were not monitored in this study.

Some differences in the microbiome of the two groups of participants were explained by the differences in the habitual diets. According to the higher intake of milk and dairy products, the participants without GID had a higher gut level of some potentially probiotic *Bifidobacterium* species, namely *B. bifidum, B. adolescentis,* and *B. longum*, in agreement with several studies showing the ability of milk proteins to increase the counts of Lactobacilli and Bifidobacteria (for a review see Zhang, Zhang, Li, & Liu, 2020). On the other hand, the tendency to higher intake of plant than animal proteins in participants with GID was coherent with the higher gut level of the genus *Prevotella*, traditionally associated with agrarian-type diet (Gorvitovskaia et al., 2016). Moreover, the higher fiber to protein ratio in the diet of

participants with GID might shape the lower ability of their gut microbiome to metabolise dietary proteins. Indeed, dietary fibers can increase bowel motility thus reducing, over a long period, the selection of microorganisms using dietary proteins to survive (Diether & Willing, 2019; Korpela, 2018). Such a framework (as discussed above) explained the lower levels of *Streptococcus* spp. (*S. oralis* and *S. salivarius*) and the higher levels of *Ruminococcus* spp. (namely *R. bacterium* D16 and *R. lactaris)*, in agreement with previous associations with faecal protease activity (Carroll et al., 2013). Contrarily, the higher level of *Bacteroides* spp. (*B. clarus* and *B. thetaiotaomicron*) in participants with GID was in disagreement with most literature reporting a high proteolytic capacity of *Bacteroides* and was in accordance with one study in Crohn's disease patients reporting an inverse correlation between *Bacteroides* and faecal tryptic activity (Midtvedt et al., 2013).

From a health viewpoint, although we selected healthy participants, **participants with GID showed specific signatures in their gut microbiome typical of gut inflammatory conditions**. The low faecal abundance of *Actinobacteria* phylum was also found in patients with functional abdominal bloating/distention (FABD) (Noh and Lee, 2020) and the high abundance of *Paraprevotella* and *Prevotella* genera was a feature positively associated with irritable bowel syndrome (IBS) and FABD (Wang et al., 2019; Noh and Lee, 2020). Similarly, high levels of *Bacteroides clarus*, *Coprococcus eutactus* and *Ruminococcus lactaris* were previously found in patients with IBS/IBD, FABD or Crohn's diseases (El Mouzan et al., 2018; Rajilić–Stojanović et al., 2011) whereas low levels of *Bifobacterium adolescentis*, *Bifidobacterium longum*, *Dialister invisus* were shown in patients with FABD or Crohn's disease vs healthy subjects (Noh and Lee, 2020; Joossens et al., 2011; Vila et al., 2018). Interestingly, a significantly higher frequency of anxious subjects was found in the participants with GID that is in agreement with the co-occurrence of GID and psychosocial symptoms in patients with intestinal diseases and in those with FGID (Barberio et al., 2021; Mukhtar et al., 2019).

Another important finding of this study is the possible discovery of ten milk derived peptides as long-term plasma biomarkers of dairy products intake, while most previous studies focused on lipids and some cyclic dipeptides (Münger et al., 2018). Interestingly, three out of the ten BAPs found significantly lower in fasting plasma from participants who habitually consumed less dairy products, were DPPIV inhibitors and the plasma levels of this type of BAPs inversely correlated with serum DPPIV activity over time after milk consumption. This association may be clinically relevant as a higher DPPIV activity is typical of people suffering with metabolic diseases (Nargis and Chakrabarti, 2018) due to the involvement of the enzyme

in the degradation and inactivation of numerous hormones, chemokines, growth factors, and neuropeptides implicated in the pathophysiological pathways (Mentlein *et al.*, 1999). Therefore, it cannot be ruled out that the higher DPPIV activity is implicated in the development of milk-related GID by degrading and inactivating endomorphin and substance-P thus weakening analgesia pathways in the body and increasing pain perception (Guieu et al., 2006).

This study also presents two limitations. First limitation is that we did not monitor a complete pattern of microbial metabolites of proteins and amino acids in biological fluids. This would have clarified the implication of the individual gut microbiome in the metabolic fate of milk proteins, however our primary objective was to clarify the metabolism in the upper gastro-intestinal tract. Secondly, we did not assess mediators of pain signalling such as neuropeptides that could be affected by the DPPIV activity. This would have clarified the implication of the enzyme in the development of milk-related GID, however this was out of the scopes of this study.

In conclusion, in this study we demonstrated that the milk-related GID in sensitive people co-occurred with a slower and less efficient digestion of milk proteins. This finding explained the blunted hormonal and endocannabinoid responses, whereas the relative circulating levels of the milk-derived opioid agonist and antagonist BAPs along with the undigested proteins in the GI lumen supported a longer transit time and the consequent GID. The less efficient digestion was explained by the lower proteolytic capacity of the gut microbiome, shaped by the small habitual consumption of dairy products and high intake of dietary fiber vs proteins. Interestingly, healthy participants with GID showed specific signatures in their gut microbiome typical of gut inflammatory conditions. The differences in gut microbiome and in the habitual diet affected plasma levels of milk derived BAPs and serum DPPIV activity in fasting subjects, being not excluded a role in the anxiety level.

The findings indicate that milk-related GID in sensitive people may be managed by personalized approaches finalized to optimize milk protein digestion through therapies with proteolytic enzymes or *ad hoc* microbiome-targeted intervention (De Filippis et al., 2018). Moreover, opportunities for new food product development can be revealed. Milk-based food products containing pre-digested milk proteins may aid to manage GID as recently shown by Laatikainen and co-workers (2020) in patients with FGID. The addition of probiotics species exhibiting proteolytic activity in the gut may further aid to mitigate both GID and anxiety in sensitive people. However, randomized controlled trials in the target population should prove

the hypothesised effect.

References

1. Agus, A., Planchais, J., & Sokol, H. (2018). Gut microbiota regulation of tryptophan metabolism in health and disease. Cell host & microbe, 23(6), 716-724.
2. Aslam, H., Ruusunen, A., Berk, M., Loughman, A., Rivera, L., Pasco, J. A., & Jacka, F. N. (2020). Unravelled facets of milk derived opioid peptides: a focus on gut physiology, fractures and obesity. International journal of food sciences and nutrition, 71(1), 36-49.
3. Barberio, B., Zamani, M., Black, C. J., Savarino, E. V., & Ford, A. C. (2021). Prevalence of symptoms of anxiety and depression in patients with inflammatory bowel disease: a systematic review and meta-analysis. The Lancet Gastroenterology & Hepatology.
4. Berthoud, H. R., Morrison, C. D., Ackroff, K., & Sclafani, A. (2021). Learning of food preferences: Mechanisms and implications for obesity & metabolic diseases. International Journal of Obesity, 45(10), 2156-2168.
5. Black, C. J., Drossman, D. A., Talley, N. J., Ruddy, J., & Ford, A. C. (2020). Functional gastrointestinal disorders: advances in understanding and management. The Lancet.
6. Böhn, L., Störsrud, S., Törnblom, H., Bengtsson, U., & Simrén, M. (2013). Self-reported food-related gastrointestinal symptoms in IBS are common and associated with more severe symptoms and reduced quality of life. Official journal of the American College of Gastroenterology| ACG, 108(5), 634-641.
7. Carlini, V. P., Varas, M. M., Cragnolini, A. B., Schiöth, H. B., Scimonelli, T. N., & de Barioglio, S. R. (2004). Differential role of the hippocampus, amygdala, and dorsal raphe nucleus in regulating feeding, memory, and anxiety-like behavioral responses to ghrelin. Biochemical and biophysical research communications, 313(3), 635-641.
8. Carroccio, A., Soresi, M., Mantia, B., Fayer, F., La Blasca, F., Seidita, A., ... & Mansueto, P. (2021). Whole Cow's Milk but Not Lactose Can Induce Symptoms inPatients with Self-Reported Milk Intolerance: Evidence of Cow's Milk Sensitivity in Adults. Nutrients, 13(11), 3833.
9. Carroll, I. M., Ringel-Kulka, T., Ferrier, L., Wu, M. C., Siddle, J. P., Bueno, L., & Ringel, Y. (2013). Fecal protease activity is associated with compositional alterations in the intestinal microbiota. PLoS One, 8(10), e78017.
10. Carroll, I. M., Ringel-Kulka, T., Ferrier, L., Wu, M. C., Siddle, J. P., Bueno, L., & Ringel, Y. (2013). Fecal protease activity is associated with compositional alterations in the intestinal microbiota. *PLoS One*, *8*(10), e78017.
11. Craig, C. L., Marshall, A. L., Sjöström, M., Bauman, A. E., Booth, M. L., Ainsworth, B. E., ... & Oja, P. (2003). International physical activity questionnaire: 12-country reliability and

validity. *Medicine & science in sports & exercise*, *35*(8), 1381-1395.

12. De Filippis, F., Vitaglione, P., Cuomo, R., Berni Canani, R., & Ercolini, D. (2018). Dietary interventions to modulate the gut microbiome—how far away are we from precision medicine. *Inflammatory bowel diseases*, *24*(10), 2142-2154.
13. Deth, R., Clarke, A., Ni, J., & Trivedi, M. (2015). Clinical evaluation of glutathione concentrations after consumption of milk containing different subtypes of β-casein: results from a randomized, cross-over clinical trial. *Nutrition journal*, *15*(1), 1-6.
14. Di Marzo, V., Verrijken, A., Hakkarainen, A., Petrosino, S., Mertens, I., Lundbom, N., ... & Taskinen, M. R. (2009). Role of insulin as a negative regulator of plasma endocannabinoid levels in obese and nonobese subjects. *European Journal of Endocrinology*, *161*(5), 715.
15. Diether, N. E., & Willing, B. P. (2019). Microbial fermentation of dietary protein: an important factor in diet–microbe–host interaction. Microorganisms, 7(1), 19.
16. Dranse, H. J., Waise, T. Z., Hamr, S. C., Bauer, P. V., Abraham, M. A., Rasmussen, B. A., & Lam, T. K. (2018). Physiological and therapeutic regulation of glucose homeostasis by upper small intestinal PepT1-mediated protein sensing. *Nature communications*, *9*(1), 1-12.
17. El Mouzan, M. I., Winter, H. S., Assiri, A. A., Korolev, K. S., Al Sarkhy, A. A., Dowd, S. E., ... & Menon, R. (2018). Microbiota profile in new-onset pediatric Crohn's disease: data from a non-Western population. Gut pathogens, 10(1), 1-10.
18. Fikree, A., & Byrne, P. (2021). Management of functional gastrointestinal disorders. *Clinical Medicine*, *21*(1), 44.
19. Freeman, H. J. (2015). Clinical relevance of intestinal peptide uptake. *World journal of gastrointestinal pharmacology and therapeutics*, *6*(2), 22.
20. Gatta-Cherifi, B., Matias, I., Vallee, M., Tabarin, A., Marsicano, G., Piazza, P. V., & Cota, D. (2012). Simultaneous postprandial deregulation of the orexigenic endocannabinoid anandamide and the anorexigenic peptide YY in obesity. International journal of obesity, 36(6), 880-885.
21. Gorvitovskaia, A., Holmes, S. P., & Huse, S. M. (2016). Interpreting Prevotella and Bacteroides as biomarkers of diet and lifestyle. Microbiome, 4(1), 1-12.
22. Guieu, R., Fenouillet, E., Devaux, C., Fajloun, Z., Carrega, L., Sabatier, J. M., ... & Marguet, D. (2006). CD26 modulates nociception in mice via its dipeptidyl-peptidase IV activity. *Behavioural brain research*, *166*(2), 230-235.
23. Guo, R., Chen, L. H., Xing, C., & Liu, T. (2019). Pain regulation by gut microbiota: molecular mechanisms and therapeutic potential. British journal of anaesthesia, 123(5),

637-654.

24. Hasan, A. A., & Hocher, B. (2017). Role of soluble and membrane-bound dipeptidyl peptidase-4 in diabetic nephropathy. J Mol Endocrinol, 59(1), R1-R10.
25. He, M., Sun, J., Jiang, Z. Q., & Yang, Y. X. (2017). Effects of cow's milk beta-casein variants on symptoms of milk intolerance in Chinese adults: a multicentre, randomised controlled study. *Nutrition journal*, *16*(1), 1-12.
26. Hillard, C. J. (2018). Circulating endocannabinoids: from whence do they come and where are they going?. *Neuropsychopharmacology*, *43*(1), 155-172.
27. Ho, S., Woodford, K., Kukuljan, S., & Pal, S. (2014). Comparative effects of A1 versus A2 beta-casein on gastrointestinal measures: a blinded randomised cross-over pilot study. *European journal of clinical nutrition*, *68*(9), 994-1000.
28. Holzer, P. (2007). Treatment of opioid-induced gut dysfunction. Expert opinion on investigational drugs, 16(2), 181-194.
29. Holzer, P. (2009). Opioid receptors in the gastrointestinal tract. Regulatory peptides, 155(1-3), 11-17.
30. Jianqin, S., Leiming, X., Lu, X., Yelland, G. W., Ni, J., & Clarke, A. J. (2015). Effects of milk containing only A2 beta casein versus milk containing both A1 and A2 beta casein proteins on gastrointestinal physiology, symptoms of discomfort, and cognitive behavior of people with self-reported intolerance to traditional cows' milk. *Nutrition journal*, *15*(1), 1-16.
31. Joossens, M., Huys, G., Cnockaert, M., De Preter, V., Verbeke, K., Rutgeerts, P., ... & Vermeire, S. (2011). Dysbiosis of the faecal microbiota in patients with Crohn's disease and their unaffected relatives. Gut, 60(5), 631-637.
32. Joosten, M. M., Balvers, M. G., Verhoeckx, K. C., Hendriks, H. F., & Witkamp, R. F. (2010). Plasma anandamide and other N-acylethanolamines are correlated with their corresponding free fatty acid levels under both fasting and non-fasting conditions in women. Nutrition & Metabolism, 7(1), 1-6.
33. Korpela, K. (2018). Diet, microbiota, and metabolic health: trade-off between saccharolytic and proteolytic fermentation. Annual review of food science and technology, 9, 65-84.
34. Kurz, A., & Sessler, D. I. (2003). Opioid-induced bowel dysfunction. Drugs, 63(7), 649-671.
35. Laatikainen, R., Salmenkari, H., Sibakov, T., Vapaatalo, H., & Turpeinen, A. (2020). Randomised Controlled Trial: Partial Hydrolysation of Casein Protein in Milk Decreases Gastrointestinal Symptoms in Subjects with Functional Gastrointestinal Disorders.

Nutrients, 12(7), 2140.

36. Lambers, T. T., Broeren, S., Heck, J., Bragt, M., & Huppertz, T. (2021). Processing affects beta-casomorphin peptide formation during simulated gastrointestinal digestion in both A1 and A2 milk. International Dairy Journal, 121, 105099.
37. Linan-Rico, A., Ochoa-Cortes, F., Beyder, A., Soghomonyan, S., Zuleta-Alarcon, A., Coppola, V., & Christofi, F. L. (2016). Mechanosensory signaling in enterochromaffin cells and 5-HT release: potential implications for gut inflammation. Frontiers in neuroscience, 10, 564.
38. Lovibond, P. F., & Lovibond, S. H. (1995). The structure of negative emotional states: Comparison of the Depression Anxiety Stress Scales (DASS) with the Beck Depression and Anxiety Inventories. *Behaviour research and therapy*, *33*(3), 335-343.
39. Mennella, I., Savarese, M., Ferracane, R., Sacchi, R., & Vitaglione, P. (2015). Oleic acid content of a meal promotes oleoylethanolamide response and reduces subsequent energy intake in humans. Food & function, 6(1), 203-209.
40. Mentlein, R. (1999). Dipeptidyl-peptidase IV (CD26)-role in the inactivation of regulatory peptides. *Regulatory peptides*, *85*(1), 9-24.
41. Meslier, V., Laiola, M., Roager, H. M., De Filippis, F., Roume, H., Quinquis, B., ... & Ercolini, D. (2020). Mediterranean diet intervention in overweight and obese subjects lowers plasma cholesterol and causes changes in the gut microbiome and metabolome independently of energy intake. *Gut*, *69*(7), 1258-1268.
42. Midtvedt, T., Zabarovsky, E., Norin, E., Bark, J., Gizatullin, R., Kashuba, V., ... & Ernberg, I. (2013). Increase of faecal tryptic activity relates to changes in the intestinal microbiome: analysis of Crohn's disease with a multidisciplinary platform. PLoS One, 8(6), e66074.
43. Mukhtar, K., Nawaz, H., & Abid, S. (2019). Functional gastrointestinal disorders and gut-brain axis: What does the future hold?. *World journal of gastroenterology*, *25*(5), 552.
44. Münger, L. H., Garcia-Aloy, M., Vázquez-Fresno, R., Gille, D., Rosana, A. R. R., Passerini, A., ... & Praticò, G. (2018). Biomarker of food intake for assessing the consumption of dairy and egg products. *Genes & nutrition*, *13*(1), 1-18.
45. Nargis, T., & Chakrabarti, P. (2018). Significance of circulatory DPP4 activity in metabolic diseases. *IUBMB life*, *70*(2), 112-119.
46. Nicklas, T. A., Qu, H., Hughes, S. O., He, M., Wagner, S. E., Foushee, H. R., & Shewchuk, R. M. (2011). Self-perceived lactose intolerance results in lower intakes of calcium and dairy foods and is associated with hypertension and diabetes in adults. *The American journal of clinical nutrition*, *94*(1), 191-198.

47. Nielsen, S. D., Beverly, R. L., Qu, Y., & Dallas, D. C. (2017). Milk bioactive peptide database: A comprehensive database of milk protein-derived bioactive peptides and novel visualization. *Food Chemistry*, *232*, 673-682.
48. Noh, C. K., & Lee, K. J. (2020). Fecal Microbiota Alterations and Small Intestinal Bacterial Overgrowth in Functional Abdominal Bloating/Distention. Journal of neurogastroenterology and motility, 26(4), 539.
49. Overduin, J., Tylee, T. S., Frayo, R. S., & Cummings, D. E. (2014). Hyperosmolarity in the small intestine contributes to postprandial ghrelin suppression. American Journal of Physiology-Gastrointestinal and Liver Physiology, 306(12), G1108-G1116.
50. Overduin, J., Tylee, T. S., Frayo, R. S., & Cummings, D. E. (2014). Hyperosmolarity in the small intestine contributes to postprandial ghrelin suppression. American Journal of Physiology-Gastrointestinal and Liver Physiology, 306(12), G1108-G1116.
51. Parashar, A., & Saini, R. K. (2015). A1 milk and its controversy-a review. *International journal of bioassays*, *4*(12), 4611-4619.
52. Pasqui, F., Poli, C., Colecchia, A., Marasco, G., & Festi, D. (2015). Adverse Food Reaction and Functional Gastrointestinal Disorders: Role of the Dietetic Approach. Journal of Gastrointestinal & Liver Diseases, 24(3).
53. Peled, S., & Livney, Y. D. (2021). The role of dietary proteins and carbohydrates in gut microbiome composition and activity: a review. Food Hydrocolloids, 106911.
54. Pimentel, G., Burton, K., Rosikiewicz, M., Freiburghaus, C., Von Ah, U., Münger, L., & Vergères, G. (2017). Blood lactose after dairy product intake in healthy men. *British Journal of Nutrition, 118*(12), 1070-1077.
55. Porzi, M., Burton-Pimentel, K. J., Walther, B., & Vergères, G. (2021). Development of Personalized Nutrition: Applications in Lactose Intolerance Diagnosis and Management. *Nutrients*, *13*(5), 1503.
56. Rajilić–Stojanović, M., Biagi, E., Heilig, H. G., Kajander, K., Kekkonen, R. A., Tims, S., & de Vos, W. M. (2011). Global and deep molecular analysis of microbiota signatures in fecal samples from patients with irritable bowel syndrome. Gastroenterology, 141(5), 1792-1801.
57. Rowland, I., Gibson, G., Heinken, A., Scott, K., Swann, J., Thiele, I., & Tuohy, K. (2018). Gut microbiota functions: metabolism of nutrients and other food components. European journal of nutrition, 57(1), 1-24.
58. Russell, W. R., Duncan, S. H., Scobbie, L., Duncan, G., Cantlay, L., Calder, A. G., ... & Flint, H. J. (2013). Major phenylpropanoid-derived metabolites in the human gut can arise

from microbial fermentation of protein. Molecular nutrition & food research, 57(3), 523-535.

59. Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., & Huttenhower, C. (2011). Metagenomic biomarker discovery and explanation. Genome biology, 12(6), 1-18.
60. Shao, S., Xu, Q., Yu, X., Pan, R., & Chen, Y. (2020). Dipeptidyl peptidase 4 inhibitors and their potential immune modulatory functions. Pharmacology & therapeutics, 209, 107503.
61. Sperber, A. D., Bangdiwala, S. I., Drossman, D. A., Ghoshal, U. C., Simren, M., Tack, J., ... & Palsson, O. S. (2021). Worldwide prevalence and burden of functional gastrointestinal disorders, results of Rome Foundation global study. *Gastroenterology*, *160*(1), 99-114.
62. Stunkard, A. J., & Messick, S. (1985). The three-factor eating questionnaire to measure dietary restraint, disinhibition and hunger. *Journal of psychosomatic research*, *29*(1), 71-83.
63. Suchy, F. J., Brannon, P. M., Carpenter, T. O., Fernandez, J. R., Gilsanz, V., Gould, J. B., ... & Wolf, M. A. (2010). NIH consensus development conference statement: Lactose intolerance and health. *NIH consensus and state-of-the-science statements*, *27*(2), 1-27.
64. Sun, Y., Asnicar, M., Saha, P. K., Chan, L., & Smith, R. G. (2006). Ablation of ghrelin improves the diabetic but not obese phenotype of ob/ob mice. Cell metabolism, 3(5), 379-386.
65. Tagliamonte, S., Laiola, M., Ferracane, R., Vitale, M., Gallo, M. A., Meslier, V., ... & Vitaglione, P. (2021). Mediterranean diet consumption affects the endocannabinoid system in overweight and obese subjects: possible links with gut microbiome, insulin resistance and inflammation. *European Journal of Nutrition*, 1-14.
66. Teschemacher, H. (2003). Opioid receptor ligands derived from food proteins. *Current pharmaceutical design*, *9*(16), 1331-1344.
67. Trzaskalski, N. A., Fadzeyeva, E., & Mulvihill, E. E. (2020). Dipeptidyl peptidase-4 at the interface between inflammation and metabolism. Clinical Medicine Insights: Endocrinology and Diabetes, 13, 1179551420912972.
68. Tulipano, G. (2020). Role of Bioactive Peptide Sequences in the Potential Impact of Dairy Protein Intake on Metabolic Health. *International Journal of Molecular Sciences*, *21*(22), 8881.
69. Van Avesaat, M., Troost, F. J., Ripken, D., Peters, J., Hendriks, H. F., & Masclee, A. A. (2015). Intraduodenal infusion of a combination of tastants decreases food intake in humans. *The American journal of clinical nutrition*, *102*(4), 729-735.

70. Vancleef, L., Van Den Broeck, T., Thijs, T., Steensels, S., Briand, L., Tack, J., & Depoortere, I. (2015). Chemosensory signalling pathways involved in sensing of amino acids by the ghrelin cell. Scientific reports, 5(1), 1-14.
71. Vestergaard, E. T., Gormsen, L. C., Jessen, N., Lund, S., Hansen, T. K., Moller, N., & Jorgensen, J. O. L. (2008). Ghrelin infusion in humans induces acute insulin resistance and lipolysis independent of growth hormone signaling. Diabetes, 57(12), 3205-3210.
72. Vila, A. V., Imhann, F., Collij, V., Jankipersadsing, S. A., Gurry, T., Mujagic, Z., ... & Weersma, R. K. (2018). Gut microbiota composition and functional changes in inflammatory bowel disease and irritable bowel syndrome. Science translational medicine, 10(472).
73. Vitaglione, P., Mennella, I., Ferracane, R., Rivellese, A. A., Giacco, R., Ercolini, D., ... & Fogliano, V. (2015). Whole-grain wheat consumption reduces inflammation in a randomized controlled trial on overweight and obese subjects with unhealthy dietary and lifestyle behaviors: role of polyphenols bound to cereal dietary fiber. *The American journal of clinical nutrition*, *101*(2), 251-261.
74. Vojdani, A. (2013). For the assessment of intestinal permeability, size matters. Altern Ther Health Med, 19(1), 12-24.
75. Walker, J. M., Mennella, I., Ferracane, R., Tagliamonte, S., Holik, A. K., Hölz, K., ... & Vitaglione, P. (2020). Melanoidins from coffee and bread differently influence energy intake: A randomized controlled trial of food intake and gut-brain axis response. Journal of Functional Foods, 72, 104063.
76. Wang, Z., Xu, C. M., Liu, Y. X., Wang, X. Q., Zhang, L., Li, M., ... & Zhu, H. Q. (2019). Characteristic dysbiosis of gut microbiota of Chinese patients with diarrhea-predominant irritable bowel syndrome by an insight into the pan-microbiome. Chinese medical journal, 132(8), 889.
77. Ware Jr, J. E., Kosinski, M., & Keller, S. D. (1996). A 12-Item Short-Form Health Survey: construction of scales and preliminary tests of reliability and validity. *Medical care*, 220-233.
78. Whelan, K., Judd, P. A., & Taylor, M. A. (2004). Assessment of fecal output in patients receiving enteral tube feeding: validation of a novel chart. *European journal of clinical nutrition*, *58*(7), 1030-1037.
79. Whelan, K., Judd, P. A., Preedy, V. R., & Taylor, M. A. (2008). Covert assessment of concurrent and construct validity of a chart to characterize fecal output and diarrhea in patients receiving enteral nutrition. *Journal of Parenteral and Enteral Nutrition*, *32*(2), 160-

168.

Chapter 4

Mediterranean diet consumption affects the endocannabinoid system in overweight and obese subjects: possible links with gut microbiome, insulin resistance and inflammation

Silvia Tagliamonte, Manolo Laiola, Rosalia Ferracane, Marilena Vitale, Maria A. Gallo, Victoria Meslier, Nicolas Pons, Danilo Ercolini, Paola Vitaglione

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Abstract

Purpose: To investigate whether a Mediterranean diet (MD) affected the plasma concentrations of endocannabinoids (ECs), N-acylethanolamines (NAEs) and their specific ratios in subjects with lifestyle risk factors for metabolic diseases. To identify the relationship between circulating levels of these compounds and gut microbiome, insulin resistance and systemic inflammation.

Methods: A parallel 8-week randomised controlled trial was performed involving 82 overweight and obese subjects aged (mean±SEM) 43±1.4 years with a BMI of 31.1±0.5 kg/m^2, habitual Western diet (CT) and sedentary lifestyle. Subjects were randomized to consume an MD tailored to their habitual energy and macronutrient intake (n=43) or to maintain their habitual diet (n=39). Endocannabinoids and endocannabinoid-like molecules, metabolic and inflammatory markers and gut microbiome were monitored over the study period.

Results: The MD intervention lowered plasma arachidonoylethanolamide (AEA, p=0.02), increased plasma oleoylethanolamide/palmitoylethanolamide (OEA/PEA, p=0.009) and OEA/AEA (p=0.006), and increased faecal *Akkermansia muciniphila* (p=0.026) independent of body weight changes. OEA/PEA positively correlated with abundance of key microbial players in diet-gut-health interplay and MD adherence. Following an MD, individuals with low plasma OEA/PEA at baseline decreased homeostatic model assessment of insulin resistance-index (p=0.01), while individuals with high plasma OEA/PEA decreased serum high sensitive C-reactive protein (p=0.02).

Conclusions: We demonstrated that a switch from a CT to an isocaloric MD affects the endocannabinoid system and increases *A. muciniphila* abundance in the gut independently of body weight changes. Endocannabinoid tone and microbiome functionality at baseline drives an individualized response to an MD in ameliorating insulin sensitivity and inflammation.

