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Functional effects of dietary proteins and bioactive peptides on satiety and metabolic response in humans

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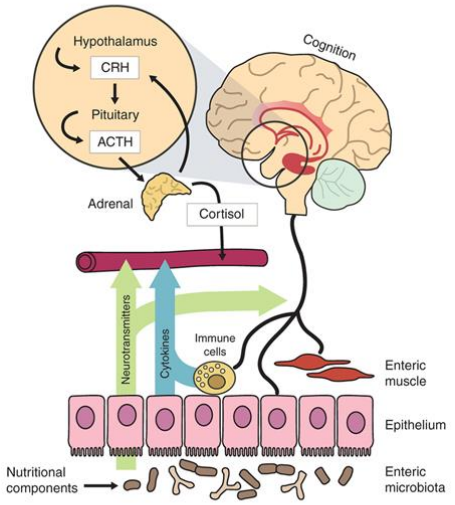
Chapter 1

Introduction

Gut-brain axis is a crucial hub of the of food intake and energy balance regulation



The neural circuits controlling food intake and emotions are finely interconnected

1.1 Oro-intestinal nutrient-sensing

The gastrointestinal tract (GIT) is the largest endocrine organ in the human body and it represents the proxy for communication between the human body and the external environment. The cells of the gut epithelium possess a subtle chemosensory system that collect the information about nutrient presence and composition in the lumen and activate other systems involved in the regulation of appetite, immune response, and gastrointestinal motility. Distress or adaptations in the communication of this sensory information may contribute to the development or maintenance of disease (Steensels & Depoortere 2018).

New findings support the existence of a functional *continuum* along the oro-intestinal tract that permanently senses ingested nutrients and non-nutrients to control ingestion, digestion, absorption, and the metabolic fate of energy nutrients. Taste buds and cells in the gut epithelium share common sensors, express similar hormones and receptors, and are connected to gustatory and vagal afferent nerve fibers involved in feeding behaviour (Roper & Chaudhari, 2017*)*. Taste perception can be considered as a novel key player participating in the regulation of gut function, likewise specific diets or agonists that target these chemosensory signalling pathways may be considered as new therapeutic targets to tune adequate physiological processes in the gut, in health and/or disease condition.

Particularly, taste buds represent the peripheral organs of taste, located mainly in the tongue epithelium. They sample the chemical makeup of foods and beverages for nutrient content, palatability and potential toxicity. The substantial diversity and redundancy of the molecular receptors for these compounds may reflect the importance of identifying nutrients and avoiding chemical threats from the environment. The molecular recognition of tastants, which occurs at the apical tips of taste bud cells, ultimately results in sensory perceptions (sweet, bitter, fat, umami, salty and sour) that guide appetite and trigger physiological processes for absorbing nutrients and adjusting metabolism.

The receptors on the chemo-sensitive apical side of taste bud cells confer specificity to gustatory stimuli. Taste receptors come in many types, including several classes of G protein-coupled receptors (GPCRs) and ion channels (Roper & Chaudhari, 2017).

After ingestion of the meal, the gut, will “taste” the macronutrient composition of the meal in order to elicit motor and secretory responses to assimilate nutrients. The chemosensory systems involved are similar to those present in oral cavity and are present on several cell types in the gut epithelium, such as enterocytes, enteroendocrine cells (EECs), tuft cells, Paneth cells, goblet cells, microfold cells, and cup cells. The epithelium plays a prominent role in the communication between the lumen, sub-epithelium, afferent nerve fibers, and the brain to trigger adaptive responses that affect

gastrointestinal function, food intake, glucose metabolism, and immune function (Stenseels & Depoortere, 2018).

Enterocytes are absorptive cells and are the major cell type lining the gut. On the apical side, they contain microvilli to enlarge the luminal contact surface and express several transporters that regulate the uptake of nutrient metabolites such as sugars, amino acids, and fatty acids (Figure 1a).

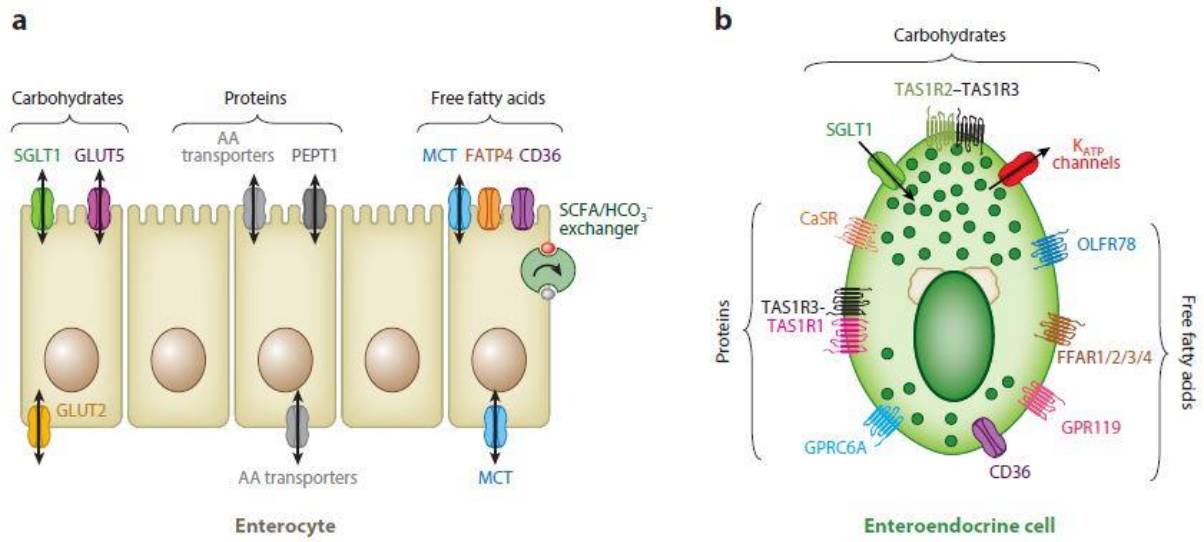


Figure 1 chemosensors in (a) enterocytes, (b) enteroendocrine cells of the gastrointestinal epithelium. Nutrients (carbohydrates, proteins, and fatty acids) are sensed by different receptors and/or transporters. Abbreviations: AA, amino acid; CaSR, calcium-sensing receptor; CD36, cluster of differentiation 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)

EECs represent the largest endocrine organ in the body. They are scattered throughout the GIT but only comprise <1% of the gut epithelium. At least 12 subtypes of EECs secrete a wide range of peptides (>20) to affect a number of physiological processes involved in the regulation of food intake and gastrointestinal motility (Roder et al., 2014) (Figure 1b).

EECs and absorptive epithelial cells are characterized by chemosensors that allow the detection of nutrient in the intestinal lumen (i.e. **nutrient-sensing**). ECCs act as chemosensory transducers that reply to dietary nutrients and other compounds by triggering the release of regulatory peptides to initiate humoral and vagal signalling cascades that convey information to the brain concerning the luminal milieu. As a consequence, a wide array of physiological responses, ranging from stimulation of gastric, intestinal, and pancreatic secretions to inhibition or stimulation of appetite and food intake, are triggered. Thus, intestinal chemosensing can regulate nutrient uptake and gut peptide secretion in order to control energy request and whole body metabolism (Stenseel & Depoortere, 2018).

Recent evidence suggests that nutrients can also directly interact with the nervous system via a neuroepithelial circuit. Moreover, peptide-secreting vesicles in EECs are contained within an axon-like basal process, called **neuropod**, that appears to guide the secretion of hormones to neurons innervating the small intestine and colon (Bohorquez et al., 2015), mainly serves to monitor the metabolic state and to relay hunger and satiety signals (Page et al., 2012). By synapsing with the vagus nerve, neuropod cells connect the gut lumen to the brainstem and they can transduce sensory stimuli in milliseconds by using glutamate as a neurotransmitter, providing a neuroepithelial circuit for fast sensory transduction (Kaelberer et al., 2018). It is hypothesized that the gut-brain neural circuit formed by neuropod cells and vagal nodose neurons could lead to: rapid computation of stimuli to distinguish their physical (e.g., volume) *versus* chemical (e.g., calorie) properties; precise sensory representation of specific gastrointestinal regions; localized plasticity encoded within the neural circuit; and timely vagal efferent feedback to modulate gastrointestinal sensory function (Kaelberer et al., 2018). The intestinal chemosensing can regulate the nutrient uptake and gut peptide secretion in order to control energy request and whole body metabolism (Janssen & Depoortere, 2013). GPCRs, expressed both in the oral cavity and in endocrine cells within the gut mucosa, coordinate the release of **hormones-like peptides** such as: ghrelin, cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), and peptide tyrosine-tyrosine (PYY $_{(3–36)}$) that regulate food intake and glucose homeostasis as well as nutrient-sensing in the gut (Steinert et al., 2017).

Ghrelin secretion is stimulated mainly by neural control levels and is correlated with hunger sensations and meal size. Feedback from small-intestinal nutrient sensing inhibits ghrelin secretion during and after meals (Steinert et al., 2017). Ghrelin exerts physiological effects on brain, stomach and pancreatic β-cells stimulating eating, gastric emptying, inhibiting insulin secretion, respectively (Janseen & Depoortere 2013).

CCK is the best-established GI endocrine satiation signal in humans. CCK may contribute to the control of meal-related glycaemia both indirectly, via its effect on gastric emptying delay, and directly via control of hepatic glucose production (Steinert et al., 2017).

GLP-1 contributes to meal-related glycaemic control by stimulating insulin secretion, inhibiting glucagon secretion, slowing gastric emptying, and reducing hepatic glucose metabolism. GLP-1 may also contribute to glycaemic control in the fasting state. GLP-1, together with glucose-dependent insulinotropic polypeptide (GIP), mediates the incretin effect by exerting dose-related, glucose-dependent insulinotropic effects on β-cells (Steinert et al., 2017).

PYY$_{(3–36)}$ is secreted in response to carbohydrates, lipids, and proteins digestion during and after meals. PYY may contribute to gastric emptying via the ileal brake mechanism, to the inhibition of eating, and to the control of meal-related glycaemia (Steinert et al., 2017).

1.2 Cross-talk between brain and gut: homeostatic/hedonic feeding and GI motility

Metabolic homeostasis is orchestrated in response to nutrient and vagal-dependent gut-initiated functions. Specifically, the sensory and motor fibres of the vagus nerve transmit intestinal signals to the CNS and exert biological and physiological responses (Waise et al., 2018).

Feeding control is a tightly regulated process at the brain level, requiring accurate information regarding the amount and nutrient content of food ingested into the GIT. Gut nutrient-sensing, hormone-derived satiety or hunger signals communicate with the CNS via the vagal afferent system, which expresses multiple receptors for orexigenic and anorexigenic peptides (Janseen & Depoortere 2013). Hormones also modulate mechanosensitive neurons that could potentially affect feeding (Kentish &Page 2014). For example, both CCK and leptin increase the firing of vagal afferent fibres (which affect feeding) that are also responsive to mechanical distension in rodents (Kentish et al., 2014). Moreover, 5-HT is secreted from gastric enterochromaffin cells in response to gastric distension to provide intake inhibitory signals by activating vagal mechanosensitive neurons, whereas ghrelin inhibits gastric tension receptors to lower the mechanosensitivity of the vagal afferent neurons (Page et al., 2007). Vagal mechanosensitive afferent activity is also modulated directly by volumetric gastrointestinal distention, which plays a pivotal role in controlling food intake behaviour triggering satiety or fullness sensations (Figure 2) (Waise et al., 2018).

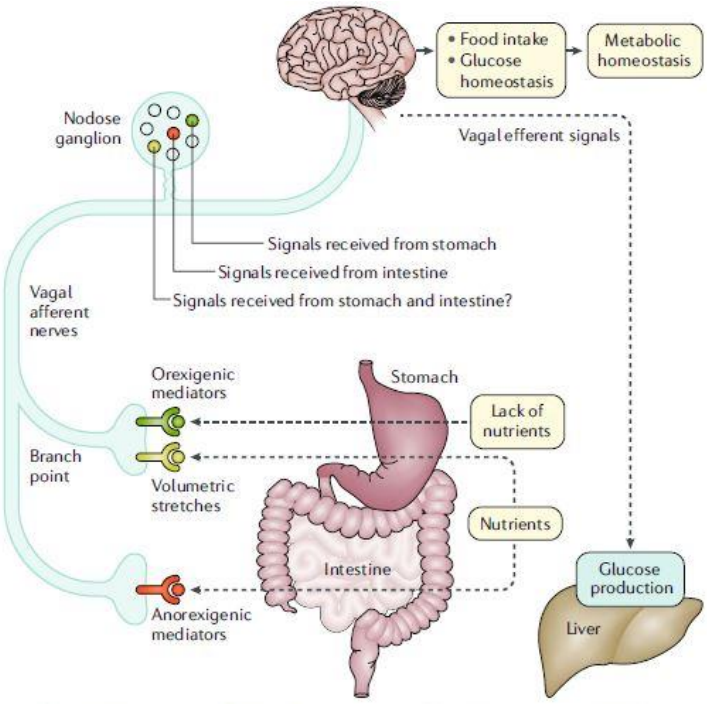


Figure 2. Before a meal, gastrointestinal- derived orexigenic mediators (ghrelin) initiate a hunger drive through the vagal afferent system. In response to a meal, nutrient- sensing and volumetric- stretch-sensing mechanisms in the stomach and the intestine trigger various signalling pathways within the vagal afferent nerves to regulate appetite and glucose homeostasis via the central nervous system. Branch points, where the parent vagal nerves intersect with the downstream neuronal branches, might facilitate the interaction of orexigenic and anorexigenic signals, thereby affecting feeding and glucose homeostatic control (Waise et al., 2018).

Extrinsic sensory pathways (vagal, thoracolumbar, lumbosacral, and viscerofugal) terminate in the gut and convey mechano- and chemosensory signals to target tissues within and outside the intestine to impact physiology and behavior (Brookes et al., 2013).

In addition, the gut is the only organ that contains its own intrinsic nervous system, the enteric nervous system (ENS). The ENS is often referred to as the second brain because it provides local control of the gastrointestinal tract and continues to function even when the primary neural connection with the vagus nerve is severed. Furthermore, the ENS contains sensory neurons, which are intrinsic primary afferent neurons that respond to mechanical and chemical stimuli and regulate the appropriate output to muscle and secretory motor neurons (Lasrado et al., 2017).

Many functions of the digestive system, and functions related to digestion, like satiety, involve both enteric innervation and the endocrine system. The ENS is one component of the neural control system of the digestive tract, working in concert with the CNS, interacting with both the gut endocrine and immune systems as well as having roles in modifying nutrient absorption and maintaining the mucosal barrier. In the small and large intestine, the ENS contains full reflex pathways that are essential to direct the movements of these parts of the digestive tract and to control fluid movement between the gut lumen and body compartments (Steensel & Depoortere, 2018).

Central regulation of food intake is a key mechanism contributing to energy homeostasis. Many neural circuits that orchestrate feeding behaviour overlap with the brain's reward circuitry both anatomically and functionally. Numerous neural pathways can simultaneously influence food intake and reward, controlling homeostatic and hedonic feeding, whereas homeostatic feeding is necessary for basic metabolic processes and survival, while hedonic feeding is driven by sensory perception or pleasure (Rossi & Stuber, 2017). Despite much progress toward understanding how certain parts of the brain contribute to either feeding or reward, questions of motivated behaviour continue to be framed in terms of homeostatic feeding (food intake that is necessary to maintain typical body weight and metabolic function) or hedonic feeding (food intake driven by sensory perception or pleasure). Together, the systems involved in hedonic and homeostatic aspects of feeding provide a means by which the nervous system can dynamically coordinate intake of 'rewarding' stimuli in order to meet metabolic demands and ensure survival (Rossi & Stuber, 2017).

Nowadays, cognitive reasoning, impulsivity, and executive self-control related to enticing food are challenged on a daily basis (Spence et al., 2016). The shift from normal "liking" and "wanting" to addictive behaviour has a pivotal role in neural mechanism for disordered eating. Stress-induced overeating can be seen as another disorder of reward mechanisms, and reward from comfort food is considered an attempt at self-medication to relieve the negative emotion and depressive state associated with chronic psychological stress (Berthoud et al., 2017).

The neural circuits controlling feeding and emotional behaviours are tightly and reciprocally connected (Sweeney & Yang, 2017). Since feeding is essential for survival, the brain has evolved multiple overlapping mechanisms to assure adequate levels of food intake during changing energy demands, involving several feeding centers distributed in the hypothalamus, hindbrain, and limbic brain regions conveying emotional information (Morton et al., 2014; Sternson et al., 2013; Schwartz & Zeltser, 2013; Williams & Elmquist, 2012). Consistently, feeding and emotions are known to be interrelated on a behavioural level (Sweeney & Yang, 2017). For example, psychiatric disorders are often associated with changes in feeding behaviour and metabolic disorders including obesity are associated with an increased risk for the development of mood and anxiety disorders (Foster et al., 2017).

The opioid signaling influences dietary behavior modulating individual 'wanting' of foods and 'liking'. Opioid peptides bind μ-opioid receptors (MORs) involved in the food reward system by attributing a value of enjoyment to the taste of food and play also a key role in modulating food intake. MORs are well known to interfere with the mechanisms of regulation of pain. In particular, they are the targets of opioids, which act as agonists on MORs to promote analgesia (De Vadder et al., 2013).

It is noteworthy that, after the brain, the body site in which MORs are most widely expressed is the gastrointestinal area (neurons of the ENS), especially the small intestine, where they have been shown to control gut motility (i.e. delayed gastric emptying and slowing of intestinal transit) and may influence food intake (Ruscitto et al., 2015).

Gastrointestinal motility is known to be associated with neurotransmitters such as serotonin, dopamine, and γ-aminobutyric acid (GABA). Particularly, biochemical signalling from the GI tract to the CNS is mediated by GABA, a primary inhibitory neurotransmitter involved in GABAergic signalling process, occurring in the intestinal epithelium. Furthermore, gut microbiota can influence CNS activity through the production of molecules that function as local neurotransmitters, including serotonin, melatonin, GABA, acetylcholine and histamine as well as affect anxiety-like behaviour, responsiveness and activation of the hypothalamic-pituitary-adrenal axis (HPA) (Arneth, 2018).

Calcium-sensing receptor (CaSR) is also present in the gastrointestinal tract, and is expressed on the apical and basolateral membranes of villous and crypt epithelial cells of the small intestine and colon, respectively, where it is involved in the regulation of intestinal homeostasis related intestinal absorption, secretion and motility. Furthermore, calcium-induced activation of CaSR trigger the regeneration of the intestinal barrier, suggesting that CaSR may be a promising target for treating intestinal inflammation (Zhang et al., 2015).

1.3 Food-derived bioactive peptides

Food is a source of bioactive compounds affecting human health. Altogether, public health professionals, consumers, food producers are becoming increasingly aware of the rapidly expanding body of epidemiological evidence linking the prevalence of diseases, such as obesity, cardiovascular disease, diabetes to dietary factors. This has led to an increased interest in the potential health effects of food derived bioactive compounds. Dietary proteins (including animals and plants sources) are characterized by peptides and amino acids encrypted within the primary structures of precursor protein molecule and both may be released either during food processing and/or during GI digestion (De Noni et al., 2009).

The process of GI digestion includes mechanical, chemical and enzymatic steps affecting the release of nutrients and promote their absorption. Bioactive peptides (BAPs) are short amino acid sequences that come from digestion of dietary protein and exert a measurable biological effect on body functions and health (Moller et al., 2008). Several food peptides are known to possess regulatory functions that can lead to health benefits as demonstrated mostly through *in vitro,* cell culture and animal studies.

Some dietary peptides show antihypertensive, antioxidant, anti-inflammatory, hypolipidemic, anticancer, antidiabetic and antimicrobial properties (Udenigwe & Fogliano, 2017).

BAPs need to be released from food matrix in the intestinal lumen to exert a biological effect and they can reach tissues through systemic circulation. Before this happens, BAPs undergo to hydrolysis during small intestinal passage and absorption. The GI tract is able to process a wide range of protein sources and the cascade of gastrointestinal proteolytic and peptidolytic enzymes very efficiently cleaves proteins into short- and medium-sized peptides as well as free amino acids. The amino acid absorption occurs in the form of di- and tripeptides at the apical side of enterocytes mediated by the proton-coupled peptide transporter 1 (PEPT1) whereas efflux of intact peptides via the basolateral membrane into systemic circulation seems to be negligible (Daniel & Zietek, 2015). Moreover, vascular endothelial tissue peptidases and soluble plasma peptidases further contribute to peptide hydrolysis, this is the reason why for most peptides the plasma half-life is limited to minutes (Foltz et al., 2010).

However, some peptides are fairly resistant to hydrolysis, and the extent and the velocity by which a dietary protein is broken down to its constituents is dependent on its composition (amino acid sequence) and on post-translational modifications such as glycosylation, which makes peptides more resistant to hydrolysis of proteases and peptidases (Daniel, 2004).

The action of dipeptidyl peptidase IV (DPP-IV) and dipeptidyl carboxypeptidase I, as rate-limiting enzymes in the GIT, determines the digestive breakdown of the peptides (Daniel, 2004).

Particularly, DPP-IV is a multifunctional type II transmembrane glycoprotein, expressed constitutively on epithelial cells of liver, intestine, kidney and in a soluble form as sCD26/DPPIV in the circulation. DPP-IV belongs to the prolyl oligopeptidase family, that preferentially remove N-terminal dipeptides from substrates and thereby either inactivates peptides and/or generates new bioactive compounds (Röhrborn et al., 2015). DPP-IV takes part in a number of biological processes as both a regulatory protease and a binding protein. The enzyme is involved in glucose homeostasis achieved by its catalytic activity against the incretin hormones GLP-1 and GIP, beyond that cleaves a number of molecules such as neuropeptides, chemokines and regulatory peptides (Lacroix & Li-chan, 2016). DPP-IV activity at intestinal mucosa level influences the amount and type of peptides passing into the bloodstream in intact form. Increased bioaccessibility and intestinal permeability, due to defective DPP-IV, can result in higher concentrations of bioactive peptides into the bloodstream.

Current research in nutrition field aims to gain insights in the physiological role of dietary constituents, among them proteins are well known for their nutritional and biological value. Different types of protein such as egg albumen, milk protein, soy protein, pea protein, and wheat gluten have

also been investigated for their potential effects on health and satiety. Proteins and peptides make up one of the main groups of food bioactive compounds, and the investigation of their nutritional value is enclosed in a new emerging field defined nutritional proteomics or nutriproteomics (Sauer & Luge, 2015). Recently, research in food science and nutrition changed the way food is considered. In fact, food is not just considerate as source of energy for the body, but it provides components with specific functions and nutritional properties, including potential benefits as well as possible adverse effects on health.

Dairy products and milk are potential sources of bioactive peptides with extra-nutritional physiological functions, influencing many regulatory systems as glucose and lipid metabolism, blood pressure, immune function, food intake and body weight (Sauer & Luge, 2015).

Furthermore, plant proteins represent valuable alternatives to animal proteins and are also cheaper and more sustainable. Still minor but nevertheless significant group of consumers, including vegetarian or vegan people, contributes to a trend of consuming plant protein sources (Capriotti et al., 2016).

Proteins influence appetite and food intake through the intestinal release of anorexigenic peptides such as PYY, GLP-1 and CCK. It is well known that taste may influence both appetite and food choice due to a strict interconnection between gustatory, metabolic and reward system (Steinert et al., 2016). Nutrient sensing in the mouth and in the gastro-intestinal tract is mediated by the same types of receptors (GPCRs) that trigger amino acid-sensing.