Clinical Trial Registry number and website: NCT03071718; www.clinicaltrials.gov

Keywords: Obesity, *Akkermansia muciniphila*, Gut microbiome, Gut barrier, Cardiovascular disease risk.

1. Introduction

The Mediterranean diet (MD) is a healthy dietary pattern, while the Western diet is not. This idea is strongly supported by epidemiological evidence pointing out inverse associations of the two dietary patterns with the incidence of metabolic diseases, obesity and related health consequences (Franquesa et al., 2019; Soltani et al., 2019). However, in the context of clinical practice the health effects that occur over a duration that is reasonable to improve intermediary clinical outcomes are not obvious. Different responses to similar diets often occur and reflect the actual status of body systems, the cross-talk ability of the involved systems and their flexibility to react to environmental (dietary) factors (Van Ommen et al., 2017). Randomized clinical trials (RCTs) designed to monitor multiple systems, including the gut microbiome, are mandatory to determine causal relationships between diet and health outcomes with the potential to unravel physiological mechanisms underpinning the effects and obtain indications for personalized nutrition and precision medicine (Zeevi et al., 2015; De Filippis et al., 2018; Kolodziejczyk et al., 2019). Several microbiome-targeted studies showed that MD consumption boosts fibre-degrading species and anti-inflammatory responses in the human body (Laiola et al., 2020; Vitale et al., 2020; Meslier et al., 2020; Ghosh et al., 2020).

Evidence indicates that the gut microbiome can affect metabolic inflammation regulating gut epithelium permeability (Tilg et al., 2020). Specifically, the mucin-degrading bacterium *Akkermansia muciniphila* is widely considered a valuable contributor to the maintenance of gut health and metabolic homoeostasis reducing obesity and related disorders such as glucose intolerance, insulin resistance, steatosis and gut permeability (Everard et al., 2013; Plovier et al., 2017; Everard et al., 2019).

The endocannabinoid system has pleiotropic functions in the body and plays a key role in the development of obesity and its comorbidities being a mediator in the relationship between the gut microbiota and host metabolism (Cani et al., 2016). Circulating endocannabinoids (ECs) and their congeners N-acylethanolamines (NAEs) are metabolically connected to diet and are tightly involved in the regulation of energy homeostasis, appetite cues, pain sensation, inflammation, immunity, obesity and dysmetabolism (Hillard, 2018; Witkamp, 2018; Silvesti & Di Marzo, 2013). Indeed, plasma ECs and NAEs concentrations, as well as NAEs ratios, are considered biomarkers of white adipose tissue distribution reflecting blood cholesterol and insulin resistance in obesity (Silvesti & Di Marzo, 2013; Fanelli et al., 2018) in addition to being involved in food liking and intake (Mennella et al., 2015[a]; Mennella et al., 2015[b]). We have recently observed several correlations between circulating NAEs, habitual diet and systemic inflammation in subjects with an ileostomy (Tagliamonte et al., 2020). However, the effect of

diet on circulating levels of ECs and NAEs in intervention studies in humans is still underexplored.

The combined high intake of slowly digestible carbohydrates, unsaturated fat, plant proteins and micronutrients in the MD as opposed to refined (quickly digestible) carbohydrates, saturated fat and animal proteins in the Western diet is responsible for the different metabolic and health effects of the two diets (Trichopoulou et al., 2014). We recently performed an 8-week RCT in 82 overweight and obese subjects with lifestyle risk factors for metabolic disease. Forty-three subjects consumed an MD individually tailored to each participant's habitual energy and macronutrient intake (MD group), and 39 subjects in the control group (CT) maintained their habitual diet that was very similar to a Western diet. A *per-protocol analysis* of data, excluding 18 participants, showed that the MD group diminished plasma cholesterol and exhibited some changes in the gut microbiome and related metabolome (Meslier et al., 2020).

In this study, we explore the impact of an isocaloric dietary shift from a Western diet to an MD on the circulating levels of ECs, NAEs, oleoylethanolamide/arachidonoylethanolamide (OEA/AEA) and oleoylethanolamide/palmitoylethanolamide (OEA/PEA) ratios. Further, by performing an *intention-to-treat analysis* of data, we shed light on the relationships of the endocannabinoid system with the gut microbiome, clinical data, and adherence to MD.

5. Methods

2.1. Study design and participants

This study is part of a randomized and controlled eight-week intervention trial whose primary outcome was to detect changes in fasting plasma lipids and faecal levels of short-chain fatty acids upon an intervention with MD, as described in a previous paper (Meslier et al., 2020). That paper also reported the details of the study design and protocol (Meslier et al., 2020). In this paper for the first time we focused on secondary outcome such as plasma ECs and NAEs, OEA/AEA and OEA/PEA ratios. Specifically, we analysed their changes in response to the dietary intervention, concomitant with gut microbiome changes and their correlations with variables related to diet (food categories, nutritional composition and adherence to the Mediterranean diet), insulin resistance (Homeostatic Model Assessment for Insulin Resistance, HOMA-IR (Matthews et al., 1985) and systemic inflammation (serum high sensitive C-reactive protein, hs-CRP). The study was conducted after the approval of the University of Naples Ethic Committee (Protocol number: 108/16). The trial was registered at www.clinicaltrials.gov

(number NCT03071718). Recruitment was performed at the Department of Agricultural Sciences and at the Department of Clinical Medicine and Surgery of the University of Naples, Naples, Italy.

The 334 potentially eligible adults, interested in study participation, were screened on the basis of the inclusion/exclusion criteria considering medical and lifestyle conditions (Meslier et al., 2020). Briefly, subjects were included if they were healthy men or women aged between 20≤age≤65 years, with BMI≥24 kg/m^2, consuming a diet characterized by an intake of fruits and vegetables ≤ 3 servings/day, no consumption of probiotics, functional foods and/or food supplements, consuming no more than 2 portions a day of whole-grain food and/or enriched with dietary fibre, having a sedentary lifestyle (MET min/week<700). Informed written consent was obtained prior to undertaking the study. Eighty-two participants were randomized between the two intervention arms of the parallel designed study and completed the study protocol. The characteristics of the participants and variations over the intervention of many clinical outcomes are reported in the Online supplementary table 1 by Meslier and co-workers.

2.2. Dietary intervention

MD participants received an isocaloric tailored MD diet based on their habitual energy and macronutrient intake assessed by a 7-day Food Diary, while CT volunteers were asked not to change their habitual diet during the 8-week intervention. Every 2 weeks the individual compliance was assessed through food diaries whereas adherence to the Mediterranean diet was assessed by Italian Mediterranean Index (IMI) (Meslier et al., 2020).

2.3. Anthropometric measurement and biochemical analysis

A trained operator measured the waist circumference midway between the lowest rib and the iliac crest by using an anthropometric tape. Hip circumference was measured around the widest portion of the buttocks, with the tape parallel to the floor. Weight from subjects wearing light clothes was measured, after voiding, to the nearest 0.1 kg on a digital scale (Model 703; Seca). Height of subjects was recorded to the nearest 0.5 cm with a stadiometer (Model 213; Seca). Body composition including total body water, fat mass, and fat-free mass, was determined at ambient temperature after voiding and after being in a supine position for at least 20 minutes, through an electric impedance analysis with a single-frequency 50-kHz bioelectrical impedance analyzer (BIA 101 RJL; Akern Bioresearch) as described in Meslier and co-workers (Meslier et al., 2020). Body mass index (BMI) was calculated as weight in kg divided by height in meters

squared. According to the World Health Organization (WHO) task force and the National Institutes of Health (NIH) guidelines, people whose BMI is between 18.5 and 24.9 kg/m^2 are considered normal weight, between 25 and 29.9 kg/m^2 are overweight, and >30 kg/m^2 are obese. Fasting blood samples were collected by venipuncture into a serum separator, EDTA-containing tubes and EDTA Aprotinin-containing tubes. All EDTA blood samples were kept chilled/on ice before processing. Plasma samples were prepared by centrifugation at 3000 rpm for 15 min at 4 °C and within 15 minutes of collection. All biological samples were rapidly frozen and stored at -80 °C until analysis. Plasma glucose, insulin, and serum hs-CRP concentrations as well as HOMA-index were assessed as described previously (Meslier et al., 2020).

2.4. Extraction of ECs and NAEs from plasma samples

The analysis of ECs and NAEs was performed in EDTA plasma samples. All samples were thawed in the fridge at 4°C before extraction and samples were kept chilled on ice during the specific extraction procedures. A solid-phase extraction (SPE) according to the method described by Marczylo and colleagues was carried out (Marczylo et al., 2009).

Plasma samples (500 µL) previously diluted 1:2 with distilled water were added with 50 µL of the internal standard 200 ng/mL solution of Arachidonoylethanolamide d8 (AEA d8) (Cayman Chemical, Ann Arbor, MI). Then the samples were vortexed and centrifuged 21100 g for 5 minutes at 4 °C.

Oasis HLB cartridges (1 cc / 30 mg Waters) were preconditioned with 1 ml of methanol and equilibrated using 1 ml of H_2O. Samples were introduced onto the cartridges and were washed with 1 ml of aqueous methanol (40%), and the monitored compounds were eluted in 1 ml of acetonitrile. The eluate was dried under nitrogen flow and reconstituted in 100 µL acetonitrile/water (50:50) before the LC–MS/MS analysis.

2.5. LC-MS/MS analysis

Chromatographic separation was performed using an HPLC apparatus provided with two micropumps, Perkin-Elmer Series 200 (Norwalk, CT, USA). The compounds were separated on a Synergi Max RP 80 column (50x2.1 mm) (Phenomenex, USA) with a setting temperature of 30 °C and a flow rate of 0.2 mL/min. The injection volume was 10 µL. The monitored compounds were eluted by a linear gradient of H_2O and 0.2% formic acid (solvent A) and CH_3CN (solvent B). According to Mennella and co-workers (Mennella et al., 2015), the eluting

gradient was set as follows: 50–79% B (10 min), 79–95% B (1 min), constant at 95% B (2 min), and finally returning to the initial conditions within 2 min. The acquisition was performed in positive ion mode on an API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Canada) in MRM (Multiple Reaction Monitoring). All the acquisition parameters are summarized in **Supplementary Table 1**.

ECs (2-arachidonoylglycerol, 2-AG; anandamide, AEA; AEAd8) and NAEs (oleoylethanolamide, OEA; linoleoylethanolamide, LEA; palmitoylethanolamide, PEA) standards were purchased from Cayman (Cayman Chemical, Ann Arbor, MI).

The limit of detection (LOD) and limit of quantification (LOQ) of the identified molecules are reported in **Supplementary Table 2**.

2.6. Faecal microbiome determination by shotgun metagenomics

A full description of the sampling, sequencing and data analysis procedures is reported in the online supplementary material by Meslier and colleagues (Meslier et al., 2020). Briefly, faecal DNA extraction was performed following IHMS SOP P7 V2 and purified DNA fragment libraries were sequenced using the Ion Proton Sequencer (ThermoFisher Scientific, Waltham, US) with a minimum of 20 million 150 bp high-quality reads generated per library. Metagenomic Species Pangenome (MSP) abundance profiles were determined and annotated as previously described (Plaza Oñate et al., 2019; Meslier et al., 2020) and the in-house FAnToMet pipeline was used to assess the functional potential modules of the intestinal gut microbiome.

2.7. Quantification of *Akkermansia muciniphila*

The abundances of *A. muciniphila* in each sample were further quantified and analysed through qPCR as described by Dao et al. (2016). Briefly, extracted DNA was quantified using Qubit Fluorometric Quantitation (ThermoFisher Scientific, Waltham, US) and amplified using primers targeting the 16S rRNA for *A. muciniphila* (forward CAGCACGTGAAGGTGGGGAC and reverse CCTTGCGGTTGGCTTCAGAT). The total 16S rRNA gene was also quantified and used as reference gene in order to normalise the abundance of *A. muciniphila* in all samples tested (bacterial universal primers forward ACTCCTACGGGAGGCAGCAG and reverse ATTACCGCGGCTGCTGG). Amplifications were performed using the StepOne Real-Time PCR System (Life Technologies) with the following thermal cycling profiles: one cycle at 95 °C for 30 s, 40 cycles at 60 °C for 30 s and

72 °C for 30s and using the SYBR Green PCR Master Mix Reagents Kit (Thermo Fisher Scientific). Negative controls (H_2O) were included in each qPCR run and each assay was performed in triplicate. The results of the amplifications were expressed as threshold cycle (Ct) values. The relative quantification of *A. muciniphila* in all samples was calculated by using the comparative Ct method (ΔCt, the difference between the Ct value of *A. muciniphila* and the Ct of total 16S rRNA gene for each sample). Lastly, the concentration of *A. muciniphila* was carried out by comparing ΔCt measures with a standard calibration curve (Y= -3.527 x log10(X) + 17.677, R2=0.97, PCR efficiency=92.11%) and by relating such values to total faecal DNA concentration.

2.8. Statistical analysis

A power analysis using the mean plasma concentrations and variations of ECs and NAEs in previous studies was performed to estimate the sample size needed to detect an effect of diet on plasma concentrations of ECs and NAEs (Fanelli et al., 2018; Fanelli et al., 2017). We calculated that 39 participants per group was sufficient to detect a minimum inter-group difference of 23% and 13% in plasma concentrations of 2-AG and AEA, 10% and 14% in plasma concentrations of PEA and OEA as well as 10% and 9% in AEA/OEA and OEA/PEA ratios, respectively, with an α error of 0.05, 80% power, and 2-sided testing. Therefore, 39 participants in CT and 43 in MD would be adequate to test the effect of MD on fasting circulating levels of ECs and NAEs.

All Statistical analyses were performed in R version 3.6.0. After being checked for normality, variables showing a significant positive skewness, were transformed in ln (x). For those variables that showed a normal distribution at the Kolmogorov-Smirnov test, Independent-samples T Test was performed to check differences between CT and MD at baseline. The 2-way ANOVA with repeated measure was performed to check within group differences over the study period. For those variables which did not show a normal distribution after logarithmic transformation, a non-parametric Wilcoxon test was performed. To test the overall correlation among ECs, NAEs, microbiome and dietary variables, pairwise Spearman's rank correlations within CT and MD at baseline, 4 week and 8 week were calculated and adjustments were performed using the Benjamini-Hochberg procedure and Spearman rho values were filtered by keeping correlations with at least one false discovery rate (FDR) of ≤0.05. The heatmap of correlation was visualised using the Hmisc package, and the function heatmap.2. Two-tailed P

values lower than 0.05 were considered significantly different. Data are expressed as means ± SEM.

Differently abundant species between MD and CT groups at either 4 or 8 weeks of dietary intervention were assessed through testRelations function of momr R package (Le Chatelier et al., 2013), as well as gut microbial modules differences at baseline between quartiles**.**

The Linear discriminant analysis (LDA) effect size (LEfSe) (Segata et al., 2011) was used to identify differentially abundant species between Q1 and Q4 groups, for a $p < 0.05$ after the Kruskal-Wallis test. Only discriminative features with an LDA score >2.0 were plotted and ranked by effect size.

3. Results

3.1. Mediterranean diet decreases plasma anandamide concentrations

The 82 participants (43 F and 39 M, average body mass index (BMI) 31.1±0.5 kg/m^2, age 43±1.4 years), of which 43 participants (22 F/21 M, average BMI 30.9±0.6 kg/m^2, age 43±1.9 years) were in MD group and the 39 participants (21 F/18 M, average BMI 31.2±2.0 kg/m^2, age 42±2.0 years) in CT group, showed high compliance with the intervention.

The intake of the main food categories monitored over the study period is reported in **Table 1.** The intakes were homogeneous between the two groups at baseline for all food categories except eggs which were consumed more by the participants in CT. Participants in MD decreased refined-grain products, snacks, oils, fats and meat intake over the study period and increased their intake of vegetables, legumes, whole-grain products and fish products after 4 and 8 weeks, whereas fruit and nuts after 8 week intervention. These changes were coherent with an increased adherence to the MD as assessed by the Italian Mediterranean Index (IMI).

No significant effect of gender on baseline plasma concentration of OEA, LEA, PEA, AEA, 2-AG, and OEA/PEA and OEA/AEA ratios in CT and MD group was found.

MD consumption lowered plasma AEA concentrations after 8 weeks compared to the baseline concentrations (-9.1%; p=0.02), while it did not affect 2-AG, linoleoylethanolamide (LEA), OEA or PEA (**Fig. 1A, B**). Interestingly, significant increases in the OEA/PEA ratio (5.9%; p=0.009) and the OEA/AEA ratio (14.6%; p=0.006) were registered after 4 and 8 weeks of MD consumption, respectively (**Fig. 1C, D**). Moreover, negative associations of plasma OEA/AEA with waist circumference (Spearman's rho=-0.462, p=<0.001) and hip circumference (Spearman's rho =-0.200, p=0.002) were observed.

Table 1. Daily food intake (g) within each category in the control (CT) and Mediterranean diet group (MD) at baseline (0 wk), 4 weeks (4 wk) and 8 weeks (8 wk).

	CT (n=39)			MD (n=43)			p-values		
g/day	**0 wk**	**4 wk**	**8 wk**	**0 wk**	**4 wk**	**8 wk**	**Δ4-0**	**Δ8-0**	**Δ8-4**
Fruit [d]	139.5 ± 22.0	195.0 ± 33.5	183.9 ± 30.9	171.3 ± 21.8 [b]	275.6 ± 25.5 [a]	271.7 ± 22.2 [a]	0.141	**0.014***	0.919
Vegetables	179.3 ± 16.7	190.4 ± 13.5	174.7 ± 16.1	203.9 ± 15.7 [b]	298.0 ± 22.5 [a]	284.4 ± 23.1 [a]	**0.010***	**0.006***	0.930
Legumes	14.1 ± 2.4	20.7 ± 3.4	15.9 ± 3.2	14.2 ± 2.5 [b]	48.3 ± 6.5 [a]	50.0 ± 8.5 [a]	**<0.001***	**<0.001***	0.240
Whole-grain products	5.6 ± 2.2	4.4 ± 2.0	5.7 ± 3.0	13.9 ± 6.1 [b]	148.8 ± 21.5 [a]	147.6 ± 21.9 [a]	**<0.001***	**<0.001***	0.902
Refined-grain products	143.6 ± 11.1	141.9 ± 19.0	142.9 ± 18.2	70.7 ± 9.2 [a]	37.2 ± 7.3 [b]	26.4 ± 5.0 [b]	**<0.001***	**0.019***	0.241
Eggs	10.8 ± 2.1‡	13.3 ± 5.0	8.2 ± 1.8	4.5 ± 1.1 [a]‡	1.7 ± 0.8 [b]	2.0 ± 0.7 [b]	0.136	0.823	**0.040***
Dairy products	94.3 ± 14.9 [b]	108.9 ± 5.9 [a]	93.3 ± 14.0 [ab]	83.2 ± 13.9	91.5 ± 14.2	96.2 ± 15.4	0.192	0.673	0.452
Fish products	29.1 ± 5.0	30.2 ± 4.8	33.3 ± 5.0	26.1 ± 5.6 [c]	49.2 ± 6.2 [a]	40.3 ± 6.0 [b]	**0.002***	0.071	**0.006***
Meat	75.3 ± 9.2 [b]	63.2 ± 7.2 [a]	72.2 ± 8.4 [ab]	55.5 ± 8.2 [a]	11.6 ± 2.3 [b]	16.9 ± 4.6 [b]	**0.003***	**0.001***	0.896
Oils & Fats	6.6 ± 2.1	7.5 ± 2.1	6.7 ± 2.0	2.5 ± 0.7 [a]	0.3 ± 0.2 [b]	1.8 ± 0.9 [a]	**<0.001***	**0.021***	0.264
Coffee	53.7 ± 8.4	51.9 ± 7.8	52.5 ± 10.2	44.5 ± 8.0 [b]	58.7 ± 8.3 [a]	50.7 ± 7.2 [a]	0.059	0.215	0.700
Fruit juices	11.8 ± 3.6 [a]	7.7 ± 5.1 [b]	11.7 ± 5.9 [ab]	3.6 ± 1.5	3.7 ± 2.6	4.6 ± 2.7	**0.048***	**0.046***	0.618
Wine	8.8 ± 1.8	9.6 ± 3.1	4.1 ± 1.5	24.5 ± 5.1	18.8 ± 8.2	19.9 ± 8.3	0.632	0.128	0.477
Snacks	76.2 ± 13.9	76.6 ± 11.3	73.7 ± 13.0	44.8 ± 6.9 [a]	20.2 ± 5.2 [b]	24.2 ± 5.1 [b]	**0.011***	**0.009***	0.492
Soft drinks	46.9 ± 12.4	33.7 ± 12.0	35.1 ± 10.8	31.7 ± 15.9	35.6 ± 23.3	18.4 ± 9.7	0.730	0.507	0.810
IMI [e]	4.5 ± 0.3 [a]	4.1 ± 0.3 [a]	3.8 ± 0.2 [b]	5.2 ± 0.3 [b]	8.0 ± 0.3 [a]	7.8 ± 0.3 [a]	**<0.001***	**<0.001***	0.664

Data are expressed as the mean ± SEM. Different letters on a row indicate significant differences between time points within groups by Wilcoxon test or 2-way ANOVA repeated measures depending on normal distribution of the data. *p<0.05 pairwise time points (Δ) between CT and MD by Mann-Whitney test or independent-sample T test.

‡ p<0.05 between MD and CT at baseline by Mann-Whitney test adjusted for energy intake.

[d] Fruits: sum of fruit and nuts;

[e] IMI, Italian Mediterranean Index.

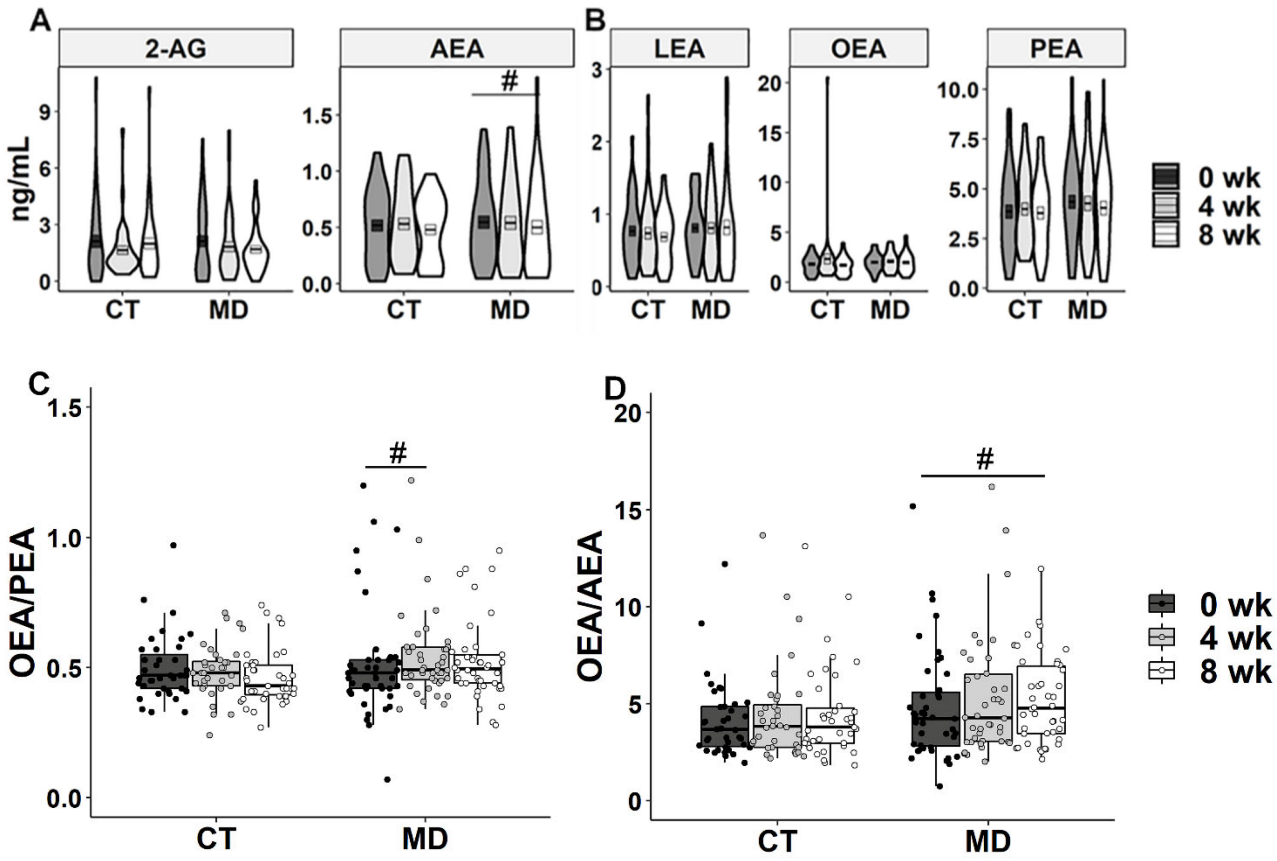

Fig. 1: Violin plots showing the distribution of plasma endocannabinoids (A), concentrations of N-acylethanolamines (B), and box plots showing the plasma OEA/PEA ratio (C) and the OEA/AEA ratio (D) in participants from the control group (CT) and the Mediterranean diet group (MD) at baseline (0 wk), 4 weeks (4 wk) and 8 weeks (8 wk). # $p<0.05$ within group by Wilcoxon test. In C and D, two outliers were removed for simpler visualization.
2-AG, 2-Arachidonoylglicerol; AEA, Arachidonoylethanolamide; LEA, Linoylethanolamide; OEA, Oleoylethanolamide; PEA, Palmitoylethanolamide.

3.2. Higher *Akkermansia muciniphila* correlates with plasma AEA concentration after the MD intervention

Consistently with results reported in our previous paper and obtained by analysing a *per protocol* selection of the population, the isocaloric MD dietary intervention led to gut microbiome changes in the overall population (Meslier et al., 2020). Indeed, we observed some MSP species such as *Roseburia faecis* and *R. hominis* significantly enriched in MD compared to CT group, after either 4 or 8 weeks, along with a rise in the fibre-degrading *Faecalibacterium prausnitzii* and several members of the *Lachnospiraceae* taxa, while *Ruminococcus torques* and

R. gnavus were CT-related species (**Supplementary Table 3** and **4**). Interestingly, significantly higher levels of *Akkermansia muciniphila* occurred in the MD compared to CT group after 8 weeks of MD consumption ($p<0.05$, **Fig. 2**), although this was not observed in the previous *per protocol* analysis (Meslier et al., 2020).

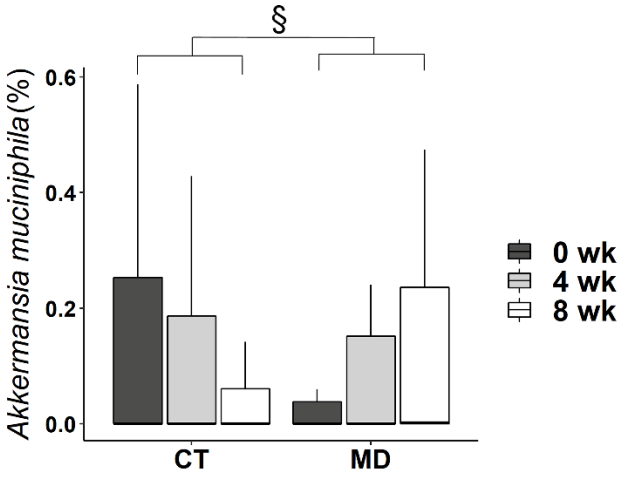

Fig. 2: Faecal *Akkermansia muciniphila* relative abundance calculated from shotgun metagenomics in participants from the control group (CT) and Mediterranean diet group (MD) at baseline (0 wk), 4 weeks (4 wk) and 8 weeks (8 wk). § $p<0.05$ pairwise time points (Δ) between CT and MD by the Mann-Whitney test.