Some metabolites and amino acids contribute to the perception of postprandial satiety. This is at the basis of the aminostatic hypothesis (Veldorst et al., 2008). Accordingly, protein leverage hypothesis suggests that individual protein intake is regulated by individual protein target, emphasizing how 'protein target' can drive energy intake (Martens & Westerterp-Plantenga, 2014). The ingestion of proteins or amino acids increases serum amino acid concentration that elicits appetite reduction; inversely, a fall of the amino acid serum concentration enhances appetite. This happens because amino acids work as satiety signal (Veldorst et al., 2008). Taste buds in the oral cavity initiate gustatory signaling that influence food liking and choice. The amino acid-sensing in the mouth is mediated by GPCRs. The heterodimer T1R1/T1R3 is the umami taste receptor that binds the L-amino acids such as L-glutammate (eliciting the umami taste) (Zhang et al., 2008). Together with T1R1/T1R3, amino acids are also sensed by gastrointestinal EECs through other GPCRs such as CasR, MORs and GPRC6A (G protein-coupled receptor family C group 6 subtype A) (**Table 1**). The binding between a certain peptide or amino acid with the specific receptor activates a signaling pathway that culminates with a release of satiating peptides (GLP-1, CCK, PYY) as well as

neurotransmitters that directly reach the brain and fire the reward area (Spreckley & Murphy, 2015; Vancleef et al., 2015).

Table 1. Amino acid taste receptors

Receptor	Localization	Binding	Function
CasR	ECCs, pancreatic cells	Aromatic amino acids L-Phe, L-Trp	Release of CCK and gastrin
GPRC6A	L-cells	Basic amino acids L-Lys, L-Arg	Release of GLP-1, increase [Ca^{2+}]
MORs	Brain, GI tract, neurons of portal vein walls	Nutropioids: Antagonist Agonist	Reduce food intake Increase food intake
T1R1/T1R3	Mouth, stomach, small intestine	L-glutammate	Umami Taste receptors

In particular, MORs are expressed both in the brain where they are involved in food reward system and in the small intestine where they control gut motility. MORs are present in the neurons of the portal vein walls and can sense blood peptides coming from dietary protein digestion. These peptides can act as MOR agonists or antagonists. The latter activate gut-brain mechanisms inducing the intestinal gluconeogenesis controlling food intake (De Vadder et al., 2013). The opioid signaling influences dietary behavior modulating individual 'wanting' of foods and 'liking'. Interestingly "**nutropioids**" can result from the digestion of dietary proteins. Casomorphin from milk casein and exorphin from gluten are opioid ligands acting as opioid agonists (Pfluger et al., 2012). Interestingly, it was recently demonstrated in animals that high-protein diets increased TAS2R/Gαtran/Gαgust expression in the pyloric mucosa possibly due to the bitter taste of compounds forming by protein digestion or to some amino acids (De Giorgio et al., 2016). This feature highlighted the chemosensory adaptation of gastro-intestinal tract to the dietary nutrients raising the hypothesis that diets may modulate both metabolism and dietary behavior through the fine network of taste receptors.

Consumption of dietary protein seems to decrease postprandial appetite and subsequent energy intake (EI) more than fat and carbohydrate. The type of protein ingested may also affect postprandial responses. A number of mechanisms have been proposed to explain this apparent satiety hierarchy of macronutrients, including higher thermogenic effect of dietary protein and post-absorptive small intestinal gluconeogenesis (which is associated with decreased EI in rats) (De Vadder et al., 2013).

Many oligopeptides coming from digestion of dietary proteins have opioid activity by acting as effectors of MORs (Pfluger et al., 2012). GIT, especially the small intestine, is the second body site, after brain, where MORs are most widely expressed and are involved in the control of gut motility and food intake (De Vadder et al., 2013).

Soymorphins-5, -6, and -7 are opioid agonist peptides derived from β-conglycinin β-subunit of soy. They are able to suppress food intake and delay bowel transit time via gut μ-opioid receptor after oral administration as well as through the activation of serotonin (5-HT1A), dopamine D2, and $GABA_B$ receptors (Kaneko et al., 2010).

Moreover, β-casomorphins (BCMs) are group of peptides with opioid properties arising by proteolytic digestion of β-casein (De Noni, 2008). Among the BCMs, BCM-7 is a typical peptide released during hydrolysis of the β-casein allelic variant A1, containing a histidine residue at position 67 (Nguyen et al., 2015). BCM-7 exerts various physiological effects, i.e., secretion of mucus, increased activity of superoxide dismutase and catalase, increased levels of prolactin, analgesic role, slows down the passage of food through the digestive system (as do other opioids) providing longer time for lactose fermentation (ul Haq, 2014).

In this scenario milk proteins are worthy of note because they are source of BAPs.

Milk proteins are often suspected to be the cause of nonspecific, undiagnosed GI symptoms in adults. The manifestation of stomach symptoms in healthy subjects is affected by meal composition, which influence gastric emptying and thereby lactose load in the gut. Also, differences in individual sensitivity and gut microflora may affect the tolerability of milk (Turpenein et al., 2016).

Opioid peptides are highly sensitive to hydrolysis by dipeptidyl peptidase IV (DPP-IV) thereby strongly limiting or preventing the transfer of these peptides in an intact form across the intestinal mucosa and the blood-brain barrier (De Noni et al., 2009). Increased intestinal permeability for digested food proteins and defective DPP-IV can result in biological active peptides circulating in the bloodstream, that can traverse the blood–brain barrier and reach the CNS (Cieślińska et al., 2015).

1.4 Intestinal permeability and GI disorders

Intestinal permeability can be defined as the facility with which intestinal epithelium allows molecules to pass through by non-mediated passive diffusion. This concept mainly refers to the passage of ions and inert molecules of low molecular weight. The intestinal transport of molecules from the intestinal lumen to the lamina propria can occur through two distinct mechanisms: paracellular diffusion through tight junctions (TJs) between adjacent intestinal epithelial cells (IECs) and transcellular transport involving endocytosis/exocytosis (transcytosis) mediated or not by membrane receptors (Ménard et al., 2010). There are a number of diseases that are known to increases

the mucosal permeation of macromolecules, such as celiac disease, Crohn's disease, type 1 diabetes and patients with food allergies (Mishra & Makharia, 2012).

A cross talk between gut and brain is consolidated. In addition, mounting evidence shows that gut microbiota can influence host appetite and eating behaviour by directly affecting nutrient sensing and appetite, for this reasons the microbiota-gut-brain axis has been coined (van de Wouw et al., 2017). The microbiota functions in tandem with the host's defences and the immune system to protect against pathogen colonisation and invasion. It also performs an essential metabolic function, acting as a source of essential nutrients and vitamins and aiding in the extraction of energy and nutrients. (Carding et al., 2015). Alterations in the bowel flora and its activities are now believed to be contributing factors to many chronic and degenerative diseases. The intestinal dysbiosis hypothesis suggests a number of factors associated with modern Western living that have a detrimental impact on the microbiota of the GIT. In comparison to diets high in overall protein, diets especially high in animal protein have specific effects on intestinal microbiota (Myers, 2004). Intestinal permeability reflects just one function of the barrier that is intimately related to and interacts with luminal contents, including the microbiota. The mucosal immune response also influences barrier integrity although changes in barrier function have been described in several gastrointestinal disorders. It is important to note that the gut microbiota has a key regulatory role in both host metabolism and central appetite, which together can modify host eating behaviour in metabolic disorders and eating disorders. (Carding et al., 2015).

Functional gastrointestinal disorders (FGIDs), are characterized by morphologic and physiological abnormalities that often occur in combination with motility disturbance, visceral hypersensitivity a well as altered mucosal and immune function, gut microbiota and CNS processes (Drossman, 2016). A complex interrelationship of predisposing genetic factors, influenced by life events as well as psychosocial factors lead to abnormalities in motility, visceral sensation, and brain – gut interactions, manifesting clinically as GI symptoms (Chey, 2013).

The relationships between celiac disease (CD), Non-Celiac Gluten Sensitivity (NCGS), and irritable bowel syndrome (IBS) remain unclear. However, it has been a matter of debate whether barrier function contributes to the development of FGIDs or if it is merely a consequence (Barbara et al., 2016).

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Chapter 2

Functional dyspepsia: new evidence for an old problem

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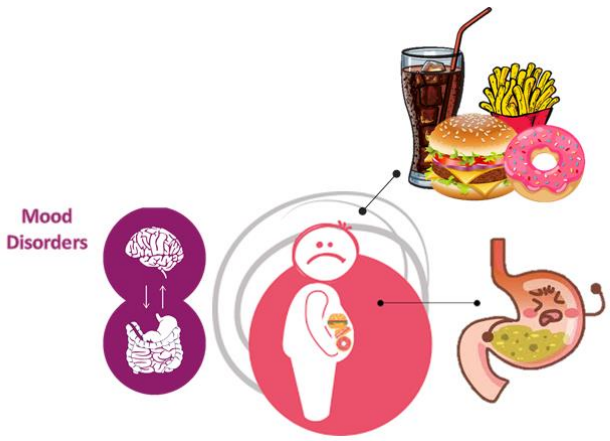

Functional dyspepsia is a multifactorial condition and associations between symptoms, functional and psychological abnormalities are still a cause of debate

Paper in submission

1.1 Functional dyspepsia: definition, incidences and main symptoms

Functional gastrointestinal disorders (FGIDs), are characterized by morphologic and physiological abnormalities that often occur in combination with motility disturbance, visceral hypersensitivity and altered mucosal and immune function, gut microbiota and central nervous system (CNS) processes.

FGDIs are one of the leading causes for referral to emergency care units and represent 40% of diagnoses in gastroenterological settings (Drossman 2016). Although only about 25% of symptomatic individuals seek medical support, the frequency of FGDIs drains substantial amounts of healthcare resources (Stanghellini 2017).

According to the Rome IV committee, FGIDs can be classified into six groups: esophageal disorders, gastroduodenal disorders, bowel disorders, centrally mediated disorders of gastrointestinal pain, gallbladder and sphincter of Oddi disorders, and anorectal disorders (Oshima & Miwa 2018).

Digestive function abnormalities and their symptoms, including functional dyspepsia (FD) and irritable bowel syndrome (IBS) are the most common FGIDs.

FD refers to upper abdominal chronic symptoms arising from the gastroduodenal region mainly triggered by ingestion of food (Talley 2017). The prevalence of FD in the community ranges between 5 and 11% (Vanheel et al.,2016).

According to the Rome IV criteria, the diagnosis of FD is based on the presence of any combination of 4 symptoms such as postprandial fullness, early satiety, epigastric pain, and epigastric burning. Moreover, to get a positive diagnosis symptoms have to be severe enough to interfere with the usual activities with a frequency of at least 3 days per week over a previous 3 months-period with an onset of at least 6 months in advance (Drossman 2016). That definition identifies patients suffering from 3 specific categories of disorders such as (1) postprandial distress syndrome (**PDS**), (2) epigastric pain syndrome (**EPS**), and (3) overlapping of PDS and EPS. PDS is characterized by meal-induced dyspeptic symptoms suggestive of a motility disturbance. EPS refers to epigastric pain or epigastric burning that do not necessarily occur after meal ingestion, can be even improved by meal, and is not associated with peptic ulcer or gastro-esophageal reflux disease. Overlapping of PDS and EPS is characterized by concomitance of meal induced dyspeptic symptoms and epigastric pain or burning (Stanghellini 2017).

According to the Rome IV definition, the prevalence of FD is higher in USA (12%) than Canada (8%) and UK (8%) with a distribution of PDS and EPS similar in the combined population and across the three countries and genders, even if a higher prevalence of FD was found in women compared to men across all age groups (Aziz et al.,2018). In particular, most of the participants with FD fulfilled criteria for PDS (61%), followed by EPS (18%) and 21% overlapping variant with both syndromes. In

addition, subjects with PDS had overlap with irritable bowel syndrome (IBS 15%, *vs.* 42% with EPS) (Aziz et al., 2018), supporting the idea that the PDS subtype of FD is distinct from other FGIDs.

A meta-analysis of 100 population-based studies comprising over 312,000 subjects showed that the pooled prevalence of uninvestigated dyspepsia was 21% (95% confidence interval, 18% to 24%) and that the risk of dyspepsia was increased in females and those with *Helicobacter pylori* infection, smokers, and nonsteroidal anti-inflammatory drug users (Talley 2017).

The prevalence of FD is higher among women than men and this correlation could be due to sex-specific biological differences in gastrointestinal function (for example, sex hormone-driven alterations in intestinal motility) or the processing of (visceral) pain in the CNS, but also to sex-specific health care behaviour (Enck et al.,2017).

1.2 Pathophysiology and biopsychosocial perspective of FGIDs

The pathophysiology of FD is multifactorial and conclusive associations between symptoms and functional as well as psychological abnormalities are still a cause of debate among experts. It is well known that FD impacts on quality of life in a manner depending from symptom severity and comorbid depression. On the other hand, psychosocial disorders such as anxiety, depression, as well as physical and emotional abuse and difficulty in coping with life events are very frequent among FD patients (Stanghellini 2017).

While a variety of peripheral candidate biomarkers related to FGIDs continue to be investigated, none appear to account for a large proportion of the symptom variance in this diversified set of syndromes. At the same time, a model for FGIDs that includes a prominent role for brain-gut interactions has been emerged over time. Brain-gut axis may explain the complex interconnections between gastrointestinal sensation, motility, immune function, and gut microbes with sensory, cognitive, and affective circuitry in the brain (Tillish 2018).

In this framework, a biopsychosocial perspective is needed to shed light on how the complex interactions of environmental, psychological, and biological factors contribute to the development and maintenance of FGIDs as well as for an appropriate treatment of these comorbidities (Van Oudenhove 2016).

The **biopsychosocial model** suggests that a complex interrelationship of predisposing genetic factors, influenced by life events as well as psychosocial factors lead to abnormalities in motility, visceral sensation, and brain – gut interactions, manifesting clinically as GI symptoms (Chey 2013).

Psychological distress is a considerable risk factor for FGIDs development and, when present, can perpetuate or exacerbate symptoms. Comorbid anxiety and depression are independent predictors of

post-infectious IBS and FD but, at the same time, also occur as a consequence of bodily symptoms and related quality of life impairment. The absence of formal psychiatric comorbidity does not exclude a role of dysfunctional cognitive and affective processes (van Oudenhove 2016), likewise represent a big bias in the context of diagnosis of FGIDs. In this frame it is essential clarify how each of these factors—the environment, the individual's own psychological states and traits, and the individual's (neuro)physiological make-up-interact to ultimately result in the generation of FGID symptoms (van Oudenhove 2016).

1.3 Involvement and metabolic interactions of microbiome-gut-brain axis in FGIDs development

A bidirectional communication takes place between gastrointestinal (GI) tract and central nervous system (CNS) through multiple pathways involving neural, endocrine, and immune cells. The gut–brain axis (GBA) allows the CNS to regulate GI functions, including motility and secretion, and the GI tract to signal sensations such as hunger, pain or discomfort to the CNS. New emotional, cognitive and behavioural functions concerning the GBA have been discovered, including affective mood, memory formation and food intake respectively (Mazzoli & Pessione, 2016). FGIDs symptoms are not as easy to localize and are influenced more by overarching effects resulting from CNS–enteric nervous system (ENS) dysregulation of symptom control pathways (Drossman 2016). Brain imaging studies using functional magnetic resonance imaging (fMRI), positron-emission tomography (PET) or other emerging imaging technologies have identified alterations in several interconnected brain networks, including sensorimotor, emotional arousal and salience networks, in patients with FGIDs (Enck et al.,2017).

Genetics and environmental factors as well as sociocultural influences may affect one's psychosocial development in terms of personality traits, susceptibility to life stresses, psychological state, and cognitive and coping skills (Holtman et al., 2017). These factors influence the susceptibility to gut dysfunction: abnormal motility or sensitivity, altered mucosal immune dysfunction or inflammation, and the microbial environment, as well as the effect of food. This complex interactions and emotional distress may feed back to perpetuate and amplify symptoms (Sahan 2018). After exposure to stress, an increased basal activation of hypothalamic-pituitary-adrenal axis (HPA) and autonomic nervous system (ANS) associated with secretion of the corticotrophin releasing hormone (CRH) lead to physical and psychological symptoms, linked to GI system as well (Sahan 2018). The mechanisms underlying gut-brain communications involve neuro-immuno-endocrine mediators. This bidirectional communication network includes the CNS, both brain and spinal cord, the ANS, the

ENS and HPA axis. The HPA axis is considered the core stress efferent axis that coordinates the adaptive responses of the organism to stressors of any kind (Holtman et al.,2017). Environmental stress, as well as elevated systemic pro-inflammatory cytokines, activate this system that, through secretion of the corticotropin-releasing factor (CRF) from the hypothalamus, stimulates adrenocorticotropic hormone (ACTH) secretion from pituitary gland that, in turn, leads to cortisol release from the adrenal glands. **Cortisol** is a major stress hormone that affects many human organs, including the brain. Thus, both neural and hormonal mediators allow brain to influence the activities of intestinal functional effector cells, such as immune cells, epithelial cells, enteric neurons, smooth muscle cells and enterochromaffin cells (Carabotti et al., 2015). Both clinical and experimental evidence suggest that gut microbiota has an important impact on GBA, interacting not only locally with intestinal cells and ENS, but also directly with CNS through neuroendocrine and metabolic pathways involving serotonergic and GABAergic signalling systems (Barbara et al., 2016).

FGIDs symptoms seem to be affected by microbiota in terms of microbial dysbiosis within the gut and its role in influencing anxiety and depressive-like behaviours (Foster & Neufeld 2013). Dysbiosis, that occurs in FGIDs, is highly associated with mood disorders as well. The break down communication of the GBA induce changes in intestinal motility and secretion, causes visceral hypersensitivity and leads to cellular alterations of the entero-endocrine and immune system. Gut microbiota may interplay with multiple of these different pathophysiological FGIDs targets and its role is supported by varying lines of evidence (Dupont 2014).

The absence of microbial colonization is associated to an altered expression and turnover of neurotransmitters in both nervous systems (Clarke et al., 2013, Stilling et al., 2014) and also to alterations of gut sensory-motor functions, consisting in delayed gastric emptying and intestinal transit altered mucosal immune function, altered gut signalling (visceral hypersensitivity) and CNS dysregulation of the modulation of gut signalling and motor function (Carabotti et al.,2015).

It was speculated that the high prevalence of psychiatric comorbidities in FGID patients reflects the fact that FGID may be a primary manifestation of brain dysfunction, or even primary somatization, with the brain driving the gut manifestations (Tanaka et al., 2011). Epidemiological data from prospective studies (Jones et al., 2012; Koloski et al., 2012; Koloski et al., 2016) suggest that in at least half of the cases, GI symptoms arise first and incident mood disorders occur later. Other studies emphasize the role of (intestinal) inflammation and cytokine response, and the gut microbiome in driving gut to brain alterations (Holtman 2017). If these findings hold true, reversing GI dysfunction, could allow targeting and potentially curing not only the FGID but also concomitant mood disorders. Moreover, mounting evidence show that duodenal low-grade inflammation may be involved in the etiopathogenesis of FD inducing mucosal immune activation, duodenal barrier dysfunction, and

sensory-motor dysfunction. An altered duodenal gut microbiota, food antigens or infection may trigger duodenal micro-inflammation in a subset of FD patients (Jung & Talley 2018).

The duodenum regulates acid secretion from the stomach and the nutrient absorption in the small intestine via local signalling pathways, and connects with the CNS, via neuronal and endocrine mediators. Duodenal eosinophils and in some cases mast cells may play a key role in immune activation in FD. Low-grade intestinal inflammation in patients with FD may provoke impairment in motor-sensory abnormalities along the gastrointestinal neural axis. Among FD patients, the risk of developing dyspeptic symptoms after a bout of gastroenteritis is 2.54 odds ratio (95% confidence, 1.76-3.65) at more than 6 months after acute gastroenteritis (Futagami et al.,2015). Albeit causation is not established, the hypothesis that FD is a disorder of small intestinal inflammation in a major subset of patients is gaining acceptance, opening the possibility of novel and targeted treatment approaches.

1.4 FGIDs and foods

Food is associated with symptom onset or exacerbation in a significant proportion of FGID patients. Currently 80% of patients report that the symptoms are aggravated by ingestion of a meal (Page & Li 2018) and particularly approximately two-thirds of patients report symptoms within 15–45 min of food ingestion (Pilichiewicz et al., 2009). Despite this, the role of food in the pathogenesis of the FGIDs has remained poorly understood. For this reason, diet has largely played an adjunctive rather than a primary role in the management of FGID patients, underestimating the role of food both in GI function and sensation as how food relates to GI symptoms in FGID patients as well (Chey 2013).

Increasing evidence show that diet contributes to functional digestive symptoms (Feinle-Bisset, 2004; Gibson et al., 2015; Pilichiewicz et al., 2009), and dietary restrictions are frequent among patients affected by FGIDs, especially those suffering from IBS (Bohn et al.,2013; Gibson et al., 2015). The most common nutrients supposed of being attributable to FGIDs symptoms are dietary fibers, dietary fats, and carbohydrates (Feinle-Bisset 2013(b); Saito et al., 2005; Yang 2012, Moayyedi et al., 2014). Nowadays, most of people adopt unhealthy dietary patterns, characterized by high consumption of fat and sugary products, sodas, snacks, breakfast cereals and other ultra-processed foods (UPFs, high density of saturated fatty acids, sugar, sodium and low content of fibers). Several studies evaluate the possibility that UPFs could be related with increased risk of IBS or contribute to the induction and/or exacerbation of digestive symptoms (Buscail et al., 2017; Khayyatzadeh et al., 2016). At this regard, Schnabel and colleagues (2018) investigated the possible association between UPFs consumption and FGIDs (Schnabel et al., 2018).

A prospective observational cohort study including 3516 adult participants was carried out. In the total population UPFs consumption accounted for 33.0% of total energy intake and the incidence of FGIDs was 10.5% for IBS, followed by functional constipation (5.4%), FD (3.9%), functional diarrhea (1.1%) respectively. Moreover, an increased proportion of UPFs in the diet was linked with a higher prevalence of IBS risk (**a**OR **Q4 vs. Q1** [95% CI]: 1.25 [1.12–1.39], $p < 0.0001$), likewise an association between increased share of UPF in the diet and higher risk of FD when concomitant with IBS was observed (Schnabel et al., 2018).

In dietary intervention studies, specific amounts of solid or liquid meals were served to determine the meal-related dyspeptic symptoms, gastric accommodation, or hormonal changes in FD patients (Lee et al., 2018). Although it is already known that FD patients tolerate only small amounts of food, evidence on the extent of nutritional intake of daily meals remains inconclusive (Feinle-Bisset 2013). Dietary recommendations in FD include eating smaller meals and avoiding high-fat meals which have been reported to aggravate clinical symptoms such as nausea and abdominal pain, more than isocaloric high-carbohydrate meals **(**Yamawaki et al.,2018).

Recent studies investigating the role of dietary habit and nutritional intake in FD patients suggest that fat ingestion influences symptom development (Goktas et al., 2016, Khodarahmi et al., 2016).

In particular, Goktas et al (2016) showed that FD subjects had symptoms mainly triggered by fried and fatty foods (27.1%), hot spices (26.4%), and carbonated drinks (21.8%) (Göktaş et al., 2016).

In general**, lipids** in the duodenum can trigger gastric filling symptoms through a direct neuronal stimulation, higher lipid sensitivity of enteroendocrine cells or nerves, increased levels of systemic or local cholecystokinin (CCK, secreted by lipid-activated enteroendocrine cells, stimulates the release of digestive enzymes and bile and induces satiety) and/or increased sensitivity to CCK involving type A CCK-receptors (Enck et al.,2017).

Evidence shows that in FD patients the intra-duodenal infused lipids (but not glucose) stimulate the stomach distension more than in healthy controls, thus suggesting a cross-sensitization between mechano-sensors and chemo-sensors (Barbera et al.,1995).

Thus early satiety and intolerance of fatty foods in FD subjects could be related to gastrointestinal hypersensitivities to distension and/or small-intestinal fat (Feinle-Bisset 2013).