Later, we further investigated and confirmed the identification of *A. muciniphila* through a quantitative real-time polymerase chain reaction (qPCR) (Spearman's Rho= 0.8, p-value = 2.2e-16, **Supplementary Fig. 1**). Plasma concentrations of OEA (Spearman's rho= -0.295, p=<0.001), PEA (Spearman's rho= -0.341, p=<0.001), LEA (Spearman's rho= -0.366, p=<0.001), and AEA (Spearman's rho= -0.319, p-value= <0.001) were negatively correlated with *A. muciniphila*, which was positively linked to the plasma OEA/PEA (Spearman's rho=0.180, p=0.005) and OEA/AEA ratios (Spearman's rho=0.259, p<0.001) (**Fig. 3**). Plasma AEA concentrations were negatively associated with *Eggerthella* sp. Furthermore, circulating levels of AEA and the OEA/PEA ratio showed opposite associations with the abundance of *Intestinimonas butyriciproducens*, *Bifidobacterium longum*, *Roseburia hominis* and *Faecalibacterium prausnitzii, Prevotella* sp. and *P. copri.* All the correlations found are detailed in **Fig. 3.**

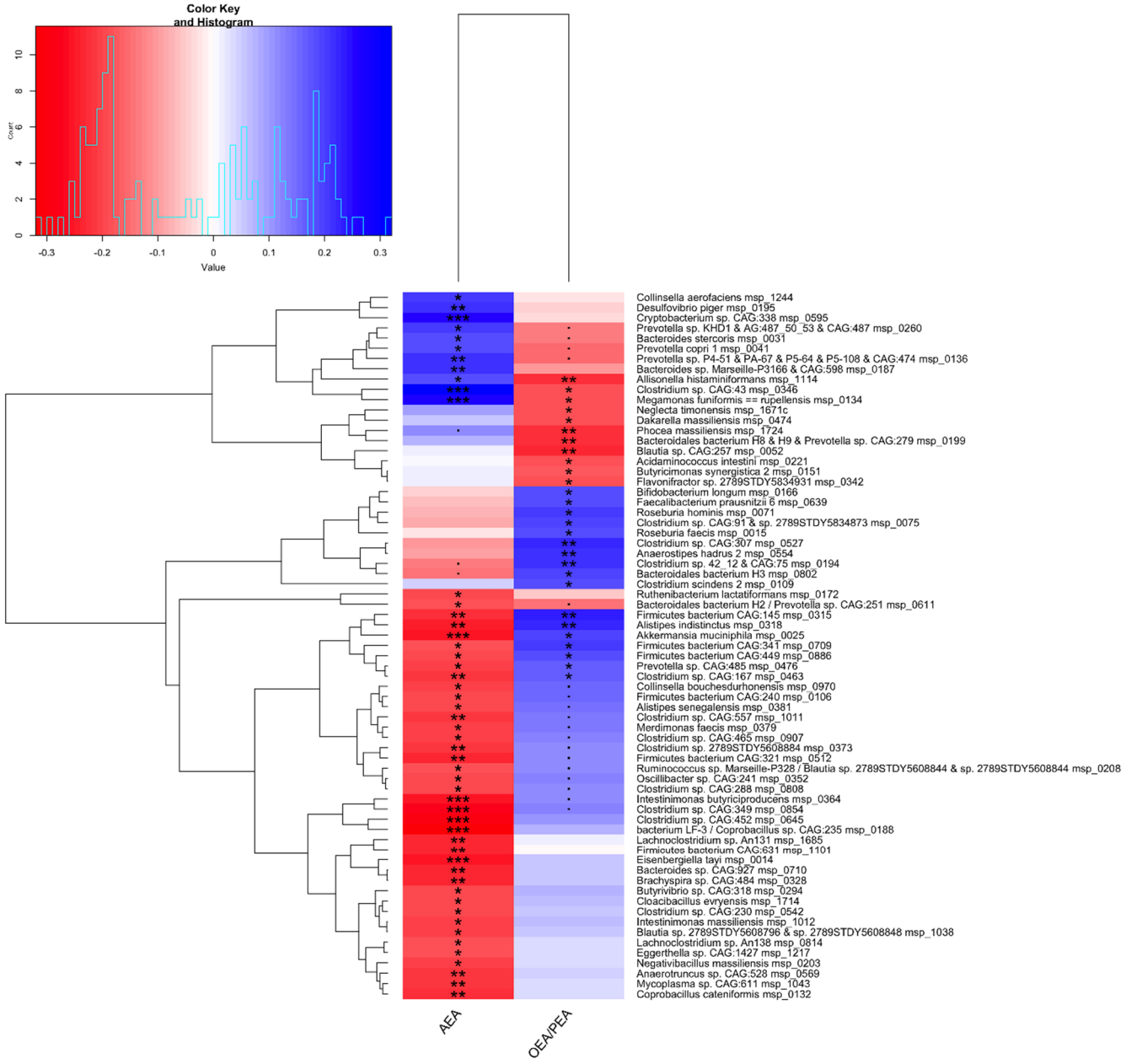

Fig. 3: Heatmap showing hierarchical Ward-linkage clustering of AEA and the OEA/PEA ratio based on Spearman's correlation with gut microbiome species. The colour scale represents the magnitude of Spearman's rho coefficient, with red indicating negative correlations and blue indicating positive correlations. Adjustments were performed using the Benjamini-Hochberg procedure, and Spearman's rho values were filtered by maintaining correlations with at least one false discovery rate (FDR) of <0.05.

▪, FDR=0.05; *, FDR<0.05; **, FDR<0.01; ***, FDR<0.001.

AEA, Arachidonoylethanolamide; OEA, Oleoylethanolamide; PEA, Palmitoylethanolamide.

3.3. Plasma OEA/PEA ratio is associated to MD adherence

OEA/PEA ratio, but not OEA/AEA ratio, was positively correlated with IMI. OEA/PEA ratio also showed positive correlation with daily intake of vegetables and negative correlation with soft drinks, snacks, meat and refined-grain products intake (**Fig. 4**). In addition, OEA/AEA was correlated negatively with eggs, soft drinks, snacks, meat and refined-grain products, fish, dairy products and legumes intake. While plasma 2-AG concentrations showed negative correlations with meat, refined-grain products, fish, dairy products and legumes intake, which conversely, were positively correlated with plasma AEA and NAEs concentrations.

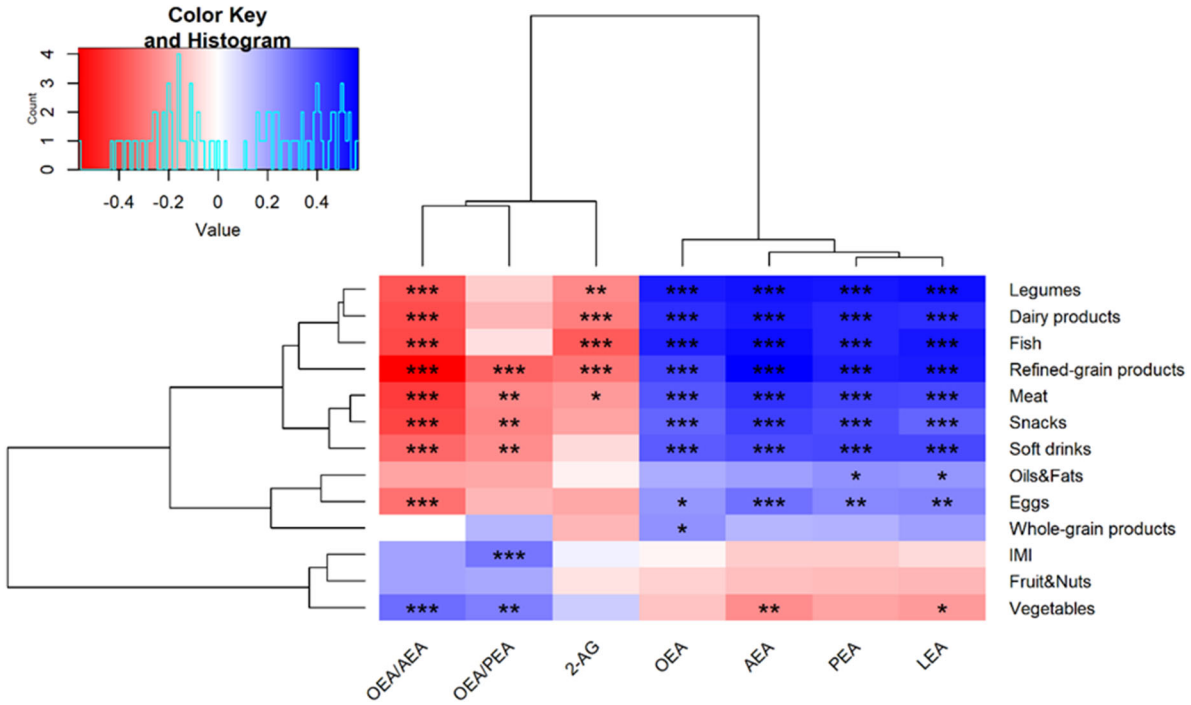

Fig. 4: Heatmap correlation matrix between plasma ECs, NAEs, OEA/PEA ratio, OEA/AEA ratio, and food categories and IMI. The colour scale represents the magnitude of Spearman's rho coefficient, with blue indicating a positive correlation and red indicating a negative correlation. *p<0.05; **p<0.01; *** p<0.001 significant Spearman's correlations and Holm correction adjusted for Energy intake.

IMI, Italian Mediterranean Index; 2-AG, 2-Arachidonoylglicerol; AEA, Arachidonoylethanolamide; LEA, Linoylethanolamide; OEA, Oleoylethanolamide; PEA, Palmitoylethanolamide.

3.4. Baseline plasma OEA/PEA ratio influences the individual response to the consumption of an MD

To test the hypothesis of whether the baseline OEA/PEA ratio affected the individual response to MD consumption, we grouped the population into quartiles of the baseline OEA/PEA ratio. MD participants in the lowest quartile (Q1) exhibited a significantly increased OEA/PEA ratio after 4 weeks (26.7%, p=0.04), while those in the highest quartile (Q4) did not exhibit a change in the OEA/PEA ratio over the study period (**Fig. 5A**). Interestingly, Q1 participants in the MD group also exhibited a diminished HOMA-index after 4 (-26.2%, p=0.01) and 8 weeks (-23.9%, p=0.01) (**Fig. 5B**).

Compared to participants in Q1, those in Q4 displayed lower plasma PEA, LEA and AEA concentrations at baseline, which remained stable during the study period, and higher plasma 2-AG concentrations; plasma OEA did not differ at baseline between participants in Q1 and Q4 and did not change over the course of the intervention (**Supplementary Fig. 2** and **Fig. 5C,D**). MD participants in Q1 had increased circulating 2-AG levels after 4 weeks (121%; p=0.02) (**Fig. 5D**), while those in Q4 had decreased serum hs-CRP (-62.6%; p=0.02) (**Fig. 5E**) and increased faecal abundance of *A. muciniphila* (**Fig. 5F**).

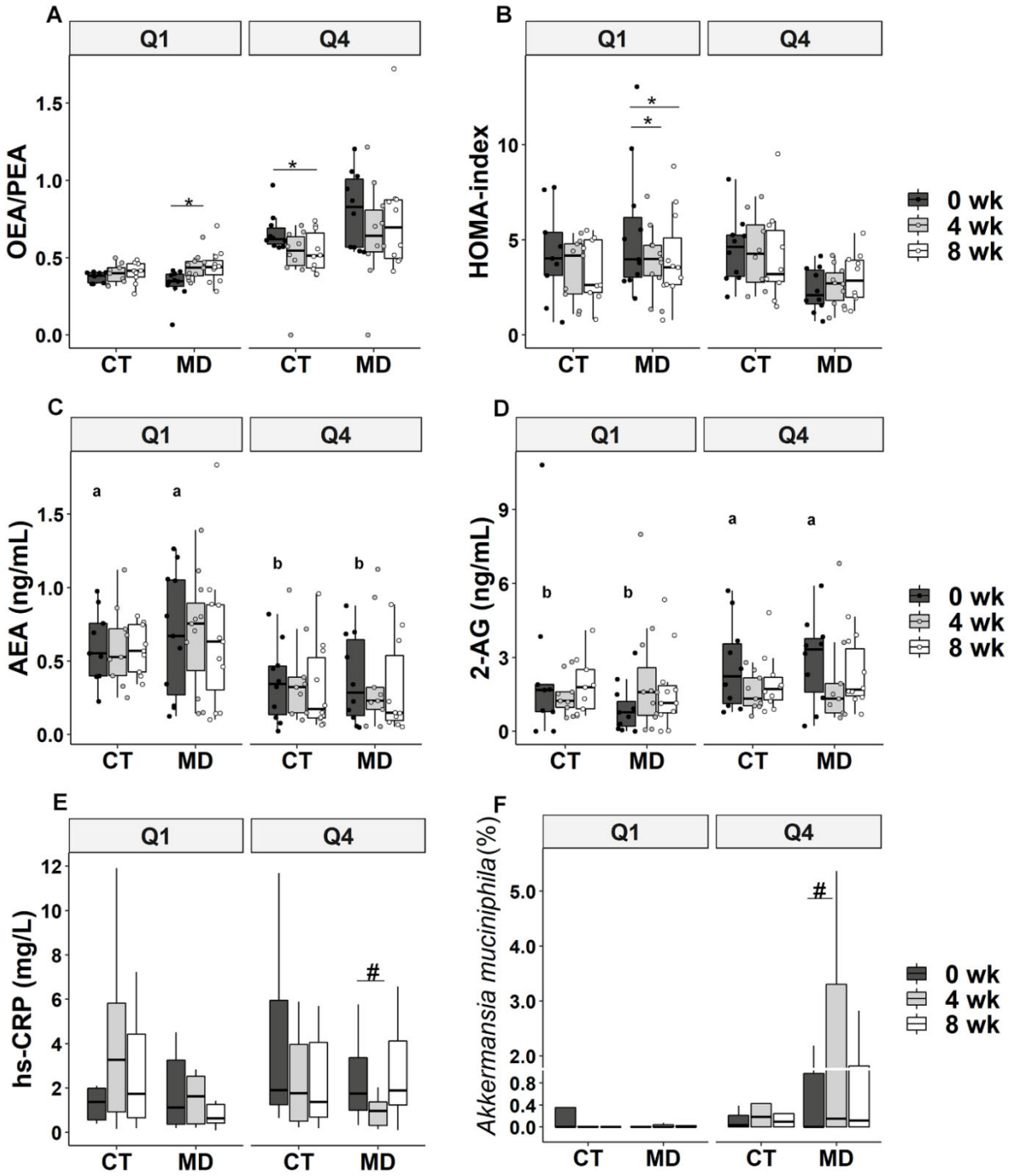

Fig. 5: Plasma OEA/PEA ratio (A), HOMA-index (B), plasma AEA concentration (C), plasma 2-AG concentration (D), serum hs-CRP concentration (E) and faecal *Akkermansia muciniphila* relative abundance (F) in participants from the control group (CT) and Mediterranean diet group (MD) in the lowest (Q1) and highest quartile (Q4) for OEA/PEA ratio at baseline (0 wk), 4 weeks (4 wk) and 8 weeks (8 wk). Q1= minimum to 25th percentile of OEA/PEA ratio at baseline (CT, n= 9; MD, n=11), Q4= 75th percentile to maximum of OEA/PEA ratio at baseline (CT, n=10; MD, n=10). Different letters on the box plots indicate $p<0.05$ between quartiles

(MD + CT) at 0 wk by independent-samples T test.

* p<0.05 within-group comparison by 2-way ANOVA with repeated measures; # p<0.05 within-group comparison by Wilcoxon test. Two outliers have been removed from panel E and 1 outlier from panel F for simpler visualization. 2-AG, 2-Arachidonoylglicerol; AEA, Arachidonoylethanolamide; OEA, Oleoylethanolamide; PEA, Palmitoylethanolamide; hs-CRP, high-sensitivity C-reactive protein; HOMA-index, Homeostatic model assessment of insulin resistance-index.

In **Fig. 6** are detailed the microbiome species that differed between Q1 and Q4 at baseline. In particular, compared to Q1 participants, Q4 participants at baseline harboured higher levels of *Roseburia faecis, Bacteroides dorei, Bacteroides intestinalis, Bifidobacterium longum* and *Anaerostipes hadrus*, *Clostridium* sp. and lower *Blautia, Coprococcus*, *Alistipes* and *C. ramosum* in the gut (**Fig. 6**).

We further explored the functional and metabolic features of microbial communities in Q1 and Q4 participants at baseline (**Supplementary Table 5**). Q4 participants showed more abundant levels of glycocholate (MF0044), glucose (MF0057) and lactose (MF0048) degradation modules.

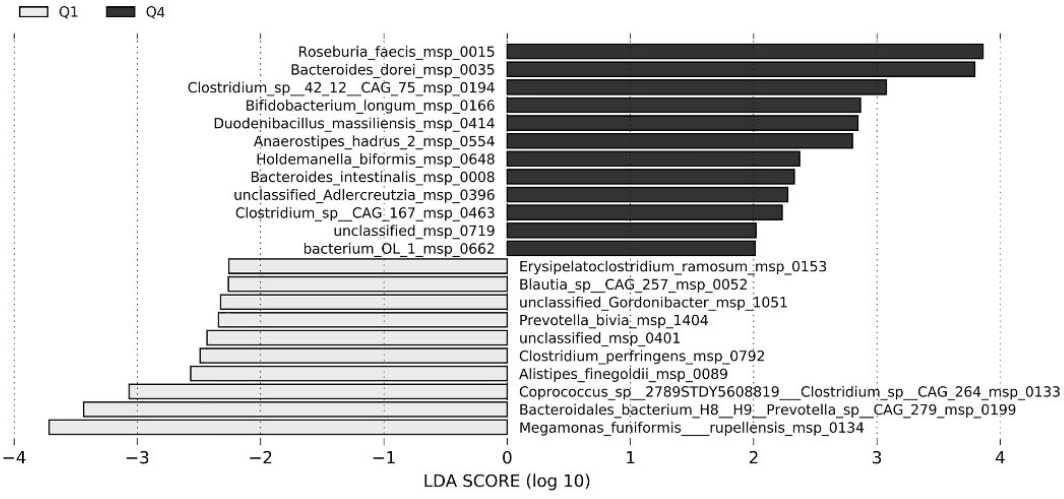

Fig. 6: Linear discriminant analysis effect size (LEfSe) showing species that were differentially abundant between Q1 (grey) and Q4 (black). Logarithmic linear discriminant analysis (LDA) score >2, p<0.05. Q1= minimum to 25th percentile of OEA/PEA ratio at baseline (CT, n= 9; MD, n=11), Q4= 75th percentile to maximum of OEA/PEA ratio at baseline (CT, n=10; MD,

n=10).

3.5. Diet composition influences plasma OEA/PEA ratio at baseline

Q1 and Q4 participants at baseline showed no different intake of energy, dietary fibres, fats, saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids, carbohydrates, proteins, sugars and alcohol as well as anthropometric variables and body composition (**Supplementary Table 6**). In contrast, Q1 participants at baseline consumed more refined-grain products, meat, oils and fats, snacks than Q4 participants (**Supplementary Table 7**). During the MD intervention, Q1 participants significantly increased their fruit, vegetables, whole-grain products, legumes, fish intake and decreased the intake of refined-grain products, oils and fats, meat, and snacks. Q4 participants in the MD group consumed more whole-grain products and less refined-grain products than those in the CT group and decreased snacks and eggs intake only after 4 weeks.

4. Discussion

In this study, we showed that an isocaloric dietary shift from a Western diet to an MD lowered plasma AEA concentration and increased the OEA/PEA and OEA/AEA ratios. The increased total polyunsaturated fatty acid intake and decreased saturated fatty acid intake caused by increased consumption of fish products and nuts as isocaloric replacements of meat and dairy products might explain the changes in the circulating lipid mediators observed in MD participants (Meslier et al., 2020; Engeli et al., 2014; Jones et al., 2014). No previous evidence of a food- or diet-induced change in the plasma OEA/PEA or OEA/AEA ratio is available. Interestingly, the OEA/PEA and OEA/AEA ratios are markers of blood cholesterol and insulin resistance (Fanelli et al., 2018), and their increase occurred concomitantly with the reduction in plasma cholesterol in our cohort (Meslier et al., 2020).

On the other hand, mounting evidence in obese subjects and patients with inflammatory bowel disease (IBD) indicates that circulating AEA is a mediator of the effects of obesity on gut barrier integrity, intestinal permeability and inflammation (Little et al., 2018; Grill et al., 2019) that underpin metabolic endotoxaemia (Cani et al., 2007). Therefore, the reduction of AEA in the MD group suggests that MD may exert metabolic and anti-inflammatory effects through its action on intestinal permeability and endocannabinoid signalling.

This hypothesis was supported by the significant increase of *A. muciniphila* faecal abundance after 8 weeks of MD compared to CT. *A. muciniphila* was not among the microbial species found to change upon intervention in the subgroup of subjects previously analysed that excluded 20 participants because of a slight increase of fruit and vegetable consumption over the run-in period compared with the consumption at the time of enrolment (Meslier et al., 2020). *A. muciniphila* is widely considered a valuable contributor to the maintenance of gut health and metabolic homoeostasis (Cani & de Vos, 2017). Indeed, *A. muciniphila* abundance decreases in several pathological conditions where intestinal dysbiosis, gut barrier functionality and endotoxaemia play key roles, such as obesity, type 2 diabetes, IBD, and hypertension (Dao et al., 2016; Yassour et al., 2016; Li et al., 2017). Contrarily, *A. muciniphila* abundance increases following pharmacological treatments, bariatric surgery and prebiotic-based interventions that ameliorate multiple aspects of nutrient metabolism and gut barrier function (Everard et al., 2013; Dao et al., 2016; Forslund et al., 2015; Greer et al., 2016).

Therefore, we consider the increased abundance of *A. muciniphila* independently of body weight change as an indicator of the alleviation of obesity-related gut barrier dysfunction.

In addition, circulating levels of AEA were negatively and OEA/PEA ratio was positively correlated with a number of other microorganisms associated with a healthy gut, such as *Intestinimonas butyriciproducens*, *Bifidobacterium longum*, *Roseburia hominis* and *Faecalibacterium prausnitzii* (Machiels et al.,2014; Wong et al., 2019; Jiang et al., 2015), or known to metabolise ellagic acid and produce antinflammatory urolitihins such as *Eggerthella* (Meslier et al., 2020). Similar findings were recorded for the abundance of *Prevotella* sp. and *P. copri*, which show contradictory association with either health conditions or intestinal and metabolic diseases (De Filippis et al., 2019; Saltzman et al., 2018), likely due to different genetic traits of the microrganisms according to strain diversity and dietary patterns (De Filippis et al., 2019; De Filippis et al., 2016).

Altogether, these findings suggest that plasma AEA concentration may be a putative marker of altered gut permeability and dysbiosis, as already shown for intestinal AEA concentrations (Cani et al., 2016). Contrarily, the OEA/PEA ratio may be an indicator of healthy gut barrier function. Indeed, participants falling in the quartile for the highest plasma levels of OEA/PEA (Q4) harboured in the gut higher abundances of fibre- or protein-degrading bacteria and species positively associated with a healthy gut barrier due to the production of short chain fatty acids such as *Bacteroides*, *Bifidobacterium*, *Clostridium*, and *Roseburia* (Wong et al., 2019; Tamanai-Shacoori et al., 2017; Alou et al., 2016). Contrarily, participants with the lowest plasma concentrations of OEA/PEA (Q1), showed higher abundances of *Blautia* and

Coprococcus taxa, *C. ramosum* and *Alistipes* previously associated with obesity, inflammation and animal-based diet rich in fat and proteins (Castaner et al., 2018; Woting et al., 2014; Roager et al., 2019). Therefore, Q4 participants, harbouring a healthy gut microbiota and displaying lower plasma AEA and higher plasma 2-AG concentrations, might possess a more functional intestinal barrier at baseline than Q1 participants. This was further supported by the differences found in functional and metabolic features of microbial communities between Q1 and Q4. The more abundant glycocholate degradation module (MF0044) in Q4might lower the intestinal concentration of glycocholic acid as occurring in healthy individuals vs those with IBD (Das et al., 2019). Similarly, the glucose (MF0057) and lactose (MF0048) degradation modules might indicate a better ability to metabolize those nutrients whose transport modules, are increased in ileal Chron's disease (Morgan et al., 2012). Interestingly, the participants in Q4 had also a lower intake of refined-grain products, oils, fats, snacks, and meat than Q1 and, switching to an MD, ameliorated the inflammatory status. Although Q4 participants in the MD group consumed more whole-grain products and less meat than those in the CT group, their snacks intake decreased only after 4 weeks. This may explain why participants in Q4 experienced the greatest ant-inflammatory benefit from the MD after 4 weeks of intervention.

The finding that Q1 participants at baseline consumed more refined-grain products, oils, fats, snacks, and meat compared to Q4 may suggest that the consumption of those foods has a role in shaping the functional and metabolic features of the gut microbiome in humans. Due to the nature and duration of the study, these results should be interpreted cautiously as a preliminary indication of the effect of high sugar, high fat, and high energy-dense food consumption on the gut microbiome.

The macronutrient composition of the diets was not different between quartiles at baseline, suggesting that other food components in snacks, meat (including processed meat), refined-grain products,oils and fat might affect the endocannabinoid system and the gut microbiome. Evidence shows that food emulsifiers affect gut microbiota composition promoting colitis and metabolic syndrome in mice (Chassaing et al., 2015) while noncaloric artificial sweetener intake can cause intestinal dysbiosis in humans (Suez et al., 2014).

This study has three main strengths. Firstly, the nutritional intervention applied allowed us to demonstrate an involvement of the endocannabinoid system in the interplay between MD consumption, the gut microbiome and health independently of changes in body weight and composition. Secondly, the influence of the baseline endocannabinoid tone in the physiological response to MD consumption provides new clinical indications for the nutritional management

of subjects at risk of metabolic diseases within the framework of personalized nutrition. Thirdly, the *intention-to-treat* analysis of the gut microbiome data evidenced the effect of the MD in increasing the abundance of *A. Muciniphila*. This finding suggests that in the present study the overall aspects of MD, other than the increased intake in fruit and vegetables considered to select subjects in the *per-protocol* analysis in Meslier and co-workers (Meslier et al., 2020), might contribute with the higher sample size in achieving the statistical differences between MD and CT as regards the diet-induced modulation of *A. muciniphila*.

On the other hand, since we did not measure gut permeability, we could not establish a causal role of gut barrier function/homeostasis in the interplay among the endocannabinoid system, the MD, and health outcomes.

In conclusion, our study demonstrates that a switch from a Western-like diet to an isocaloric MD affects the endocannabinoid system and increases *A. muciniphila* abundance in the gut, independent of body weight changes in subjects with lifestyle risk factors for metabolic disease. Moreover, individual endocannabinoid tone at baseline drives a personalized response to the MD ameliorating insulin sensitivity or systemic inflammation. Specifically, individuals with a low plasma OEA/PEA ratio (high AEA) exhibit an increase of plasma OEA/PEA and 2-AG, ameliorating insulin sensitivity, while those with a high plasma OEA/PEA ratio (low AEA) show an increase in intestinal *A. muciniphila* and a reduction in systemic inflammation.

A high plasma OEA/PEA ratio and low AEA concentration reflect a more functional gut microbiome characterized by species associated with gut homeostasis and barrier integrity. Finally, we provide preliminary evidence that the individual endocannabinoid system is shaped by habitual intake of meat, refined-grain products, oils, fat and snacks affecting gut microbiome functionality and its responsiveness to dietary changes.

Altogether our findings shed light on endocannabinoid system implication in the interplay between diet, gut and health, revealing opportunities for clinical practice in the context of personalized nutrition. Specifically, data suggests that the measure of plasma OEA/PEA ratio and AEA concentration might mirror individual gut microbiome and intestinal barrier functionality determining the individual responsiveness towards metabolic and/or anti-inflammatory changes following a dietary intervention with an MD.

Conflict of interest statement

On behalf of all authors, the corresponding author states that there is no conflict of interest.