Hypersensitivity to mechanical stimulation of the stomach is frequent in patients with FD, however, the underlying mechanisms for this hypersensitivity are unclear. There is some evidence that transient receptor potential (TRP) channels may be involved in the visceral hypersensitivity associated with FGIDs (Balemans et al., 2017). In particular, the TRP vanilloid receptor 1 (TRPV1) expressed on vagal and spinal sensory nerve endings in the gut wall and activated by low pH, high temperature, painful stimuli, exogenous irritants such as capsaicin (active component of hot chilli peppers;

Caterina et al., 1997) and endocannabinoids like anandamide seems the most involved (Zygmunt et al., 1999). Indeed, the consumption of spicy capsaicin containing food was positively associated with scores of stomach fullness in FD patients (Lee et al., 2018) and a hypersensitivity to capsaicin in patients with FD compared to healthy controls has been recently reported (Hammer et al., 2018). The chemical hypersensitivity to capsaicin characteristic of FD patients was used to develop a simple and minimally invasive oral test to identify subjects with FD **(**Hammer et al., 2018).

Carbohydrates have been also associated to FGIDs as abdominal symptoms can be specifically induced by challenges with sugar (lactose or fructose), sorbitol, and oligosaccharides (fructans) alone or in combination and because exclusion diet-re-challenge tests in patients with FGIDs identified many cereal-based foods as those responsible to induce symptoms (Sheperd et al., 2013).

Short-chain carbohydrates containing up to 10 monosaccharide units vary in their digestibility and subsequent absorption. Those that are poorly absorbed exert osmotic effects in the intestinal lumen, attract water, and are rapidly fermented by bacteria with consequent gas production (Sheperd et al., 2013). As all dietary poorly absorbed short-chain carbohydrates have similar and additive effects in the intestine, a concept has been developed to regard them collectively as FODMAPs (fermentable oligosaccharides, disaccharides, monosaccharides and polyols) and to evaluate a dietary approach that restricts them all. Observational and comparative studies, and randomized-controlled trials support that FODMAPs trigger gastrointestinal symptoms in patients with functional bowel disorders, and that a diet low in FODMAPs offers considerable symptom relief in the majority of patients who use it (Yamawaki et al.,2018). Dairy or lactose intolerances and hypersensitivity to sour, acid-secreting or irritant foods (for example, citrus, spices, coffee or alcohol) could also play a role in stimulating GI symptoms. In addition, protein-rich foods could have an effect as thy can contain hidden fat (not readily identified by patients) or cause specific immune responses (Enck et al.,2017). Studies using magnetic resonance imaging (MRI) confirm that when FODMAPs are administered to healthy volunteers, small bowel distension occurs due to increased small bowel water content. However, water retention in the intestine cause discomforts only in patients with FGIDs and altered sensory functions (Holtman et al.,2017).

If FODMAPs are poorly absorbed and have been shown to induce symptoms in FGIDs, likewise gluten intolerance, even in the absence of coeliac disease, needs to be considered.

In fact, IBS and FD patients, without evidence for celiac disease based on serological markers and histology, experience substantial improvement in symptoms upon withdrawal of gluten from their diet (Holtman 2017). The relationships between celiac disease (CD), “gluten-sensitivity,” and IBS remain unclear, with various studies reporting increased or expected rates of CD among IBS subjects

(Barbara et al.,2016). An overlap between IBS and Non-Celiac Gluten Sensitivity (NCGS) has been detected. NCGS is a syndrome characterized by intestinal and extra-intestinal symptoms related to the ingestion of gluten-containing food, in subjects that are not affected by either celiac disease or wheat allergy (Catassi et al.,2015).

Epidemiology studies on IBS provide an indirect estimation of intestinal NCGS frequency. According to recent population-based surveys performed in Northern Europe, the prevalence of IBS in the general adult population was 16%–25% (Breckan et al., 2012; Krosgaard et al., 2013). In a selected series of adults with IBS, the frequency of NCGS, documented by a double-blind, placebo-controlled challenge, was 28% (Biesiekierski et al., 2011). Another study showed that 276 out of 920 (30%) subjects with IBS-like symptoms, according to the Rome II criteria, suffered from wheat sensitivity or multiple food hypersensitivity, including wheat sensitivity (Carroccio et al., 2012). Should a consistent proportion of IBS patients be affected with NCGS, the prevalence of NCGS in the general population could well be higher than CD (1%) (Catassi et al., 2015).

A recent systematic review of Duncanson et al., (2018) consolidated the already known relationship between dietary fats and FD. The apparently disparate set of foods reported as inducing symptoms are each high in either fermentable carbohydrate (some soft drinks, fruit, fruit juice, watermelon, milk), wheat/gluten (grain/pasta/wheat products, takeout/processed foods) or natural food chemicals (fruit, fruit juice, red pepper, soft drink, tea). The findings in relation to lactose and fructose intolerance suggest that FODMAPs may induce symptoms via the small or large bowel. From this review conducted from January 1982 to February 2016 only 16 studies out of 6451 studies met the inclusion criteria and they dealt with investigation about the effect of nutrients, foods and food components in adults FD patients. Of note, of this 16 studies only 5 were case-control studies and 1 randomized controlled trial (RCT) characterized by a crossover gluten or placebo challenge.

In two years, despite the relevance of food triggering FGIDs-symptoms, there is still the lack of data in literature related to RCT. Previous findings highlighted that FD is, at least in part, a disorder related to food ingestion *per se*, in which symptoms can be induced by specific foods or food components and not simply a postprandial gastrointestinal motility disorder. This highlights the need for well-designed clinical studies that involve randomising patients to a wheat/gluten-free diet as well as controlling for FODMAP/fat content, aiming to investigate specific dyspeptic symptom associations. Furthermore, several lines of evidence suggest the involvement of the intestinal microbiota in the pathogenesis of FGIDs in general and IBS in particular. Gastrointestinal infections are strong risk factors for the development of FD and IBS, enhancing the intestinal permeability (leaky gut), within lifestyle and diet that are crucial determinants of microbiota composition and function in humans (Barbara et al., 2016). The impact of diet on the microbiota can be direct, through changes in its

composition or total energy supply, or indirect, via the induction of changes in intestinal transit time or intraluminal pH. Interestingly, the impact of diet on the microbiota is also highly dependent on the intestinal location: the conversion of complex indigestible carbohydrates is the driving force for the microbiota in the colon, whereas the fast uptake and conversion of sugars deriving from digestible polysaccharides mainly impact the microbiota in the small intestine (Barbara et al., 2016).

Conclusion

FGIDs are prevalent and debilitating GI disorders. The heterogeneity of these illnesses hints at a complex multifactorial pathophysiology including a role for the endocrine, immune, and nerve systems that are finely regulated by the microbiome-gut-brain axis.

However, the molecular and cellular mechanisms behind the symptomatology are not yet fully elucidated.

Although FD has been traditionally considered as a motility or acid-related disorder, mounting evidence shows that a subtle duodenal inflammation (notably, duodenal eosinophilia in a subset of patients with PDS), increased duodenal mucosal permeability, a disturbed duodenal microbiota, impaired ENS reflexes and systemic alterations (for example, increased levels of circulating cytokines and small-intestinal T cells) may be majorly implicated, thus shifting the interest from the stomach to the upper small intestine.

In this frame the role of foods, diets and dietary components on etiology of the disease and symptomatology occurrence need to be developed. Large nutrition surveys with a prospective design could provide information on dietary habits of FGID subjects and clarify whether dietary preferences reflect the subconscious exclusion of foods to which subjects are intolerant. Moreover, long-term randomized controlled intervention trials are needed to identify foods that can reduce or even enhance the discomforts.

Finally, some evidence shows that functional and molecular alterations of factors involved in intestinal permeability might explain FGIDs pathophysiology and symptom generation. More researches focused on this area may provide innovative and non-invasive biomarkers valuable in both diagnosis and assessment of drug and/or dietary therapy response in FGIDs patients.

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Chapter 3

Appetite and gastro-intestinal hormone response to a gluten free meal in celiac and healthy subjects: a pilot study

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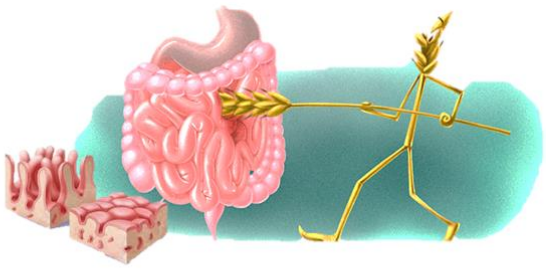

An adaptive hormone response may influence post-prandial appetite and insulin resistance over a long period in celiac subjects

Paper in submission

Abstract

Celiac disease (CD) is an immune-mediated inflammatory enteropathy triggered by gluten in genetically susceptible individuals. Gastrointestinal (GI) hormone response related to appetite and glucose metabolism are still under-investigated in patients with CD. This study aimed at shedding light on the appetite sensations, glycaemia and hormone response induced by a complex meal in celiac subjects.

Twenty-two female subjects with CD, 9 at the diagnosis (CDD) and 13 under a gluten free diet (CDGF), and 10 healthy subjects (HS) were enrolled in a single day intervention study. All subjects consumed a test meal, recorded their appetite sensations and blood was collected over three hours after meal consumption.

A lower decrease of hunger in CDD compared to CDGF and HS after meal intake was recorded. No difference of fullness and satiety between the groups was observed. CDD had lower insulin and GIP than CDGF and HS. Both CDD and CDGF experienced a lower post-prandial response of glucose than HS.

Data suggested that patients with coeliac disease show an impaired glucose absorption after more than 12 months of gluten-free diet. Postprandial GIP may play a significant role in appetite cues and insulin response to a complex meal.

1.Introduction

The incidence of celiac disease (CD) has significantly increased over the recent decades (Lohi 2007). A spectrum of disorders often coexists in patients with CD (Lauret 2013). It has been suggested that gluten consumption, along with gut permeability and inflammation, are factors in the development of type 1 diabetes (Smyth 2008).

Type 1 diabetes mellitus develops in 1.6% to 16.4% of patients with CD (Al-Bawardy 2017). The co-occurrence of insulin-dependent diabetes mellitus and celiac disease is linked to similar genetic background associated with the HLA (Human Leukocyte Antigen) class II genes on chromosome 6p21 (Smyth 2008; Cronin 1997).

Furthermore, mounting evidence showed that gluten-free diet (GFD) increased the risk of overweight or obesity in patients with CD who were normal weight or overweight at the diagnosis. That fact was associated to the improved intestinal absorption induced by GFD and to unhealthy dietary behaviour of CD patients who preferred foods rich in lipids, sugars and proteins (Mariani et al., 1998; Dickey and Kearney, 2006; Valletta 2010) and decreased dietary fiber intake (Mariani et al., 1998; Dickey and Kearney, 2006; Ferrara 2009). It is well-known that eating habits and preference result from metabolic, hedonic and cognitive factors (Rossi 2017).

The metabolic factors associated to GF food consumption in CD patients were under-investigated. Post-prandial physiological mechanisms influence when and how much people can eat at the next eating occasion through modulation of appetite sensations (Berthoud 2017).

Glucose plays a metabolic role in the control of hunger, satiety and the regulation of body energy balance (Chaput & Tremblay, 2009). A decreased glucose utilization or decreased intracellular glucose concentrations rather than the absolute level of blood glucose, work as the stimulus for meal initiation.

The glucose uptake acts on the short-term control of hunger and food intake, argued by glucostatic theory of appetite control (Mayer 1953). The latter suggests that reduced glucose utilization in critical brain regions leads to perception and expression of hunger. Experimental evidence, together with glucostatic theory, suggests that pre-prandial hypoglycaemia, a reduced availability of glucose for metabolism and a decreased level of body carbohydrate stores or liver glycogen are stimuli for increased food intake (Chaput & Tremblay 2009). Both insulin and incretins play a key role in glucose variations. Glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are major incretins with considerable effects on glucoregulatory functions (Edholm 2010). Edholm et al., (2010) showed that GIP dose-dependently reduces, postprandial glucose increment mainly through an increased insulin release and no effect on the gastric emptying rate. On the other hand,

GLP-1 displays dual effects on postprandial glucose control: an initial slowing of gastric emptying, and a subsequent increase of insulin release (Edholm 2010).

Previous literature focused on the dosage of ghrelin in patients with celiac disease (CD), in particular describing a higher level of this peptide in CD patients at diagnosis and a lower level on a gluten free diet (Jarocka-Cyrta 2010, Rocco 2008), probably due to the changes in the nutritional state (Lanzini et al., 2006) or to the reduction of a systemic inflammation after diagnosis (Malandrino et al 2008).

Ghrelin is a 28 amino acids hormone-like peptide mainly secreted by oxyntic glands of the gastric fundus. It has a pivotal role on the regulation of food intake eliciting the appetite (Schwartz MW et al 2000) and accelerates gastric emptying (Levin et al 2005), acting both to peripheral and central level. Therefore, circulating ghrelin concentration is high in fasting state and decreases in the postprandial period (le Roux 2006). Conversely, glucagon like peptide 1 (GLP-1) decreases food intake, increases satiety, inhibits the acid secretion in the stomach and decelerates the gastric emptying (Gutzwiller et al 1999). This peptide is secreted by L-cells of distal ileum as well as in the gut and its release is mainly stimulated by both fats and carbohydrates intake.

In this frame, a gap of knowledge still exists in the literature on the GI peptide response to a meal in CD patients in association to appetite sensations.

The aim of this study was to evaluate the post-prandial appetite sensations induced by a mixed meal in celiac subjects at diagnosis and on a GFD and to clarify the role of gastro-intestinal response.

To this purpose a single day intervention study was conducted in celiac and healthy subjects who consumed a test meal, recorded their appetite sensations and were submitted to blood drawings over three hours after meal consumption. Blood samples were analyzed to monitor gastro-intestinal hormone response related to appetite and glucose metabolism.

2.Material & methods

2.1 Subjects

Enrollment of CD patients was carried out at the CD outpatient clinic of the University of Salerno and the Federico II of Naples. Eligible subjects were adult women, (aged 18-40 years) with normal weight (BMI 18.5-25 kg/m^2) and with celiac disease (CD), diagnosed according to the current Guidelines (Ludvigsson 2014). CD diagnosis was based on the presence of gastrointestinal symptoms, Marsh $\geq$ 2 histology and antigliadin antibodies up to the year 1999 and in the presence of Marsh 2 histology and with both anti-tissue transglutaminase (a-tTG) IgA > 7 U/ml and positive anti-endomysial (EMA) antibodies from the year 2000. Subjects with a diagnosis of CD were eligible to participate into the study. CD patients were at diagnosis (CDD), if they had received the CD diagnosis

over a month before the enrollment and CD on a GFD (CDGF) if they were on a GFD from at least 12 months.

A control group of healthy women was also recruited among the patients' friends and the hospital staff. The inclusion criteria were the same as for the celiac patients as regards age and BMI but the CD and food allergies/intolerance as well as all gastro-intestinal diseases were excluded.

For both groups of subjects (CD and healthy) those with eating disorders evidenced by Three Factor Eating Questionnaire (Stunkard & Messik 1985) were excluded.

2.2 Study design

It was a one-day study based on a test meal. The day before the experimental session subjects were asked to have a dinner consisting of a standardized meal including meat, vegetable, bread, and fruit, within 9 p.m. Fasting subjects were invited to reach the laboratory at 8 am. After checking for the health condition and absence of menstruation, and after 10 minutes of resting, a cannula needle was put in the arm vein. After 5 minutes of resting, a first blood sample was collected (0 min, baseline). Then a test meal was offered and blood samples were collected 30 min, 60 min, 120 min and 180 min after food intake.

At each time point before blood collection, individual appetite sensations (including hunger, fullness and satiety) by visual analogue scale (VAS) were also recorded.

The nutritional composition of the meal is reported in **Table 1**. It included: 100 g of gluten free bread, 50 g of ham, 30g of cheese (stracchino type), 10g of butter, a yogurt and one apple.

Table 1. **Nutritional composition of the meal test.**

Meal test	Protein (g)	Fat (g)	SFA (g)	MUFA (g)	PUFA (g)	CHO (g)	Fiber (g)	Energy (kcal)
Gluten free bread 100g	3.2	2.5	0.4	0	0	45.3	6.3	229
Ham 50g	13.4	1.6	0.5	0.1	0.1	0	0	68
Cheese 30g	5.6	7.5	4.7	2.4	0.3	0	0	90
Butter 10g	0.1	8.3	4.9	2.4	0.3	0.1	0	75.8
Yogurt 125g	4.8	4.9	2.6	1.1	0.2	5.4	0	82.5
Apple 150g	0.5	0.2	0	0	0	20.6	3	79.5
Total	**27.43**	**25.00**	**13.0645**	**5.993**	**0.7795**	**71.34**	**9.3**	**624.8**

2.3 Blood sample collection and preparation

For each time point, two different vacutainer® tubes of 4 mL each were used to collect plasma samples. A protease inhibitor mix, consisting of dipeptidylpeptidase IV (DPP-IV) inhibitor (Millipore's DPP-IV inhibitor; St Charles, MO, USA), protease inhibitor cocktail (Sigma, St. Louis, MO, USA) and 4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF, Pefabloc® SC, Roche-diagnostics), was immediately added only into the blood destined to the GI peptide. After sample preparation, tubes were centrifuged at 4000 g for 10 minutes and plasma samples were aliquoted and frozen at -80°C, within 30 min from collection.

Plasma samples were analyzed for gastrointestinal (GI) peptides concentration. Luminex Technology (Bio-Plex; Bio-Rad, Nazareth, Belgium) was used to determine GI peptides. A magnetic bead panel kit provided by Milliplex® (Merck Millipore, Millipore S.p.A.) allowed the simultaneous determination of the following four hormones: ghrelin, glucagon-like peptide-1 (GLP-1), glucose-dependent insulinotropic peptide (GIP), and insulin. The sensitivity levels of the assay (in pg/mL) were: ghrelin 2.0; GIP 0.6; GLP-1 7.0; insulin 58.0. The intra-assay variation (%CV) was 2% for ghrelin, 3% for GIP and insulin; 7% for GLP-1. The inter-assay variation (%CV) was 5% for GIP; 6% for insulin; 8% for ghrelin; 10% for GLP-1.

2.4 Statistical analysis

The analysis of variance (ANOVA) was performed to evaluate the differences between groups at each time points for both biochemical analysis and appetite scores. Since no difference at baseline for appetite scores was found, the results were analyzed and expressed as the absolute changes from the baseline. The subjective appetite sensations, the glycaemia and the response of hormones were tested for the effect of time as factor by the ANOVA for repeated measures. The linear trapezoidal rule was used to calculate the total area under the curves (AUC) for the appetite sensations and biochemical markers. Results are expressed as means ± SEM and were considered statistically significant for $p < 0.05$. Statistical analyses were performed using Statistical Package for Social Sciences (version 16.0; SPSS, Inc., Chicago, IL, USA).

3. Results

3.1 Subjects

Twenty-two CD patients (9 at diagnosis, CDD, and 13 on a GFD, CDGF) and 10 healthy subjects (HS) participated into the study. Their general characteristics are reported as mean±SD in **Table 2**. The three groups were homogenous for age (p=0.09) and BMI (p=0.3). In CDGF the time passed since the start of gluten free diet was more than 5 years in 6 subjects, 3 years in 4 subjects, 2 years in 2 subjects and 1 year in 1 subject.

Table 2. Characteristics of study participants. CDD: CD patients at diagnosis; CDGF: CD on a GFD; HS: healthy subjects. Data was reported as mean ± standard deviation.

	CDD	**CDGF**	**HS**	***p* value**
Age (years)	29.4±2.3	30.6±3.6	31.6±4.0	0.1
BMI (kg/m^2)	21.93±20.1	22.17±1.9	22.0±1.9	0.3
a-tTG (U/ml)	142.83±28.2	2.84±0.02	−	<0.001

3.2 Appetite

No significant difference of hunger, fullness and satiety sensations at baseline between the groups was found. **Figure 1** shows the variations from baseline of appetite ratings over time. Data showed a lower decrease of hunger in CDD compared to CDGF and HS after food intake.

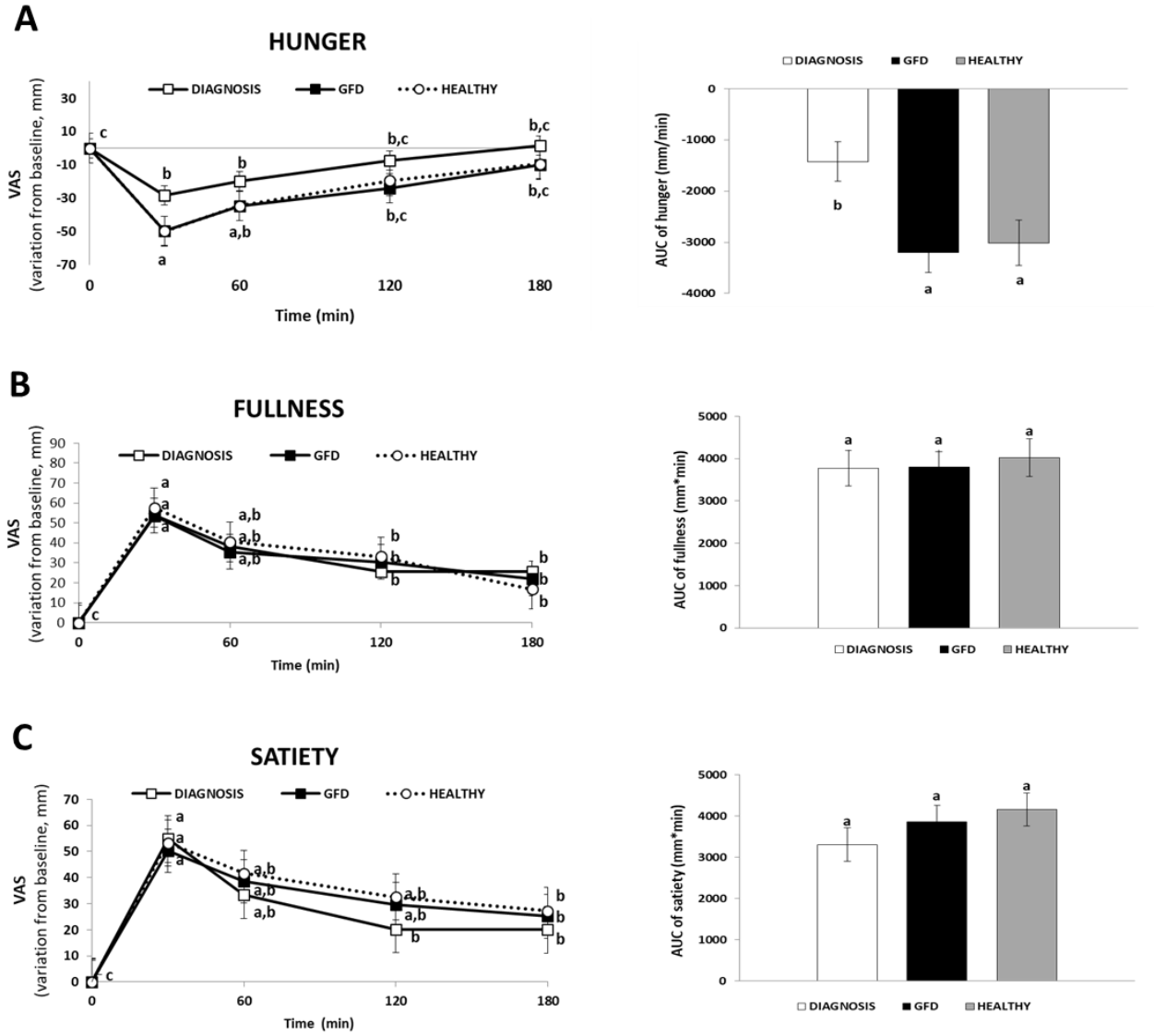

Figure 1: Post-prandial appetite. Variations from baseline and area under the curve (AUC) of hunger (A), fullness (B) and satiety (C) feelings recorded during the study. Different letters indicate significant differences between groups (ANOVA and post-hoc Tukey test, $p<0.05$).

3.3 Hormone responses

Figure 2 shows the time-concentration curve, the variation from baseline and the AUC of circulating ghrelin, insulin, GIP, and GLP-1.

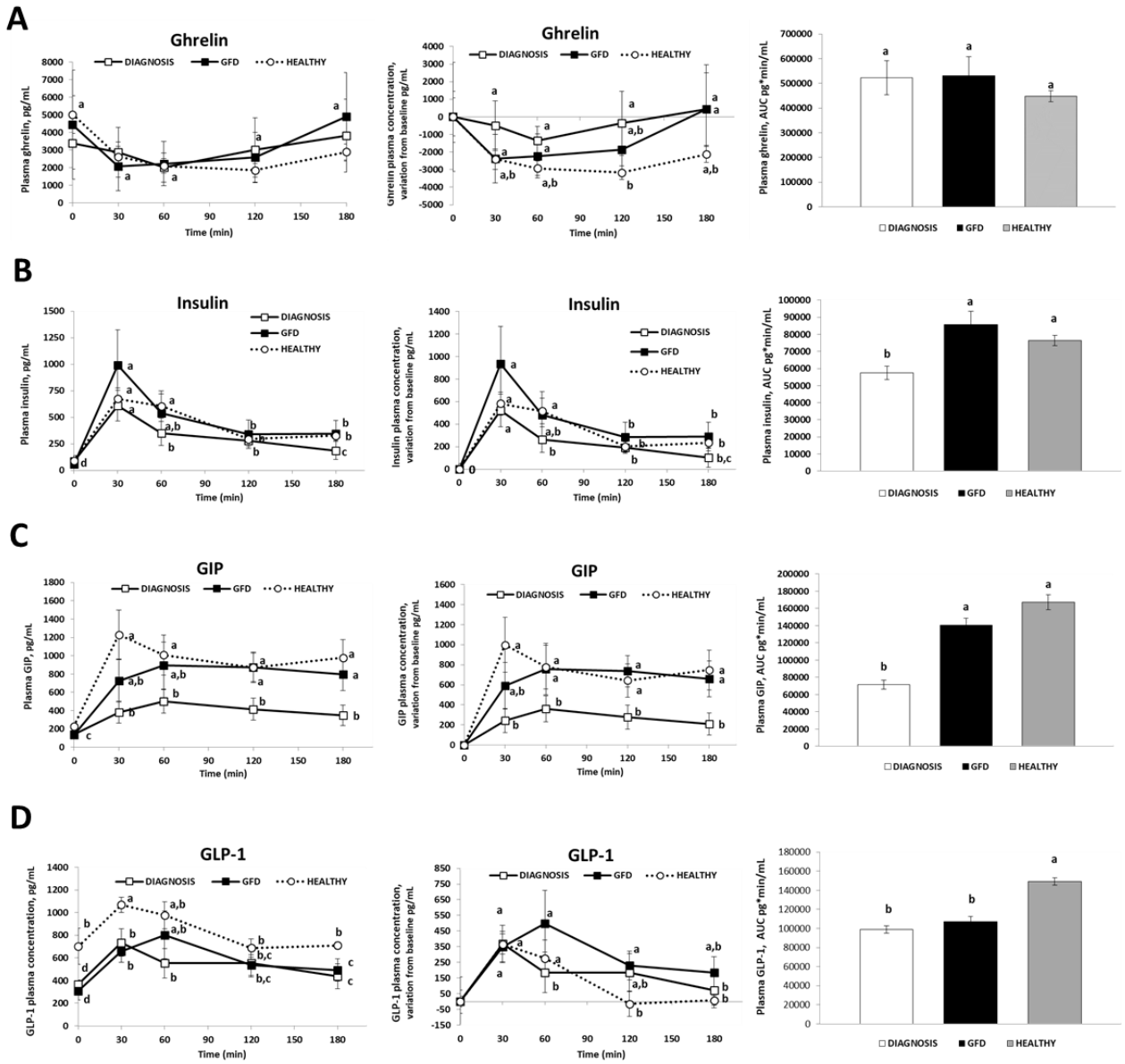

Figure 2: Post-prandial gastro-intestinal hormone response. Concentration-time response, v**ariation from baseline** and AUC of plasma concentration of ghrelin (A), insulin (B), GIP (C), GLP-1 (D) over the study, into the three groups of subjects. Different letters indicate significant differences between groups (ANOVA and post-hoc Tukey test, $p<0.05$).

Data showed that CDD tended to have lower reductions of ghrelin in the post-prandial phase than CDGF and HS. No significant different AUC of plasma ghrelin over the three hours post-lunch

between groups was observed. CDD showed a lower response of insulin than CDGF and HS. Celiac subjects (both CDD and CDGF) showed a lower concentration of plasma GLP-1 at baseline and over the post-prandial phase than HS.

Significant difference AUC of both plasma incretins GIP and GLP-1 over the three hours post-lunch was observed respectively.

3.4 Blood glucose

Figure 3 shows blood glucose concentration-time curve over the study and the AUC of glucose in the three groups. Data showed that celiac subjects (both CDD and CDGF) experienced lower post-prandial response of glucose than HS ($p<0.05$).

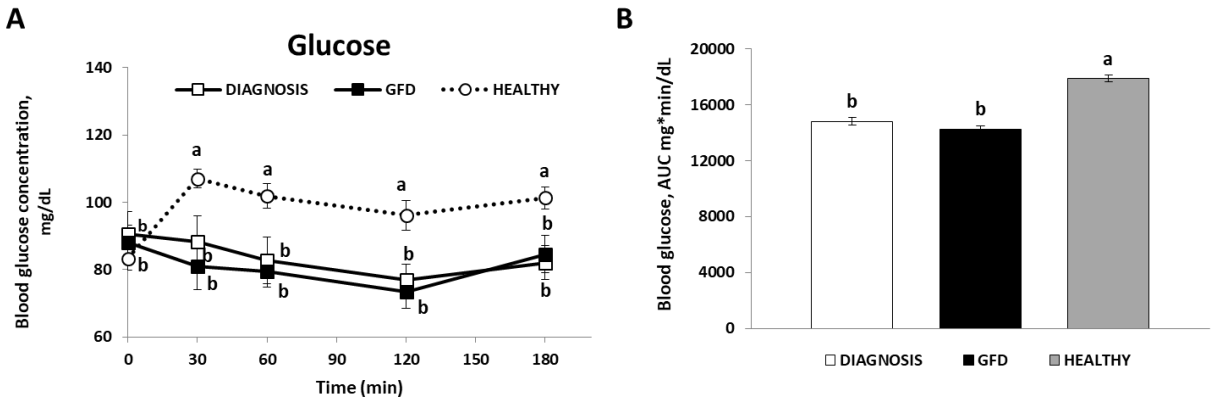

Figure 3: **Post-prandial blood glucose.** A) Concentration-time response over three hours post-meal consumption in subjects with CD at diagnosis (CDD) and on a GFD (CDGF) and in healthy subjects (HS); B) AUC of blood glucose over three hours post-meal in the three groups of subjects. Different letters on the bars indicate significant differences between groups (ANOVA and post-hoc Tukey test, $p<0.05$)

4. Discussion

To the best of our knowledge, this is the first study investigating the post-prandial effect of a mixed gluten-free meal on appetite sensations, blood glucose response and gastro-intestinal hormone response in patients with CD at the diagnosis (before starting a GFD), on a GFD (under a GFD since at least 12 months) and in healthy subjects.

An important finding of this study was that in the post-prandial phase CDD showed a sustained hunger sensation and a reduced response of plasma GIP and insulin compared to CDGF and HS. In CDD, the lower hunger reduction could be explained by the lower GIP response than in CDGF and HS, in association with the lower GLP-1 than HS. Indeed, GIP and GLP-1, delay gastric emptying at physiological concentration (Marathe 2013). Therefore, a lower response of GIP in CDD, in addition to the low GLP-1 (as in CDGF vs HS), could determine a faster gastric emptying and, in turn, a faster returning of hunger. This picture is in accordance with the similar ghrelin responses recorded in the three groups and the evidence by Rocco and co-workers (2008) that the delayed gastric emptying in CD patients was not associated with reduced ghrelin levels (Rocco et al., 2008).

Another important finding was the lower post-prandial glycaemia of celiac subjects, both CDD and CDGF, than HS. That finding was in agreement with the known condition for celiac patients of a decreased glucose absorption as observed in an old study by measuring jejunal transmural potential differences (Read et al., 1976) and as more recently demonstrated by Laforenza and co-workers (2010). These authors reported in duodenal biopsies of active celiac patients a reduced expression of the main solute transporters such as the Na^+/glucose co-transporter 1 (SGLT1), the H^+/oligopeptide transporter 1 (PEPT1) and Na^+/H^+ exchanger 3 (NHE3) thus revealing a clear mechanism underpinning impaired glucose, oligopetide and sodium absorption in those patients. Moreover, they showed that tissue expression of the transporters was completely restored in celiac patients consuming a gluten-free diet for at least 12 months. Our data suggested that this was not the case for our subjects even if they were all under a gluten free diet for a time longer than 12 months. However, the different hormonal response found into the two groups let us hypothesize the occurrence of concomitant mechanisms underpinning post-prandial glycaemia. In CDD, additionally to a supposed impaired glucose absorption, the low glycaemia was coherently associated with low responses of the incretins GIP and GLP-1 as well as of insulin. Contrarily, CDGF showed post-prandial insulin and GIP curves that were similar to HS. It is well known that GIP can reduce postprandial blood glucose by prolonging insulin release or, alternatively, by improving glucose uptake directly in the liver or other peripheral tissues, as well as by inhibiting glucose output from the liver (Edholm et al., 2010). Therefore, supposing a similar absorption rate of glucose, hormonal data suggested that an increased sensitivity of insulin and GIP in CDD compared to CDGF and HS might occur; this might explain the similar glucose curve of CDD and CDGF irrespective from GIP and insulin responses. In other words, GIP and insulin were more effective in glucose uptake at cellular level in CDD than CDGF. Such a phenomenon may be a physiological adaptive response of the body to the condition of malabsorption typical of CDD and is coherent with mechanisms occurring after weight loss (Asmar et al., Nutrition & Diabetes, 2016).

Interestingly, celiac subjects (both CDD and CDGF) showed a lower blood GLP-1 concentration than HS at every time point as previously shown by Papastamataki and co-workers (2014) in fasting children with celiac disease. Since no difference for post-prandial blood glucose between CDD and CDGF was found, similar circulating level of GLP-1 in the two groups supported a main role of this peptide in glucose management as well as the hypothesis (above discussed) of a similar expression/activity of SGLT-1 in CDD and CDGF in our study (Oguma et al., 2015).

5. Conclusions

This study shows the evolution of appetite sensations, blood glucose and the gastro-intestinal peptide response in celiac disease patients compared to healthy subjects over three hours after the consumption of a gluten free meal.

Main data showed that CDD experienced a sustained hunger sensation and a reduced response of plasma GIP and insulin compared to CDGF and HS and celiac patients (both CDD and CDGF) showed a lower post-prandial glycaemia and blood GLP-1 than HS.

A longer study is needed to verify the possible metabolic adaptations and homeostatic mechanisms that which may occur in celiac compared to healthy subjects.

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Chapter 4

Milk protein enriched beverage reduces post-exercise energy intakes in women with higher levels of cognitive dietary restraint

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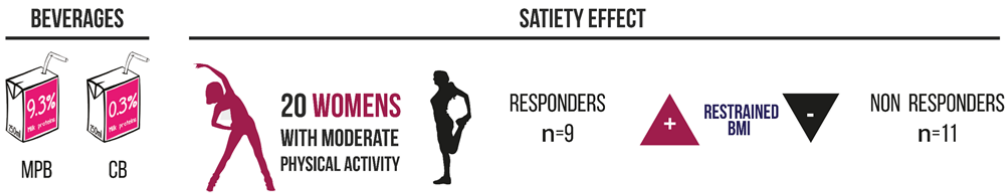

Satiating effect of dairy proteins modulating mechanisms underpinning both energy and reward homeostasis

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Abstract

Objective: The aim of this study was to assess the satiating efficacy of milk proteins compared to carbohydrates in twenty women during post-exercise period.

Methods: A milk protein-enriched beverage (MPB), and an isocaloric carbohydrate-enriched beverage (CB) containing respectively 9.3 g and 0.3 g of milk proteins per 100 mL beverage, were developed and tested in a satiety study with 20 free-living healthy and normal weight women. The participants drank 250 mL of the two beverages after an aerobic exercise session, filled daily food diaries and rated their appetite on visual analogue scale (VAS), in two days over three consecutive weeks. A psychometric evaluation of eating behaviour by three-factor eating questionnaire (TFEQ) was obtained.

Results: No differences in appetite feelings and energy intakes between MPB and CB were found in the study population. However, 9 participants were significantly less hungry (-9% *vs* +15%, p 0.03) and ate later (208 min *vs* 127 min, p 0.03) and less (-10% *vs* +8% daily energy intake, p 0.01) when they had MPB than CB. These women had a slightly higher BMI and were more restrained than the others.

Conclusions: Data showed that MPB compared to CB could modify daily eating habits by enhancing satiety in women with a stronger cognitive control of eating behaviour.

Keywords: milk proteins; appetite control; energy intake; eating behaviour; functional food.

1. Introduction

Milk is recognised among foods with high biological and nutritive value. Some evidence showed promising effects of dairy consumption on body weight and composition. A key role for such effects was mainly attributed to protein content (Abargouei, Janghorbani, Salehi-Marzijarani, & Esmaillzadeh, 2012; Chen, Pan, Malik, & Hu, 2012).

Bovine milk proteins are about 32 g per litre and comprise caseins (~80%) and whey proteins (~20%) (Haug, Høstmark, & Harstad, 2007). They are both high quality proteins containing all the essential amino acids but they have a different digestion and absorption behaviour: caseins (slow proteins) empty the stomach later and elicit a more sustained but prolonged increase of postprandial blood amino acids concentration than whey (fast proteins) (Boirie et al., 1997). Both protein types can delay gastric emptying. Furthermore, caseins induce the secretion of anorexygenic hormones such as cholecystokinin (CCK) and glucagon-like peptide (GLP-1) over a long term period (for a review see Bendtsen, Lorenzen, Bendsen, Rasmussen, & Astrup, 2013). Conversely, whey proteins due to glycomacropeptide and α-lactoalbumin as well as tryptophan (produced by digestion), induce CCK and serotonin secretion in the short-term period (Schellekens et al., 2014).

A full consensus among researchers on the satiating effect of dairy proteins as well as on possible active dose or best way of consumption (i.e. through foods or beverages), was not yet established. Some studies tested the satiety effect of dairy proteins as constituent of yogurts or beverages. In those studies, amount of proteins ranged from 5g to 51g and they were included in portions with different size and energy value. Satiety effect was compared with similar isocaloric foods providing lower amount of proteins (Bertenshaw et al., 2009; Burton-Freeman, 2008; Douglas et al., 2012; El Khoury et al., 2014; Ortinau, et al., 2012) or carbohydrates as total replacement of proteins (Chungchunlam et al., 2014). Overall results showed different effects on appetite sensations, energy intake and delay of subsequent meal independently from the protein content of the foods. Such discrepancies among studies were due to the different protocols adopted and to the absence of a proper control food overcoming the interferences of fats and carbohydrates (Bendtsen et al., 2013). Interestingly, a common outcome from the literature could be highlighted: women were more sensitive to proteins-rich foods than men (Bertenshaw et al., 2009; Burton-Freeman, 2008; Chungchunlam et al., 2014; El Khoury et al., 2014).

Milk whey fraction is also a rich source of essential amino acids mainly branched chain amino acids which have a pivotal role in muscle metabolism and protein synthesis (Børsheim et al., 2002). Whey amino acid and electrolyte composition makes milk a good alternative to traditional sport drinks (for a review see Roy, 2008). Bovine milk has a short term hydration potential higher than still water and

commercial oral rehydration solutions (Maughan et al., 2016) and is more efficient to enhance the replacement of lost fluid following exercise than carbohydrate–electrolyte drinks (Desbrow, Jansen, Barrett, Leveritt, & Irwin, 2014; Watson, P., Love, T., Maughan, R., and Shirreffs, 2008).

Previous evidence in the literature about the positive satiety effects of milk proteins in women and the potentiality of milk as a valuable sport drink (as discussed above) supported this study. It aimed to elucidate the satiety efficacy of milk proteins, added at 9% in a beverage, in comparison to an isocaloric beverage containing carbohydrates. To this purpose, the two beverages were developed and a randomized cross-over study involving women practicing exercise regularly was carried out.

2. Materials and Methods

2.1 Beverages

Two types of milk-based beverage with a different protein/carbohydrate ratio were developed. Both beverages were prepared from water diluted UHT (Ultra High Temperature) skim milk. That was added with milk proteins (Nutrilac-Be-8123, purchased by Arla Foods Ingredients Broups P/S, Vibj, Denmark) and a low-calorie sweetener in the case of the milk protein enriched beverage (MPB) whereas carbohydrates (25% maltodextrin, Dry MD 01910, Cargill®,Minneapolis, United States; 25% corn starch, PolarTex™ 06739, Cargill®, Minneapolis, United States; 50% sucrose) were added in the case of the carbohydrate enriched beverage (CB). **Table 1** reports nutrition facts of MPB and CB.The amount of milk proteins added in MPB provided the highest amount of proteins was possible without affecting beverage consistency. Starch and sugars in CB were added to balance both the calories and the consistency provided by milk proteins in MPB. Sweetness in MPB was adjusted using steviol glycosides (PURECIRCLE SDN BHD, Negeri Sembilan, Malaysia). The formulation of the beverages was optimised in order to make them as much as possible similar for taste, flavour, texture and colour.

The beverages were UHT treated and were provided in 250 mL PET (polyethylene terephthalate) bottles that were singularly codified with an alphanumeric code. Each volunteer received 3 bottles of the two beverages on the week before starting the protocol.

2.2 Amino acid composition of beverages

Macronutrient content of MPB and CB was assessed by official methods of analysis of the Association of Official Analytical Chemists (Horwitz, W., Chichilo, P., & Reynolds, 1970).

Amino acid composition of the beverages was determined by Liquid chromatography–high-resolution mass spectrometry (LC/HRMS) as previously described (Troise, Fiore, Roviello, Monti, & Fogliano, 2015). Two ml of beverage were centrifuged at 16000 × g for 10 min and the supernatants were separated and diluted (1:10) with a H_2O/CH_3CN (50/50) 1% formic acid solution.

2.3 Subjects

Recruitment was performed at the Department of Agricultural Sciences of University of Naples "Federico II" among students and staff. Eligible subjects were women, with a BMI in the range 18-24.5 kg/m^2 and with a moderately active lifestyle, as indicated by a physical activity level ranging between 600 and 1500 metabolic equivalents (MET)-min/week (Jettè et al., 1990). Subjects were excluded from the study if they had a BMI≥24.6 kg/m^2 or ≤17.9 kg/m^2, if they did not have planned

and structured physical activity (low physical activity, <600 MET-min/week) or they had high levels of physical activity (>1500 MET-min/week), if they had irregular menstrual cycle and any chronic illness such as diabetes or hypertension, if they were taking any prescription medication, or any type of supplement, or any performance-enhancing or recreational drug, or if they were under a controlled dietary regimen, or they lost body weight (changes ≥ 2 kg) over the previous three months, or they were pregnant or lactating women, or they were participating in other clinical trials.

The physical activity was assessed through the International Physical Activity Questionnaire (IPAQ) (Hagströmer, Oja, & Sjöström, 2006) and the overall physical activity (3 hours per week) was expressed as MET.

All participants also completed the three-factor eating questionnaire (TFEQ) that measures 3 different aspects of eating behavior such as restraint, disinhibition and hunger (Stunkard & Messick, 1985).

The study protocol was approved by the Ethic Committee of University of Naples and the eligible subjects were enrolled in the study after signing an informed written consent.

2.4 Study design

A cross-over randomized double blind study was performed for three consecutive weeks, including two fixed experimental days per week, with one day wash-out in between (**Figure 1**).

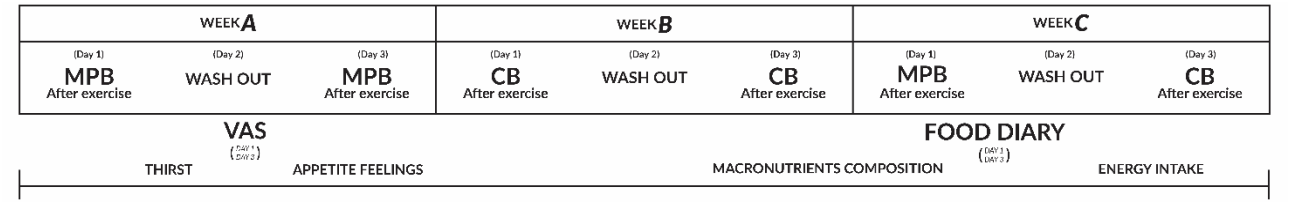

Figure 1. Study design indicating the experimental days and the randomization of beverages as well as the instruments and the measured outcomes over the study period.

For each participant along the three weeks the experimental days were always the same week days. Once enrolled subjects were randomized for the order of beverage consumption over the three weeks. All subjects tested each beverage on three experimental days. On each experimental day immediately after exercise subjects consumed the beverage set out by the randomization. Subjects habitually exercised between 5:00 pm and 7:00 pm and they were required not to change time, type, intensity and volume of exercise over the study. Each woman started the protocol over the week following her menstruation in order to normalize the inter-individual impact of menstrual cycle on appetite. The volunteers were asked to reach the laboratory over the week before the start to get the coded beverages and a personalized calendar of consumption of the beverages. To evaluate the compliance to the protocol, at the end of each week, volunteers came to the laboratory and gave back the bottles; the

leftovers in the bottles were weighed. Moreover, they filled the IPAQ in order to monitor the level of physical activity over the past week.

Dietary intake, appetite feelings, thirst, and beverage liking were recorded by subjects during all the experimental days. Diets and time of each meal were recorded in 24h weighed food diaries which were used to calculate energy intakes (EI) and macronutrient composition of each meal as well as the time of eating.

Appetite sensations (hunger, fullness, satiety and desire to eat) were rated on a 100 mm visual analogue scale (VAS), anchored on the left as ‘‘not at all’’ and on the right as ‘‘extremely’’ in five specific moments during the experimental days: immediately before breakfast, at midmorning (11:00 a.m.), immediately after lunch, before exercise and after exercise and beverage consumption. At the latter time point subjects also rated on specific VAS questionnaires their thirst and their liking (for sweetness, acidity, fatty taste, consistency, pleasantness and overall palatability) of the beverage they just consumed.

Thirst was evaluated by VAS questionnaires. The subjects responded to the question, “How thirsty do you feel right now?” by marking on a line 100 mm in length with intersecting lines anchored at 0 mm for “not at all” and at 100 mm for “extremely thirsty” (Marks et al., 1988; Stachenfeld et al.,1996; Stachenfeld et al., 1997).

2.5 Statistical analysis

Power analysis indicated that seventeen participants were sufficient to detect a minimum difference of 837 kJ (200 kcal) in EI with a power of 80% and an α=0.05 (Vitaglione P, Lumaga RB, Montagnese C, Messia MC, Marconi E, 2010). Twenty subjects were enrolled taking into account possible dropouts.

Subjective appetite ratings and the EI were evaluated for differences by two-way repeated measures analysis of variance (ANOVA). When appropriate, differences between means were tested using the Student's t-test.

Statistical analysis of data was carried out using SPSS (version 21, SPSS Inc. Chicago, IL, USA). Statistical significance was set at a level of α=0.05 (5 %).

3. Results

3.1 Beverages

Table 1 shows the composition of both MPB and CB per 100 mL. The beverages were isocaloric and differed for the proteins/carbohydrates ratio that was 3.7 in MPB and 0.03 in CB.

Table 1. Nutritional composition of the Milk Protein (MPB) and of the Carbohydrate (CB) – enriched beverage per 100 mL. Values are reported as means ± standard deviations.