References

1. Alou, M. T., Lagier, J. C., Raoult, D. (2016). Diet influence on the gut microbiota and dysbiosis related to nutritional disorders. Human Microbiome Journal, 1, 3-11.
2. Cani, P. D., Amar, J., Iglesias, M. A., Poggi, M., Knauf, C., Bastelica, D., Neyrinck, A. M., Fava, F., Tuohy, K. M., Waget, A., et al. (2007). Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes, 56(7), 1761-1772.
3. Cani, P. D., and de Vos, W. M. (2017). Next-generation beneficial microbes: the case of Akkermansia muciniphila. Frontiers in microbiology, 8, 1765.
4. Cani, P. D., Plovier, H., Van Hul, M., Geurts, L., Delzenne, N. M., Druart, C., Everard, A. (2016). Endocannabinoids—at the crossroads between the gut microbiota and host metabolism. Nature Reviews Endocrinology, 12(3), 133.
5. Castaner, O., Goday, A., Park, Y. M., Lee, S. H., Magkos, F., Shiow, S. A. T. E., Schröder, H. (2018). The gut microbiome profile in obesity: a systematic review. International journal of endocrinology, 2018.
6. Chassaing, B., Koren, O., Goodrich, J. K., Poole, A. C., Srinivasan, S., Ley, R. E., Gewirtz, A. T. (2015). Dietary emulsifiers impact the mouse gut microbiota promoting colitis and metabolic syndrome. Nature, 519(7541), 92-96.
7. Dao, M. C., Everard, A., Aron-Wisnewsky, J., Sokolovska, N., Prifti, E., Verger, E. O., Kayser, B. D., Levenez, F., Chilloux, J., Dumas, M. E., et al. (2016). Akkermansia muciniphila and improved metabolic health during a dietary intervention in obesity: relationship with gut microbiome richness and ecology. Gut, 65(3), 426-436.
8. Das, P., Marcišauskas, S., Ji, B., Nielsen, J. (2019). Metagenomic analysis of bile salt biotransformation in the human gut microbiome. BMC genomics, 20(1), 517.
9. De Filippis, F., Pasolli, E., Tett, A., Tarallo, S., Naccarati, A., De Angelis, M., Neviani, E., Cocolin, L., Gobbetti, M., Segata, N., et al., (2019). Distinct genetic and functional traits of human intestinal Prevotella copri strains are associated with different habitual diets. Cell host & microbe, 25(3), 444-453.
10. De Filippis, F., Pellegrini, N., Laghi, L., Gobbetti, M., Ercolini, D. (2016). Unusual sub-genus associations of faecal Prevotella and Bacteroides with specific dietary patterns. Microbiome, 4(1), 1-6.
11. De Filippis, F., Vitaglione, P., Cuomo, R., Berni Canani, R., Ercolini, D. (2018). Dietary interventions to modulate the gut microbiome—how far away are we from precision medicine. Inflammatory bowel diseases, 24(10), 2142-2154.

12. Engeli, S., Lehmann, A. C., Kaminski, J., Haas, V., Janke, J., Zoerner, A.A., Luft, F.C., Tsikas, D. and Jordan, J. (2014). Influence of dietary fat intake on the endocannabinoid system in lean and obese subjects. Obesity, 22(5), E70-E76.
13. Everard, A., Belzer, C., Geurts, L., Ouwerkerk, J. P., Druart, C., Bindels, L. B. Guiot, Y., Derrien, M., Muccioli G. G., De Vos, W. M. (2013). Cross-talk between Akkermansia muciniphila and intestinal epithelium controls diet-induced obesity. Proceedings of the national academy of sciences, 110(22), 9066-9071.
14. Everard, A., Plovier, H., Rastelli, M., Van Hul, M., de Wouters d'Oplinter, A., Geurts, L., Druart, C., Robine, S., Delzenne, N., Muccioli, G. G. et al., (2019). Intestinal epithelial N-acylphosphatidylethanolamine phospholipase D links dietary fat to metabolic adaptations in obesity and steatosis. Nature communications, 10(1), 1-17.
15. Fanelli, F., Mezzullo, M., Belluomo, I., Di Lallo, V. D., Baccini, M., Gasparini, D. I., Casadio, E., Mastroroberto, M., Vicennati, V., Gambineri, A., Morselli-Labate, A. M., et al. (2017). Plasma 2-arachidonoylglycerol is a biomarker of age and menopause related insulin resistance and dyslipidemia in lean but not in obese men and women. Molecular metabolism, 6(5), 406-415.
16. Fanelli, F., Mezzullo, M., Repaci, A., Belluomo, I., Gasparini, D. I., Di Dalmazi, G., Mastroroberto, M., Vicennati, V., Gambinieri, A., Pasquali, R., et al. (2018). Profiling plasma N-Acylethanolamine levels and their ratios as a biomarker of obesity and dysmetabolism. Molecular metabolism, 14, 82-94.
17. Forslund, K., Hildebrand, F., Nielsen, T., Falony, G., Le Chatelier, E., Sunagawa, S., Prifti, E., Vieira-Silva, S., Gudmundsdottir, V., Arumugam, M., et al. (2015). Disentangling type 2 diabetes and metformin treatment signatures in the human gut microbiota. Nature, 528(7581), 262-266.
18. Franquesa, M., Pujol-Busquets, G., García-Fernández, E., Rico, L., Shamirian-Pulido, L., Aguilar-Martínez, A., Medina, F., Serra-Majem, L., Bach-Faig, A. (2019). Mediterranean diet and cardiodiabesity: A systematic review through evidence-based answers to key clinical questions. Nutrients,11(3), 655.
19. Ghosh, T. S., Rampelli, S., Jeffery, I. B., Santoro, A., Neto, M., Capri, M., Giampieri, E., Jennings, A., Candela, M., Turroni, S., Zoetendal, E. G., et al. (2020). Mediterranean diet intervention alters the gut microbiome in older people reducing frailty and improving health status: the NU-AGE 1-year dietary intervention across five European countries. Gut, 69(7), 1218-1228.
20. Greer, R. L., Dong, X., Moraes, A. C. F., Zielke, R. A., Fernandes, G. R., Peremyslova, E., Vasquez-Perez, S., Schoenborn, A. A., Gomes, W. P., Ferreira, S. R., et al. (2016). Akkermansia

muciniphila mediates negative effects of IFNγ on glucose metabolism. Nature communications, 7(1), 1-13.

21. Grill, M., Högenauer, C., Blesl, A., Haybaeck, J., Golob-Schwarzl, N., Ferreirós, N., Thomas, D., Gurke, R., Trötzmüller, M., Gallé, B.,g et al. (2019). Members of the endocannabinoid system are distinctly regulated in inflammatory bowel disease and colorectal cancer. Scientific reports, 9(1), 1-13.
22. Hillard, C. J. (2018). Circulating endocannabinoids: from whence do they come and where are they going?. Neuropsychopharmacology, 43(1), 155-172.
23. Jiang, W., Wu, N., Wang, X., Chi, Y., Zhang, Y., Qiu, X., Hu, Y., Liu, Y. (2015). Dysbiosis gut microbiota associated with inflammation and impaired mucosal immune function in intestine of humans with non-alcoholic fatty liver disease. Scientific reports, 5, 8096.
24. Jones, P. J., Lin, L., Gillingham, L. G., Yang, H., & Omar, J. M. (2014). Modulation of plasma N-acylethanolamine levels and physiological parameters by dietary fatty acid composition in humans. Journal of lipid research, 55(12), 2655-2664.
25. Kolodziejczyk, A. A., Zheng, D., Elinav, E. (2019). Diet–microbiota interactions and personalized nutrition. Nature Reviews Microbiology, 1-12.
26. Laiola, M., De Filippis, F., Vitaglione, P., Ercolini, D. (2020). A Mediterranean diet intervention reduces the levels of salivary periodontopathogenic bacteria in overweight and obese subjects. Applied and Environmental Microbiology, 86(12).
27. Le Chatelier, E., Nielsen, T., Qin, J., Prifti, E., Hildebrand, F., Falony, G., Arumugam, M., Batto, J., Almeida, M., Leonard, P., et al. (2013). Richness of human gut microbiome correlates with metabolic markers. Nature, 500(7464), 541-546.
28. Li, J., Zhao, F., Wang, Y., Chen, J., Tao, J., Tian, G., Wu, S., Liu, W., Cui, Q., Zhang, W. et al., (2017). Gut microbiota dysbiosis contributes to the development of hypertension. Microbiome, 5(1), 1-19.
29. Little, T. J., Cvijanovic, N., DiPatrizio, N. V., Argueta, D. A., Rayner, C. K., Feinle-Bisset, C., Young, R. L. (2018). Endocannabinoids and Cannabinoid Receptors as Regulators of Endocrine Functions and Tissue Metabolism: Plasma endocannabinoid levels in lean, overweight, and obese humans: relationships to intestinal permeability markers, inflammation, and incretin secretion. American Journal of Physiology-Endocrinology and Metabolism, 315(4), E489.
30. Machiels, K., Joossens, M., Sabino, J., De Preter, V., Arijs, I., Eeckhaut, V., Ballet, V., Claes, K., Immerseel, F. V., Ferrante, M., et al. (2014). A decrease of the butyrate-producing species Roseburia hominis and Faecalibacterium prausnitzii defines dysbiosis in patients with ulcerative colitis. Gut, 63(8), 1275-1283.

31. Marczylo, T. H., Lam, P. M., Nallendran, V., Taylor, A. H., Konje, J. C. (2009). A solid-phase method for the extraction and measurement of anandamide from multiple human biomatrices. Analytical biochemistry, 384(1), 106-113.
32. Matthews, D. R., Hosker, J. P., Rudenski, A. S., Naylor, B. A., Treacher, D. F.,Turner, R. C. (1985). Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia, 28(7), 412-419.
33. Mennella, I., Ferracane, R., Zucco, F., Fogliano, V., Vitaglione, P. (2015). Food liking enhances the plasma response of 2-arachidonoylglycerol and of pancreatic polypeptide upon modified sham feeding in humans. The Journal of nutrition, 145(9), 2169-2175.
34. Mennella, I., Savarese, M., Ferracane, R., Sacchi, R., Vitaglione, P. (2015). Oleic acid content of a meal promotes oleoylethanolamide response and reduces subsequent energy intake in humans. Food & function, 6(1), 203-209.
35. Meslier, V., Laiola, M., Roager, H. M., De Filippis, F., Roume, H., Quinquis, B., Pasolli, E. Rivellese, A., Dragsted, L. O., Vitaglione, P., et al. (2020). Mediterranean diet intervention in overweight and obese subjects lowers plasma cholesterol and causes changes in the gut microbiome and metabolome independently of energy intake. Gut, 69(7), 1258-1268.
36. Morgan, X. C., Tickle, T. L., Sokol, H., Gevers, D., Devaney, K. L., Ward, D. V., Reyes, J. A., Shah, S. A., LeLeiko, N., Bousvaros, A., et al. (2012). Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. Genome biology, 13(9), R79.
37. Plaza Oñate, F., Le Chatelier, E., Almeida, M., Cervino, A. C., Gauthier, F., Magoulès, F., Ehrlich, D. S., Pichaud, M. (2019). MSPminer: abundance-based reconstitution of microbial pan-genomes from shotgun metagenomic data. Bioinformatics, 35(9), 1544-1552.
38. Plovier, H., Everard, A., Druart, C., Depommier, C., Van Hul, M., Geurts, L., Chilloux, J., Ottman, N., Duparc, T., Lichtenstein, L. et al., (2017). A purified membrane protein from Akkermansia muciniphila or the pasteurized bacterium improves metabolism in obese and diabetic mice. Nature medicine, 23(1), 107-113.
39. Roager, H. M., Vogt, J. K., Kristensen, M., Hansen, L. B. S., Ibrügger, S., Mærkedahl, R. B., Bahl, M. I., Lind, M. V., Nielsen, R. L., Gøbel, R. J., et al. (2019). Whole grain-rich diet reduces body weight and systemic low-grade inflammation without inducing major changes of the gut microbiome: a randomised cross-over trial. Gut, 68(1), 83-93.
40. Saltzman, E. T., Palacios, T., Thomsen, M., Vitetta, L. (2018). Intestinal microbiome shifts, dysbiosis, inflammation, and non-alcoholic fatty liver disease. Frontiers in microbiology, 9, 61.
41. Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., Huttenhower, C. (2011). Metagenomic biomarker discovery and explanation. Genome biology, 12(6), 1-18.

42. Silvestri, C., and Di Marzo, V. (2013). The endocannabinoid system in energy homeostasis and the etiopathology of metabolic disorders. Cell metabolism, 17(4), 475-490.
43. Soltani, S., Jayedi, A., Shab-Bidar, S., Becerra-Tomás, N., Salas-Salvadó, J. (2019). Adherence to the Mediterranean Diet in relation to all-cause mortality: a systematic review and dose-response meta-analysis of prospective cohort studies. Advances in Nutrition, 10(6), 1029-1039.
44. Suez, J., Korem, T., Zeevi, D., Zilberman-Schapira, G., Thaiss, C. A., Maza, O., Israeli, D., Zmora, N., Gilad, S., Kuperman, Y., et al. (2014). Artificial sweeteners induce glucose intolerance by altering the gut microbiota. Nature, 514(7521), 181-186.
45. Tagliamonte, S., Gill, C. I., Pourshahidi, L. K., Slevin, M. M., Price, R. K., Ferracane, R., Lawther, R., O'Connor, G., Vitaglione, P. (2020). Endocannabinoids, endocannabinoid-like molecules and their precursors in human small intestinal lumen and plasma: does diet affect them? European Journal of Nutrition, 1-13.
46. Tamanai-Shacoori, Z., Smida, I., Bousarghin, L., Loreal, O., Meuric, V., Fong, S. B., Bonnaure-Mallet, M., Jolivet-Gougeon, A. (2017). Roseburia spp.: a marker of health?. Future Microbiology, 12(2), 157-170.
47. Tilg, H., Zmora, N., Adolph, T. E., Elinav, E. (2020). The intestinal microbiota fuelling metabolic inflammation. Nature Reviews Immunology, 20(1), 40-54.
48. Trichopoulou, A., Martínez-González, M. A., Tong, T. Y., Forouhi, N. G., Khandelwal, S., Prabhakaran, D., Mozaffarian, D., de Lorgeril, M. (2014). Definitions and potential health benefits of the Mediterranean diet: views from experts around the world. BMC medicine, 12(1), 112.
49. van Ommen, B., van den Broek, T., de Hoogh, I., van Erk, M., van Someren, E., Rouhani-Rankouhi, T., Anthony, J. C., Hogenelst, K., Pasman, W., Boorsma, A., et al. (2017). Systems biology of personalized nutrition. Nutrition Reviews, 75(8), 579-599.
50. Vitale, M., Giacco, R., Laiola, M., Della Pepa, G., Luongo, D., Mangione, A., Salamone, D., Vitaglione, P., Ercolini, D., Rivellese, A. A. (2020). Acute and chronic improvement in postprandial glucose metabolism by a diet resembling the traditional Mediterranean dietary pattern: Can SCFAs play a role?. Clinical Nutrition.
51. Witkamp, R. (2018). The role of fatty acids and their endocannabinoid-like derivatives in the molecular regulation of appetite. Molecular aspects of medicine, 64, 45-67.
52. Wong, C. B., Odamaki, T., Xiao, J. Z. (2019). Beneficial effects of Bifidobacterium longum subsp. longum BB536 on human health: Modulation of gut microbiome as the principal action. Journal of functional foods, 54, 506-519.

53. Woting, A., Pfeiffer, N., Loh, G., Klaus, S., Blaut, M. (2014). Clostridium ramosum promotes high-fat diet-induced obesity in gnotobiotic mouse models. MBio, 5(5), e01530-14.
54. Yassour, M., Lim, M. Y., Yun, H. S., Tickle, T. L., Sung, J., Song, Y. M., Lee, K., Franzosa, A. E., Morgan, X. C., Lander, E. S., et al.- (2016). Sub-clinical detection of gut microbial biomarkers of obesity and type 2 diabetes. Genome medicine, 8(1), 1-14.
55. Zeevi, D., Korem, T., Zmora, N., Israeli, D., Rothschild, D., Weinberger, A., Ben-Yacov, O., Lador, D., Avnit-Sagi, T., Suez, J., et al. (2015). Personalized nutrition by prediction of glycemic responses. Cell, 163(5), 1079-1094.

Chapter 5

Endocannabinoids, endocannabinoid-like molecules and their precursors in human small intestinal lumen and plasma: does diet affect them?

Silvia Tagliamonte, Chris I.R. Gill, L. Kirsty Pourshahidi, Mary M. Slevin, Rosalia Ferracane, Roger Lawther, Gloria O'Connor, Paola Vitaglione

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Abstract

Purpose: To determine the small intestinal concentration of endocannabinoids (ECs), *N*-acylethanolamines (NAEs) and their precursors *N*-acylphosphatidylethanolamines (NAPEs) in humans. To identify relationships between those concentrations and habitual diet composition as well as individual inflammatory status.

Methods: An observational study was performed involving 35 participants with an ileostomy (18W/17M, aged 18-70 y, BMI 17-40 kg/m^2). Overnight fasting samples of ileal fluid and plasma were collected and ECs, NAEs and NAPEs concentrations were determined by LC-HRMS. Dietary data was estimated from self-reported 4-day food diaries.

Results: Regarding ECs, *N*-arachidonoylethanolamide (AEA) was not detected in ileal fluids while 2-Arachidonoylglycerol (2-AG) was identified in samples from two participants with a maximum concentration of 129.3 µg/mL. In contrast, mean plasma concentration of AEA was 2.1 ± 0.06 ng/mL and 2-AG was 4.9 ± 1.05 ng/mL. NAEs concentrations were in the range 0.72-17.6 µg/mL in ileal fluids and 0.014-0.039 µg/mL in plasma. NAPEs concentrations were in the range 0.3-71.5 µg/mL in ileal fluids and 0.19-1.24 µg/mL in plasma being more abundant in participants with obesity than normal weight and overweight. Significant correlations between the concentrations of AEA, OEA and LEA in biological fluids with habitual energy or fat intakes were identified. Plasma PEA positively correlated with serum C-reactive protein.

Conclusions: We quantified ECs, NAEs and NAPEs in the intestinal lumen. Fat and energy intake may influence plasma and intestinal concentrations of these compounds. The luminal concentrations reported would allow modulation of the homeostatic control of food intake via activation of GPR119 receptors located on the gastro-intestinal mucosa.

Keywords: Lipid mediators; nutrient sensing; ileal fluids; gastrointestinal receptors; N-acylethanolamines; ileostomists.

2. Introduction

Endocannabinoids (ECs) and their structural congeners *N*-acylethanolamines (NAEs), also known as "endocannabinoid-like molecules", are endogenous lipid mediators involved in a wide range of biological pathways regulating appetite, nutrient metabolism, energy balance and inflammation (Witkamp, 2018; Simon & Cota, 2017). ECs include 2-arachidonoylglycerol (2-AG) and *N*-arachidonoylethanolamide (AEA) (Alexander et al., 2017) while the most studied NAEs are *N*-oleoylethanolamide (OEA), *N*-palmitoylethanolamide (PEA), *N*-linoleoylethanolamide (LEA) and *N*-stearoylethanolamide (SEA) (Wang et al., 2017; Witkamp, 2016).

Circulating NAEs and AEA as well as 2-AG are formed from membrane precursors such as *N*-acylphosphatidylethanolamines (NAPEs) and diacylglycerol, through the activity of NAPE-specific phospholipase D (NAPE-PLD) and diacylglycerol lipase (DAGL), respectively. Other enzymes hydrolyse NAEs and ECs to fatty acids and ethanolamines (Fowler et al., 2017; Bisogno, 2008; Wei et al., 2006).

ECs elicit their biological activities through the cannabinoid type 1 (CB1) and type 2 (CB2) receptors which are located mainly in the brain but also in peripheral tissues, such as the intestine, liver, skeletal muscle, vascular endothelium, reproductive tissues, and tissues of the immune system (Witkamp, 20016). NAEs regulate food intake, glucose homeostasis and inflammation through activation of G protein-coupled receptors (GPCRs), peroxisome proliferator-activated receptors (PPAR-α), and transient receptor potential vanilloid receptors (TRVP) (Izoo & Sharkey, 2010; Overton et al., 2008; Balvers et al., 2013). Some of these receptors are located on cells lining in the gastro-intestinal tract (GIT). For example, GPR119 is expressed on cells in the stomach, small intestine and colon (Hansen & Diep, 2009) and, upon NAEs-mediated activation, it elicits the secretion of the insulin-regulating peptides, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) (Cheng et al., 2015; Lan et al., 2012). PPAR-α is expressed in the small intestine, kidneys, liver, heart and brown adipose tissue and increases satiety through its ligand OEA (Fu et al., 2003; Lo Verme et al., 2005; Piomelli, 2013). In contrast, 2-AG and AEA stimulate food intake through the CB1 receptors (Di Patrizio & Piomelli, 2015).

Mounting evidence in subjects with inflammatory bowel disease and obesity indicates that circulating ECs are key mediators in the interplay between gut, microbiota and metabolic health (Little et al., 2018; Grill et al., 2019; Chelakkot et al., 2018; Cani et al., 2007; de La Serre et al., 2010). Moreover, plasma ECs and NAEs are considered biomarkers of white adipose tissue distribution and insulin resistance in obesity (Di Marzo et al., 2009; Blüher et al., 2006; Côté et al., 2007; Engeli et al., 2005; Matias et al., 2012; Sipe et al., 2010; Matias et al., 2006; Silvestri & Di Marzo, 2013; Fanelli et al.,

2018) and, as NAPEs, they are tightly connected with diet, especially dietary fat (Witkamp, 2018) but also proteins (Tischmann et al., 2019; Drummen et al., 2020).

On the other hand, ECs, NAEs, and NAPEs are present in several foods (De Luca et al., 2019), they increased in saliva upon food mastication in humans (Kong et al., 2016; Mennella et al., 2018) and the direct injections of NAPEs in the duodenum reduced food intake in mice (Gillum et al., 2018). Altogether, this evidence raised the question of whether the luminal content of ECs, NAEs, and NAPEs may be sufficient to elicit biological effects through the receptors located in the GIT.

Therefore, we determined the concentrations of ECs, NAEs and NAPEs in ileal fluids and plasma collected during an observational study in participants with ileostomy and investigated their relationship with habitual macronutrient and energy intake as well as with individual inflammatory status.

6. Methods

6.1. Study design

This study is part of an observational study (16/NI/0267) whose primary outcome was the assessment of bone mineral density (by Dual-energy X-ray absorptiometry) in ileostomy patients. Secondary outcomes included: anthropometric measurements, habitual diet, blood lipid profile, blood inflammatory marker, blood polyunsaturated fatty acids, blood and ileal endocannabinoids, and physical activity. The study was conducted with the prior approval of the Office for Research Ethics Committees Northern Ireland (ORECNI), the University of Ulster Ethical Committee and with the informed consent of participants and in accordance with the Declaration of Helsinki. The trial was registered at www.clinicaltrials.gov (NCT04143139). Participants (male and non-pregnant females) recruited to the study were aged 18-70 years and had previously undergone an ileostomy and were 2+ years post-operative at time of recruitment. Participants were excluded if they were outside the desired age range or had their surgical procedure <2 years before.

Following an overnight fast, the participants visited the clinic of Ulster University in Coleraine (UK) and provided ileal fluid sample and blood samples.

All participants provided questionnaires including a 4-day estimated food diary to establish habitual dietary intake completed within ±14 days of sampling, subsequently analysed using Nutritics Nutrition Analysis Software. A validated Recent Physical Activity Questionnaire (RPAQ) (Besson et al., 2009) was also completed by each participant to capture self-reported habitual activity levels at home, work and in leisure-time. The total physical activity energy expenditure (PAEE) was estimated by summing up the individual energy expenditure due to each activity domain (home, work and recreational).

6.2. Preparation of biological samples

In order to avoid handling bias with ileal fluids we used methodologies consistent with precautions as described in Karu *et al.* (2018) for metabolomic analysis in faeces. The ileal fluid samples were collected and processed within 30 min as described in Mc Dougall *et al.* (2014). In brief, volumes and pH values of the ileal fluids were recorded, before dilution with ice-cold distilled water as required, dependent on the viscosity, and before the fluid was homogenized in a chilled Waring blender for 30 s.

Fasting blood samples were collected by venipuncture into serum separator and EDTA-containing tubes. To avoid EDTA blood samples handling bias that may lead to *ex vivo* biotransformation of monitored compounds, we used recommendations of Gurke and co-workers (2019). All EDTA blood samples were kept chilled/on ice before processing. Plasma samples were prepared by centrifugation at 3000 rpm for 15 min at 4 °C and within 15 minutes of collection. Serum samples were prepared by allowing blood to clot for 30 min at room temperature then centrifugation at 3000 rpm for 15 min at 4°C.

Once prepared, ileal fluids, serum and plasma samples were aliquoted and immediately frozen at -80 °C. All samples were kept frozen at Ulster University according to Human Tissue Act (HTA) standards until further analysis.

2.3 Extraction of ECs, NAEs and NAPEs from ileal and plasma samples

Simultaneous extraction of ECs, NAEs and NAPEs from ileal fluids and plasma were performed using the method by Bligh and Dyer with brief modifications (Bligh & Dyer, 1959). All samples were thawed in the fridge at 4°C before extraction and samples were kept chilled on ice during the specific extraction procedures (Gurke et al., 2019). The ileal fluids samples were diluted prior the extraction to enhance the efficiency of solvent extraction as previously reported in faeces (Karu et al., 2018; Gregory et al., 2012).

Ileal fluids (100 µL) previously diluted 1:10 with distilled water and plasma samples (500 µL) were added to 50 µL of the internal standard 200 ng/mL solution of Arachidonoylethanolamide d8 (AEA d8) (Cayman Chemical, Ann Arbor, MI). A volume of 1.5 mL of $CHCl_3/CH_3OH$ (2:1 *v/v*) was added to the sample that was vortexed for 20 seconds and centrifuged at 14800 rpm for 10 minutes at 4 °C. Then, the supernatant was collected in a glass tube and the pellet was extracted with $CHCl_3/CH_3OH$ (2:1) twice. KCl 0.07 M (2 mL) was added to the collected phase and the lower layer (chloroform phase) was evaporated under nitrogen flow and reconstituted in 100 µL acetonitrile/isopropanol/water (60:35:5) prior the LC-HRMS analysis.

The extraction recovery was 86% in ileal fluids and 71% in plasma samples.

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

LC-HRMS analysis was performed by adapting the method by Gregory *et al.* (2012). Data were collected by using an Accela U-HPLC system consisting of a quaternary pump and a thermostated autosampler (10 °C) coupled to an Exactive Orbitrap MS provided with a heated electrospray interface (HESI) (Thermo Fisher Scientific, San Jose, CA). The compounds were separated on a Kinetex 2.6 μ C_{18} 100 A column (100 mm × 2.1 mm) (Phenomenex, Torrance, CA) with setting temperature at 45 °C and eluted by a linear gradient of a 40:60 water/acetonitrile mixture (5 mM ammonium formate 0.1% formic acid) (solvent A) and 90:10 isopropanol/acetonitrile (5 mM ammonium formate and 0.1% formic acid) (solvent B) with a flow rate of 200 μL/min and volume injection of 10 μL. According to Gregory *et al.* eluting gradient was set as follows: 32 % B from 0 to 1.5 min, 32–45 % B from 1.5 to 4 min, 45–52 % B from 4 to 5 min, 52-58 % B from 5 to 8 min, 58-66 % B from 8 to 11 min, 66-70 % B from 11 to 14 min, 70-75 % B from 14 to 18 min, 75-97 % B from 18 to 21 min and kept at 97% B until 25 min (Gregory et al., 2012). MS detection was performed in positive and negative ion mode in the *m/z* 120-1200 mass range: spray voltage was 3.5 kV (positive mode) and 3.0 kV (negative mode), capillary voltage 30V, heater temperature 300 °C, capillary temperature at 350 °C, sheath gas 35 and auxiliary gas 15 arbitrary units, respectively.

Compounds were identified and quantified against authentic standards by using exact mass value up to the fifth decimal digit (± 5 ppm mass tolerance). 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). N-Arachidonoylphosphatidylethanolamine standard was purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Inc., USA). SEA was expressed as equivalents of PEA. NAPEs were found in the chromatographic region between 19-23 min and were detected in negative ions mode as $[M-H]^-$. In the chromatographic region between 6-9 min, the NAEs were detected in positive ion mode as $[M+H]^+$. **Supplementary Table 1** reports the molecular formula, theoretical and experimental mass, the mass accuracy and the retention time of identified compounds. The limit of detection (LoD) and limit of quantification (LoQ) of the identified molecules are reported in **Supplementary Table 2.**

2.5 Biochemical analysis

Circulating lipid profiles (serum triglycerides, total and HDL cholesterol) were quantified on the iLab 650 Clinical Chemistry auto-analyzer (Instrumentation Laboratory, Massachusetts, USA) using a

commercially available assay. LDL cholesterol was calculated using the Friedewald formula (Tremblay et al., 2004). Serum C-reactive protein (CRP) was measured at the clinical chemistry department of St James' Hospital Dublin.

Total lipid was extracted from serum according to a modified version of Folch et al. (1957) where chloroform and methanol were used as the extracting solvents in a 2:1 ratio, with 250 µL of plasma extracted in 5 mL of extracting solvents (Strain et al., 2008), the wash solution was 3:47:48 (chloroform:methanol:water). Fatty acid methyl esters were detected and quantified for six key polyunsaturated fatty acids (PUFA) – linoleic acid (LA), arachidonic acid (AA), α-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) using the gold-standard technique of gas chromatography–mass spectrometry (7890A-5975C; Agilent) using heptadecanoic acid (C17:0) as the internal standard, as previously described (Strain et al., 2008). In brief, to a 250 µL plasma sample, 18 µL internal standard (heptadecanoic acid (C17:0)) was added and then chloroform:methanol (2:1) to a final volume of 5 mL. The solution was vigorously mixed, 1 mL DDH_2O added, then centrifuged at 2500 g for 5 min. The upper layer was discarded, the remaining solution washed twice with chloroform:methanol:water (3:48:47) and the sample filtered, then evaporated to dryness (at 70 ^oC, under nitrogen). Toluene (100 µL) and borontrifluoride methanol (500 µL) were added and the solution maintained at 100 ^oC for 1 hour. Subsequently, 250 µL of hexane and 800 µL DDH_2O was added to the cooled sample and mixed. The supernatant was then removed and evaporated to dryness (at 50 ^oC, under nitrogen). Extractions were reconstituted in 180 µL ethyl acetate before analysis on GC/MS which was completed in split mode (split ratio 1:20), with a BPX70 capillary GC column (SGE Analytical Science) (length 30 m, internal diameter 250 µm and film thickness 0.25 µm), using helium as the carrier gas (constant flow at 1.0 mL/min). Samples were injected using an automatic liquid sampler (ALS) (injection volume 1 µL) at a temperature of 130 °C, this was then ramped at 15 °C/min to 200 °C and then at 30° C/min to 250 °C where it was held for 5 min. Mass Spectrometry was operated in positive ion mode using an electron ionisation (EI) source. The mass range was set to 50–500 Da and acquisition was performed by Total Ion Chromatogram (TIC). We identified the individual PUFA – LA, AA, ALA, EPA, DPA and DHA – by their retention time and corresponding qualifier ions with reference to those of commercially available fatty acid standards (Sigma Aldrich, UK). These were quantified by use of an internal standard, heptadecanoic acid (C17:0) (Sigma Aldrich, UK) and corresponding PUFA target ions (quantifiers). For the purpose of this paper we have defined: Total PUFA as the sum of LA, ALA, AA, EPA, DPA, DHA; n-3 fatty acids as the sum of ALA, EPA, DPA and DHA; n-6 fatty acids as the sum of LA and AA; n-6:n-3 ratio as the ratio between n-6 and n-3 fatty acids.

2.6 Anthropometric measurements

Height and weight were measured using standardized procedures to determine Body Mass Index (BMI, kg/m^2). Standing height (cm) was measured to the nearest 0.5 cm using a calibrated stadiometer (SECA, Model 220, Germany). Body weight (kg) was recorded without footwear or heavy clothing and was measured to the nearest 0.1 kg using portable scales (Seca; Brosch Direct Ltd, Peterborough, UK). Participants were considered with normal weight when the BMI was in the range 18.5-24.9 kg/m^2, with overweight when the BMI was in the range 25.0-29.9 kg/m^2 and with obesity when the BMI was higher than 30 kg/m^2.

2.7 Statistical analysis

Statistical analyses were performed in R version 3.6.0. After being checked for normality, significantly skewed variables were transformed in ln (x) with k values. For those that showed a normal distribution at the Kolmogorov-Smirnov test, Student's t test was performed to check differences between sexes. One-way ANOVA and Bonferroni adjustment for multiple comparisons were performed to check differences between compounds in the overall population. For the variables which did not show a normal distribution after logarithmic transformation, a non-parametric Mann-Whitney test was performed. Two-tailed P values lower than 0.05 were considered significantly different. To test correlations between concentrations of NAPEs, NAEs and ECs in biological fluids with the dietary intake, all variables were transformed in ln(x) and a Pearson correlation test was performed to check the best model to fit the curve. In all cases logarithmic model was selected and results obtained were reported. Data are expressed as means ± SEM.

3. Results

3.1 Study participants

The participant flow is shown in **Fig. 1**. Thirty-five participants with ileostomy were recruited and completed the study. The characteristics of the participants including general information and anthropometry, serum lipids, PUFA and CRP as well as the mean dosages of each type of medication taken by 27 subjects, are reported in **Table 1**. Differences between sexes were found for height and weight that were higher, as well as serum HDL-cholesterol concentration that were lower, in men than women. The participants had a mean BMI of 26.9 ± 0.9 kg/m^2, specifically there was 1 subject (1 man) who was underweight, 11 subjects (8 women and 3 men) with normal weight (NW), 12 (7 women and 5 men) with overweight (OW) and 11 (3 women and 8 men) with obesity (OB). Compared to NW, OB showed higher triglycerides (1.19 ±0.15 mmol/L vs 0.6 ±0.06 mmol/L, p=0.002) and lower HDL-cholesterol concentrations (0.99 ± 0.08 mmol/L vs 1.32 ± 0.06 mmol/L, p=0.013). No

significant difference between BMI classes for general characteristics and CRP was observed.

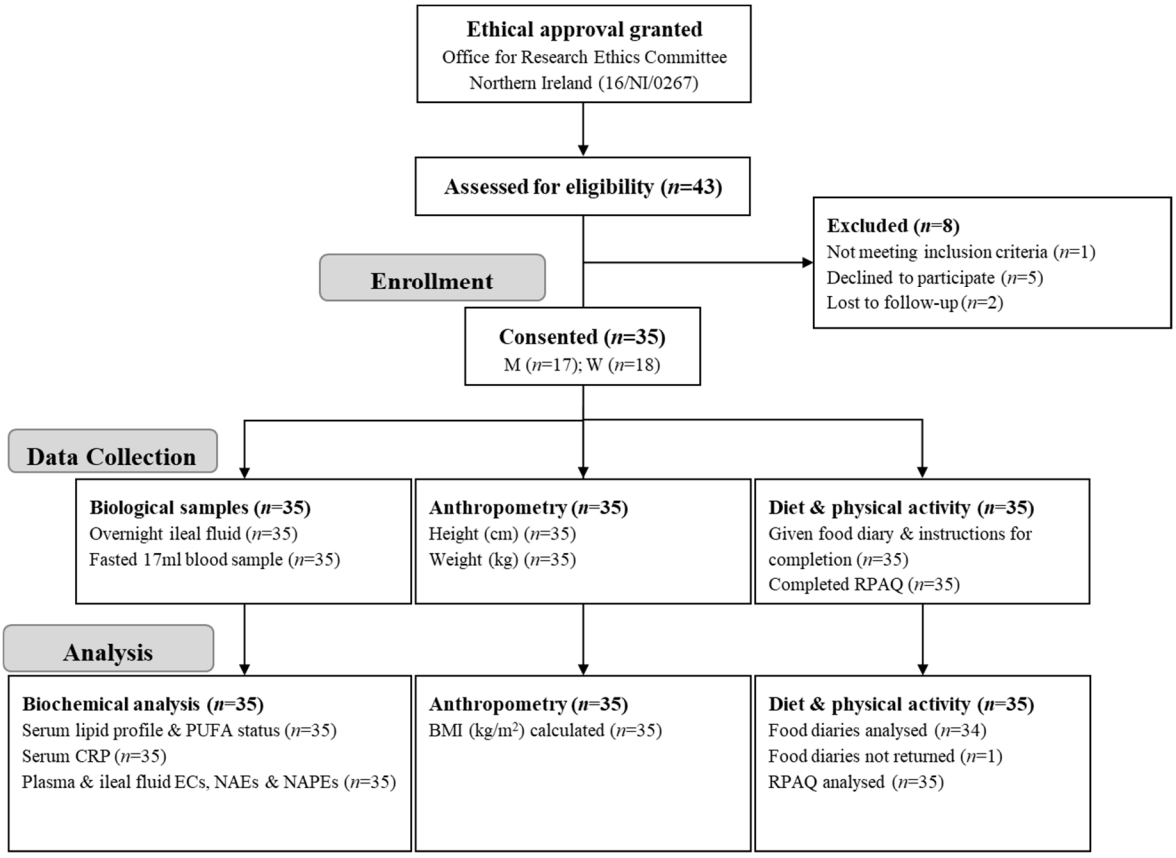


Fig. 1: Participants flow.

Table 1. Characteristics of the study participants with ileostomy. [a]

	All	Women	Men	p-value[b]
General characteristics and anthropometry	**(n = 35)**	**(n = 18)**	**(n = 17)**	
Age (years)	51.1 ± 2.4	50.8 ± 3.8	51.4 ± 3.0	0.898
Height (cm)	168.1 ± 1.3	162.7 ± 1.2	173.9 ± 1.4	**<0.001**
Weight (kg)	76.4 ± 3.1	68.3 ± 3.1	85.0 ± 4.9	**0.006**
BMI (kg/m²)	26.9 ± 0.9	25.9 ± 1.2	28.0 ± 1.5	0.266
Ileal fluids pH	6.1 ± 0.1	6.1 ± 0.1	6.1 ± 0.1	0.908
Ileal fluids net weight (g)	234.6 ± 15.9	229.1 ± 15.4	240.5 ± 28.9	0.732
PA energy expenditure (MET hours/day) [c]	21.6 ± 3.0	16.2 ± 1.8	27.3 ± 5.8	0.087
Serum lipids	**(n = 35)**	**(n = 18)**	**(n = 17)**	
Triglycerides (mmol/L)	0.92 ± 0.08	0.92 ± 0.12	0.92 ± 0.12	0.840
Total Cholesterol (TC, mmol/L)	3.52 ± 0.14	3.69 ± 0.19	3.33 ± 0.19	0.190
LDL-Cholesterol (mmol/L)	1.94 ± 0.12	2.02 ± 0.17	1.86 ± 0.16	0.509
HDL-Cholesterol (mmol/L)	1.16 ± 0.05	1.26 ± 0.04	1.05 ± 0.08	**0.030**
LA (mg/mL)	1.10 ± 0.04	1.16 ± 0.05	1.04± 0.05	0.131
ALA (mg/mL)	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.245
AA (mg/mL)	0.28 ± 0.02	0.28 ± 0.02	0.27 ± 0.02	0.815
EPA (mg/mL)	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.226
DPA (mg/mL)	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.918
DHA (mg/mL)	0.03 ± 0.00	0.04 ± 0.01	0.02 ± 0.00	0.093
Total PUFA (mg/mL) [d]	1.45 ± 0.05	1.52 ± 0.07	1.37 ± 0.07	0.144
n-6:n-3 ratio [e]	28.67 ± 3.15	28.41 ± 4.62	28.93 ± 4.40	0.451
Inflammatory status	**(n = 35)**	**(n = 18)**	**(n = 17)**	
CRP (mg/L)	3.30 ± 0.66	3.71 ± 1.18	2.87 ± 0.57	0.719
Medications (mg/kg b.w./day)				
Antihistamine (n=5; 2W, 3M)	0.28 (0.15-0.42)	0.29 (0.15-0.42)	0.27 (0.21-0.35)	
Antidiarrheal (n=5; 2W, 3M)	0.22 (0.08-0.39)	0.17 (0.16-0.17)	0.25 (0.08-0.39)	
Antihypertensive (n=4; 1W, 3M)	1.42 (0.06-3.10)	1.65	1.34 (0.06-3.10)	
Antacids (n=7; 3W, 4M)	0.43 (0.19-0.86)	0.51 (0.42-0.66)	0.38 (0.19-0.86)	
Antidepressant (n=5; 2W, 3M)	0.49 (0.24-1.07)	0.39 (0.32-0.47)	0.56 (0.24-1.07)	
Cholesterol lowering (n=6; 2W, 4M)	0.49 (0.33-0.66)	0.62 (0.58-0.66)	0.42 (0.33-0.51)	

BMI, Body mass index; PA, Physical activity; LA, Linoleic acid; ALA, Alpha-linolenic acid; AA,

Arachidonic acid; EPA, Eicosapentaenoic acid; DPA, Docosapentaenoic acid; DHA, Docosahexaenoic acid; PUFA, Polyunsaturated fatty acids; CRP, C-reactive protein.

[a] Data are expressed as means ± SEM. Medications are expressed as means (range) of dosages (mg/kg body weight/day) taken by total (n) subjects; n women (W), n men (M).

[b] $p<0.05$ in bold indicates significant difference between Women and Men, by Student's t-test or Mann-Whithney test depending on data normal distribution.

[c] Self-reported data collected using a validated physical activity (PA) questionnaire (Besson et al., 2009).

[d] Total PUFA: sum of LA, ALA, AA, EPA, DPA, DHA.

[e] n-6:n-3 ratio: (sum of LA and AA)/(sum of ALA, EPA, DPA and DHA).

3.2 Concentration of ECs, NAEs and NAPEs in ileal fluids

Fig. 2 shows the ileal concentrations of monitored NAEs and NAPEs in overall population and some significant differences between sexes and BMI classes.

Mean concentration of NAEs and NAPEs were not significantly different (3346.94 ± 561.39 ng/mL vs 4896.99 ± 2043.01 ng/mL).

LEA was the most abundant NAE and it was, like OEA, significantly higher in men than women while PEA concentration was significantly higher in OW than NW.

No difference between sexes in ileal concentrations of PEA, SEA and NAPEs or between BMI classes in ileal LEA, OEA, SEA and NAPEs concentrations were found (**Supplementary Fig. 1)**.

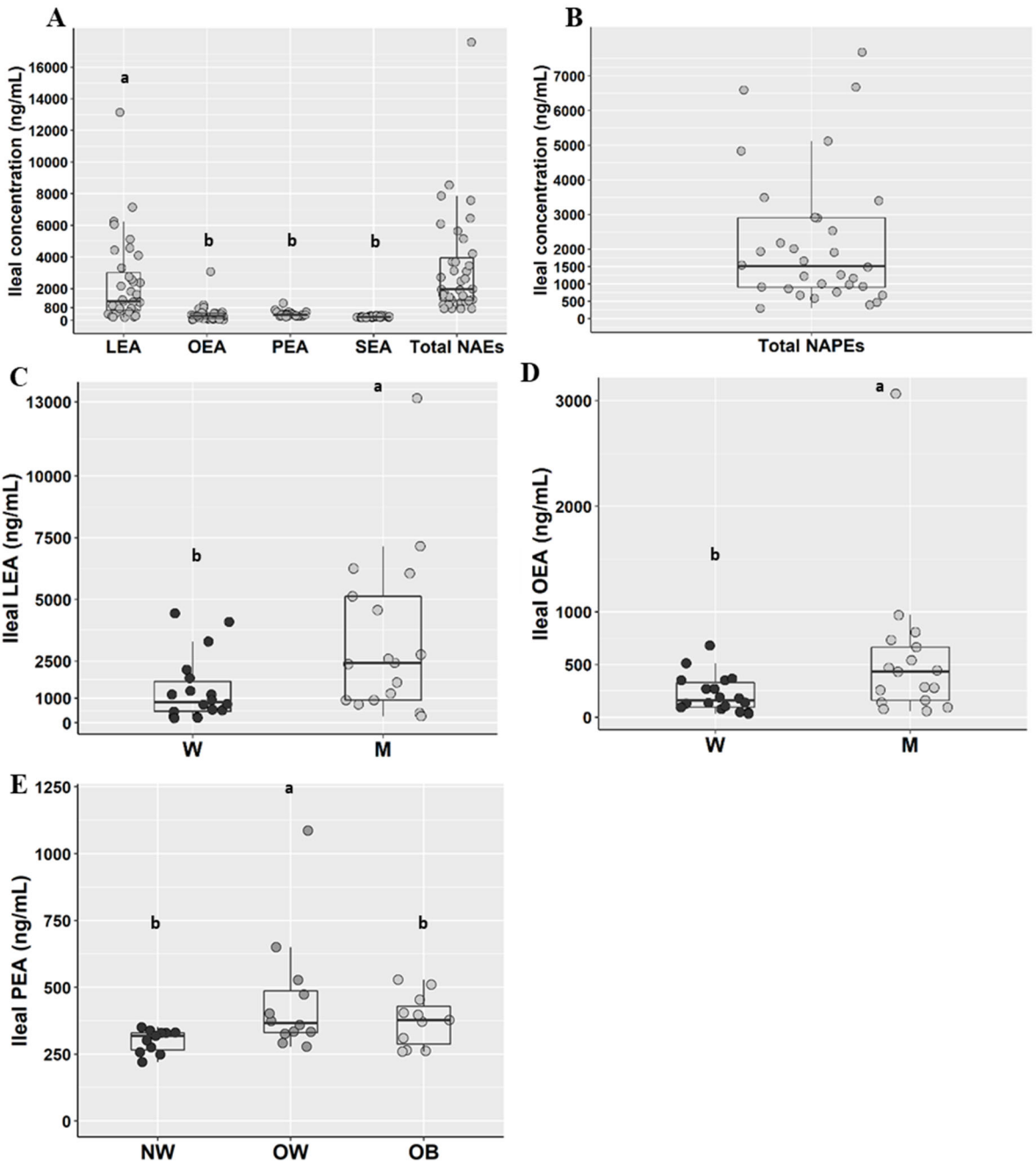


Fig. 2: *N*-acylethanolamines (A) and *N*-acylphosphatidylethanolamines (B) concentrations in ileal fluids from the overall population (n=35), LEA (C) and OEA (D) concentrations in ileal fluids from men (M, empty dots; n=17) and women (W, solid dots; n=18) and PEA(E) concentrations in ileal fluids from participants with normalweight (NW; n=11), overweight (OW; n=12) and obesity. Different letters on the box plots indicate p-value<0.05 by One-way ANOVA and Bonferroni adjustement for multiple comparisons or by Student's t test. Total NAEs include the sum of LEA, OEA, PEA and SEA. LEA, Linoylethanolamide; OEA, Oleoylethanolamide; PEA, Palmitoylethanolamide; SEA, Stearoylethanolamide; NAEs, *N*-acylethanolammines; NAPEs, *N*-

acylphosphatidylethanolamines. The box plots show the data distribution based on first quartile, median and third quartile.

3.3. Plasma concentration of ECs, NAEs and NAPEs

Fig. 3 shows the plasma concentrations of all monitored compounds in the population. In contrast to ileal fluids, AEA and 2-AG were frequently detected and in similar concentrations in plasma. PEA was the most abundant NAE followed by SEA, and similar concentration of OEA and LEA.

No difference in plasma NAEs between sexes and between BMI classes was observed (**Supplementary Fig. 2**). Contrarily, circulating levels of plasma NAPEs were higher in OB compared to NW subjects (**Fig. 3**).

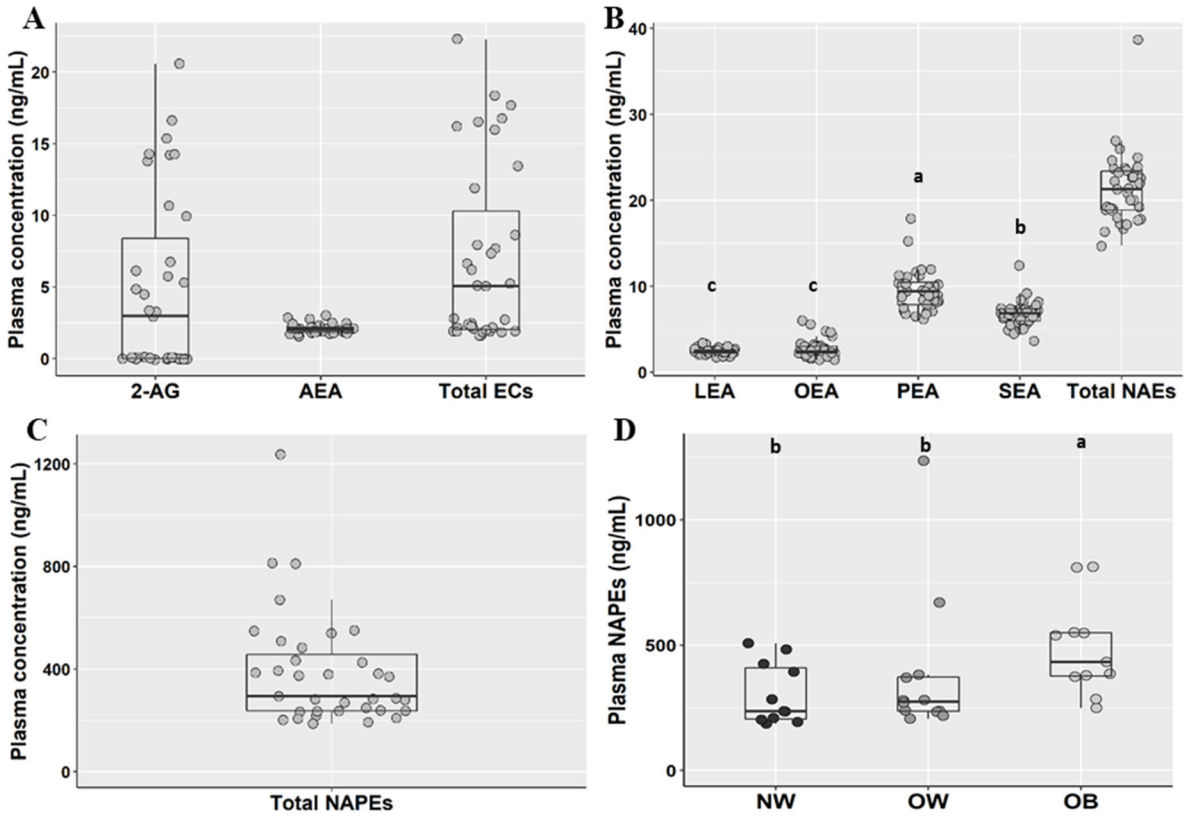


Fig. 3: Plasma Endocannabinoids (A), *N*-acylethanolammines (B) and *N*-acylphosphatidylethanolamines (C) concentrations in the overall population (n=35) and *N*-acylphosphatidylethanolamines (D) plasma concentrations from participants with normalweight (NW; n=11), overweight (OW; n=12) and obesity (OB, n=11). Different letters on the box plots indicate p-value<0.05 by One-way ANOVA and Bonferroni adjustement for multiple comparisons. Total ECs include the sum of 2-AG and AEA; Total NAEs include the sum of LEA, OEA, PEA and SEA. 2-AG, 2-Arachidonoylglicerol; AEA, Arachidonoylethanolamide; ECs, Endocannabinoids;

LEA, Linoylethanolamide; OEA, Oleoylethanolamide; PEA, Palmitoylethanolamide; SEA, Stearoylethanolamide; NAEs, *N*-acylethanolammines; NAPEs, *N*-acylphosphatidylethanolamines. The box plots show the data distribution based on first quartile, median and third quartile.

Interestingly, positive logarithmic correlations between plasma CRP with PEA (r=0.411, p=0.014) and BMI (r=0.363, p=0.032) were found (**Fig. 4**). Although, subjects who took supplements had lower serum CRP concentrations than those who did not (2.66 ± 0.95 and 3.83± 0.92, p=0.024) (**Supplementary Fig. 3**), supplement consumption did not affect plasma ECs, NAEs and NAPEs concentration.

No significant association between plasma concentration of the monitored compounds and age with the exception of a negative association between AEA plasma and age in men (r= -0.522, p=0.032) independently of BMI, was found.

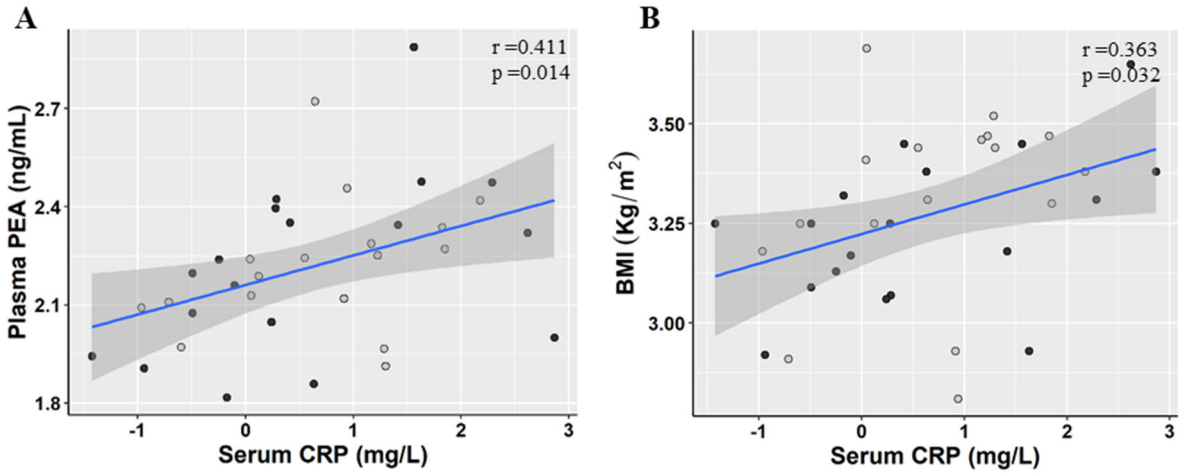


Fig. 4: Correlation between individual plasma concentrations of PEA (A), BMI (B) and individual serum CRP. Men (M, n=17) are indicated with empty dots and women (W, n=18) with solid dots. R and p-value are assessed by Pearson correlation on ln transformed variables. PEA, Palmitoylethanolamide; CRP, C-reactive protein; BMI, Body Mass Index.

3.4 Diet and correlations with plasma and ileal fluids of ECs, NAEs and NAPEs

Table 2 shows daily energy value and macronutrient composition of diets self-reported by the participants. Women reported significantly lower energy, carbohydrate, protein and fat intakes than men but a similar repartition of energy among macronutrients; only the % energy from dietary fiber was higher in women than in men.