	Milk Protein Beverage (MPB)		**Carbohydrate Beverage (CB)**	
Energy (kcal)	47.0		47.0	
	g	***%Energy***	***g***	***%Energy***
Fat	0.03±0.001	0.60	0.03	0.60
Carbohydrates	2.50 ±0.01	21.3	11.0±0.4	93.6
starch	-		*5.60*±0.3	
sugars	*2.50*±0.01		*5.40*±0.1	
Protein	9.30±0.2	79.1	0.30±0.01	2.50
added milk proteins	9.00±0.2		-	
Sodium	0.06±0.01		0.06±0.01	
Calcium	0.06±0.02		0.01±0.01	

Table 2 shows the amino acid content of both beverages. MPB contained about 9.3 folds higher amount of total amino acids (2608.9 μg/mL vs 279.38 μg/mL, p=0.001).

Beverage formulations were selected in a preliminary sensory test on the basis of individual overall liking; beverages getting similar preference scores on VAS-based questionnaires were used in the study (*data not shown*).

Table 2. Amino acid composition of the Milk Protein (MPB) and of the Carbohydrate (CB) – enriched beverage. Data was reported as mean (μg/ml) ± SEM.

Amino acids	**MPB** Mean ± SEM (μg/ml)	**CB** Mean ± SEM (μg/ml)
TRP	52.70 ± 0.24	5.54 ± 0.10
ILE-LEU	63.30 ± 1.87	14.83 ± 0.24
PHE	65.32 ± 1.35	8.63 ± 0.31
TYR	95.11 ± 1.35	9.99 ± 0.04
VAL	611.05 ± 34.04	51.86 ± 2.08
ALA	455.15 ± 63.09	31.53 ± 1.83
PRO	101.27 ± 4.53	13.69 ± 0.31
GLN	140.08 ± 2.59	27.92 ± 0.74
GLU	302.25 ± 2.46	27.72 ± 0.11
ASN	30.54 ± 0.23	3.08± 0.16
ARG	219.32 ± 4.33	14.19 ± 0.24
LYS	127.08 ± 1.98	12.81 ± 0.18
HIS	78.33 ± 1.51	5.59 ± 0.04
MET	38.60 ± 0.35	2.58± 0.09
SER	171.40 ± 8.27	29.76 ± 0.28
THR	57.40 ± 1.77	19.67 ± 0.22
TOTAL	2608.9 ± 16.19	279.38 ± 3.55

3.2 Human trial

Table 3 reports the characteristics of the 20 women who participated into the study.

Participants always drank whole portions of the provided beverages after their habitual exercise sessions, which were always at the same time (between 5:00 p.m. and 7:00 p.m.) and had the same intensity over the study weeks.

Table 3. General characteristics of participants

	Mean±SD	**Range**
Age (years)	24±7	19 - 42
Body weight (kg)	57.9±6.2	46 - 67
BMI (kg/m^2)	21.4±1.9	18.6 - 24.4
F1 (Restraint)	11.9±3.7	5 - 18
F2 (Disinhibition)	6.2±3.1	2 - 13
F3 (Hunger)	3.8±4.0	0 - 13
MET (min/week)	1188±129.4	1080 - 1440

Table 4 shows mean energy intake and nutrient composition of meals consumed during the experimental days, over the time period before and after exercise as well as each beverage consumption.

Table 4. Energy intake and nutrient composition of meals consumed before and after exercise and beverage consumption. Data are reported as means of the three experimental days per beverage ± SEM (n=20).

	Milk Protein Beverage (MPB)			**Carbohydrate Beverage (CB)**		
	Before*	***After**#*	***Total***	***Before****	***After**#*	***Total***
		Mean ± SEM			**Mean ± SEM**	
Energy intake (kcal)	892.03±36.34	463.06±48.12	**1355.10±40.38**	905.08±23.83	472.29±43.59	**1377.37±45.8**
Protein (g)	31.84±2.03	29.09±4.07	**60.93±2.84**	35.53±2.61	26.39±2.89	**61.92±2.92**
%kcal	*14*	*25*	*18*	*16*	*22*	*18*
Lipids (g)	32.48±1.52	21.14±2.38	**53.62±1.90**	33.84±1.76	21.54±2.57	**55.38±2.05**
%kcal	*33*	*41*	*36*	*34*	*41*	*36*
Saturated Fat (g)	10.79±0.39	6.01±1.18	16.79±0.49	10.20±0.48	6.17±1.13	16.37±0.56
Monounsaturated Fat (g)	13.20±0.82	10.19±1.10	23.39±1.02	13.25±0.93	10.71±1.27	23.97±1.12
Polyunsaturated Fat (g)	4.40±0.22	2.97±0.49	7.37±0.28	4.42±0.30	3.17±0.53	7.60±0.34
Carbohydrate (g)	117.95±3.58	37.98±6.18	**155.93±3.23**	115.96±4.10	42.35±7.27	**158.31±3.82**
%kcal	*53*	*33*	*46*	*51*	*36*	*46*
Starch (g)	57.13±2.96	25.09±4.43	82.22±2.80	88.09±3.25	28.23±5.45	88.09±3.25
%kcal	*27*	*23*	*26*	*17*	*25*	*27*
Sugars (g)	46.64±0.91	9.97±2.23	56.60±0.81	38.95±0.81	9.83±2.69	48.78±0.70
%kcal	*22*	*9*	*17*	*18*	*9*	*15*
Dietary fiber (g)	8.30±0.42	3.81±0.84	**12.11±0.41**	8.94±0.54	3.53±0.70	**12.47±0.49**

* included breakfast, mid-morning snack, lunch, mid-afternoon snack.
# included dinner and after-dinner snacks.

Data showed no significant difference between energy intake and nutrient composition of the meals between the days over the same time period (before and after beverage) and between beverages.

Figure 2 shows mean scores of appetite (hunger, fullness, satiety and prospective food consumption) sensations and thirst recorded by participants (n=20) over the experimental days when they drank MPB or CB.

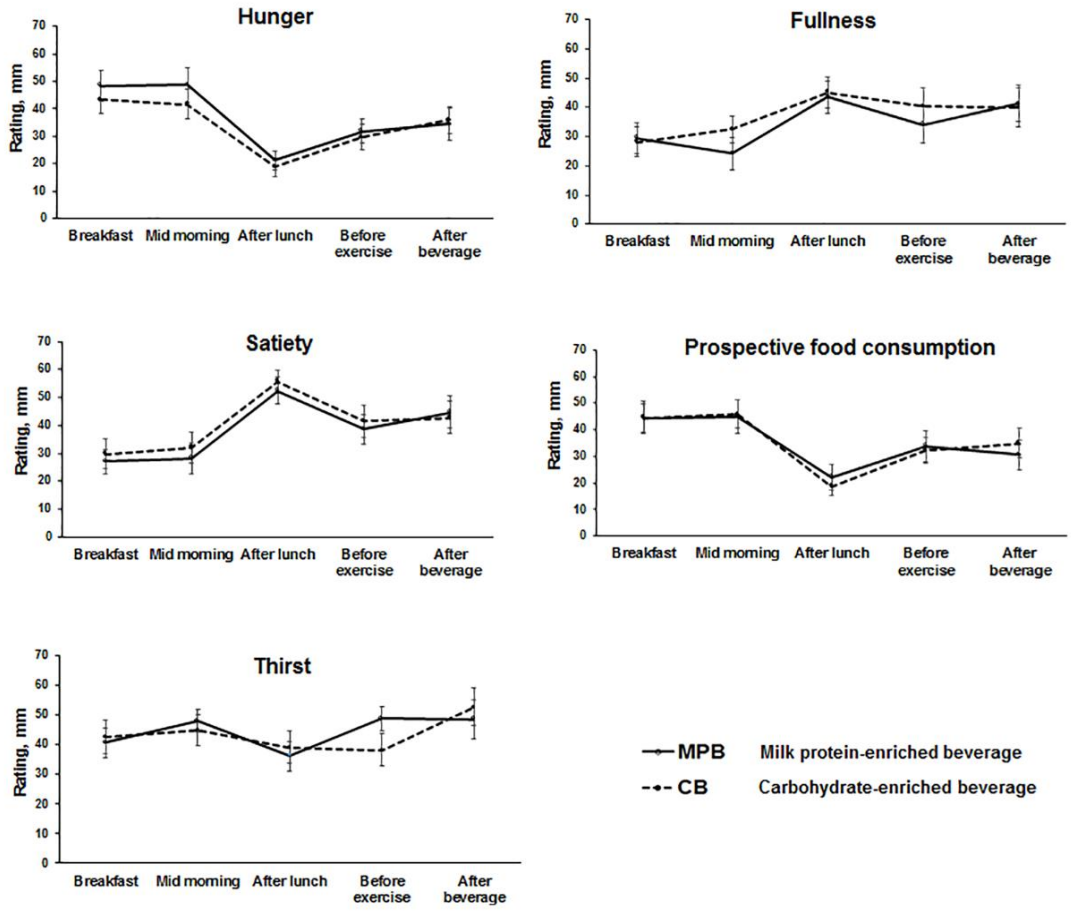

Figure 2: mean scores of appetite (hunger, fullness, satiety and prospective food consumption) sensations and thirst recorded by the participants (n=20) over the three experimental days when they drank MPB or CB.

No significant difference for any of appetite sensations or thirst at any moment of the day before and after the consumption of each type of beverage was found.

However, considering individual daily energy intake during the time period before the exercise/beverage separately from the energy intake after the exercise/beverage, data showed that one group of 9 participants out of 20 ate significantly less (-10% vs +8% daily energy intake, p=0.01) when they had MPB after exercise than CB. The same participants were also significantly less hungry (-9% vs +15%, p=0.03) and ate later (208 min vs 127 min, p=0.03) over the post-exercise period after MPB compared to CB; conversely, they did not show any difference for fullness, satiety and prospective food consumption. Those 9 participants were named Responders (R) whereas the other 11 were the Non-Responders (NR) hereinafter.

By a post hoc power analysis we calculated that 9 participants in this study were enough to guarantee a power of 86.8% with an α-error of 0.05, and 2-sided testing.

Figure 3 and **Figure 4** show energy intake and hunger before and after both beverages in R and NR. No difference of total daily energy intake between R and NR when they drank MPB or CB was found.

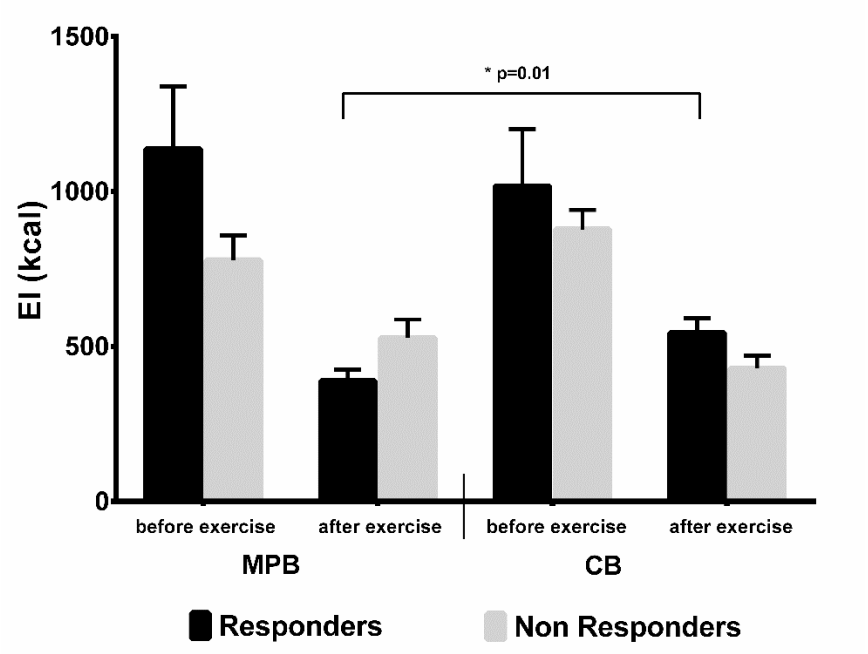

Figure 3: Energy intake (EI) of Responders (n=9) and Non-Responders (n=11) before and after post-exercise consumption of MPB or CB (line over the bars indicate significant difference $p<0.05$ between MPB and CB in Responders after exercise).

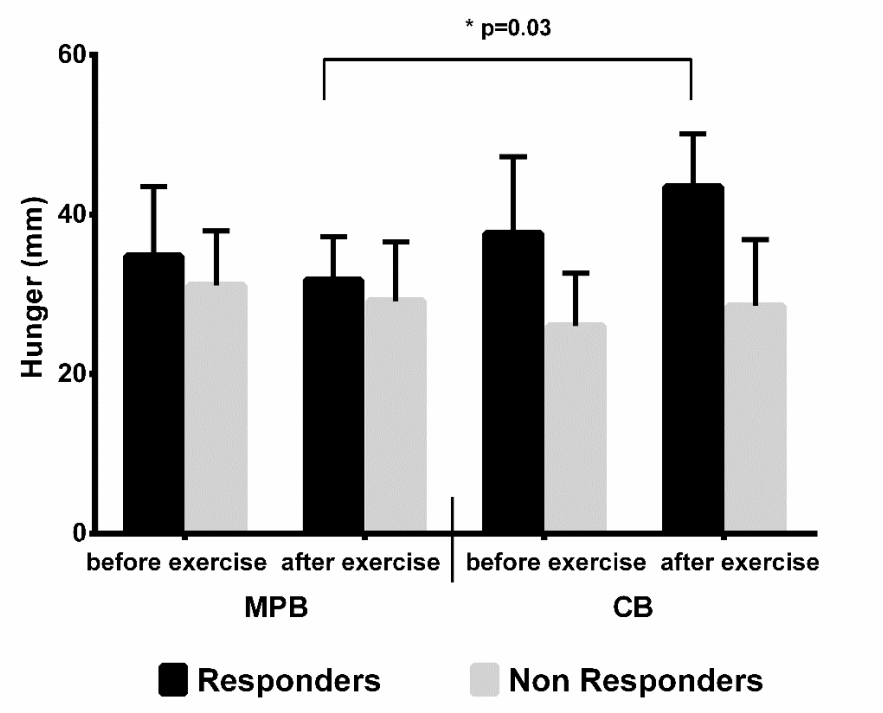

Figure 4: Hunger rating of Responders (n=9) and Non-Responders (n=11) before and after post-exercise consumption of MPB or CB (line over the bars indicate significant difference $p<0.05$).

Data showed that NR compensate the calorie intake over the time period following exercise and beverage consumption. The higher EI at the meals (mainly dinners) over the evenings after MPB than CB was attributable to lower energy intakes at the meals (mainly lunches) before exercise time on the experimental days when NR drank MPB compared to CB (346.3 kcal MPB vs 437.5 kcal CB, $p=0.02$). On the contrary, R subjects had similar energy intakes during the time period before the consumption of MPB and CB (558.2 kcal MPB vs 526.3 kcal CB, $p=0.59$), therefore their reduction of caloric intake during the period following the post-exercise consumption of MPB was not due to a compensation effect.

3.3 Thirst

No significant differences for thirst sensation of all subjects over the test day (before breakfast, midmorning, before lunch, before exercise, after exercise/beverage) with CB and MPB (0.89 ± 1.43, $p=0.54$) were found. However, within R group, thirst sensation post-exercise and after CB consumption significantly increased compared to the pre-exercise time ($p<0.001$) whereas when subjects drank MPB, no significant difference of thirst before and after exercise was found ($p=0.53$). This finding was likely to be due to a lower thirst of R subjects before exercise over the days they

had CB compared to MPB (p=0.01). No differences of thirst in NR group neither before or after beverage consumption were found.

3.4 Beverage liking

Data about beverage liking for sweetness, acidity, fatty taste, consistency, pleasantness and overall palatability showed no differences between MPB and CB when all the participants or the two groups separately (R and NR) were considered (p>0.05). Therefore, the hedonic value of beverages could not have influenced the results of hunger or energy intakes at dinner in both R and NR.

3.5 Characteristics of Responders and Non-Responders

Table 5 shows general characteristics of population categorized as R and NR. Data showed that the two subgroups of subjects differed only for their eating behaviour. In fact, R showed a higher mean dietary restraint factor than NR i.e. R used to cognitively control their food intake compared to NR. In particular, the 9 R subjects showed a restraint score between 12 and 18 whereas among 11 NR, 8 subjects showed a restraint score in the range 5 - 11 while the remaining 3 subjects showed a score of 12, 15, and 16.

Table 5. General characteristics of R and NR (* p<0.05 vs NR)

FEATURES	RESPONDERS	NON-RESPONDERS
Number	9	11
Age (years)	28±9	21±2
Body weight (kg)	59.1±6.3	57.1±6.3
BMI (kg/m^2)	22.0±2.0	21.0±1.9
F1 (Restraint)	14.4*±2.4	10.2±3.6
F2 (Disinhibition)	5.1±2.3	7.0±3.4
F3 (Hunger)	2.7±3.5	4.7±4.5
MET (min/week)	1233.8±154.2	1152.0±99.2

4. Discussion

In this study, a new protein milk rich beverage was developed and tested for its effects on satiety in women post-exercise. Data showed that 9 out of the 20 participants felt significantly less hungry and ate less and later when they drunk MPB compared to CB after exercise. These subjects were called Responders and scored higher Restraint value in the Three-Factor Eating Questionnaire than the other participants (Non-Responders).

Previous studies showed an energy compensation effect after consumption of milk-based drinks offered to the participants in different volumes (Rolls & Roe, 2002). In the present study, the two tested beverages were isocaloric, the volume of administration was fixed (250 mL) and only women at the same phase of menstrual cycle participated into the study. A further strength of the study was the repeated consumption of the beverages for three-times during a three-week period while in previous studies beverages were tested by a single-acute consumption (Burton-Freeman, 2008; Chung Chun Lam, Moughan, Awati, & Morton, 2009; Desbrow et al., 2014; Hall, Millward, Long, & Morgan, 2003; Hursel, van der Zee, & Westerterp-Plantenga, 2009; Nieuwenhuizen et al., 2009; Shirreffs, Watson, & Maughan, 2007; Veldhorst et al., 2009a; Veldhorst et al., 2009b).

Previous literature let us to hypothesize that MPB could increase satiety because of the high content of milk proteins including both caseins and whey proteins. The satiating effect of caseins could be induced by the hormonal response triggered by the circulating amino acids derived from digestion (Martens & Westerterp-Plantenga, 2014; M. Veldhorst et al., 2008; Veldhorst et al., 2009b). On the other hand, whey proteins were found more effective on satiety when compared to carbohydrates (Bellissimo et al., 2008; Bertenshaw, Lluch, & Yeomans, 2008). That effect was associated to whey content of the amino acids (Chungchunlam, S. M., Henare, S. J., Ganesh, S., & Moughan, 2016), as well as to glycomacropeptide (Chung Chun Lam et al., 2009) and α-lactalbumin which are able to enhance serum tryptophan (Nieuwenhuizen et al., 2009; Orosco et al., 2004), the precursor of serotonin (Schellekens et al., 2014). Serotonin could act on brain reward centres and reduce food intake by modulating food wanting (Born, Martens, Lemmens, Goebel, & Westerterp-Plantenga, 2013; Fromentin et al., 2012; Journel, Chaumontet, Darcel, Fromentin, & Tome, 2012).

Data of this study suggested that the MPB consumption (providing 23 g milk proteins compared to the 0.8 g of CB) reduced individual energy intakes in R by modulating mechanisms underpinning both energy and reward homeostasis. Moreover, data showed that individual restraint status was a key factor in satiating effect induced by MPB vs CB.

The psychometric characterization of participants showed that those subjects having a more restrained attitude, i.e. a stronger control of their eating behaviour, were more susceptible to the satiating effect

of milk proteins compared to carbohydrates. That may explain the discrepant findings between literature studies on the satiating effect of dairy proteins (Bertenshaw et al., 2008; de Graaf, Hulshof, Weststrate, & Jas, 1992; Douglas, Ortinau, Hoertel, & Leidy, 2013; Marmonier, Chapelot, & Louis-Sylvestre, 2000) or between men and women into the same study (Burton-Freeman, 2008; Chung Chun Lam et al., 2009). Chung Chun Lam and co-workers (2009) found that the consumption of ~40 g of whey proteins modified dietary behaviour in women participating into the study but not in men; the restraint score of those women was higher (double) than men. That women are more restrained than men is what is usually occurring among people. Pohle-Krauza et al. (2008) showed that a rigid restraint status modulated the satiety response to phenylalanine possibly through effects of reproductive hormones (Pohle-Krauza et al., 2008). In the present study, women during all the menstrual cycle (the study lasted for three weeks and started the week after the menstruation) were involved, whereas the differences in the restraint status were a clear cut factor determining the satiating effect of MPB *vs* CB. This hypothesis is also supported by a fMRI study demonstrating that individual cognitive restraint was associated with inhibition of brain responses to hedonic food stimuli (Hollmann et al., 2012). Independent evidence suggested that cognitive control played a pivotal role to counterbalance a lack of homeostatic mechanisms or to reinforce physiological reduction of food wanting induced by high protein intake through the reward system. This is a key concept to implement personalized nutrition strategies and consumer-centred design of new food. More restrained people usually have a higher BMI than less restrained people because they use to alternate periods of excessive food consumption with periods of restriction, during which they try to monitor and regulate food intake through self-imposed rules (Bublitz, Peracchio, & Block, 2010). A milk protein-enriched beverage is more effective for energy control in this type of subjects and it may be a useful functional food for their body weight management.

Data of the present study also showed in responders subjects the ability of MPB to maintain post-exercise thirst similar to that perceived before exercise compared to CB despite the supposed dehydration induced by exercise. This finding was in line with literature studies showing that milk or milk-based beverages were more effective for post-exercise rehydration than traditional sports drinks and plain water (Shirreffs et al., 2007; Watson, P., Love, T., Maughan, R., and Shirreffs, 2008) or in comparison with carbohydrate–electrolyte drinks (Desbrow et al., 2014). Moreover, James et al. (2012) demonstrated that by replacing 2.5% of a 6% carbohydrate drink with milk proteins (and maintaining constant the energy density and electrolyte content of the beverages) an increased fluid retention was obtained (James et al., 2012).

It is worth of notice that in this study the subjects did not have similar thirst before exercising on the experimental days with MPB and CB, thus the initial thirst might have exalted the effect of MPB on thirst.

The main limitations of this study were the lack of measurements of individual hormonal and neural signals as well as of hydration status during the experiments. The data available allowed us to hypothesize the mechanisms underlying satiety effect and thirst according to previous literature without clarifying the physiological pathways. On the other hand, the study design here adopted and based on a free living condition optimized the reliability of the findings compared to the laboratory environment that would have been necessary for studying the physiological mechanisms underpinning satiety and thirst.

5. Conclusions

In conclusion, compared to a carbohydrate rich beverage the milk protein-enriched beverage here developed was able to influence hunger sensation and reduce energy intake at subsequent meal in women having a strong cognitive control of eating behaviour in order to regulate their food intake for body weight control. In few words, data suggested that a milk protein-rich product may be useful to control food intake in dietary restrained women. Future studies should evaluate the effect on appetite, energy intakes, body weight and/or body composition of a long-term consumption of MPB in overweight/obese subjects with different eating behaviour.

6. Conflict of interest

The authors declare no conflict of interest. EB and RD are employees of a dairy company.