Table 2. Nutritional composition of habitual diets of all the study participants and by sexes. [a]

	All (n=34)	Women (n=18)	Men (n=16)	p-value[b]
Energy, kJ	8292.8 ± 364.2	7176.5 ± 393.7	9548.7 ± 473.4	**<0.001**
kcal	1982.0 ± 87.1	1715.2 ± 94.1	2282.2 ± 113.1	
Carbohydrates, g	221.9 ± 9.3	195.8 ± 9.9	251.3 ± 13.0	**0.002**
% Energy	*45.3 ± 1.0*	*46.2 ± 1.4*	*44.3 ± 1.4*	*0.334*
Dietary fiber, g	17.8 ± 0.8	17.7 ± 1.2	17.9 ± 1.0	0.914
% Energy	*1.9 ± 0.1*	*2.1 ± 0.2*	*1.6 ± 0.1*	***0.006***
Proteins, g	85.5 ± 4.6	73.0 ± 5.4	99.5 ± 6.1	**0.002**
% Energy	*17.4 ± 0.7*	*17.0 ± 0.8*	*17.9 ± 1.2*	*0.519*
Fats, g	79.7 ± 5.0	67.2 ± 5.4	93.7 ± 7.4	**0.006**
% Energy	*35.4 ± 1.1*	*34.7 ± 1.6*	*36.2 ± 1.4*	*0.476*
SFA, g	30.2 ± 2.5	24.6 ± 2.7	36.5 ± 4.0	**0.011**
MUFA, g	29.0 ± 1.9	23.9 ± 2.0	34.8 ± 2.6	**0.002**
PUFA, g	12.4 ± 0.9	11.0 ± 1.1	13.9 ± 1.5	0.107
Total n-3 FA, g	1.2 ± 0.2	1.0 ± 0.2	1.4 ± 0.4	0.325
Total n-6 FA, g	5.8 ± 0.7	6.3 ± 1.0	5.3 ± 1.0	0.458
Trans fats, g	**1.1 ± 0.1**	**1.0 ± 0.2**	**1.1 ± 0.1**	**0.584**

SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids; n-3 FA, Omega-3 fatty acids; n-6 FA, Omega-6 fatty acids.

[a] Data are expressed as mean ± SEM, obtained from self-reported 4-day food diaries.

[b] $p<0.05$ in bold indicates significant difference between women and men, by Student's t-test.

Self-reported dietary intakes and plasma concentrations of PUFAs were similar between sexes (**Table 1**). Overall dietary intake of n-3 PUFAs was lower (1.2 ± 0.21 g) compared to the intake of n-6 PUFAs (5.8 ± 0.68 g). Reflecting a limited consumption of foods like oily fish in the cohort with only 6 out of 34 participants reporting intake (group average 55 ± 60 g/day) and only 2 consumed canned oily fish (group average 23.1 ± 9.7 g/day). Whereas foods rich in n-6 PUFAs such as chicken (22/34 participants, group average 65.7 ± 61.2 g/day) and eggs (20/34 participants, group average 21.8g ± 12.1 g/day) were consumed in greater quantities and by more participants. Only 9 participants consumed nuts and seed (group average 17.6 ± 16.0 g/day) a rich source of MUFAs. The relative amounts (g) of self-reported food consumption for all participants in the study are shown in **Supplementary Table 3**.

A logarithmic correlation analysis between ileal and plasma concentrations of ECs, NAEs and NAPEs with habitual self-reported energy and nutrient intakes, was performed. Results showed that ileal concentrations of LEA positively correlated with individual energy intake whereas plasma concentrations of LEA, OEA and AEA inversely correlated with it. Moreover, we found that ileal concentrations of LEA and OEA positively correlated with fat intake whereas plasma concentrations of LEA, OEA, AEA were negatively correlated. Specifically, plasma concentrations of LEA and AEA negatively correlated with saturated fat intake; PEA negatively correlated with n6, n3 series and PUFA intake; OEA negatively correlated with PUFA and AEA with MUFA intake. Moreover ileal LEA and OEA were positively associated with saturated fat and trans fat intake. All the correlations found are detailed in **Supplementary Table 4.**

3.5 Correlations between serum PUFA and ECs, NAEs and NAPEs

Plasma LEA and 2-AG concentrations were negatively associated with AA while SEA was positively associated with DHA and n3 fatty acids. Concentrations of NAPEs in ileal fluids were positively associated with plasma EPA and DPA. All the correlations found are shown in **Supplementary Table 5.**

4. Discussion

The implications of the endocannabinoid system and its role in human health has become an area of increasing interest over the last decade (Witkamp, 2016). To date, only a limited number of studies have actually measured ECs, NAEs and NAPEs in human biological fluids with circulating ECs reported in the range 0.2-9.4 ng/mL (12 studies), NAEs ranging from 0.5-64.6 ng/mL (10 studies), and NAPEs ranging from 120.7-800.0 ng/mL (2 studies) (Sipe et al., 2010; Tischmann et al., 2019; Drummen et al., 2020; Röhrig et al., 2019; Mennella et al., 2014; Mennella et l., 2015). While ECs

are acknowledged key mediators in the interplay between gut, microbiota and metabolic health, to the best of our knowledge their levels and those of NAEs and NAPEs in the gastro-intestinal tract are largely unknown with only one recent human study reporting fecal concentration for single NAPE (Santoru et al., 2017). In this study, for the first time the concentration of ECs, NAEs and NAPEs within the GIT are measured, via ileal fluid, revealing the amount of the compounds that would be colon-available upon leaving the small intestine. We determined that ileal NAEs and particularly OEA (383.72 ± 88.78 ng/mL), LEA (2360.10 ± 454.74 ng/mL) and PEA (377.72 ± 26.08 ng/mL) were on average 1.5-13 times higher than that required to elicit a physiological response through the intestinal receptors. Indeed, the agonist activity (EC50) of such compounds on GPR119 ranges from 65 ng/mL to 1.0 μg/mL for OEA, 180 ng/mL for LEA and 250 ng/mL for PEA (Hansen et al., 2011; Overton et al., 2006); OEA also displayed agonist activity on PPAR-α with an EC50 of 39 ng/mL (Fu et al., 2003).

In the present study, ileal samples were collected from stoma bags filled during the overnight fasting period and consequently may contain some food residues from the last meal consumed by the participants prior to the fasting period. Therefore, food eaten in that meal might have directly influenced the lipid type and amount in ileal fluids whose composition is more susceptible to individual digestion rate than fasting plasma samples. This may explain the differences in the lipids' profiles between ileal fluids and plasma and why, for example, LEA is the most abundant NAE in the ileal samples while it is the less abundant in plasma. Indeed, we hypothesize that dietary NAEs and NAPEs (De Luca et al., 2019) may result from digestive processes in the proximal GIT following mastication (Kong et al., 2016; Mennella et al., 2018), being into the alimentary canal postprandially at concentrations even higher than those found in this study. Such an effect could trigger GLP-1 secretion by enterocytes through activation of intestinal GPR119 thus contributing to the homeostatic regulation of food intake. Which is consistent with the findings from Chen and co-workers (Chen et al., 2017) showing that feeding mice with NAPE-synthesizing bacteria increased delivery of NAEs/NAPEs in the intestinal lumen and significantly reduced food intake and adiposity induced by a high fat diet. In the case of intestinal NAPEs, their activity necessitates conversion into NAEs through the action of intestinal NAPE-hydrolyzing phospholipase D (Chen et al., 2014). NAEs and NAPEs therefore appear to be more effective in eliciting a leptogenic effect from the intestinal lumen than in other tissues. Consequently, more robustly designed studies are needed to elucidate the physiological relevance of diet-induced increases in intestinal NAEs and NAPEs postprandially.

It should be noted, that correlations between ileal and plasma LEA and OEA with energy and fat intakes concomitant with the sex differences suggested that an individual's diet may influence NAEs intestinal and plasma concentrations. Dosoky and co-workers reported that fecal concentrations of

NAPEs (NAEs precursors) in mice fed with a plant-based diet were significantly higher than in mice fed a lard-based high fat diet (Dosoky et al., 2017), which is in line with our observations that NAPEs are more abundant in plant compared to animal foods (De Luca et al., 2019).

In agreement with Balvers and co-workers we found that total plasmatic NAPEs were about 13- to 32-folds higher than NAEs (Balvers et al., 2017) and were more abundant in OB than NW and OW. Plasma concentrations of ECs and NAEs in the ileostomy cohort (average BMI 26.9 ± 0.9) were in the same order of magnitude to those previously measured in overweight and obese subjects with intact GI tracts (Little et al., 2018; Matias et al., 2012; Sipe et al., 2010; Fanelli et al., 2018). In contrast we did not find any association between 2-AG and AEA with BMI and this is consistent with the reported heterogeneity of observations in the literature whereby plasma 2-AG has been positively correlated with BMI (Blüher et al., 2006; Côté et al., 2007) and yet no in other studies (Little et al., 2018; Jumpertz et al., 2011). Similarly, AEA plasma concentrations were elevated in obese subjects compared to normal weight subjects (Little et al., 2018; Engeli et al., 2005; Fanelli et al., 2018) yet other studies report no association with BMI (Blüher et al., 2006; Côté et al., 2007).

Other characteristics than BMI which are often disregarded in the populations studied such as the body composition, the number of women in menopause or the use of drugs or dietary supplements, may be responsible of the different findings between literature studies.

We found sex correlations between the plasma concentrations of AEA, OEA and PEA with individual age and only our observations in women were in agreement with Fanelli *et al.* (2018). However, serum free fatty acids were not positively associated with NAEs and AEA in contrast to a previous study in healthy women (Joosten et al., 2010). The heterogeneity observed is likely due in part to the different age and BMI ranges of the study populations used.

Few studies currently in the literature, reported on whether drugs affected plasma ECs and NAEs other than in relation to treating depression, whereby circulating 2-AG was halved while AEA was unchanged in patients with major depression compared to healthy subjects (Hill et al., 2009; Hauer et al., 2013) and antidepressants use did not change plasma EC nor NAE concentrations (Hauer et al., 2013). In our study, AEA plasma concentrations were about 2- to 4-folds higher than those of depressed patients (Hill et al., 2009; Hauer et al., 2013), whereas, 2-AG plasma concentrations were consistent (Hauer et al., 2013) or ~15-folds lower than patients with depression (Hill et al., 2009).

Inflammatory status (CRP) was positively correlated with the nutritional status as assessed by BMI and was consistent with previous studies (Choi et al., 2013). On the other hand, the positive association we found between plasma concentrations of PEA and CRP, independently of BMI, was in agreement with animal and human studies showing that circulating and tissue PEA concentrations increased in subjects suffering from inflammatory diseases such as neuropathic and inflammatory

pain or ulcerative colitis (Darmani et al., 2005). Furthermore, a recent study reported elevated concentrations of a NAPE, namely 18:1/16:1/18:0, in the faeces of patients suffering from ulcerative colitis in comparison to healthy controls (Santoru et al., 2017).

This study has some limitations. Firstly, the habitual diet and physical activity energy expenditure were estimated by self-reported data from participants using previously validated survey instruments, which is not as reliable as direct observations. Secondly, since we did not measure total energy expenditure we could not estimate if participants were in energy balance. If subjects were not in energy balance, this might alter the implications of our observations. Finally, the possible presence of residues of the dinner in the ileal fluids makes any attempt to identify the relationships between ileal and plasma content challenging in this study.

In conclusion, we assessed, for the first time, the concentrations of ECs, NAEs and NAPEs present in ileal fluids from participants with ileostomy and thus established the amounts likely to be entering the colon in individuals with an intact GIT. Men had higher ileal concentrations of OEA and LEA than women which positively correlated with the self-reported dietary higher energy and fat intake. An ongoing intervention study will address whether the dietary intake of NAPEs and NAEs affect the concentrations of NAEs in the intestinal lumen postprandially and if they contribute to the homeostatic control of food intake.

Conflict of interest statement

On behalf of all authors, the corresponding author states that there is no conflict of interest.

References

1. Alexander, S.P.H., Christopoulos, A., Davenport, A.P., Kelly, E., Marrion, N.V., Peters, J.A., Faccenda, E., Harding, S.D., Pawson, A.J., Sharman, J.L., Southan, C., Davies, J.A. (2017). The Concise Guide to PHARMACOLOGY 2017/18: G protein-coupled receptors. Br J Pharmacol 174:S17-S129.
2. Balvers, M. G. J., Verhoeckx, K. C. M., Meijerink, J., Wortelboer, H. M., Witkamp, R. F. (2013). Measurement of palmitoylethanolamide and other N-acylethanolamines during physiological and

pathological conditions. CNS Neurol Disord-Dr 12:26-33.

3. Balvers, M. G., Wortelboer, H. M., Witkamp, R. F., Verhoeckx, K. C. (2013). Liquid chromatography–tandem mass spectrometry analysis of free and esterified fatty acid N-acyl ethanolamines in plasma and blood cells. Anal Biochem 434:275-283.
4. Besson, H., Brage, S., Jakes, R. W., Ekelund, U., & Wareham, N. J. (2009). Estimating physical activity energy expenditure, sedentary time, and physical activity intensity by self-report in adults. Am J Clin Nutr 91:106-114.
5. Bisogno, T. (2008). Endogenous cannabinoids: structure and metabolism. J Neuroendocrinol 20:1-9.
6. Bligh, E.G., Dyer, W.J. (1959). A rapid method for total lipid extraction and purification. Can J Biochem Physiol 37:911-917.
7. Blüher, M., Engeli, S., Klöting, N., Berndt, J., Fasshauer, M., Bátkai, S., Pacher, P., Schön, M. R., Jordan, J., Stumvoll, M. (2006). Dysregulation of the peripheral and adipose tissue endocannabinoid system in human abdominal obesity. Diabetes 55:3053-3060.
8. Cani, P. D., Amar, J., Iglesias, M. A., Poggi, M., Knauf, C., Bastelica, D., Neyrinck, A. M., Fava, F., Tuohy, K. M., Waget, A., et al. (2007). Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes, 56:1761-1772.
9. Chelakkot, C., Ghim, J., Ryu, S. H. (2018). Mechanisms regulating intestinal barrier integrity and its pathological implications. Exp Mol Med, 50:1-9.
10. Chen, Z., Guo, L., Zhang, Y., Walzem, R. L., Pendergast, J. S., Printz, R. L., Morris, L. C., Matafovona, E., Stien, X., Kang, L. et al. (2014). Incorporation of therapeutically modified bacteria into gut microbiota inhibits obesity. J Clin Invest 124:3391-3406.
11. Chen, Z., Zhang, Y., Guo, L., Dosoky, N., de Ferra, L., Peters, S., Niswender, K. D., Davies, S. S. (2017). Leptogenic effects of NAPE require activity of NAPE-hydrolyzing phospholipase D. J Lipid Res 58:1624-1635.
12. Cheng, Y.-H., Ho, M.-S., Huang, W.-T., Chou, Y.-T., King, K., (2015). Modulation of glucagon-like peptide-1 (GLP-1) potency by endocannabinoid-like lipids represents a novel mode of regulating GLP-1 receptor signaling. J Biol Chem 290:14302-14313.
13. Choi, J., Joseph, L., Pilote, L. (2013). Obesity and C-reactive protein in various populations: a systematic review and meta-analysis. Obes Rev 14:232-44.
14. Côté, M., Matias, I., Lemieux, I., Petrosino, S., Alméras, N., Després, J.P., Di Marzo, V., (2007). Circulating endocannabinoid levels, abdominal adiposity and related cardiometabolic risk factors in obese men. Int J Obes 31(4):692-9.
15. Darmani, N. A., Izzo, A. A., Degenhardt, B., Valenti, M., Scaglione, G., Capasso, R., Sorrentini,

I., Di Marzo, V. (2005). Involvement of the cannabimimetic compound, N-palmitoyl-ethanolamine, in inflammatory and neuropathic conditions: review of the available pre-clinical data, and first human studies. Neuropharmacology 48:1154-1163.

16. de La Serre, C. B., Ellis, C. L., Lee, J., Hartman, A. L., Rutledge, J. C., Raybould, H. E. (2010). Propensity to high-fat diet-induced obesity in rats is associated with changes in the gut microbiota and gut inflammation. Am. J. Physiol. Gastrointest. Liver Physiol., 299: G440-G448.
17. De Luca, L., Ferracane, R., Vitaglione, P. (2019). Food database of N-acyl-phosphatidylethanolamines, N-acylethanolamines and endocannabinoids and daily intake from a Western, a Mediterranean and a vegetarian diet. Food Chem 300:125218.
18. Di Marzo, V., Côté, M., Matias, I., Lemieux, I., Arsenault, B.J., Cartier, A., Piscitelli, F., Petrosino, S., Alméras, N., Després, J.P. (2009). Changes in plasma endocannabinoid levels in viscerally obese men following a 1 year lifestyle modification programme and waist circumference reduction: associations with changes in metabolic risk factors. Diabetologia 52:213.
19. Di Patrizio, N.V., Piomelli, D. (2015). Intestinal lipid–derived signals that sense dietary fat. J Clin Invest. 125(3): 891–898.
20. Dosoky, N. S., Guo, L., Chen, Z., Feigley, A. V., Davies, S. S. (2017). Dietary Fatty Acids Control the Species of N-Acyl-Phosphatidylethanolamines Synthesized by Therapeutically Modified Bacteria in the Intestinal Tract. ACS Infect Dis 4:3-13.
21. Drummen, M., Tischmann, L., Gatta-Cherifi, B., Cota, D., Matias, I., Raben, A., Adam, T., Westerterp-Plantenga, M. (2020). Role of endocannabinoids in energy-balance regulation in participants in the postobese state—a PREVIEW Study. J. Clin. Endocrinol. Metab. 105 (7): e2511–e2520.
22. Engeli, S., Böhnke, J., Feldpausch, M., Gorzelniak, K., Janke, J., Bátkai, S., Pacher, P., Harvey-White, J., Luft, F. C., Sharma, A. M., Jordan, J. (2005). Activation of the peripheral endocannabinoid system in human obesity. Diabetes 54:2838-2843.
23. Fanelli, F., Mezzullo, M., Repaci, A., Belluomo, I., Gasparini, D. I., Di Dalmazi, G., Mastroroberto, M., Vicennati, V., Gambineri, A., Pasquali, R., et al. (2018). Profiling plasma N-Acylethanolamine levels and their ratios as a biomarker of obesity and dysmetabolism. Mol Metab 14:82-94.
24. Folch, J., Lees, M., Stanley, G. S. (1957). A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226: 497-509.
25. Fowler, C. J., Doherty, P., Alexander, S. P. (2017). Endocannabinoid turnover. In Adv Pharmacol (Vol. 80, pp. 31-66).

26. Fu, J., Gaetani, S., Oveisi, F., Verme, J. L., Serrano, A., de Fonseca, F. R., Rosengarth, A., Luecke, H., Di Giacomo, B., Tarzia, G., Piomelli, D. (2003). Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-α. Nature 425:90.
27. Gillum, M., Zhang, D., Zhang, X., Erion, D., Jamison, R., Choi, C., Dong, J., Shanabrough, M., Duenas, H., Frederick, D., et al. (2008). N-acylphosphatidylethanolamine, a gut-derived circulating factor induced by fat ingestion, inhibits food intake. Cell 135:813-824.
28. Gregory, K. E., Bird, S. S., Gross, V. S., Marur, V. R., Lazarev, A. V., Walker, W. A., Kristal, B. S. (2012). Method development for fecal lipidomics profiling. Anal Chem 85:1114-1123.
29. Grill, M., Högenauer, C., Blesl, A., Haybaeck, J., Golob-Schwarzl, N., Ferreirós, N., Thomas, D., Gurke, R., Trötzmüller, M., Gallé, B.,g et al. (2019). Members of the endocannabinoid system are distinctly regulated in inflammatory bowel disease and colorectal cancer. Sci. Rep., 9:1-13.
30. Gurke, R., Thomas, D., Schreiber, Y., Schäfer, S. M. G., Fleck, S. C., Geisslinger, G., Ferreirós, N. (2019). Determination of endocannabinoids and endocannabinoid-like substances in human K3EDTA plasma–LC-MS/MS method validation and pre-analytical characteristics. Talanta 204:386-394.
31. Hansen, H. S., Diep, T. A. (2009). N-acylethanolamines, anandamide and food intake. Biochem Pharmacol 78:553-560.
32. Hansen, K. B., Rosenkilde, M. M., Knop, F. K., Wellner, N., Diep, T. A., Rehfeld, J. F., Hansen, H. S. (2011). 2-Oleoyl glycerol is a GPR119 agonist and signals GLP-1 release in humans. J Clin Endocr Metab 96:E1409-E1417.
33. Hauer, D., Schelling, G., Gola, H., Campolongo, P., Morath, J., Roozendaal, B., Hamumi, G., Karabatsoakis, A., Atsak, P., Vogeser, M., Kolassa, I. T. (2013). Plasma concentrations of endocannabinoids and related primary fatty acid amides in patients with post-traumatic stress disorder. PLoS ONE 8(5):e62741.
34. Hill, M. N., Miller, G. E., Carrier, E. J., Gorzalka, B. B., Hillard, C. J. (2009). Circulating endocannabinoids and N-acyl ethanolamines are differentially regulated in major depression and following exposure to social stress. Psychoneuroendocrinology 34:1257-1262.
35. Izzo, A.A., Sharkey, K.A. (2010). Cannabinoids and the gut: new developments and emerging concepts. Pharmacol Therapeut 126:21-38.
36. Joosten, M. M., Balvers, M. G., Verhoeckx, K. C., Hendriks, H. F., Witkamp, R. F. (2010). Plasma anandamide and other N-acylethanolamines are correlated with their corresponding free fatty acid levels under both fasting and non-fasting conditions in women. Nutr Metab, 7:1-6.
37. Jumpertz, R., Guijarro, A., Pratley, R. E., Piomelli, D., Krakoff, J. (2011). Central and peripheral endocannabinoids and cognate acylethanolamides in humans: association with race, adiposity,

and energy expenditure. J Clin Endocr Metab 96:787-791.

38. Karu, N., Deng, L., Slae, M., Guo, A. C., Sajed, T., Huynh, H., Wine, E., Wishart, D. S. (2018). A review on human fecal metabolomics: Methods, applications and the human fecal metabolome database. Anal Chim Acta 1030:1-24.
39. Kong, X., Ferracane, R., De Luca, L., Vitaglione, P. (2016). Salivary concentration of N-acylethanolamines upon food mastication and after meal consumption: Influence of food dietary fiber. Food Res Int 89:186-193.
40. Lan, H., Lin, H. V., Wang, C. F., Wright, M. J., Xu, S., Kang, L., Hedrick, J.A., Kowalski, T. J. (2012). Agonists at GPR119 mediate secretion of GLP-1 from mouse enteroendocrine cells through glucose-independent pathways. Br J Pharmacol 165:2799–2807.
41. Little, T. J., Cvijanovic, N., Di Patrizio, N. V., Argueta, D. A., Rayner, C. K., Feinle-Bisset, C., Young, R. L. (2018). Plasma endocannabinoid levels in lean, overweight, and obese humans: relationships to intestinal permeability markers, inflammation, and incretin secretion. Am J Physiol Endocrinol Metab 315:E489-E495.
42. Lo Verme, J., Gaetani, S., Fu, J., Oveisi, F., Burton, K., Piomelli, D., (2005). Regulation of food intake by oleoylethanolamide. Cell Mol Life Sci 62:708.
43. Matias, I., Bisogno, T., Di Marzo, V. (2006). Endogenous cannabinoids in the brain and peripheral tissues: regulation of their levels and control of food intake. Int J Obes 30: S7-S12.
44. Matias, I., Gatta-Cherifi, B., Tabarin, A., Clark, S., Leste-Lasserre, T., Marsicano, G., et al., (2012). Endocannabinoids measurement in human saliva as potential biomarker of obesity. PLoS ONE 7:e42399.
45. McDougall, G. J., Conner, S., Pereira-Caro, G., Gonzalez-Barrio, R., Brown, E. M., Verrall, S., Stewart, D., Moffet, T., Ibars, M., Lawther, R., et al. (2014). Tracking (Poly) phenol components from raspberries in ileal fluid. J Agr Food Chem 62:7631-7641.
46. Mennella, I., Di Monaco, R., Balazy, A., Ferracane, R., Miele, N. A., Cavella, S., & Vitaglione, P. (2018). Salivary endocannabinoids and N-acylethanolamines upon mastication of a semisolid food: implications in fat taste, appetite and food liking. Food Funct 9:476-484.
47. Mennella, I., Ferracane, R., Zucco, F., Fogliano, V., Vitaglione, P. (2015). Food liking enhances the plasma response of 2-arachidonoylglycerol and of pancreatic polypeptide upon modified sham feeding in humans. The Journal of nutrition, 145(9), 2169-2175.
48. Mennella, I., Savarese, M., Ferracane, R., Sacchi, R., Vitaglione, P. (2014). Oleic acid content of a meal promotes oleoylethanolamide response and reduces subsequent energy intake in humans. Food & function, 6(1), 203-209.
49. Overton, H. A., Fyfe, M. C. T., Reynet, C. (2008). GPR119, a novel G protein-coupled receptor

target for the treatment of type 2 diabetes and obesity. Br J Pharmacol 153:S76-S81.

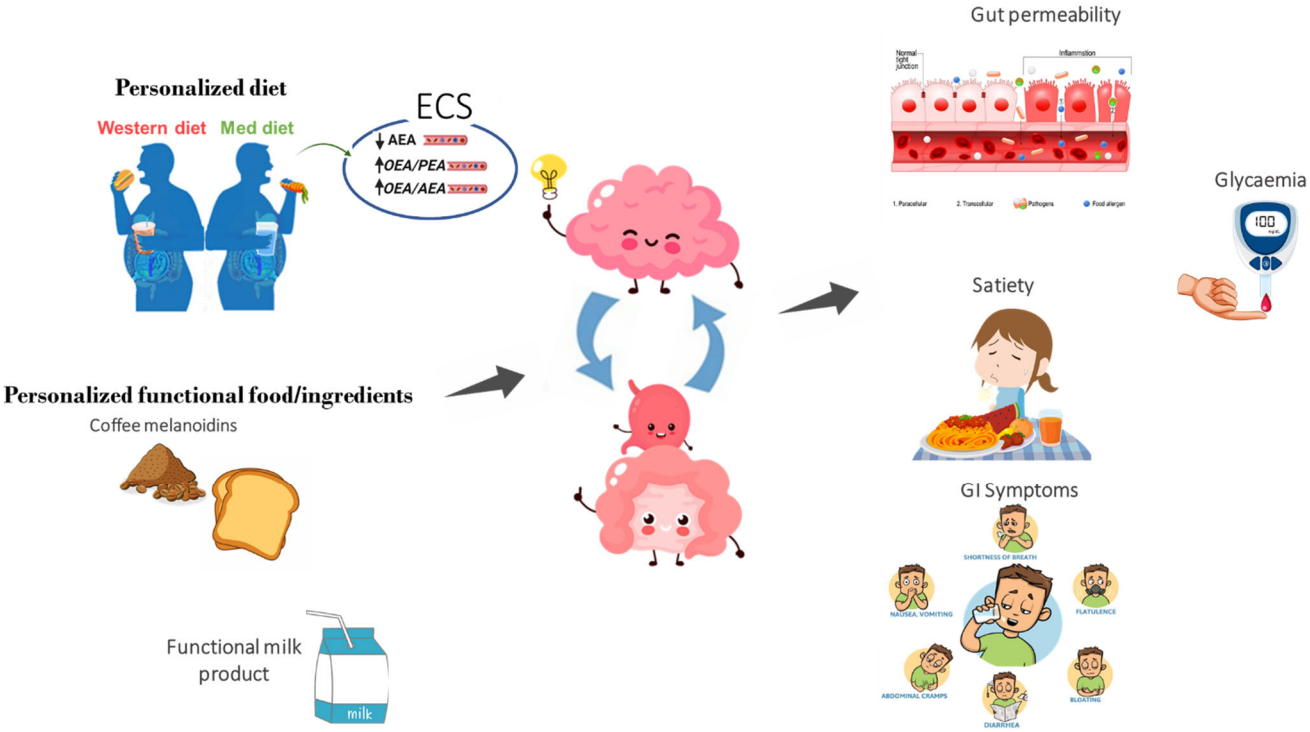
50. Overton, H.A., Babbs, A.J., Doel, S.M., Fyfe, M.C., Gardner, L.S., Griffin, G., Jackson, H.C., Procter, M.J., Rasamison, C.M., Tang-Christensen, M., et al. (2006). Deorphanization of a G protein-coupled receptor for oleoylethanolamide and its use in the discovery of small-molecule hypophagic agents. Cell Metab 3:167-175.
51. Piomelli, D. (2013). A fatty gut feeling. Trends Endocrin Met 24:332-341.
52. Röhrig, W., Achenbach, S., Deutsch, B., Pischetsrieder, M. (2019). Quantification of 24 circulating endocannabinoids, endocannabinoid-related compounds, and their phospholipid precursors in human plasma by UHPLC-MS/MS. Journal of Lipid Research, 60(8), 1475-1488.
53. Santoru, M. L., Piras, C., Murgia, A., Palmas, V., Camboni, T., Liggi, S., Ibba, I., Lai, M. A., Orrù, S., Blois, S., et al. (2017). Cross sectional evaluation of the gut-microbiome metabolome axis in an Italian cohort of IBD patients. Sci Rep 7:9523.
54. Silvestri, C., and Di Marzo, V. (2013). The endocannabinoid system in energy homeostasis and the etiopathology of metabolic disorders. Cell Metab. 17:475-490.
55. Simon, V., Cota, D. (2017). Mechanisms in endocrinology: endocannabinoids and metabolism: past, present and future. Eur. J. Endocrinol., 176(6), R309-R324.
56. Sipe, J. C., Scott, T. M., Murray, S., Harismendy, O., Simon, G. M., Cravatt, B. F., & Waalen, J. (2010). Biomarkers of endocannabinoid system activation in severe obesity. PLoS ONE 5(1): e8792.
57. Strain, J. J., Davidson, P. W., Bonham, M. P., Duffy, E. M., Stokes-Riner, A., Thurston, S. W., Wallace, J.M.W., Robson, P.J., Shamalaye, C.F., Georger, L.A., et al. (2008). Associations of maternal long-chain polyunsaturated fatty acids, methyl mercury, and infant development in the Seychelles Child Development Nutrition Study. Neurotoxicology 29:776-782.
58. Tischmann, L., Drummen, M., Gatta-Cherifi, B., Raben, A., Fogelholm, M., Hartmann, B., Holst, J. J., Matias, I., Cota, D., Joris, P. J., et al. (2019). Effects of a high-protein/moderate-carbohydrate diet on appetite, gut peptides, and endocannabinoids—a PREVIEW study. Nutrients 11(10):2269.
59. Tremblay, A. J., Morrissette, H., Gagné, J. M., Bergeron, J., Gagné, C., & Couture, P. (2004). Validation of the Friedewald formula for the determination of low-density lipoprotein cholesterol compared with β-quantification in a large population. Clin Biochem 37:785-790.
60. Wang, Y., Balvers, M., Hendriks, H., Wilpshaar, T., van Heek, T., Witkamp, R., Meijerink, J. (2017). Docosahexaenoyl serotonin emerges as most potent inhibitor of IL-17 and CCL-20 released by blood mononuclear cells from a series of N-acyl serotonins identified in human intestinal tissue. BBA-Molecular and Cell Biology of Lipids 1862:823-831.
61. Wei, B. Q., Mikkelsen, T. S., McKinney, M. K., Lander, E. S., Cravatt, B. F. (2006). A second

fatty acid amide hydrolase with variable distribution among placental mammals. J Biol Chem 281:36569-36578.