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Chapter 5

Bioactive peptides and amino acids from casein and soy enhance barrier integrity in a Caco-2 model of intestinal inflammation

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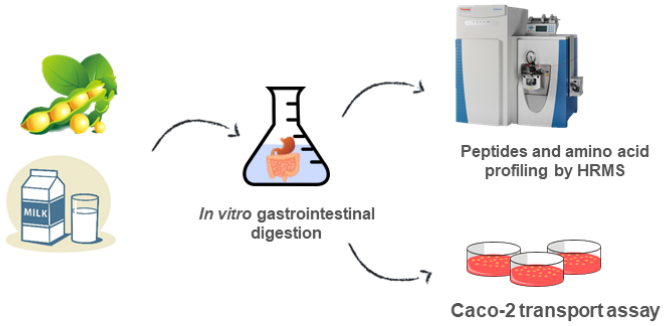

Bioactive peptides/amino acids may regulate the intestinal barrier structure/functions and restore epithelial cells' properties

Paper in preparation for submission

Abstract

Many oligopeptides coming from digestion of dietary proteins show opioid activity by acting as effectors of μ-opioid receptors (MORs). MORs are widely expressed in the gastrointestinal tract (GIT), especially the small intestine. β-casomorphins and soymorphins are a group of peptides with opioid properties arising by proteolytic digestion of β-casein and β-conglycinin β-subunit of soy respectively.

To act systemically the bioactive peptides (BAPs) should be absorbed from the GIT and then reach the bloodstream in an active form. Activity inside the GIT may include induction of mucus secretion, analgesic role, and delay of bowel transit time.

A gap of knowledge still exists on the effects of peptides/amino acids coming from digestion of casein and soy protein hydrolysates on barrier permeability in a condition of inflamed intestine. In this study, casein (CH) and soy (SH) protein hydrolysates were subjected to an *in vitro* simulated gastrointestinal digestion (SGID). Digested samples were administered to differentiated intestinal epithelial cells (Caco-2) in a normal condition and in an inflamed condition that was obtained by TNF-α administration into the culture. Markers of monolayer integrity (TEER) and cells viability (MTT and SRB) were measured in cell culture.

Results of the assays demonstrated that the administration of CH and SH did not have toxic effects on cells and determined a response that was compatible with a full functionality of the cells.

In conclusion, this study demonstrated that both hydrolysates may be protective towards intestinal inflammation, possibly acting on tight-junctions.

1.Introduction

Caco-2 cells derived from human colon adenocarcinoma are a well-established model to evaluate the intestinal permeability and physiological response to food-derived peptides (Vieira et al., 2017).

Upon a 3 weeks cultivation, the Caco-2 cells undergo a spontaneous differentiation leading to the formation of a cell monolayer displaying several morphological and functional characteristics of a mature enterocyte like microvillus structure, carrier-mediated transport system for di/tri-peptides, amino acids and glucose, tight junctions at the apical side and expression of several kinds of brush-border enzymes. Therefore, Caco-2 cells can mimic all functions of the intestine epithelium including nutrient absorption (Yang et al., 2016)**.**

Dietary peptides can be directly absorbed or digested in the duodenum by pancreatic peptidases to form smaller peptides and/or amino acids. Some peptides are able to modulate intestine functions including nutrient absorption (Capriotti et al., 2016) such as casein phosphopeptides from milk that enhance mineral absorption, and soybean peptides suppressing dietary cholesterol absorption. Other peptides namely opioid peptides (including beta-casomorphin-7 and soymorphin-5,-6,-7) can regulate intestinal motility and glutamine-containing peptides can even prevent and/or repair damage caused by oxidative stress and inflammatory reactions (De Noni 2009,. Kaneko et al., 2010, Shimuzu et al., 2017)

The human intestine allows the absorption of nutrients while also functioning as a barrier, which prevents antigens and pathogens entering the mucosal tissues and potentially causing disease. Increased intestinal permeability is implicated in autoimmune, inflammatory, and atopic diseases, which can manifest both locally (within the intestinal mucosa) and systemically.

A chronic inflammatory condition of the intestine, as found in inflammatory bowel disease and celiac disease, is characterized by a leaky intestinal barrier (Suenaert et al., 2002). Physiological conditions like stress have also been shown to increase epithelial permeability. The intercellular junctional complex has a crucial role in the maintenance of barrier integrity that can be deranged by pro-inflammatory cytokines, such as as tumour necrosis factor-α (TNF-α). It can increase epithelial tight junction (TJ) permeability and contribute to intestinal inflammation in Crohn's disease and other inflammatory conditions (Ye et al., 2006; Ulluwishewa et al., 2011). Soybean proteins have been considered as a functional food ingredient. Particularly, the peptides from β-conglycinin could be absorbed by Caco-2 cell monolayers and the permeates could exert antioxidant and cytoprotective effects (Amigo-Benavent et al., 2014).

In recent years, studies have established the important roles of glutamine in regulating the functions of TJ proteins (Yang et al., 2017). In human Caco-2 cells, glutamine deprivation or inhibition of glutamine synthetase significantly decreased the trans-epithelial resistance and the expression of TJ

proteins, and improved intestinal immunity (Ren et al., 2014a, 2014b, 2014c; Yin et al., 2014). The intestinal permeability can be modulated by proteins and amino acids. For example, an arginine-rich protein decreases whereas L-alanine increases para-cellular flow (Jochems et al., 2018). Deficiency in amino acids such as tryptophan, arginine, glutamine, and cysteine can reduce immune cells activation. These amino acids have been shown to play unique roles in maintaining the integrity, growth, and function of the intestine, and in normalizing inflammatory cytokines secretion (Kong et al., 2018)

The trans-epithelial transport routes of peptides vary for different peptides. Three major mechanisms for peptide transport have been identified in the intestine: i) peptide transporter-1 (PepT1), mediating the transport for di- and tri-peptides, ii) paracellular passive transport, via intercellular junctions and iii) transcytosis (Daniel & Zietek, 2015). Dipeptidyl peptidase IV (DPP-IV), represents the rate-limiting enzymes in the GIT for bioactive peptides absorption as it can finalize their hydrolysis thus (thus inactivating them) or even generate new ones (Daniel et al, 2004; Röhrborn et al., 2015). Several distinctive factors, such as the molecular size, hydrophobicity and charge, influence the permeability of peptides across the intestinal epithelium (Wang & Li 2018).

Recent findings in Caco-2 cell lines demonstrated that amino acids and peptides may exert anti-inflammatory activity and protect intestinal barrier functions, by blocking TNF-α-induced inflammatory responses through allosteric activation of calcium-sensing receptor (Zhang & Mine 2018).

In this framework a gap of knowledge still exists on the effects of peptides/amino acids coming from digestion of casein and soy protein hydrolysates on barrier permeability in a condition of inflamed intestine.

In this study, casein (CH) and soy (SH) protein hydrolysates were subjected to *in vitro* simulated gastrointestinal digestion (SGID). Digested samples were administered to differentiated intestinal epithelial cells (Caco-2) in a normal condition and an inflamed condition that was mimicked by TNF-α administration. Markers of cell monolayer integrity (TEER) and cells viability were measured in cell culture.

2. Materials and methods

2.1 Protein hydrolysates

Casein (CH) and isolate soy protein (SH) hydrolysates were provided by a. Costantino & C. spa (Favria, Torino, Italy).

Nutrition facts of both ingredients are shown in **Table 1**.

Table 1. Nutrition facts of casein (CH) and isolate soy protein (SH) hydrolysates.

Composition g/100 g	CH	SH
Proteins	85	83.7
Carbohydrates	8.1	3.0
Total fats	ND	4.3
of which saturated	ND	31.8%
Fibers	0.7	1.1
Sugars	ND	ND
Salt	3.5	4.88
Energy value (kcal/kJ)	374 kcal/1588 kJ	388 kcal/1642 kJ

2.2 Protein hydrolysate characterization

Amino acid profile of the protein hydrolysates was determined by Liquid chromatography–high-resolution mass spectrometry (LC/HRMS) as previously described by Troise et al., (2015), whereas the profile of food-derived BAPs was determined by a newly developed LC-Orbitrap-high-resolution mass spectrometry (HRMS) method.

Hydrophobic peptides-markers targeted for a simultaneous detection were β-casomorphin-7, arising from β-casein of bovine milk, and Soymorphin-5,-6,-7, coming from β-conglycinin β-subunit of soy.

Both casein and soy protein hydrolysates were suspended in water, centrifuged at 4 °C, for 15 min at 21100 g and 100 µL was spiked with a known amount of internal standard (BCM-7, 100 ng/ml final concentration, Bachem , Zurich, Switzerland). Food samples were diluted up to 1 ml and loaded onto Oasis HLB 30 mg cartridges (Waters, Wexford, Ireland) previously equilibrated with 1 ml of each acetonitrile and 0.1% formic acid in water. Cartridges were washed twice with 0.1 % formic acid and BCMs and hydrophobic bioactive peptides were eluted with 1 ml of 0.1% formic acid in acetonitrile. Collected samples were dried by using a Savant centrifugal evaporator (Thermo Fisher Scientific, Bremen, Germany). Finally, samples were dissolved in 100 µL of water and 5 µL was injected. Chromatographic separation of hydrophobic-BAPs was achieved by using an Accela 1250 U-HPLC (Thermo Fisher, Bremen Germany) equipped with a Luna Polar C18 column (50 x 1.0 mm, 1.6 µm, Phenomenex, Torrance, CA) and a guard column of the same phase, both at 40° C. Mobile phases consisted of 0.1% v/v formic acid (A) and 0.1% v/v formic acid in acetonitrile (B) with the following gradient (min/%B): (0/2), (0.50/2), (4.5/90), (5.5/90). The flow rate was 100 µl/min and the column temperature was 40°C. The U-HPLC system was directly interfaced to an Exactive Orbitrap HRMS (Thermo Fisher Scientific, Bremen, Germany) and analytes were detected through a heated electrospray interface (HESI-II) operating in the polarity switch mode and scanning the ions in the

m/z range of 60–1300. The resolving power was set to 75000 full widths at half maximum (FWHM, *m/z* 200) resulting in a scan time of 1 s.

The recovery test was performed by spiking hydrolysates with a known amount of BCM-7 (final concentration 0.5, 50, 500 ng/ml), a fixed amount of internal standard and taking into account the overestimation due to BCMs already present in the milk casein hydrolysate samples. Recovery was calculated plotting the area of the current associated to each *m/z* of target analyte in the matrix towards the concentration of the standard in the calibration curve.

The tolerance range for mass accuracy of BCMs (i.e. the experimental mass of each analyte had to fall within the maximum permitted tolerance) was fixed at 5 ppm (Michalski et al., 2012). Data were recorded and analysed using Xcalibur software version 2.2 (Thermo Fisher Scientific, Bremen Germany).

2.3 Simulated gastrointestinal digestion

In vitro gastrointestinal digestion was performed on 1 g of **CH** and **SH** respectively, according to Minekus et al., (2014). Each sample was digested in triplicates and a blank sample (including all digestion fluids without hydrolysate) was run in parallel.

At intestinal phase, an aliquot from each sample was collected and centrifuged at 2500 g for 10 min at 4°C. Supernatants were collected, separated and diluted (1:10) with methanol, centrifuged at 21100 g for 5 min at 4°C and dried with nitrogen flow.

Samples were stored at -25°C until analysis.

2.4 Cell culture

The human colon adenocarcinoma cell line (Caco-2) obtained from American Type Culture Collection were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with Glutamax (Gibco, Life Technologies, Carlsbad, CA, USA), 10% heat-inactivated fetal bovine serum (FBS, Greiner Bio One, Wemmel, Belgium), 1% penicillin–streptomycin (Pen Strep, Gibco, Life Technologies) and 1% non-essential amino acids (NEAA, Gibco, Life Technologies) (growth medium). The cells were grown in 75 cm^2 canted neck tissue culture flasks (Sarstedt Co., Essen, Belgium) and incubated at 37 °C in a humidified atmosphere of 10% CO_2 in air (Memmert CO2 incubator, Memmert GmbH & Co., Nurnberg, Germany). The medium was replaced three times per week and cells were subcultured at 80-90% confluence using trypsinisation. Briefly, the cells were washed with 4 mL PBS (Gibco, Life Technologies) and were detached from the bottom of the flask

with 2 mL of 0.25% trypsin-EDTA wash (Sigma-Aldrich, Steinheim, Germany) and subsequently incubated at 37 °C and 10% CO2 in air for 3–5 min.

A volume of suspension, depending on the cell concentration and passage number, was seeded to new flasks, and the final volume was made up to 4 mL with the DMEM medium with Glutamax, 10% FBS, 1% NEAA, and 1% Pen Strep (subculturing procedure).

2.5 Measurement of cytotoxicity

Caco-2 cells were seeded in 96-well plates, allowed to differentiate for 14 days after confluence.

Each digested sample was previously re-suspended in distilled water, filter sterilized (Millex-GP, 0,22 µm, Merck) and diluted (1/25) in exposure medium (DMEM supplemented with 1% Pen Strep, 1%NEAA).

Digested and diluted CH and SH (1/25) as well as blank of digestion (all digestion fluids without ingredients, DigBlank) were administered to Caco-2 cells.

To analyse the cytotoxic effects of digested samples on Caco-2 cells after 4 hours of treatment, the 3- (4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay was applied.

Briefly, 200 µL of MTT (Sigma-Aldrich) dissolved in PBS (5 mg/mL) was added to cells and incubated for 2 h at 37°C to convert MTT to formazan. After 2 h, the medium was removed, formazan crystals were dissolved in DMSO, and absorbance was recorded at 570 nm with a Bio-Rad multiplate reader (Bio-Rad Laboratories, Hercules, CA, USA). Furthermore, the measurement of cellular protein content was evaluated using the SRB assay as described in De Vreese et al. (2016). MTT and SRB assays were performed with 5 biological replicates (5 wells per condition).

Control wells (untreated cells, exposure medium only) were included in the plate design.

The results for both assays were expressed as percentage compared to untreated cells (viability).

The same conditions described above were used when the inflammatory-condition was mimicked. Briefly Caco-2 cells were seeded in 96-well plates, allowed to differentiate for 14 days after confluence. TNF-α (10 ng/mL) was prepared in exposure medium and administrated to cells (1h). After 1h, the cells were rinsed and rested in exposure medium (1h) and then treated with digested sample (4h). Cytotoxicity was evaluated with MTT and SRB assay. Control wells (untreated cells, exposure medium only) were included in the plate design.

The results for both assays were expressed as percentage compared to untreated cells (viability).

2.6 Transepithelial transport study

Caco-2 cells were grown and trypsinized as described above, suspended in growth medium and seeded in 12-Transwell ® plates (0.4 µm pore diameter, 12 mm insert, Elscolab, Kruibeke, Belgium)

at a density of 10^6 cell/well, with 500 µL of growth medium in the apical side and 2 mL of growth medium in the basolateral side. Cells were allowed to grow and differentiate to confluent monolayers for 21 days post seeding. Medium was changed three times per week. Passage numbers of the cells used in this study were less than 50.

Transepithelial electrical resistance (TEER) was measured using an automated tissue resistance measurement system (REMS, World Precision Instruments, Hertfordshire, UK), to ensure that the monolayers exhibit the properties of a tight biological barrier. Only monolayers with a TEER value higher than 400 $\Omega \cdot cm^2$ were used for the experiments (Wu et al., 2017).

The experimental design of transport assay is shown in **Figure 1.**

Digested CH and SH and DigBlank were suspended, filter sterilized (Millex-GP, 0,22 µm, Merck) and diluted (1/25) in exposure medium and then administered at the apical side (500 µL) of Caco-2 cells. Untreated cells (cells in culture medium, without digested samples) were used as control.

In order to investigate the cellular responses under high-grade inflammation-induced conditions, Caco-2 cells were preincubated for 1 h with TNF-α (10 ng/mL exposure medium) at basolateral side. A healthy/control batch (without TNF-α) were included in the study design (**Figure 1**).

Monolayer integrity was tested by TEER before treatments (day 1, growth medium), before digested treatment with/without TNF-α (day 2, exposure medium). Then the exposure medium was replaced by growth medium, and the monolayers were incubated (24h) in order to measure irreversible damage to the cell monolayer (day 3) (**Figure 1**).

Transport experiments were performed with 4 biological replicates (4 wells per condition) whereas TEER of each well was measured with 3 technical replicates.

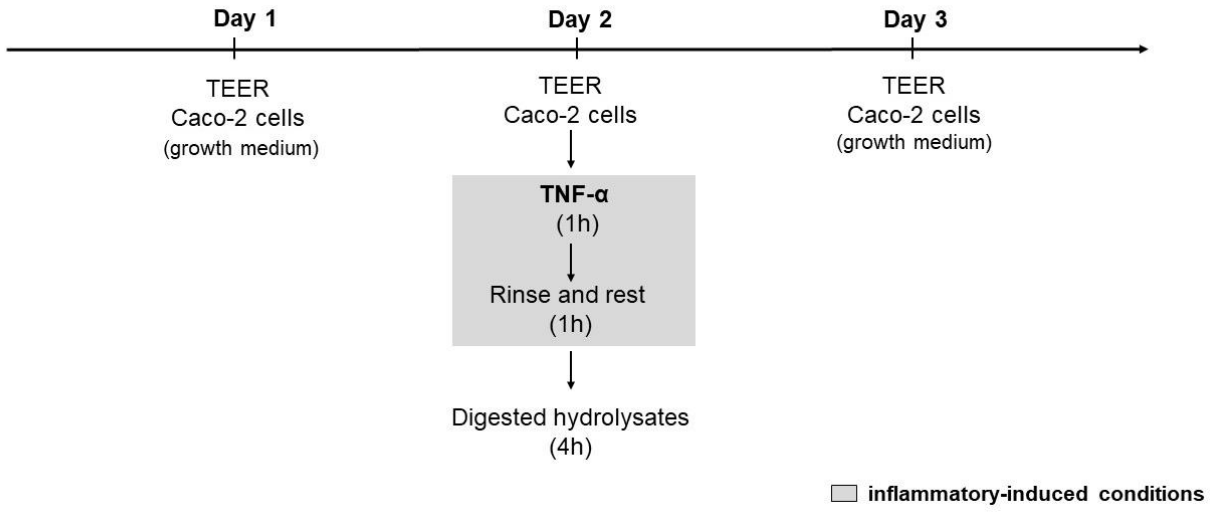

Figure 1. Experimental design of transport assay in healthy/inflamed condition

2.7 Statistical analysis

MTT and SRB analyses were performed with 5 biological replicates (5 wells per condition).
TEER and transport experiments were performed with 4 biological replicates (4 wells per condition) and 3 technical replicates (3 times each well).
Data are expressed as mean values ± standard deviation (SD), whereas error bars in all the figures of MTT and SRB indicate relative standard deviation (RSD).
To determine differences between treatments, one-way ANOVA followed by a Tukey post hoc test ($p < 0.05$) was applied using SPSS software (version 21.0, SPSS, Inc. Chicago, IL, USA).
Statistical significance was set at a level of $\alpha = 0.05$ (5%).

3. Results

3.1 Amino acid profile determination

Amino acid (AA) profile of casein and soy protein hydrolysates after SGID were evaluated.
Figure 2 shows the AA content of CH and SH at each step of digestion (both gastric and intestinal phase). Figure 2 A and B shows the overall amino acid profile of digested CH and SH respectively, whereas Figure 2 C and D include essential amino acid (EAA) of digested CH and SH respectively. AA content of each hydrolysate at each digestion step is shown as Mean (ng/ml) ±standard deviation (SD).
Data show a trend of reduction at intestinal phase vs gastric phase of most of AA. Conversely, an increase of γ-amino butyric acid (GABA) at intestinal phase of CH was recorded (**Figure 2 A**), whilst lysine and arginine of SH increase at intestinal phase (**Figure 2 B and C**).

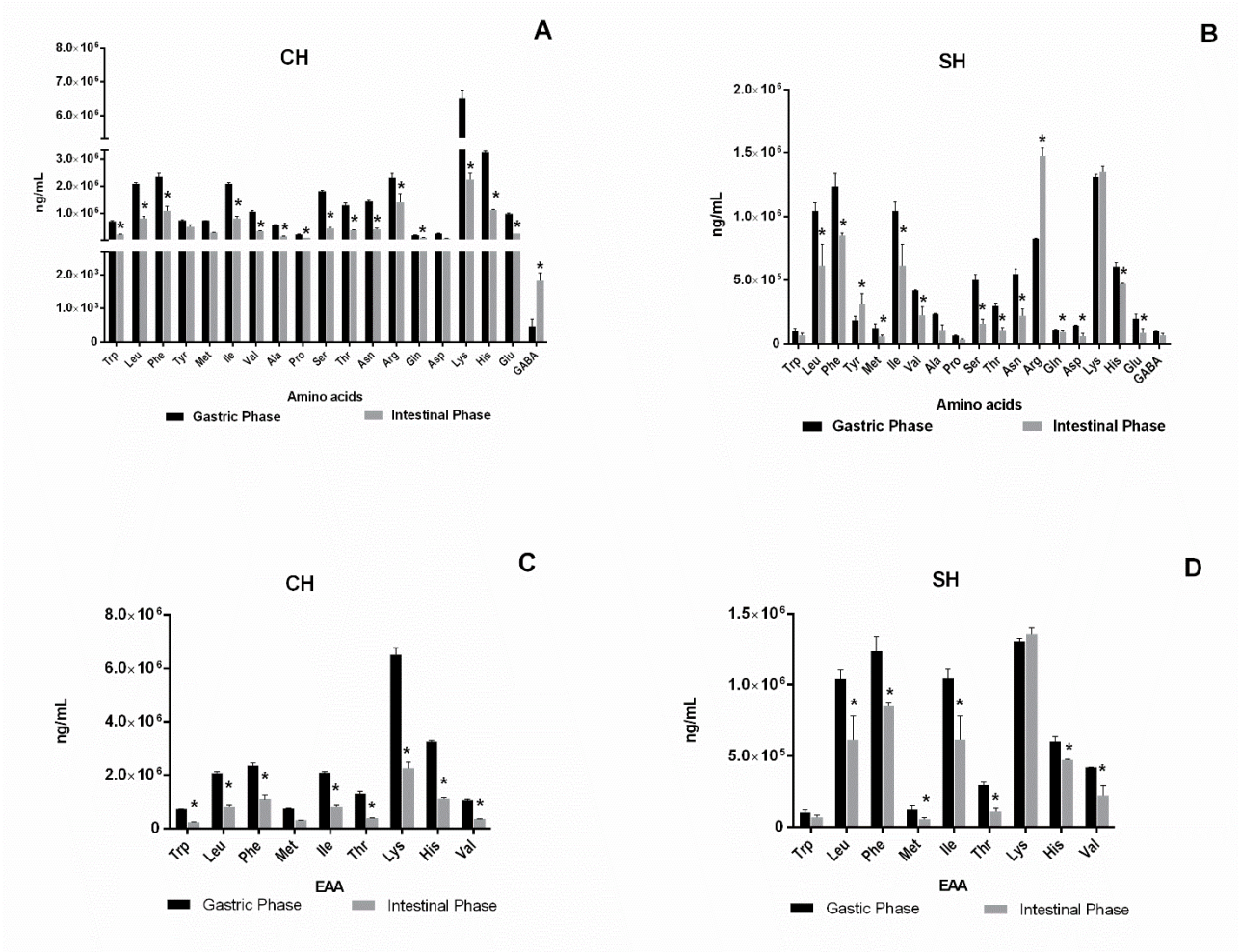

Figure 2. Amino acid (AA) profile of casein and soy protein hydrolysates after SGID. Data are shown as mean ±standard deviation (SD). Statistical differences considering gastric vs intestinal phase for each AA was reported as * ($p<0.05$). A: AA profile of digested CH; B: AA profile of digested SH; C and D: zoom on essential amino acid (EAA) of CH and SH respectively.

3.2 Hydrophobic bioactive peptides detection

Hydrophobic biomarker β-casomorphin-7 (BCM-7) and soymorphin-5,-6,-7 were identified, and their fate during SGID were characterized.

Figure 3 showed the fate of BCM-7 at each step of digestion. Data are represented as mean (g/ml) ±standard deviation (SD).

Particularly, an increase of rate of BCM-7 was recorded from hydrolysate (CH) to gastric phase (84,6%) as well as at intestinal phase (up to 23,8% then gastric phase).