62. Witkamp, R. (2016). Fatty acids, endocannabinoids and inflammation. Eur J Pharmacol 785:96-107.
63. Witkamp, R. (2018). The role of fatty acids and their endocannabinoid-like derivatives in the molecular regulation of appetite. Mol Aspects Med 64:45-67.

Chapter 6

General discussion



Diet plays a key role in the development of chronic diseases and obesity (Jayedi et al., 2020) and in shaping human gut microbiome (Cuevas-Sierra et al., 2019). However, findings from nutritional RCTs indicates that individuals can have a different response to the same food or diet. Therefore, in the clinical practice 'one-size-fits-all' nutritional approach is inappropriate and personalized approaches are necessary (Heinzmann et al., 2012, Zeevi et al., 2015).

Findings from human clinical trials, including well characterized categories of subjects and monitoring multiple systems and outcomes, including dietary habits, gut microbiome and systemic metabolome, are mandatory to clarify the physiological mechanims and design personalized nutritional plan tailored to optimize individual nutritional status, prevent disease and design functional food (De Filippis et al., 2018; Kolodziejczyk et al., 2019).

The experiments carried out in the present thesis unraveled the physiological mechanisms underpinning satiety and post-prandial wellbeing in healthy subjects, and they clarified the implications of the endocannabinoid system (ECS) in obesity and metabolic diseases prevention. A summary of the studies and the major findings and conclusions are showed in **Table 1**.

Table 1. Overview of the experiments performed in the thesis.

Chapter	*Objectives*	*Methods and Subjects*	*Outcomes*	*Major findings*	*Conclusions*
2	To investigate the satiating efficacy of coffee melanoidins-enriched bread (CM) and bread melanoidins-enriched bread (BM) compared to a conventional bread (CT) in humans	*Study:* randomized crossover, meal test designed *Measures:* • Blood biomarkers (postprandial, 0-180 min) • Energy intakes (EI, 24h) • Appetite *Subjects:* • Healthy • 14 - 7M/7F • Age, y: 26.4 (19-49) • BMI kg/m^2: 22.2±0.14	• ECs (n=2) • NAEs (n=3) • GI peptides (n=8) • Neuropeptides (n=7) • Appetite ratings • EI	• CM and BM ↓ EI; • CM *vs* BM ↓ glucose, insulin, α-MSH, orexin-A, β-endorphin; • No effect on NAEs	Coffee melanoidins at breakfast reduces daily energy intake modulating postprandial glycemia and gut-brain modulators.
3	To investigate the implication of cow's **milk** protein **digestion** and related physiological responses, gut microbiome, and gut permeability in the occurrence of **gastrointestinal discomforts** (GID) after milk consumption **in healthy subjects.**	*Study:* Observational parallel study and meal study *Measures:* • Gut microbiome • Dietary habits • Gut permeability (GPT) • Blood biomarkers (postprandial, 0-360 min) • Urinary biomarkers (postprandial) • Gastrointestinal symptoms • Appetite *Subjects:* • Healthy • 39 – 19 NHMC (9M/10F); 20 HMC (10M/10F)	• Gut microbiome (GM) • Dietary habits • 24h urinary lactulose, mannitol and sucralose • GI peptides (n=4); • Glycaemia • Aminoacids (n=20) • Bioactive peptides (31 BAPS) • DPPIV conc & activity • ECs (n=2) • NAEs (n=4) • Urinary indoxyl sulfate • Gastrointestinal symptoms ratings • Appetite ratings	***NHMC vs HMC:*** • > anxiety • < intake dairy products • > dietary fiber/protein ratio • <*Bifidobacteria*, > *Prevotella* • < proteolytic activity in GM • Aspects of GM associated with intestinal health ***Milk consumption in NHMC:*** • ↑ GID • ↓ BAPS • blunted ghrelin & AEA • ↓ insulin • ↑ glucose & DPPIV activity • ↓ hunger late in the day • ↓ indoxyl sulfate	A slower and lower digestion of milk proteins explained the occurrence of GID in healthy subjects independently from the gut permeability. A lower proteolytic activity of the gut microbiome shaped by the habitual diet likely contributed to the findings. -

		• Age, y: NHMC (24.2 ± 0.9); HMC (25 ± 0.7) • BMI kg/m^2: NHMC (23.5 ± 0.7); HMC (25.6 ± 0.8)			
4	To investigate whether a **Mediterranean diet** (MD) affected the plasma concentrations of **ECs**, **NAEs** and their ratios, in **subjects at risk of CVD**. To evaluate the relationship between those levels, gut microbiome, insulin resistance and inflammatory status.	*Study:* 8-week randomized controlled trial *Measures:* • Blood biomarkers • Gut microbiome • 7-day food diary • Anthropometry *Subjects:* • Overweight/obese • 82 – 43 MD (21M/22F; 39 CT (18M/21F) • Age, y: MD (43±1.9); CT (42±2.0) • BMI kg/m^2: MD= 30.9 ±0.6; CT= 31.2±2.0)	• ECs (n=2) • NAEs (n=3) • hs-CRP • Glucose • Insulin • Gut microbiome	**MD** • ↓ AEA • ↑ OEA/PEA & OEA/AEA • ↑ *Akkermansia muciniphila* • Positive Association between OEA/PEA, IMI and healthy gut microbiome. • Subjects with low OEA/PEA (high AEA) ↓ HOMA-index. Subjects with high OEA/PEA (low AEA) ↑ *A. muciniphila* & ↓ CRP.	Endocannabinoid system is shaped by MD increasing *A. muciniphila* abundance in the gut independently of body weight changes. EC tone and microbiome functionality at baseline drives an individualised response to an MD ameliorating insulin sensitivity and inflammation.
5	To quantify **ECs**, **NAEs** and **NAPEs** levels in the **small intestine.** **To** valuate the relationship between those levels, habitual diet and inflammatory status in an **ileostomy inflammatory model**.	*Study:* Observational study *Measures:* • Blood biomarkers • Ileal fluids biomarkers • 4-day food diary *Subjects:* • Ileostomists • 35 - 17M/18F • Age, y: 51.1 (18-70) • BMI kg/m^2: 26.9 ± 0.9	***Ileal fluids:*** • ECs (n=2) • NAEs (n=4) • NAPEs (n=19) ***Plasma:*** • ECs (n=2) • NAEs (n=4) • NAPEs (n=19) • hs-CRP • Lipids (n=8)	***Ileal fluids:*** • NAEs= 0.72–17.6 µg/mL; • NAPEs= 0.3–71.5 µg/ mL; • Positive association between LEA, energy and fat intake. • Positive association between OEA and fat intake ***Plasma:*** • NAEs= 0.014–0.039 µg/mL; • NAPEs= 0.19–1.24 µg/mL; • Negative association between LEA, OEA and AEA and energy and fat intake. • Positive association between PEA and CRP.	Diet may infuence plasma and intestinal concentrations of NAEs and NAPEs. The amount of NAEs in the intestinal lumen may be physiological relevant to exert their agonist activity on GPR119 receptor lining on the gastro-intestinal mucosa.

Nutrient digestion: food for satiety and post-prandial wellbeing

Dietary fiber and food-derived bioactive peptides are responsible of many health benefits in humans acting from the gastrointestinal tract (GIT), shaping and/or interacting with the gut microbiome and other body systems (Lordan et al., 2020; Sanders et al., 2019; Awika et al., 2018; Caron et al., 2017; Park et al., 2015). Therefore, they are considered promising ingredients for functional foods.

Specifically, dietary fibers are well recognized for the satiating effect in humans. They are categorised into soluble and insoluble fibers. The physiological effects of soluble dietary fibers can be imputed to its physico-chemical properties, such as viscosity, gel formation or fermentability in the colon (Salleh et al., 2019). Viscous soluble dietary fibres are more effective in inducing satiety compared to non-viscous soluble dietary fibres. Indeed, they increase digesta viscosity delaying gastric emptying, slowing digestion and the absorption of nutrients, and reducing enzyme diffusion (Salleh et al., 2019). Finally, viscous dietary fibres are fermented in the colon resulting in the production of short chain fatty acids (SCFAs). SCFAs are known to increase satiety and reduce food intake by modulating the secretion of various appetite-regulating peptides (PYY, GLP-1 and CCK) throughout the colon (Delzenne et al., 2005; Keenan et al., 2006; Salleh et al., 2019).

Interestingly, undigestible compounds can be formed over food processing due to the chemical interaction and reactions occurring in the food matrix among food components. This phenomenon is responsible for instance for the increase of resistant starch in starchy foods subjected to high-pressure treatment in combination with high temperature treatment (Nagy et al., 2021). Resistant starch pass through the human digestive tract, skips digestion and absorption, is fermented by the gastrointestinal microbiota that produce SCFAs, well known modulators of satiety and antiglycemic response through the gut (Nagy et al., 2021).

Besides resistant starch, other types of molecules are formed over heating of specific food matrices and they behave as dietary fibers upon digestion in human body. This is the case of melanoidins which are high molecular weight compounds present in heat-treated and highly processed foods including bakery products, coffee, milk, beer, cocoa, soy sauce and vinegar. They are formed at the final stages of the Maillard reaction during thermal processing of foods containing precursors including reducing sugars and amino groups of proteins, polypeptides and oligopeptides (Troise et al., 2016). From a nutritional viewpoint, the presence of Maillard reaction products in some foods (like milk) may denote a poor-quality food product due to the

reduced protein digestibility and the known effect of advanced glycation end-products that negatively affect health and being implicated in aging, diabetes and atherosclerosis (ALjahdali & Carbonero, 2019). However, food melanoidins have attracted great attention as a functional food ingredient being recognized as good contributors to the digestion-resistant polymers that reach the colon and are fermented by the local microbiota (Fogliano and Morales, 2011). Indeed, it has been estimated that dietary intake of melanoidins account for 10 g/day and consumption of bread and coffee majorly contribute to that value (Fogliano and Morales, 2011). Interestingly, bread and coffee melanoidins are chemically different. Bread crust melanoidins derive mainly from gluten proteins and starch (Morales et al., 2012) and remain insoluble throughout the gastrointestinal tract (Helou et al., 2017). On the other hand, coffee melanoidins are formed mainly by polysaccharides and have a limited ability to form gel structures in the stomach and the intestine (Nunes & Coimbra, 2007). Coffee melanoidins are known for their antioxidant, antimicrobial and anticancer properties. Indeed, the chemical structure of coffee melanoidins has all the features of an "antioxidant dietary fiber": they can elicit a "radical-sponge action" accross the GIT and enter the colon where they are partly fermented by colonic microorganisms (Vitaglione et al., 2012).

Although melanoidins share physicochemical, biochemical and biological properties with dietary fibers (Fogliano & Morales, 2011), the interaction of melanoidins with the gut-brain axis underpinning appetite sensations in humans was never explored.

These aspects were investigated in the randomized crossover meal test study described in the **Chapter 2.** It explored the satiating efficacy of coffee melanoidins (CM) and bread melanoidins (BM) used as food ingredients in a conventional bread (CT). Twentyone gut-brain modulators of appetite including 8 gastrointestinal peptides, 2 endocannabinoids, 3 *N*-acylethanolamines and 7 neuropeptides were monitored post-prandially (0-180 min) along with energy intakes and appetite ratings accross the day. Data showed that bread enriched with CM and BM were effective in reducing the daily energy intake by 26% and 18%, respectively. However, differences among melanoidins sources were found. Compared to BM, CM was more effective in lowering blood glucose peak, insulin, α-melanocyte stimulating hormone, orexin-A, β-endorphin, while blunting the response of three *N*-acylethanolamines.

Therefore, in **Chapter 2** study, **coffee melanoidins were validated as effective functional food ingrendient to reduce individual food intake and postprandial glycaemia** by using a multi-system approach integrating *in vivo* homeostatic and non-homeostatic postprandial responses. These results pave the way to future RCT to study the long-term effect of CM bread in obese subjects at risk of CVD and insulin resistance to control body weight and to improve

metabolic responses. In **Figure 1** the hypothisised mechanisms according to findings of Chapter 2 and other evidence are schematized.

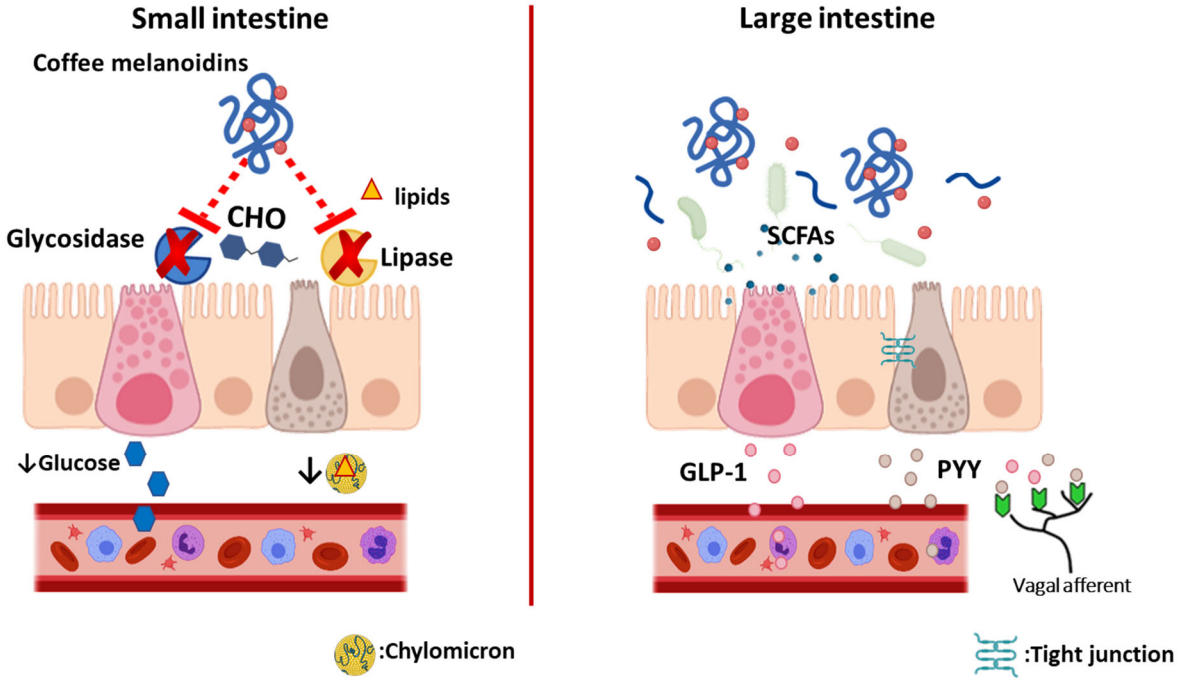


Figure 1. Schematic representation of hypothesized intestinal mechanisms underpinning the effect of coffee melanoidins as functional ingredients for weight management. In small intestine, coffee melanoidins may inhibit carbohydrate and lipids digestive enzymes decreasing postprandial glycaemia (Chapter 2) and the upake of lipids as suggested in the animal study by Vitaglione and co-workers (2019). In the long term, these phenomena could cause improvement of insulin sensitivity and body weight management, also resulting from the effect occurring in the large intestine. There, coffee melanoidins are fermented by the gut microbiota producing short-chain fatty acids (SCFAs) and delivering CM polyphenols. This may determine changes in the gut microbiome (Iriondo-DeHond et al., 2021) leading to improvement of gut permeability, through reinforcement of the tight junctions (Vitaglione et al., 2019) that cause reduced endotoxiemia (Cani, 2016) and ameliorated metabolic responses. SCFAs can stimulate GLP-1 and PYY secretion, boosting insulin response and satiety by entering to the bloodstream and/or activating the central nervous system through recepors located on the neural net in the lamina propria (Delzenne et al., 2005; Salleh et al., 2019).

Briefly, it could be hypothisized that the postprandial inhibition of glycosidases and lipase by coffee melanoidins in the small intestinal lumen (Walker et al., 2020; Vitaglione et al., 2019)

may reduce carbohydrate and lipid digestion blunting blood glucose and insulin peak as well as the intestinal uptake of lipids. These effects may improve insulin sensitivity and reduce liver fat deposition in the long term in obese people consuming a high fat diet. The undigested nutrients sensed in the distal small intestine may induce the "ileal brake" increasing the satiety sensation and leading people to eat less (Willbring et al., 2021). Moreover, coffee melanoidins fermentation in the large intestine may result in increased levels of SCFAs in the intestinal lumen (Iriondo-DeHond et al., 2021) which can improve intestinal barrier integrity as well as, stimulate GLP-1 and PYY production (Delzenne et al., 2005; Salleh et al., 2019) increasing satiety, insulin sensitivity, fat oxidation and energy expenditure (Delzenne et al., 2005; Salleh et al., 2019).

The use of coffee melanoidins as functional ingredients is also interesting in the perspective of food technology as it allows re-utilizing agricultural byproducts, as recently reviewed by Iriondo-DeHond et al., 2021. The use of coffee melanoidins in food formulation requires attention to the sensory aspects of food.

The sensory and reward aspects of the food as well as, the individual beliefs on the effects of a food are crucial in functional food design and development as they affect digestive process already during food consumption and early after meal through the nervous system (Świąder et al., 2021; Singer et al., 2016).

Therefore, clarifying the physiological mechanisms occurring in a specific target population when consuming a food may be helpful in the design of more effective functional food using that food matrix.

For instance, many people suffering from gastrointestinal discomforts (GID) after consuming some food, believe that it is “few digestible” and in order to relief from those discomforts, often reduce or even exclude from the diet that food. This behaviour may generate incorrect dietary pattern and in turn malnutrition or even chronic disease.

This scenario is quite common for milk. Infact many healthy people suffer with milk-related GID and avoid its consumption even in absence of lactose-intolerance leading to a prevalence of self-perception of lactose intolerance that has been estimated between 8 and 20% (Porzi et al., 2021; Nicklas et al., 2011). Moreover, these people often exclude cow’s milk and dairy products from their diet missing the opportunity to get from milk important nutrients such as calcium, vitamin D, some B vitamins and bioactive peptides coming from protein digestion; such a behaviour increases in these people the risk of developing hypertension and diabetes (Nicklas et al., 2011). In this respect, growing interest among the scientific community deals

with the potential implications of food-derived bioactive peptides (BAPs) coming upon dietary protein digestion on weight loss, glycemia management and, gastrointestinal diseases (Caron et al., 2017; Ma et al., 2018). In the past few decades, a wide range of food-derived BAPs have been identified and associated with multiple health benefits (Chakrabarti et al., 2018). However, the commercial application of those BAPs as nutracetical and functional food requires well-designed clinical trials to provide the substantial evidence for potential health claims due the high inter-individual gastro-intestinal digestibility and absorption rate (Chakrabarti et al., 2018, Virgilio et al., 2019). Indeed, some evidence showed that the amount BAPs in the human intestinal lumen following casein and whey protein ingestion was characterized by high standard deviations (above 50%) across all peptide sizes and digestion times, which suggests a large variability in protein digestion among the subjects (Walther et al., 2019), which may trigger postprandial GI symptoms in some subjects (Laatikainen et al., 2020). In this context, the scientific debate on the milk-induced GID in humans is moving toward some BAPs with opioid properties deriving from the proteolysis of β-casein, in particular β-casomorphin-7 (BCM7) (Jianquin et al, 2015; Deth et al., 2015, He et al., 2017). It is believed that the circulating levels of the peptide BCM7, might explain the higher GID following A1 than A2 milk consumption through activation of μ-opioid receptors leading to reduced gastrointestinal transit time (Jianqin et al, 2015; Deth et al., 2016; He et al., 2017). However, studies that clearly demonstrate a causal role of milk containing A1 β-casein and BCM7 implications in GID are missing. Finally, most of the studies focused only on BMC7 neglecting other BAPs formed during digestion of milk (for a review see Nielsen et al., 2017) which may cross the intestinal barrier modulating gastrointestinal motility, digestive processes and inflammatory responses *in vivo*.

In **Chapter 3**, the fate of milk protein-derived BAPs postprandially was investigated in a meal test study with 19 participants with GID and non habitual milk consumers (NHMC) and 20 participants without GID and habitual milk consumers (HMC) who drank 250 mL milk in fasting condition. The participants were lactose tolerant and well characterized for the diet, the gut permeability, psychological symptoms and the gut microbiome.

Data suggested that a slower and lower digestion of milk proteins occurred in people with milk related GID. This could be influenced by the habitual diet that was higher in fiber vs protein and low in animal proteins vs plant proteins. Indeed, diet could shape the gut microbiome towards a lower proteolytic capability that could retard protein digestion from a food like milk, independently from the gut permeability. The study mainly clarified the upper gastro-intestinal digestion of proteins. A more detailed analysis of microbial metabolites of proteins and amino acids in urine would have clarified the implication of the individual gut microbiome in the metabolic fate of milk proteins.

Therefore, **the findings of this study revealed short-term and long-term opportunities in the frame of new food development tailored to people with cow's milk related GID.**

It is likely that consumption of milk-based beverages formulated with predigested milk proteins may be effective to manage GID, as recently shown by Laatikainen and co-workers (2020) in patients with functional gastrointestinal disorders. On the other hand, restablishing gut microbiome ability to digest proteins through milk-based products containing probiotics species known to have proteolytic activity in the gut or using personalized microbiome-targeted intervention may be an effective long-term strategy for this people. Indeed, it is likely that habitual consumption of such a milk product could optimize gut microbiome to manage milk digestion aiding people not to miss the nutritional benefits of consuming milk.

According to the findings in **Chapter 3** and previous evidence, a functional milk product may be designed for people suffering with GID and acting through the mechanisms proposed in the **Figure 2**.

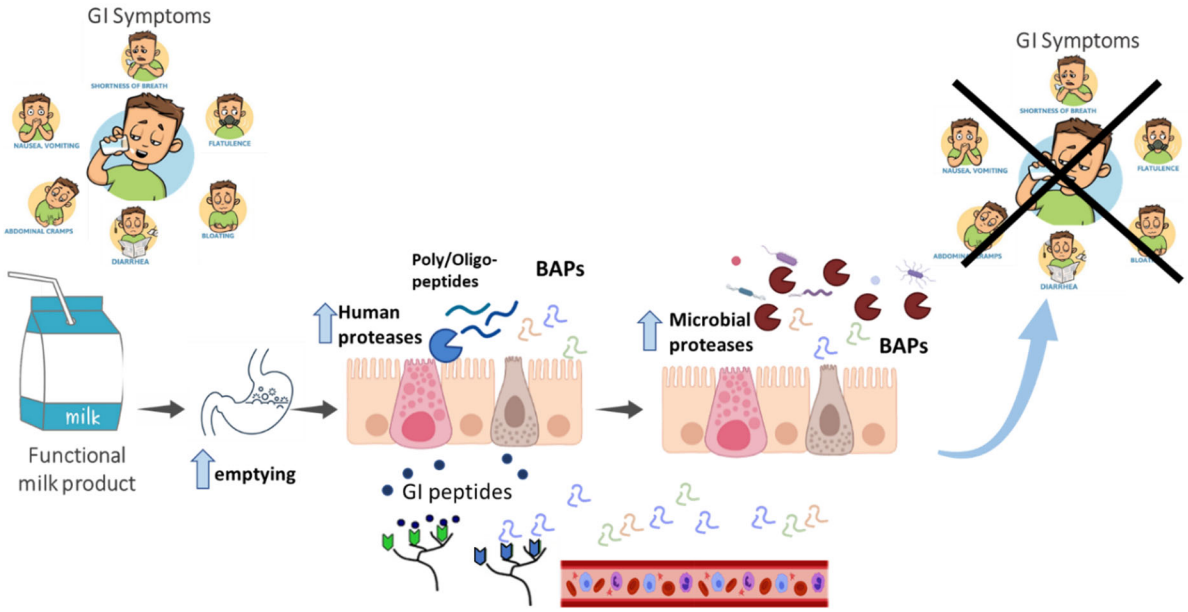


Figure 2. Schematic representation of potential physiological targets of a milk product to resolve GID in sensitive people. Targets may be stomach emptying, protein digestion in the small intestine, and protein digestion in the lage intestine. A product containing pre-digested milk proteins may lead to a faster gastric emptying and peptide sensing in the small intestine that cause faster bioactive peptide (BAPs) absorption and metabolic responses (Dranse et al., 2018; Overduin et al., 2014). These include the effects of gastro-intestinal peptides and BAPs by entering the bloodstream or by activating/blocking μ-opioid receptors on the neural circuitry present on myenteric and submucosal neurons and on immune cells in the lamina propria (Holzer, 2009). The activity of BAPs through μ-opioid receptors result in faster gastric emptying, lower abdominal distension, bloating, discomfort, and gastroesophageal reflux (Holzer, 2009; Kurz and Sessler, 2003). A product determining a gut microbiome change that boost microbial peptidases activity in the large intestine may be also effective in completing protein digestion of food material that escapes human digestion, thus solving GID occurring later after milk consumption.