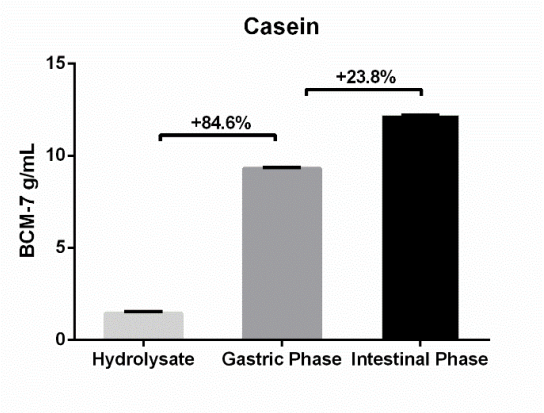

Figure 3. Fate of BCM-7 at each step of digestion.

Data relative to soymorphin-5,-6,-7 are shown as the mean value of area ± SD (**Figure 4**).

An overall reduction at each step of digestion of soy-biomarker was recorded when compared to hydrolysate initial content. Particularly, soymorphin-7 was completely hydrolysed at intestinal phase when compared to hydrolysate, whereas soymorphin-6 increased at gastric phase and then a reduction was observed at intestinal phase.

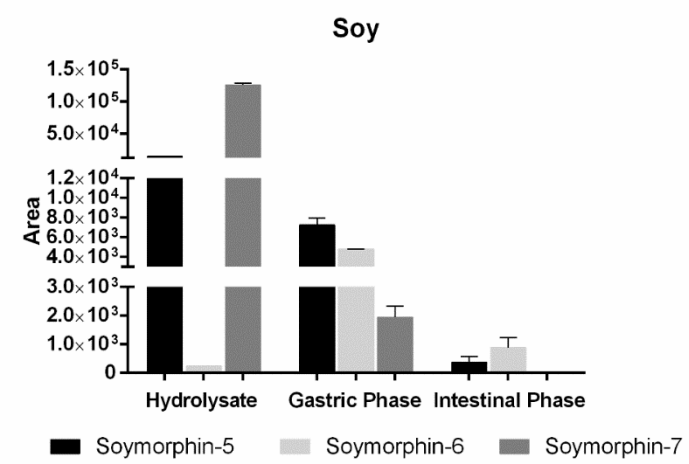

Figure 4. Fate of soy-biomarker at each step of digestion.

3.3 Cytotoxicity

Results of MTT (**Figure 5**) and SRB (**Figure 6**) assays on differentiated Caco-2 cells over 4h of treatment with digested protein hydrolysates in healthy/inflamed condition are shown as percentage of Viability ± Relative standard deviation (RSD).

Data of MTT and SRB assays show that the administration of CH and SH had not toxic effects on cells.

Figure 5 shows that the administration of Casein hydrolysate at dilution 1/25 (CH) and soy hydrolysate 1/25 (SH) positively influenced cells viability particularly in inflamed cell condition**.** **Figure 6** shows, a significant decrease of protein content of the cells treated with CH (vs the untreated ones) in inflamed condition; data from MTT assay excluded that the observed reduction was due to cell death.

Protein content of the healthy cells were significantly influenced by SH probably due to a more efficient uptake of peptides and amino acids coming from *in vitro* digestion **(Figure 6).** MTT data confirmed a very good cell viability over the experiments.

Digested casein and soy protein hydrolysates positively influenced cells viability. It seemed that Caco-2 became metabolically more active when they were treated with digested hydrolysates in inflamed condition (**Figure 5**).

This data suggest a possible protective role of both digested hydrolysates in an inflamed condition was mimicked.

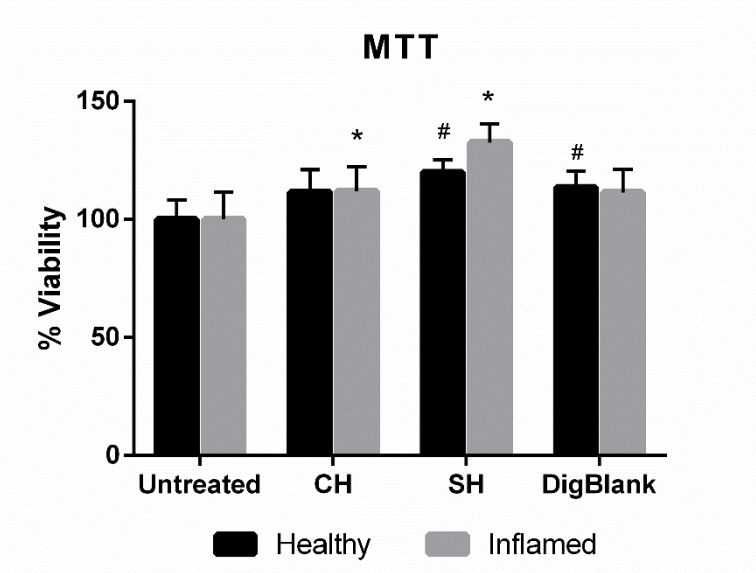

Figure 5. Results (Viability ±RSD) of MTT assay after 4h of incubation in healthy (black bars) and inflamed condition (grey bars). Abbreviations: digested casein protein hydrolysates (CH), digested soy protein hydrolysate (SH), blank of digestion (DigBlank) all administered at dilution of 1/25. Untreated cells (Untreated, only medium).
Statistical differences compared to untreated cells in healthy conditions are denoted as # ($p < 0.05$) whereas statistical differences compared to untreated cells in inflamed conditions are denoted as * ($p<0.05$)

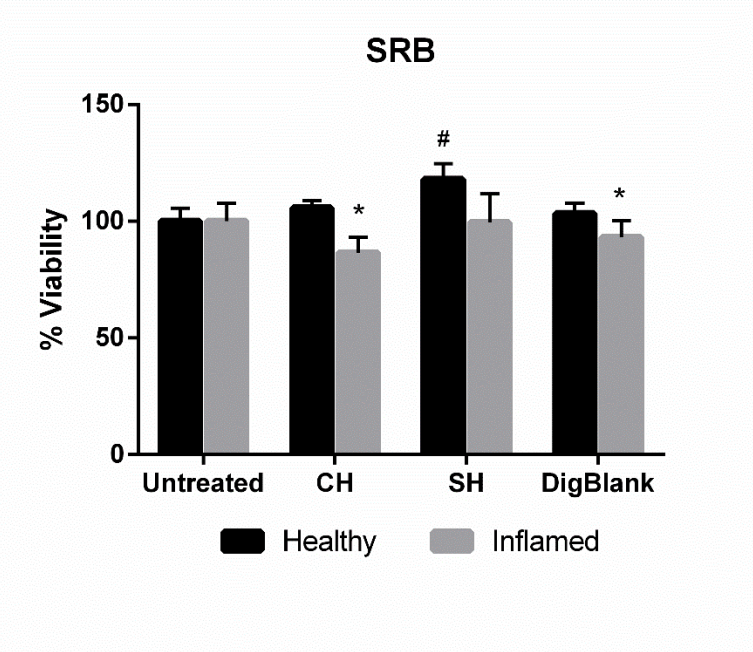

Figure 6. Results (Viability ±RSD) of SRB assay after 4h of incubation in healthy (black bars) and inflamed condition (grey bars). Abbreviations: digested casein protein hydrolysates (CH), digested soy protein hydrolysate (SH), blank of digestion (DigBlank) all administered at dilution of 1/25. Untreated cells (Untreated, only medium).
Statistical differences compared to untreated cells in healthy conditions are denoted as # ($p < 0.05$) whereas statistical differences compared to untreated cells in inflamed conditions are denoted as * ($p<0.05$).

3.4 TEER

Table 2 shows TEER ($\Omega \cdot cm^2$) values of Caco-2 cells treated with digested casein and soy protein hydrolysates (CH and SH respectively, dilution 1/25) and blank of digestion (DigBlank 1/25) during different experimental days, whereas TEER values of inflamed condition are reported in **Table 3**. Data are shown as **Mean ± SD**; numbers in brackets represent the TEER values relative to the initial TEER value, expressed as percentages. Different letters in the rows represent statistically significant differences ($p < 0.05$).

Table 2. TEER ($\Omega \cdot cm^2$) evaluation of Caco-2 cells treated with digested casein and soy protein hydrolysate in **healthy condition**. Different letters in the rows represent statistically significant differences ($p < 0.05$).

	Day 1	**Day 2**	**Day 3**
Untreated cells	1952.3±31.1 [a]	1998.4±35.0 [a] (102.4%)	1976.1±30.3 [a] (101.2%)
CH 1/25	1920.8±33.8[a]	1929.4±37.8 [ab] (100.4%)	1964.4±8.0 [b] (102.3%)
SH 1/25	1961.3±8.11 [a]	1995.1±18.7 [a] (101.7%)	2017.3±12.6 [a] (102.9%)
Dig Blank 1/25	1945.2±21.1 [a]	1964.2±15.5 [a] (100.6%)	1928.9±16.5 [a] (99.5%)

Table 3. TEER ($\Omega \cdot cm^2$) evaluation of Caco-2 cells treated with digested soy and casein hydrolysate in inflamed condition. Different letters in the rows show statistically significant differences ($p < 0.05$).

	Day 1	**Day 2**	**Day 3**
Untreated cells	1641.5±78.3 [a]	1849.5±32.7 [b] (112.7%)	1894.5±31.5 [b] (115.4%)
CH 1/25	1749.1±45.8 [a]	1924.9±59.0 [b] (110.0%)	1943.0±56.9 [b] (111.1%)
SH 1/25	1673.8±62.1 [a]	1944.9±25.9 [b] (116.2%)	1963.4±19.0 [b] (117.3%)
Dig Blank 1/25	1690.7±54.5[a]	1934,9±47,6 [b] (114.4%)	1938.6±45.6 [b] (114.7%)

TEER data showed no irreversible damage at the normal and inflamed cell monolayer when treated with digested samples. Particularly, CH enhanced TEER ($p<0.05$) compared to the baseline values of normal cells (**Table 2.** day 1). A possible increase of TEER values between day 1 (preliminary TEER evaluation) and day 2 (effective day of treatment) is due to change of medium (**Table 3**). No significant change in TEER values between day 2 and 3 were reported, showing that the hydrolysate may play a role on tight junction integrity in high grade of inflammation state.

4. Discussion

Caco-2 cells derived from human colon adenocarcinoma are a well-established model to evaluate the intestinal permeability and physiological response to food-derived peptides (Vieira et al., 2017).
Increased intestinal permeability is implicated in autoimmune, inflammatory, and atopic diseases, which can manifest both locally (within the intestinal mucosa) and systemically.
A TNF-α-induced increase in intestinal epithelial TJ permeability was proposed to be an important pro-inflammatory mechanism contributing to intestinal inflammation in Crohn's disease and other inflammatory conditions (Ye et al., 2006).
Several food bioactive components were reported to exert anti-inflammatory activity. Recently, milk-derived hydrolysates have been reported to exhibit anti-inflammatory activity in endothelial cells (Marcone, et al., 2015), whereas casein-derived peptides and amino acids improved Caco-2 TEER, primarily through the increased expression of the TJ proteins (Beutheu et al.,2013, Li et al., 2004; Yasumatsu &. Tanabe 2010).
Several studies have reported that a variety of amino acids or oligopeptides, tryptophan, γ-glutamyl peptides, polylysin and protamine, were able to block TNF- α-induced inflammatory responses in both Caco-2 cell lines through allosteric activation of CaSR (Zhang & Mine 2018).
Furthermore, the role of glutamine in regulating the functions of TJ proteins is well established, whereas dietary L-arginine supplementation improved intestinal recovery following mucosal injury in rats.
Barrier enhancing effects by protein hydrolysates were shown by Visser et al., (2010 and 2012), who fed diabetes prone rats with casein hydrolysates for 150 days and measured the barrier function. This study showed that casein hydrolysate intake decreased the epithelial permeability compared to a diet with sole amino acids. This benefit was evidenced by a normalization of the TJ mRNA expression, and an up-regulation of the regulatory and anti-inflammatory cytokine Interleukin (IL)-10. Multiple bioactive tri- and tetrapeptides derived from soy and whey have been found to induce anti-inflammatory effects after being taken up into the cell (Dalmasso et al., 2008; Kovacs-Nolan et al., 2012; Oyama et al., 2003) by PepT1, that is expressed in the small intestine, but during inflammation, is also up-regulated in the colon (Adibi et al., 2003). Treatment with the soy peptides KVP (lysine, valine, proline) and VPY (valine, proline, tyrosine) and the whey peptide IPAV (isoleucine, proline, alanine, valine) all showed a decrease in the production of the pro-inflammatory cytokines IL-6, IL-8, and TNF-α in Caco-2 cells. This effect disappeared when PepT1-activity was inhibited, indicating that transport via PepT1 is necessary for the anti-inflammatory effects. Peptides derived from many different food protein sources are known to bind opioid receptors (Stefanucci et al., 2018). Although

endogenous opioid peptides have a main function as neurotransmitters, they are also known to modulate innate and acquired immune responses (Liang et al., 2016). These effects have also been described in immune cells after hydrolysate administration. Therefore, it cannot be excluded that protein hydrolysates also modulate the immune system via opioid receptors.

Overall, this experimental evidence supports the potential of casein and soy protein hydrolysates to positively influence the barrier permeability in inflamed condition of the gastrointestinal tract.

5. Conclusion

This study showed that casein and soy protein hydrolysates may restore inflammation-induced intestinal barrier dysfunction of Caco-2 cells. In the presence of inflammatory stimulus, the protein hydrolysates ameliorate and preserve the intestinal barrier possibly acting at TJ level.

Simulated gastrointestinal digestion of the hydrolysates increase the bioavailability of BAPs and amino acids, enhancing the chance of these compounds to be absorbed and to exert a biological effect.

Taken together, these results suggest that BAPs and amino acids can regulate the intestinal epithelial barrier structure and functions, restoring the properties (and functions) of intestinal epithelial cells.

Future studies should evaluate this activity *in vivo.*

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Chapter 6

Potential functionality of protein hydrolysates for glycaemia control

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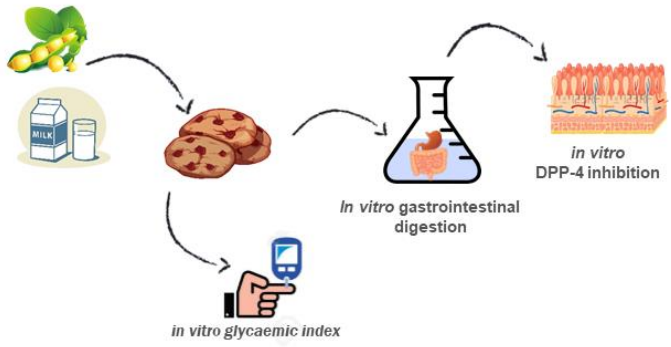

Casein and soy protein hydrolysates may be functional ingredients for glycaemia control through inhibition of DPP-IV activity

Paper in submission

Abstract

Dietary proteins may contain some bioactive peptides (BAPs) encrypted within their primary structures. BAPs may be delivered in the food during processing and/or in the gastro-intestinal tract during food digestion and can exert some biological effects beyond nutrition such as antimicrobial, anti-thrombotic, antihypertensive, opioid and immunomodulatory effects. BAPs modulating blood glucose response are promising ingredients for functional foods development. They work through inhibition of the enzyme dipeptidyl peptidase IV (DPP-IV) that modulate glucose homeostasis by cleaving glucagon-like peptide-1 and glucose-dependent insulinotropic polypeptide.

The aim of this study was to evaluate the potential activity of casein (CH) and soy (SH) protein hydrolysates as well as of CH and SH enriched biscuits (CHB and SHB) on post-prandial glucose response *in vitro*.

Control biscuits (ConB) without protein hydrolysates and two types of CH and SH-enriched biscuits providing 4.5% (CHB1 and SHB1) and 13% (CHB2 and SHB2) of each hydrolysate were developed. CH, SH, CHB1, CHB2, SHB1 and SHB2 were subjected to *in vitro* simulated gastrointestinal digestion and the ability of the digested samples to inhibit DPP-IV activity was assessed. *In vitro* glycaemic index (GI) of the biscuits was also measured.

Data showed that CH and SH behaved as mixed and competitive inhibitor of DPP-IV with an IC50 of 2.59 mg/ml and 3.56 mg/ml ($p<0.05$), respectively, when tested alone. No significant difference between digested biscuits for the inhibition of DPP-IV activity was observed. The GI of the biscuits was in the order ConB> CHB1>SHB1>CHB2>SHB2. This study suggested that CH and SH maybe functional ingredients for glycaemia control through inhibition of DPP-IV activity. A food matrix effect could hide the bioactivity of CH and SH at the doses used in the biscuits during *in vitro* enzymatic digestion.

1.Introduction

A large body of research focuses on bioactive food ingredients and compounds i.e. those able to provide health benefits in consumers and specifically in people at risk to develop chronic diseases. Among promising bioactive compounds, particular interest was recently gained by some food-derived peptides for the ability to regulate glycaemia, possibly through inhibition of the enzyme Dipeptidyl Peptidase IV (DPP-IV). DPP-IV is a multifunctional type II transmembrane glycoprotein, expressed constitutively on epithelial cells of liver, intestine, kidney and in a soluble form as sCD26/DPPIV in the circulation. DPP-IV belongs to the prolyl oligopeptidase family, that preferentially remove N-terminal dipeptides from substrates and thereby either inactivates peptides and/or generates new bioactive compounds (Röhrborn et al., 2015). DPP-IV takes part in a number of biological processes as both a regulatory protease and a binding protein. The enzyme is involved in glucose homeostasis achieved by its catalytic activity against the incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), beyond that cleaves a number of molecules such as neuropeptides, chemokines and regulatory peptides (Lacroix & Li-chan 2016). Of the twelve classes of glucose-lowering drugs currently available for the management of diabetes, inhibitors of DPP-IV are among the newest agents to have been introduced to the type 2 diabetes pharmacopeia, preventing the degradation of gut-derived hormones that play a pivotal role in glycaemic homeostasis (Filippatos et al., 2014).

Bioactive peptides (BAPs) are short amino acid sequences that coming from processing and/or digestion of dietary protein, which exert a measurable biological effect on body functions and health (Mo and Roos, 2008). Several food peptides are known to possess regulatory functions that can lead health benefits as demonstrated mostly through *in vitro*, cell culture and animal studies. Some dietary peptides show antihypertensive, antioxidant, anti-inflammatory, hypolipidemic, anticancer, antidiabetic and antimicrobial properties (Udenigwe and Fogliano, 2017).

BAPs and amino acids trigger GLP-1 secretion (Chang et al., 2013; Haberer et al.,2011; Steinert et al., 2014), involved into meal-related glycaemic control by stimulating insulin secretion (i.e., acting as an incretin), inhibiting glucagon secretion, slowing gastric emptying, and reducing hepatic glucose metabolism (Steinert et al., 2016)

Diet is well recognized to play an important role in the prevention and management of diabetes and particularly, protein hydrolysates are recognized as a potent source of BAPs. In fact, consuming protein hydrolysates containing these peptides might be helpful in the management of many western diseases like inflammatory bowel diseases, allergies, and diabetes (Kiewiet et al., 2018).

Mounting evidence reported putative associations between the consumption of certain foods, or their constituents, and the incidence of diabetes (Lacroix & Li-Chan, 2014a). Moreover, compelling findings from *in vitro* as well as animal and clinical studies have shown that some dietary factors, such as peptides and phenolic compounds, can help regulate blood glucose levels (Lacroix & Li-Chan, 2014a). Recent research suggested that one of the plausible mechanisms of action underlying the anti-diabetic effect of various food commodities could reside in the ability to inhibit the DPP-IV enzyme (Lacroix & Li-Chan, 2012). Dietary factors that are natural sources of DPP-IV inhibitors could potentially complement pharmacotherapy in the regulation of blood glucose levels. A variety of common food, including milk, egg, fish, corn, as well as amaranth, quinoa and hemp, have been explored to produce protein hydrolysates and peptides with DPP-IV-inhibiting properties (Nongonierma et al., 2015; Nongonierma et al.,2017). To date, proteins from cow's milk have been the most extensively investigated sources of DPP-IV inhibitors *in vitro*, in the form of hydrolysates of dairy ingredients, casein and whey fractions of milk (Lacroix & Li-Chan 2012*;* Nongonierma et al., 2017). Additionally, protein digests produced by *in vitro* simulated gastrointestinal digestion have also been reported to be able to inhibit DPP-IV activity (Lacroix & Li-Chan 2016), therefore suggesting that DPP-IV inhibitory peptides might be generated *in vivo* during the digestion process.

The relevance of studying whole foods instead of single nutrients is becoming clear as potential nutrient–nutrient interactions may affect the metabolic response to the whole food compared to its isolated nutrients.

In this frame, the aim of this study was to evaluate the potential activity of casein (CH) and soy (SH) protein hydrolysates as well as CH and SH enriched biscuits (CHB and SHB) on post-prandial glucose response *in vitro*.

2.Material and methods

2.1 Protein hydrolysates

Food-grade protein hydrolysates from bovine casein (CH) and soy isolate (SH), were donated by A. Costantino & C (Italy). Nutritional facts for each hydrolysate is provided in **Table 1.**

Table 1. Casein and soy protein hydrolysate composition

(g/100g)	**Casein (CH)**	**Soy (SH)**
Protein	82,9	86,4
Carbohydrate	8.1	3.0
Total Fats	ND	4.3
Of which *saturated*	ND	21.8%
Fibers	0.7	1.1
Sugars	ND	ND
Salt	3.5	4.9
Energy Value (kcal/ kJ)	374/1588	388/1642
pH (2%m/v)	5,9	5,5
Loss on drying (%)	4,4	3,7

2.2 Model biscuits design

Control biscuits (ConB) without protein hydrolysates and two types of CH and SH-enriched biscuits providing 4.5% (CHB1 and SHB1) and 13% (CHB2 and SHB2) of each hydrolysate were developed. The lower percentage hydrolysate formulations (4.5% CH/SH) refer to biscuits that comply with the claim of "source of protein" (at least 12% of the energy comes from protein), and the biscuits with a higher percentage of hydrolysate (13% CH/SH) comply with the "high protein" claim (at least 20% of the energy comes from protein) according to the European Food Safety Authority (EFSA Commission, 2012).

Model biscuits were prepared according to the formulations proposed by Kong et al., (2016). For the preparation of the model biscuit: flour 00' (Divella S.p.A, Italy), bottled still water, baking powder (Lievito dolci Paneangeli, Cameo S.p.A) and sugar (Eridania Italia S.p.A) were used.

CH and SH-enriched biscuit were prepared adding CH and SH respectively in different amount. The formulations are shown in **Table 2,** whereas **Table 3** shows the final nutritional content of the ConB, 4.5% CH and SH-enriched biscuits (CHB1, SHB1) and 13% CH and SH- enriched biscuits (CHB2, SHB2).

Table 2. Hydrolysate-enriched biscuit (4.5% and 13%) and control biscuit (ConB) recipe

Ingredients (g)	4.5% Hydrolysate	13% Hydrolysate	ConB
Flour	68.0	58.4	73.1
Hydrolysate	5.1	14.7	-
Sugar	21.9	21.9	21.9
Water	17.5	17.5	17.5
Baking powder	0.6	0.6	0.6

Table 3. Nutritional content of the ConB, 4.5% CH and SH-enriched biscuits (CHB1, SHB1) and 13% CH and SH- enriched biscuits (CHB2, SHB2).

100 g	CHB1	CHB2	SHB1	SHB2	ConB
Energy (kcal)	387.2	382.4	392.3	392.3	382.2
Protein (g)	12.7	20.0	13.1	20.9	8.7
Fat (g)	0.9	0.8	1.2	1.5	1.0
Saturated fats (g)	0.2	0.1	0.2	0.3	0.2
Carbohydrate (g)	79.2	71.1	79.8	71.3	83.1
Fibers (g)	2.2	1.4	2.5	1.2	3.2
Sugars (g)	24.0	23.6	24.3	24.0	24.1
Starch	53.0	46.1	53.0	46.1	55.8
Sodium (mg)	280	640	361	872	84
%Energy from protein	13.12	20.92	13.34	21.30	8.99

The biscuits were prepared at a laboratory scale, and baked using a small/pilot scale convection baking oven. According to the method after the dough preparation it was kneaded for 10 minutes, then a sheet of 2 mm thickness and 3.5 cm diameter with a rolling pin was formed and circular biscuits cut using a cookie cutter. Biscuits were baked at 190° C for 15 min in a convection oven. After this, they were cooled, processed into powder using a food processor and stored for further analysis.