Dietary nutrients: long-term effects on health through gut-microbiome

Human digestion is not 100% efficient process. Part of nutrients escapes digestion and enter the colon. The percentage of undigested nutrients reaching the colon may depend on food processing (as discussed above), individual gut permeability and gastrointestinal transit (Riccio et al., 2019; Müller et al., 2018; Teodorowicz et al., 2017). The undigested nutrients shape the gut microbiome and can affect host's health through its implication in dietary nutrient digestion (as discussed above). The microbiota can interact with gut and brain via various routes including the immune system, tryptophan metabolism, the vagus nerve and the enteric nervous system, involving microbial metabolites such as SCFAs, branched chain amino acids, and peptidoglycans affecting gut homeostasis, postprandial response to food, behavioral states and cognition (Cryan et al., 2019). Mounting evidence suggests that a crosstalk between gut microbiome and endocannabinoid system exists (Forte et al., 2020). Bidirectional interconnections exist between diet, ECS and gut microbiome affecting long-term individual energy balance, gut inflammation, and neuroinflammation (Forte et al., 2020, Witkamp, 2018). The implication of ECS in metabolic benefits driven by MD through the gut microbiome and the effect of diet on the levels of ECS mediators entering the colon, were unexplored.

In **Chapter 4** the changes of ECS mediators along with gut microbiome in a population at risk of diseases (with overweight and obesity) who underwent a 2-months dietary shift from a Western-type diet to a Mediterranean-type diet were studied.

Results showed that the switch from Western diet to MD shaped microbiota composition and functionality driving a personalized response in ameliorating insulin sensitivity, systemic inflammation and gut health mediated by the ECS. The concomitant assessment of individual gut permeability would have clarified wether MD exerted its anti-inflammatory effect through improving gut permeability.

These findings reveal opportunities for clinical practice in the context of personalized nutrition paving the way for RCT, specifically designed to build and validate models integrating ECS to shape gut microbiome. These models may be helpful to drive a personalized response towards an amelioration in inflammation and insulin sensitivity (**Figure 3**). Indeed, the ECS is an important regulator of intestinal functions and the microbiota-gut-brain axis as it is involved in the physiological modulation of the GI tract (Sharkey et al., 2016; Forte et al., 2020).

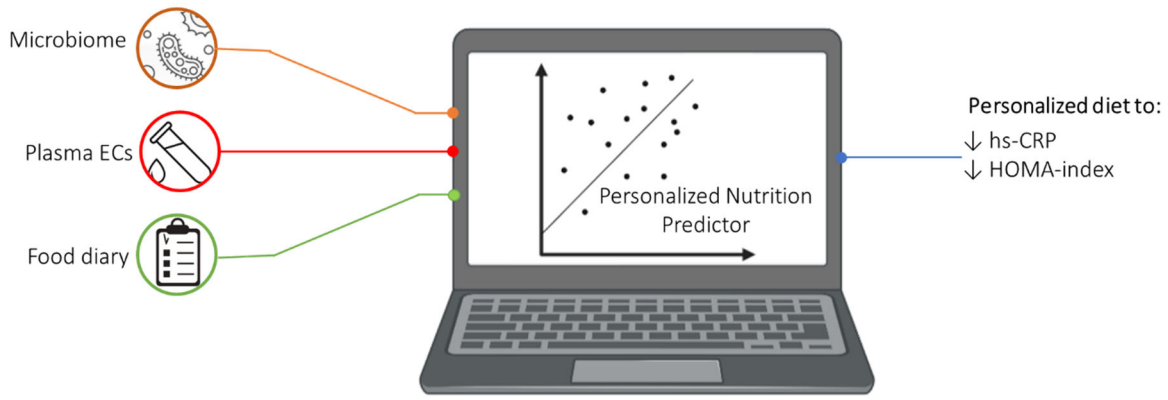


Figure 3. Illustration of important outcomes for future RCT studies finalized to build integrative ECS-models to predict gut microbiome response to diet for lowering systemic inflammation and ameliorating insulin sensitivity. After being devised a machine-learning algorithm that integrates plasma ECs, dietary habits and gut microbiome, the model could be validated in a blinded randomized controlled dietary intervention based on this algorithm to predict hs-CRP and HOMA-index response.

In **Chapter 5** the effect of diet on the levels of ECS mediators entering the colon and eventually interacting with the gut microbiome was exlored. To this purpose, human ileostomy model was used. Participants with ileostomists provided blood and ileal samples and for the first time the levels of ECs, NAEs and NAPEs in those fluids were measured along with habitual diet and inflammatory status.

The ileostomy model offers a method for direct, accurate and quantitative determination of small bowel excretion since rapid changes in response to food can be measured. Therefore, ileostomy-based bioavailability studies provide a unique insight into events taking place in the GI tract (Andersson, 1992; Dobani et al., 2021). In **Chapter 5** study, ileal samples were collected from stoma bags filled during the overnight fasting period, which likely contained some food residues from the last meal consumed. The amount of NAEs in the intestinal lumen was on average 1.5–13 times higher than that required to elicit a physiological response via the intestinal receptors GPR119 and PPAR-α. Therefore, it was likely that 1) the amount of NAEs entering the colon was affected by lipids, NAEs and NAPEs present in food (De Luca et al., 2018) and, 2) the post-prandial levels may be even higher than those found in fasting subjects. Specifically designed meal study with ileostomy subjects consuming a meal high or low in NAEs and NAPEs, providing blood and ileal fluids as well as appetite ratings postprandially,

may be useful to clarify the effect of dietary ECs, NAEs and NAPEs in modulating appetite and energy intakes through the receptors lining on GI mucosa. Such a study could reveal opportunities for new products able to elicit satiety through the activity of NAEs and NAPEs from the intestinal lumen. A schematic representation of a hypothisised mechanism underpinning the effect of dietary NAEs and NAPEs on satiety is reported in **Figure 4**. Specifically, the working hypothesis is that dietary NAEs and NAPEs are delivered during digestion in the upper gastro-intestinal tract and modulate appetite and energy metabolism by binding the receptors located on the intestinal mucosa.

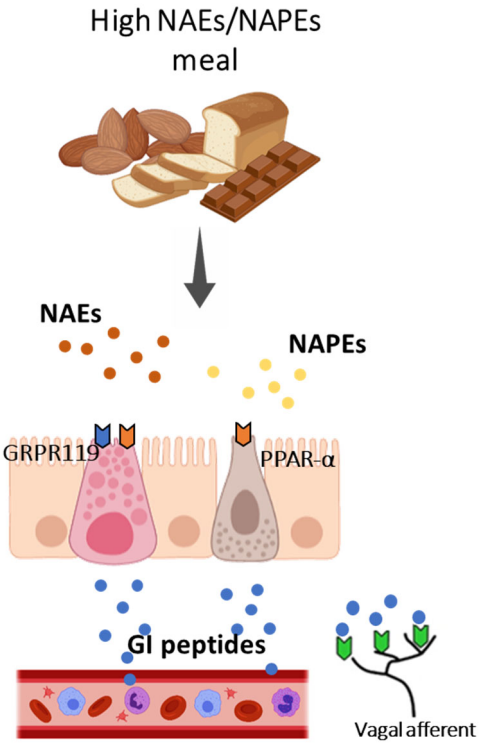


Figure 4. Schematic representation of hypothisised mechanism underpinning satiety effect of a high NAEs and NAPEs diet. High NAEs/NAPEs meal designed by using the food database previously published (De Luca et al., 2019) may deliver NAEs and NAPEs in the small intestinal lumen. They could trigger GLP-1, CCK and PYY secretion by enterocytes through activation of intestinal GPR119 and PPAR-α and stimulate satiety (Witkamp et al., 2018).

CONCLUSIONS

The work described and discussed in the present thesis unreveal the following relevant conclusions in the frame of food innovation and/or human physiology-health/wellbeing-diet relationships.

- ✓ Coffee melanoidins included in bread, increases satiety and lower post-prandial glycaemia in healthy people, therefore, CM can be a functional food ingredient for appetite control. The effects are mainly mediated by the postprandial responses of NAEs, and several neuropeptides. Findings of this study and previous evidence in animals suggest the use of CM as food ingredient for the management of body weight and insulin resistance. Digestive enzyme inhibition in the small intestine and fermentation in the large intestine may be the key physiological mechanisms.
- ✓ Cow's milk proteins are digested less and slower in people with milk related GID. Therefore, milk proteins may not cause GID anymore in sensitive people if they are included in food in a pre-digested form. Pharmacological therapies with proteolytic enzymes or personalized dietetic strategies that boost human and gut microbial proteolytic enzymes may be effective to control milk related GID. A habitual diet high in dietary fibre/protein ratio may shape gut microbiome towards a low proteolytic capacity that can affect digestibility of protein-rich meals and trigger discomforts in sensitive people.
- ✓ A switch from a Western-like diet (low in fruits, vegetables, legumes and high in animal products) to an isocaloric MD (high in fruits, vegetables, legumes and low in animal products) can affect the ECS and shape the gut microbiome by increasing *A. muciniphila* abundance in the gut independently of body weight changes. Changes in ECS are associated with personalized response to the MD ameliorating insulin sensitivity, systemic inflammation, and gut health. These findings reveal opportunities in the context of personalized nutrition laying the foundation for RCT specifically designed to build and validate integrative ECS-model to shape gut microbiome. Such models may be helpful to drive an individual response towards an amelioration of inflammation and insulin sensitivity.
- ✓ Dietary fat affects ECS mediators in circulation and intestinal fluids entering the colon and interacting with the gut microbiome. Moreover, the levels of NAEs in the intestinal lumen may be physiologically relevant to modulate appetite through the GPR119 receptors lining on the GI mucosa. These findings are still preliminary for application in food innovation. Specifically designed study should address whether dietary intake of EC mediators (NAEs and NAPEs) affect the concentrations of NAEs in the intestinal lumen postprandially modulating the homeostatic control of food intake.

Altogether results from this thesis using a multi-system approach integrating postprandial and long-term biological responses to meals or diets and including markers from gut-brain axis and

gut microbiome, in different types of people (healthy, with discomforts, at risk of disease, with ileostomy), revealed evidence and opportunities for designing functional food tailored to specific consumers categories and personalized dietary strategy.

References

1. ALjahdali, N., & Carbonero, F. (2019). Impact of Maillard reaction products on nutrition and health: Current knowledge and need to understand their fate in the human digestive system. *Critical reviews in food science and nutrition*, *59*(3), 474-487.

2. Andersson, H. (1992). The ileostomy model for the study of carbohydrate digestion and carbohydrate effects on sterol excretion in man. *European journal of clinical nutrition*, *46*, S69-76.

3. Awika, J. M., Rose, D. J., & Simsek, S. (2018). Complementary effects of cereal and pulse polyphenols and dietary fiber on chronic inflammation and gut health. *Food & function*, *9*(3), 1389-1409.

4. Cani, P. D. (2016). Interactions between gut microbes and host cells control gut barrier and metabolism. *International journal of obesity supplements*, *6*(S1), S28-S31.

5. Caron, J., Domenger, D., Dhulster, P., Ravallec, R., & Cudennec, B. (2017). Protein digestion-derived peptides and the peripheral regulation of food intake. *Frontiers in endocrinology*, *8*, 85.

6. Chakrabarti, S., Guha, S., & Majumder, K. (2018). Food-derived bioactive peptides in human health: Challenges and opportunities. *Nutrients*, *10*(11), 1738.

7. Cryan, J. F., O'Riordan, K. J., Cowan, C. S., Sandhu, K. V., Bastiaanssen, T. F., Boehme, M., ... & Dinan, T. G. (2019). The microbiota-gut-brain axis. *Physiological reviews*.

8. Cuevas-Sierra, A., Ramos-Lopez, O., Riezu-Boj, J. I., Milagro, F. I., & Martinez, J. A. (2019). Diet, gut microbiota, and obesity: links with host genetics and epigenetics and potential applications. Advances in Nutrition, 10(suppl_1), S17-S30.

9. De Filippis F, Vitaglione P, Cuomo R, Berni Canani R, Ercolini D (2018) Dietary interventions to modulate the gut microbiome— how far away are we from precision medicine. Infamm Bowel Dis 24(10):2142–2154.

10. De Luca L, Ferracane R, Vitaglione P (2019) Food database of N-acyl-phosphatidylethanolamines, N-acylethanolamines and endocannabinoids and daily intake from a Western, a Mediterranean and a vegetarian diet. *Food Chemistry,* 300:125218.

11. Delzenne, N. M., Cani, P. D., Daubioul, C., & Neyrinck, A. M. (2005). Impact of inulin and oligofructose on gastrointestinal peptides. British Journal of Nutrition, 93(S1), S157-S161.

12. Deth, R., Clarke, A., Ni, J., & Trivedi, M. (2015). Clinical evaluation of glutathione concentrations after consumption of milk containing different subtypes of β-casein: results from a randomized, cross-over clinical trial. *Nutrition journal*, *15*(1), 1-6.

13. Dobani, S., Latimer, C., McDougall, G. J., Allwood, J. W., Pereira-Caro, G., Moreno-

Rojas, J. M., ... & Gill, C. I. (2021). Ex vivo fecal fermentation of human ileal fluid collected after raspberry consumption modifies (poly) phenolics and modulates genoprotective effects in colonic epithelial cells. *Redox biology*, *40*, 101862.

14. Dranse, H. J., Waise, T. Z., Hamr, S. C., Bauer, P. V., Abraham, M. A., Rasmussen, B. A., & Lam, T. K. (2018). Physiological and therapeutic regulation of glucose homeostasis by upper small intestinal PepT1-mediated protein sensing. *Nature communications*, *9*(1), 1-12.

15. Fogliano, V., & Morales, F. J. (2011). Estimation of dietary intake of melanoidins from coffee and bread. *Food & function*, *2*(2), 117-123.

16. Forte, N., Fernández-Rilo, A. C., Palomba, L., Di Marzo, V., & Cristino, L. (2020). Obesity affects the microbiota–gut–brain axis and the regulation thereof by endocannabinoids and related mediators. *International journal of molecular sciences*, *21*(5), 1554.

17. Hall, K. D., Ayuketah, A., Brychta, R., Cai, H., Cassimatis, T., Chen, K. Y., ... & Zhou, M. (2019). Ultra-processed diets cause excess calorie intake and weight gain: an inpatient randomized controlled trial of ad libitum food intake. *Cell metabolism*, *30*(1), 67-77.

18. He, M., Sun, J., Jiang, Z. Q., & Yang, Y. X. (2017). Effects of cow's milk beta-casein variants on symptoms of milk intolerance in Chinese adults: a multicentre, randomised controlled study. *Nutrition journal*, *16*(1), 1-12.

19. Heinzmann, S. S., Merrifield, C. A., Rezzi, S., Kochhar, S., Lindon, J. C., Holmes, E., & Nicholson, J. K. (2012). Stability and robustness of human metabolic phenotypes in response to sequential food challenges. Journal of proteome research, 11(2), 643-655.

20. Helou, C., Anton, P. M., Niquet-Leridon, C., Spatz, M., Tessier, F. J., & Gadonna-Widehem, P. (2017). Fecal excretion of Maillard reaction products and the gut microbiota composition of rats fed with bread crust or bread crumb. *Food & function*, *8*(8), 2722-2730.

21. Holzer, P. (2009). Opioid receptors in the gastrointestinal tract. Regulatory peptides, 155(1-3), 11-17.

22. Iriondo-DeHond, A., Rodríguez Casas, A., & Del Castillo, M. D. (2021). Interest of Coffee Melanoidins as Sustainable Healthier Food Ingredients. *Frontiers in Nutrition*, 733.

23. Jayedi A, Soltani S, Abdolshahi A, Shab-Bidar S (2020) Healthy and unhealthy dietary patterns and the risk of chronic disease: an umbrella review of meta-analyses of prospective cohort studies. Br J Nutr, 124(11): 1133-1144.

24. Jianqin, S., Leiming, X., Lu, X., Yelland, G. W., Ni, J., & Clarke, A. J. (2015). Effects of milk containing only A2 beta casein versus milk containing both A1 and A2 beta casein proteins on gastrointestinal physiology, symptoms of discomfort, and cognitive behavior of people with self-reported intolerance to traditional cows' milk. *Nutrition journal*, *15*(1), 1-16.

25. Keenan, M. J., Zhou, J., McCutcheon, K. L., Raggio, A. M., Bateman, H. G., Todd, E., ... & Hegsted, M. (2006). Effects of resistant starch, a non-digestible fermentable fiber, on reducing body fat. Obesity, 14(9), 1523-1534.

26. Kolodziejczyk, A. A., Zheng, D., Elinav, E. (2019). Diet–microbiota interactions and personalized nutrition. Nat Rev Microbiol, 1–12

27. Kurz, A., & Sessler, D. I. (2003). Opioid-induced bowel dysfunction. Drugs, 63(7), 649-671.

28. Laatikainen, R., Salmenkari, H., Sibakov, T., Vapaatalo, H., & Turpeinen, A. (2020). Randomised Controlled Trial: Partial Hydrolysation of Casein Protein in Milk Decreases Gastrointestinal Symptoms in Subjects with Functional Gastrointestinal Disorders. *Nutrients*, *12*(7), 2140.

29. Leshem, A., Segal, E., & Elinav, E. (2020). The gut microbiome and individual-specific responses to diet. *Msystems*, *5*(5), e00665-20.

30. Lordan, C., Thapa, D., Ross, R. P., & Cotter, P. D. (2020). Potential for enriching next-generation health-promoting gut bacteria through prebiotics and other dietary components. *Gut Microbes*, *11*(1), 1-20.

31. Ma, Y., Yan, W., Ding, S., Fei, Y., Liu, G., & Fang, J. (2018). Effects of bioactive peptide on inflammatory bowel disease, focus on signal transduction and intestinal microbiota. *Current pharmaceutical design*, *24*(24), 2782-2788.

32. Müller, M., Canfora, E. E., & Blaak, E. E. (2018). Gastrointestinal transit time, glucose homeostasis and metabolic health: modulation by dietary fibers. Nutrients, 10(3), 275.

33. Nagy, R., Máthé, E., Csapó, J., & Sipos, P. (2021). Modifying Effects of Physical Processes on Starch and Dietary Fiber Content of Foodstuffs. *Processes*, *9*(1), 17.

34. Nicklas, T. A., Qu, H., Hughes, S. O., He, M., Wagner, S. E., Foushee, H. R., & Shewchuk, R. M. (2011). Self-perceived lactose intolerance results in lower intakes of calcium and dairy foods and is associated with hypertension and diabetes in adults. The American journal of clinical nutrition, 94(1), 191-198.

35. Nielsen, S. D., Beverly, R. L., Qu, Y., & Dallas, D. C. (2017). Milk bioactive peptide database: A comprehensive database of milk protein-derived bioactive peptides and novel visualization. *Food Chemistry*, *232*, 673-682.

36. Nunes, F. M., & Coimbra, M. A. (2007). Melanoidins from coffee infusions. Fractionation, chemical characterization, and effect of the degree of roast. *Journal of Agricultural and Food Chemistry*, *55*(10), 3967-3977.

37. Overduin, J., Tylee, T. S., Frayo, R. S., & Cummings, D. E. (2014). Hyperosmolarity in

the small intestine contributes to postprandial ghrelin suppression. American Journal of Physiology-Gastrointestinal and Liver Physiology, 306(12), G1108-G1116.

38. Park, Y. W., & Nam, M. S. (2015). Bioactive peptides in milk and dairy products: a review. *Korean journal for food science of animal resources*, *35*(6), 831.

39. Porzi, M., Burton-Pimentel, K. J., Walther, B., & Vergères, G. (2021). Development of Personalized Nutrition: Applications in Lactose Intolerance Diagnosis and Management. Nutrients, 13(5), 1503.

40. Riccio, P., & Rossano, R. (2019). Undigested food and gut microbiota may cooperate in the pathogenesis of neuroinflammatory diseases: a matter of barriers and a proposal on the origin of organ specificity. Nutrients, 11(11), 2714.

41. Salleh, S. N., Fairus, A. A. H., Zahary, M. N., Bhaskar Raj, N., & Mhd Jalil, A. M. (2019). Unravelling the effects of soluble dietary fibre supplementation on energy intake and perceived satiety in healthy adults: evidence from systematic review and meta-analysis of randomised-controlled trials. *Foods*, *8*(1), 15.

42. Sanders, M. E., Merenstein, D. J., Reid, G., Gibson, G. R., & Rastall, R. A. (2019). Probiotics and prebiotics in intestinal health and disease: from biology to the clinic. *Nature reviews Gastroenterology & hepatology*, *16*(10), 605-616.

43. Sharkey, K. A., & Wiley, J. W. (2016). The role of the endocannabinoid system in the brain–gut axis. *Gastroenterology*, *151*(2), 252-266.

44. Singer, B. F., Bryan, M. A., Popov, P., Scarff, R., Carter, C., Wright, E., ... & Robinson, T. E. (2016). The sensory features of a food cue influence its ability to act as an incentive stimulus and evoke dopamine release in the nucleus accumbens core. *Learning & Memory*, *23*(11), 595-606.

45. Świąder, K., & Marczewska, M. (2021). Trends of Using Sensory Evaluation in New Product Development in the Food Industry in Countries That Belong to the EIT Regional Innovation Scheme. *Foods*, *10*(2), 446.

46. Teodorowicz, M., Van Neerven, J., & Savelkoul, H. (2017). Food processing: The influence of the maillard reaction on immunogenicity and allergenicity of food proteins. Nutrients, 9(8), 835.

47. Troise, A. D., Buonanno, M., Fiore, A., Monti, S. M., & Fogliano, V. (2016). Evolution of protein bound Maillard reaction end-products and free Amadori compounds in low lactose milk in presence of fructosamine oxidase I. *Food chemistry*, *212*, 722-729.

48. Virgilio, N., De Donno, R., Bandini, E., Napolitano, A., Fogliano, V., & Vitaglione, P. (2019). Milk protein enriched beverage reduces post-exercise energy intakes in women with

higher levels of cognitive dietary restraint. *Food Research International*, *118*, 58-64.

49. Vitaglione, P., Fogliano, V., & Pellegrini, N. (2012). Coffee, colon function and colorectal cancer. *Food & function*, *3*(9), 916-922.

50. Vitaglione, P., Mazzone, G., Lembo, V., D'Argenio, G., Rossi, A., Guido, M., ... & Morisco, F. (2019). Coffee prevents fatty liver disease induced by a high-fat diet by modulating pathways of the gut–liver axis. *Journal of nutritional science*, *8*.

51. Walther, B., Lett, A. M., Bordoni, A., Tomás-Cobos, L., Nieto, J. A., Dupont, D., ... & Vergères, G. (2019). GutSelf: Interindividual variability in the processing of dietary compounds by the human gastrointestinal tract. *Molecular nutrition & food research*, *63*(21), 1900677.

52. Wilbrink, J., Masclee, G., Klaassen, T., van Avesaat, M., Keszthelyi, D., & Masclee, A. (2021). Review on the Regional Effects of Gastrointestinal Luminal Stimulation on Appetite and Energy Intake:(Pre) clinical Observations. *Nutrients*, *13*(5), 1601.

53. Witkamp, R. (2018). The role of fatty acids and their endocannabinoid-like derivatives in the molecular regulation of appetite. Mol Aspects Med 64:45-67.

54. Zeevi, D., Korem, T., Zmora, N., Israeli, D., Rothschild, D., Weinberger, A., Ben-Yacov, O., Lador, D., Avnit-Sagi, T., Suez, J., et al. (2015). Personalized nutrition by prediction of glycemic responses. Cell, 163(5), 1079-1094.

Curriculum Vitae

Name/Surname	Silvia Tagliamonte
Address	Via Rotabile– Formia (LT)
Nationality	Italian
Phone	+39 3284496520
e-mail	silvia.tagliamonte@unina.it
Date/Place of birth	2nd November 1994, Gaeta (LT)

Education

- November 2018. Beginning of **PhD in Food Science (XXXIV):** ***"Food digestibility, a physiological approach for new product development"***.

Department of Agricultural Sciences, University of Naples "Federico II", Italy.

Under the supervision of **Prof. Paola Vitaglione** (paola.vitaglione@unina.it).

- September 2017 – December 2017. **Erasmus Programme**

Abertay University*, Food, Nutrition and Health*,

Dundee, Scotland.

- October 2016 – October 2018. **Master's Degree in Food Science and Technology**

Department of Agricultural Sciences, University of Naples "Federico II", Italy.

Thesis Title: ***"The effect of Mediterranean diet on plasma N-acylethanolamines and Endocannabinoids"***

Under the supervision of **Prof. Paola Vitaglione** (paola.vitaglione@unina.it).

- October 2013 – July 2016. **Bachelor's degree in Food Technology.**

Department of Agricultural Sciences, University of Naples "Federico II", Italy.

Thesis Title: *"**Physiology of gastrointestinal enteroendocrine cells"***

Under the supervision of **Prof. Paola Vitaglione** (paola.vitaglione@unina.it).

- September 2008 – July 2013. **High school degree.**

Scientific high school.

Liceo scientifico *Ascanio Landi,* Velletri (Roma).

Foreign languages & skills

- English Level: C1;
- Spanish Level: B1;
- Application of R software for biostatistical analysis, plotting and clustering (Very good);
- Multi-system data analysis and clinical data integration (Very good);
- HPLC, LC-MS/MS and LC-HMRS analysis (Good);
- Microsoft Office for data manipulation and presentation (Word, Excel, Acrobat and Power Point) (Excellent).

Participation to international research projects

- European project under the Joint Action “Healthy diet for a heathy life" (HDHL), JPI-Intestinal Microbiomics. Project titled “Diet-INduced Arrangement of the gut Microbiome for Improvement of Cardiometabolic health” - Acronym “DINAMIC”. Project Coordinators: Prof. Thomas Clavel and Dirk Haller;

- Interational Research Project titled “Identification of Physiological Biomarkers of Gastro-intestinal Discomforts Induced by Milk Consumption- Acronym “MIDI”. Project Coordinator: Prof. Paola Vitaglione

Publications in peer-reviewed international journals

- **Tagliamonte, S.**, Gill, C. I., Pourshahidi, L. K., Slevin, M., Price, R. K., Ferracane, R., Vitaglione, P. (2020). Endocannabinoids, endocannabinoid-like molecules and their precursors in human small intestinal lumen and plasma: does diet affect them?. European Journal of Nutrition,1-13. https://doi.org/10.1007 /s00394-020-02398-8.
- Walker, J. M., Mennella, I., Ferracane, R., **Tagliamonte, S.**, Holik, A. K., Holz, K., Vitaglione, P. (2020). Melanoidins from coffee and bread differently influence energy intake: A randomized controlled trial of faod intake and gut-brain axis response. Journal of Functional Foods, 72, 104063. https://doi .org/10.1016/j. jff.2020.104063.
- **Tagliamonte, S.**, Laiola, M., Ferracane, R., Vitale, M., Gallo, M. A., Meslier, V., ... & Vitaglione, P. (2021). Mediterranean diet consumption affects the endocannabinoid system in overweight and obese subjects: possible links with gut microbiome, insulin resistance

and inflammation. *European Journal of Nutrition*, 1-14. https://doi.org/10.1016/j.numecd.2021.08.022.

- **Tagliamonte, S.**, ….., Vitaglione P. Digestion of milk proteins affects the gastro-intestinal discomforts after cow's milk consumption in healthy subjects independently from gut permeability: implications of gut microbiome. Prepared for a submission to *Gut*, 2022.

Poster and oral presentations in international conferences proceedings

- **Tagliamonte, S.** "Food digestibility: a physiological approach for new product development". Proceedings of the XXIV Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology, Florence, 2019**.**
- **Tagliamonte, S.**, Gill, C. I., Pourshahidi, L. K., Slevin, M., Lawther, R., O'Connor, G., ... & Vitaglione, P. "Endocannabinoids and endocannabinoid-like molecules are present in foods, blood and ileal fluids from ileostomy subjects: insight into possible metabolic implications". 13th European Nutrition Conference (FENS), Dublin, 2019.
- **Tagliamonte, S.** "Food digestibility: a physiological approach for new product development". First Virtual Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, 2021.
- **Tagliamonte, S.**, Barone Lumaga, R., Vitaglione, P. "Gut permeability and quality of life in healthy subjects reporting milk consumption related gastro-intestinal discomforts. XLI National Congress (SINU), 2021.
- **Tagliamonte, S.**, Barone Lumaga, R., Vitaglione, P. "Profiling the intestinal permeability and wellbeing status of healthy subjects reporting gastro-intestinal discomforts upon milk consumption". Virtual International Conference on Food Digestion (INFOGEST), 2021.