2.4 Simulated gastrointestinal digestion

In vitro gastrointestinal digestion was performed with 5 g of each CHB1, SHB1, CHB2, SHB2, ConB respectively, according to Minekus et al., (2014). Each sample was prepared in 50 ml falcon tubes, digested in triplicates together with a blank of digestion (sample including all digestion fluids without biscuit). Samples were incubated in a shaking water bath at 37°C at 100 rpm, in dark conditions. At intestinal phase, an aliquot from each sample was collected (digesta), centrifuged at 21100 g for 10 min at 4°C and then freeze dried. Samples were stored at -25°C until further analysis.

For the simulated gastrointestinal digestion (SGID), the following reagents were used: α-amylase (from porcine pancreas, ≥10 U/mg solid, Sigma Aldrich), pepsin (from porcine gastric mucosa ≥250 U/mg, Sigma Aldrich), bile salts (Sigma Aldrich), pancreatin (from porcine pancreas, 4 X USP, Sigma Aldrich).

2.5 DPP-IV inhibition assay

CH and SH were reconstituted in 100 mM Tris-HCl buffer, pH 8.0, at initial concentrations ranging from 0.25 to 25 mg/ml, as well as the freeze-dried digesta of each biscuit, considering the initial protein content of the biscuits per ml of digesta.

The DPP-IV inhibition assay was carried out as described by Lacroix & Li-Chan, (2013). Briefly, 25 μL of test sample was pre-incubated with 25 μL of substrate Gly-Pro-p-nitroanilide (Gly-Pro-pNA, Sigma-Aldrich) 12 mM at 37 °C for 10 min, in a low-binding 96-well plate (Greiner, clear polystyrene wells flat bottom, Sigma-Aldrich). Then, 50 μL of DPP-IV (0.02 units/ml, from porcine kidney, Sigma-Aldrich) was added, followed by an incubation at 37 °C for 30 min. The enzymatic reaction was stopped by the addition of 100 μl of 1M sodium acetate buffer (Sigma-Aldrich) pH 4.0. The absorbance of the released pNA was measured at 405 nm using a microplate reader (Multiskan GO, Thermo Fisher Scientific). The negative control contained 100 mM Tris–HCl buffer pH 8.0 and the reaction substrate Gly-Pro-pNA, whereas Diprotin A (Enzo Life), competitive inhibitor of DPP-IV, was used as a positive control. Each test sample and both positive and negative control were analyzed in triplicate.

The percentage of DPP-IV inhibition was defined as the percentage of DPP-IV activity inhibited by a given concentration of hydrolysate (protein basis) and was calculated using the absorbance values corrected with blanks (Tris-HCl buffer only).

DPP-IV IC_{50} values (concentrations of hydrolysate/digesta required to cause a 50% inhibition of the enzyme activity) were determined by plotting the percentage of inhibition as a function of the concentration of test compound.

Lineweaver–Burk analysis was used to study the mode of inhibition according to Nongonierma & FitzGerald, (2013).The initial rate of the reaction (pNA released from Gly-Pro-pNA) was measured at different Gly-Pro-pNA concentrations ranging between 0.2 and 0.6 mM in the presence and absence of the DPP-IV peptide inhibitors at their IC_{50} concentration.

2.6 Glycemic index determination

The glycemic index (GI) determination was performed according to Kim & White (2012) and the following reagents were used: pancreatin (EC 232.468.9, from porcine pancreas, activity 4 × USP/g, Sigma-Aldrich), amyloglucosidase (EC 3.2.1.3, 3300 U/ml, Megazyme total starch kit), glucose oxidase/peroxidase reagent (Sigma Aldrich), o-dianisidine dichloride (Sigma Aldrich), anhydrous glucose (Carlo Erba) and absolute ethanol (Merck). The enzyme solution for digestion was prepared as follows: 1.8 g of pancreatin was dispersed in 4 mL of distilled water and centrifuged at 310 g for 10min. The supernatant was mixed with 1.6 mL of diluted amyloglucosidase (1.28 mL of amyloglucosidase., 3300 U/mL, diluted to 1.6 mL of distilled water), and distilled water (400 ul) was added until a final volume of 5 mL.

All biscuit sample processed into powder and a control (white bread made from wheat flour) were weighed to 100 mg into 50 mL tubes, in triplicate, with 10 glass beads (5 mm diameter) added to each tube. A solution containing 2 ml of 0.05 M hydrochloric acid and 10 mg of pepsin was added to each tube and incubated at 37 °C in a shaking water bath for 30 min. Then, 4 ml of sodium acetate buffer (0.5 M, pH 5.2) was added to each tube; the freshly prepared enzyme solution (1 mL) was added after 1 min intervals. The mixtures were then incubated at 37 °C in a shaking water bath. Aliquots (100 μl) were taken at 0, 30, 60, 90, 120, and 180 min intervals and mixed with 50% ethanol (1 ml).

The samples were centrifuged at 21100 g for 10 min, and the hydrolyzed glucose content of the supernatant was measured by using the glucose oxidase-peroxidase assay. Total starch hydrolysis (%) was calculated and the GI was then estimated by using the following equation according to Goñi, Garcia-Alonso, & Saura-Calixto, (1997):

$$GI = 39.71 + 0.549HI.$$

The area under the curve (AUC) is calculated for each interval of sampling during the digestion part of the assay. Then, the total AUC is calculated for each sample and then the hydrolysis index (HI) is estimated as follow:

$$HI = AUC\ (sample) / AUC\ (reference\ control)$$

For the glucose determination in the GI digestion samples, the protocol established by Sigma Aldrich in its Glucose Assay Kit was followed.

All analyses were done in triplicate.

The absorbance of each tube against a reagent blank at 540 nm and the glucose was calculated by preparing a standard curve containing glucose from 10 to 100 ug/ml. According Goñi et al., (1997), to transform the glucose into starch, a factor of 0.9 was used.

2.7 Statistical analysis

Data about IC_{50} and Lineweaver–Burk were evaluated for differences by two-way repeated measures analysis of variance (ANOVA).

Data on the replicate preparations of GI were analyzed by using the analysis of variance (ANOVA). When appropriate, differences between means were tested using the Student's t-test.

Statistical analysis of data was carried out using SPSS (version 21, SPSS Inc. Chicago, IL, USA). Statistical significance was set at a level of $\alpha = 0.05$ (5%).

3. Result

3.1 Mode of inhibition of DPP-IV

The inhibition of the enzyme activity of DPP-IV by CH and SH was evaluated. For each hydrolysate tested, the IC_{50} value was used as criteria to compare the effectiveness of the inhibition of each sample. Data showed that CH has an IC_{50} of 2.59 mg/ml whereas SH displayed an IC_{50} of 3.56 mg/ml (**Table 4**). The lower IC_{50} is, the more effective the hydrolysate is in inhibiting the enzyme, since this means that less concentration of hydrolysate is required to achieve the inhibition. In this frame, CH is more effective ingredient in terms of inhibition.

In order to look into the type of inhibition presented by CH and SH, a Lineweaver-Burk model assay was evaluated for both. Included in this test was DiProtin A, which is used as a reference for competitive inhibition (**Table 4**).

Data showed that either way CH and SH are able to inhibit the activity of DPP-IV; particularly SH acts in a competitive manner whilst CH behaved as mixed/not competitive inhibitor ($p<0.05$).

Table 4. IC_{50} value (mean±standard deviation) and mode of inhibition* ($p<0.05$) of CH and SH estimated by *Lineweaver–Burk* plot. Diprotin A was used as competitive inhibitor of DPP-IV.

	IC_{50} ±SD (mg/ml)	**Mode of inhibition**
CH	2.59±0.34	*Mixed/not competitive
SH	3.56±0.42	*Competitive
Diprotin A	0.02±0.01	*Competitive

The samples of biscuit were digested and the extractable digesta fraction was separated and analyzed for its inhibitory activity against DPP-IV.

No significant difference between digested biscuits (CHB1, SHB1, CHB2, SHB2, ConB) for the inhibition of DPP-IV activity was observed ($p>0.05$), probably due to more factors of the food matrix that interfered, decreasing the inhibitory activity.

3.2 GI determination

Data from GI determination showed that the GI of all the biscuits was lower than white bread, which is represented as a reference with an index of 100 (**Figure 1**). Only SHB2 showed significantly lower GI than the others ($p<0.05$). According to GI data, when the percentage of protein was higher, the GI was lower, and it confirmed that the soy hydrolysate biscuits had lower GI than the casein ones.

From these results, it can be concluded that the soy hydrolysate-enriched biscuits generated a lower GI probably due to a higher protein content. Of note, SH had a slightly higher protein content in comparison to the 13% casein hydrolysate biscuit because of the weight loss of baking and freeze drying (17.3% and 16.3%, respectively). This might have caused the difference with respect to the casein 13% biscuit.

Furthermore, CHB2 (but not CHB1) differed from the ConB. The biscuits containing the lower amount of hydrolysates had a higher carbohydrate percentage, that was mainly composed of sugars and starch (and a small amount of fibers), that are the source of the glucose measured in the GI index assay. Thus it could be expected that the higher their content is, the higher is the *in vitro* GI of the food product.

In general, the biscuits with 4.5% hydrolysate did not contain significantly less carbohydrates than the control biscuits, and in turn the ~13% formulations had almost 10% less carbohydrates.

Together with the results from the GI analysis, it can be highlighted that the highest soy formulation gave a lower GI out of all the samples tested

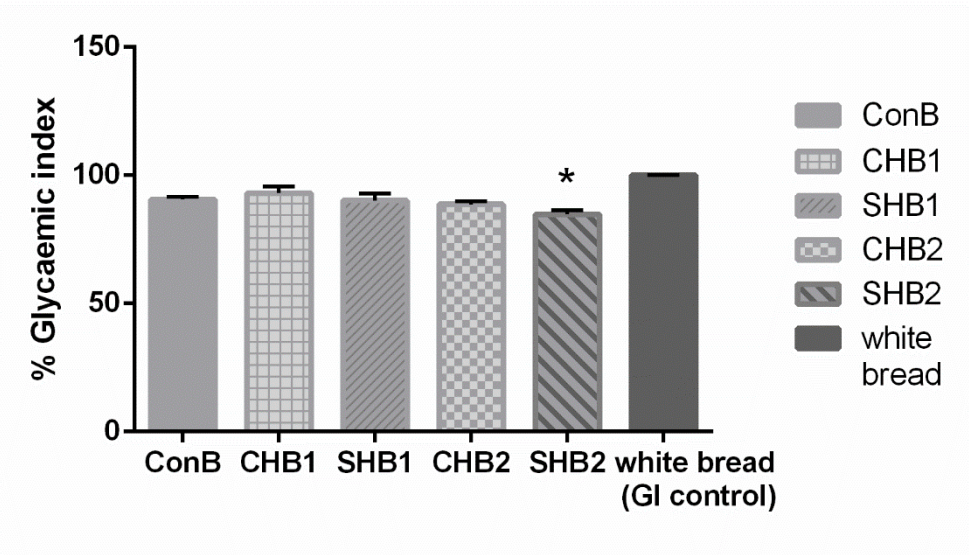

Figure 3. in vitro glycaemic index (GI) of biscuit samples. Statistical differences are denoted as * ($p<0.05$). White bread was used as positive control of the assay.

4. Discussion

While the production of DPP-IV inhibitors from dietary proteins and the identification of active peptide sequences have been the subject of a large body of research, little is currently known on the bioavailability of these bioactive molecules, involved in glycaemia control (Dailey & Moran 2013). GLP-1 is one of the most relevant molecular targets for the prevention and treatment of diabetes. There has been increasing interest in the possible ability of dietary factors to treat diabetes modulating the secretion of endogenous GLP-1(Tsuda 2015).

Several studies showed that amino acids such as glutamine and arginine are able to inhibit DPP-IV enzyme (Samocha-Bonet et al., 2011, Clemmensen et al., 2013, Greenfield et al., 2009). Glutamine is also reported to increase the concentrations of GLP-1, GIP, and insulin in humans (Samocha-Bonet et al., 2011, Clemmensen et al., 2013, Greenfield et al., 2009). Furthermore, GLP-1 secretion is also stimulated by branched-chain amino acids, including leucine and isoleucine, in NCI-H716 cells (Chen & Reimer 2009). Moreover, GLP-1 secretion is enhanced by skim milk and casein but not by whey (Chen & Reimer 2009); proteins from codfish, egg, and wheat, as well as egg protein hydrolysate can trigger GLP-1 release (Geraedts et al., 2011). Pioneering research on protein hydrolysates reported the GLP-1 secretion-enhancing effect and hyperglycemia-suppressing effect of the hydrolysate of zein, a maize-derived protein (Higuchi et al., 2013)**.**

Fractionation of DPP-IV-inhibiting protein hydrolysates and digests and analysis of the most potent fractions by mass spectrometry have allowed the identification of a number of peptide sequences

contributing to the observed inhibitory activity, that vary widely in terms of their length (2-17 amino acids long), amino acid composition and potency (half-maximal inhibitory concentration (IC50) values ranging from 5 μM to >20 000 μM.

Similar to the hydrolysates whose IC50 values ranged from μg/mL to mg/mL the identified DPP-IV inhibitory peptides show much weaker effect on DPP-IV activity than the synthetic DPP-IV inhibitors currently used for the treatment of type 2 diabetes (IC50 in the nM range) (Hunziker, Henning, & Peters, 2005).

Accordingly, casein hydrolysate used in this study showed a lower IC50 value of 2.59 mg/ml than soy protein hydrolysate with IC50 of 3.56 mg/ml. This might indicate that the casein hydrolysate has a BAPs/ amino acids profile that is more efficient in the inhibition of DPP-IV.

These BAPs can be found in the sequences of commonly consumed proteins such as those from milk and soy, and were shown to inhibit the DPP-IV enzyme; particularly the tripeptide IPI (also known as diprotin A), which can be found in the sequence of k-casein, is a well-known DPP-IV inhibitor and the most potent (IC50 = ~4 μM) of currently known peptides.

The consumption of specific food products, including dairy proteins, has been reported in a number of epidemiological and observational studies to be associated with a reduced incidence of type 2 diabetes linked to the ability of the relative BAPs involved in blood glucose metabolism and/or to stimulate the secretion of gut-derived hormones (Lacroix & Li-Chan 2014b)

At this regard, exhaustive studies conducted in rodents and humans have demonstrated that high protein (HP) diets improve glucose homeostasis. Particularly, increased HP intake over several weeks improves metabolic parameters, including body weight, adiposity, insulin sensitivity, glycated hemoglobin levels, and food intake in both humans and rodents (Arciero 2008, Lacroix 2004; Pichon 2006). Five weeks of HP feeding in diabetic patients improved glucose tolerance even when individuals maintained a stable weight (Gannon et al., 2003) and pair-feeding to match energy intake and body weight improved glucose homeostasis in rats fed a HP diet (Blouet et al., 2006). This suggests that HP diets regulate glucose homeostasis independent from effects on food intake and/or body composition. Notably, acute HP feeding is also effective at lowering blood glucose levels and improving glycemic control in individuals with diabetes (Nuttal et al., 1984, Manders et al., 2005). It has been postulated that these improvements in glucose control result from decreased dietary carbohydrate content; however, the addition of protein to a meal reduces the post-prandial glucose response compared to a meal consisting of equal carbohydrate content alone (Claessens et al., 2009; Nuttal et al., 1984) and consumption of a pre-meal protein beverage reduces post-meal glycemia (Gunnerud et al., 2012). This suggests that the glucoregulatory influence of acute HP intake results from the presence of protein itself. Clinical studies have shown

that dietary protein intake or intestinal protein administration increases circulating levels of gut peptides, including cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), and peptide YY (PYY) (van der Klaauw et al., 2013)

The binding of many foods-derived peptides with competitive DPP-IV inhibitors has been described at the active site of DPP-IV, mainly through interactions with a hydrophobic pocket in the enzyme. However, a secondary binding site for DPP-IV inhibitors has also been described, located close to the active site and results in a mixed-type inhibition of DPP-IV (Lacroix & Li-Chan, 2016). For example, a milk protein-derived dipeptide Trp-Val behaved as a non-competitive inhibitor of DPP-IV as well as several milk derived-peptides showed no competitive or mixed type of inhibition near the active site of DPP-IV (Nongonierma & FitzGerald, 2013). This might have been the case for the CH tested in this study, since it did not clearly act as a competitive inhibitor of the enzyme. When evaluating the mode of inhibition of the raw hydrolysates, SH presented a type of inhibition more similar to that of a competitive inhibitor whereas Casein seemed to have a mixed/noncompetitive type of inhibition. This again can be attributed to differences in the peptide profile and specific sequences present in the two types of hydrolysates, since they come from different protein sources.

It is known that bioactive peptides are produced from proteins by fermentation of food using proteolytic starter cultures, during the manufacture of protein hydrolysates, during *in vivo* digestion of dietary proteins, or during *in vitro* digestion, using various proteolytic enzymes (Capriotti et al., 2015). Regarding the changes they might undergo during digestion, although some of the peptides with inhibitory activity may reach the small intestine unchanged, it is likely that many of the peptides identified to have *in vitro* DPP-IV inhibitory activity will be broken down *in vivo* during the digestion process (Lacroix & Li-Chan, 2016). Even though it was possible to observe an inhibition of the original hydrolysates when tested directly in the presence of DPP-IV, there are other phenomena that can occur to the peptides present in the raw material such as the processing to prepare the biscuits (sugar-amino acid interactions during baking process) and the simulated digestion with a series of gastric enzymes that can affect not only their structure but also their availability.

The possible causes of a GI lowering effect of proteins in a carbohydrate containing foods can be : an increase in viscosity of the digesta (that negatively affects enzymatic action and mobility of solutes), an increase in digested solids, the presence of hypo-glycaemic phytochemicals and, the increased protein percentage with respect to a non-protein containing version (Dove et al., 2011).

Finally, it can be hypothesized that the biscuits developed in this study have a potential to eventually be a type of food product that can deliver a benefit in the maintenance of glucose homeostasis and other related mechanisms.

In this study, there was no significant difference between IC50 of the digested biscuit, indicating that when used in a food matrix and digested, the hydrolysates might vary their initial capacity to inhibit DPP-IV. The glycaemic index (GI) of the biscuits shows that the biscuit formulation that had more clear decrease in the GI was the biscuit containing soy at 13% hydrolysate. This shows that the addition of proteins in a biscuit formulation in a higher percentage (13% or possibly more) can potentially decrease the GI of the biscuit and with this generate a food product that can possibly contribute to a lower peak in postprandial glycaemia.

It is important to evaluate not only the potential health benefit of a food ingredient but also try to confirm its potential effect after it has been incorporated into a food matrix.

5. Conclusion

This study tested CH and SH as functional ingredients for glycaemia control, through inhibition of DPP-IV activity.

Data showed that when included in a food matrix these ingredients loosed their inhibitory activity against DPP-IV. This finding might be due to a food matrix effect, i.e. to other components present in the food that could hide the bioactivity of CH and SH at the doses used in the biscuits.

A higher amount of CH and SH in the biscuits may be necessary to trigger a reduction of DPP-IV activity and a potential effect in glucose homeostasis.

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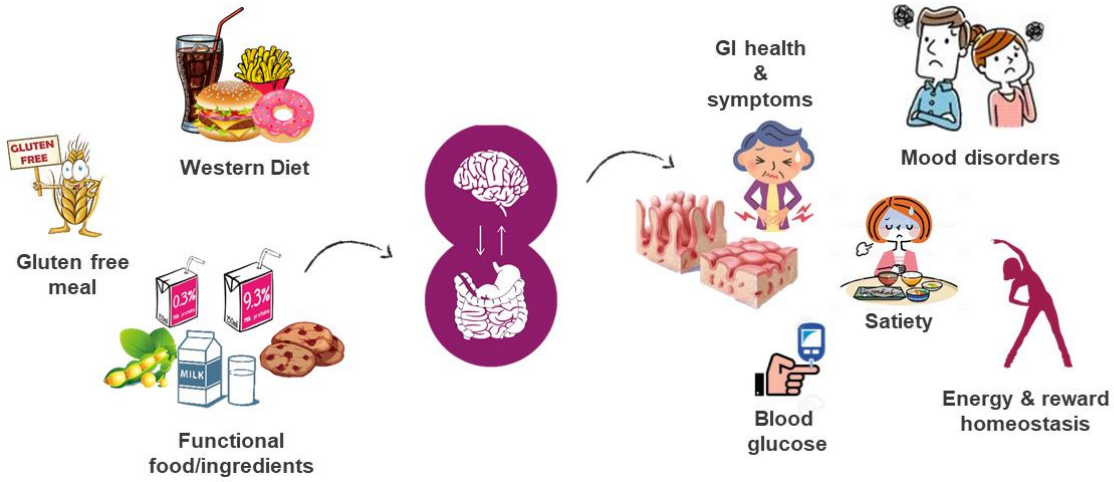
Chapter 7

Conclusions and future perspectives



Gut-brain axis is a crucial hub of the food intake and energy balance regulation.

Dichotomy between homeostatic and non-homeostatic/hedonic systems should be replaced by a larger, highly interactive system that unifies homeostasis with reward, cognition, and emotion.

Understanding the integrative role of the CNS in energy and reward homeostasis has become increasingly important considering the prevalence of obesity and functional gastrointestinal disorders (FGIDs). The GIT is the largest endocrine organ in the human body and it represents the gateway for communication between the human body and the external environment. Distress (i.e. inflammation) or adaptations in the communication of sensory information may contribute to the development or maintenance of disease. Several diseases such as celiac disease, Crohn's disease, type 1 diabetes and some food allergies, are known to increase the GIT mucosa permeation of macromolecules.

As reported in **Chapter 2** FGIDs are classified as morphologic and physiological abnormalities often occurring in combination with motility disturbance, visceral hypersensitivity, as well as altered mucosa, immune function, gut microbiota and CNS processes. The incidence of FGIDs among population is about 11%. Some of the symptoms reported for the functional dyspepsia are similar to those of non-celiac gluten sensitivity such as postprandial fullness, early satiety, epigastric pain, and epigastric burning. These symptoms may appear isolated or combined after consumption of specific foods. The nutritional consequence of this symptomatology is that people arbitrarily exclude from diet the foods which are mainly associated with the symptoms such as spicy foods, high-fat meals, dairy products or cereal-based foods. Life-lasting dietary exclusions may cause nutritional deficiency as well as metabolic adaptation that may be even worst of the previous symptomatology. This is the reason why in the case of a specific food/nutrient-induced disorder the usual dietetic approach is the exclusion from the diet of the specific food/nutrient for a certain period and a slow re-introduction in the follow-up period.

The exclusion from the diet of gluten represents the cure only for people with celiac disease but literature was still lacking on the knowledge of the metabolic response to a gluten-free meal in patients with celiac disease compared to healthy subjects. The human study reported in **Chapter 3** aimed at filling that gap of knowledge. From a dietetic perspective, the effect of a meal is more relevant in exerting metabolic responses than a single nutrient/food. Celiac subjects (CD) at the diagnosis or on a gluten free diet since 12 months and healthy subjects consumed a standard gluten-free meal and the post-prandial blood glucose and hormone response as well as appetite feelings were monitored. The main results of the study was that CD showed a lower postprandial blood glucose response than healthy subjects with a lower response of GLP-1, GIP and insulin. The different hormonal status was associated with a different evolution of the post-prandial hunger sensation that was higher in CD at diagnosis than in the others. These findings suggested that CD subjects after more than 1 year on a

gluten free diet did not recover a complete functionality of the intestine and this might determine an adaptive hormone postprandial response that may influence post-prandial appetite sensations and insulin resistance over long period.

From a food science and nutrition perspective, some bioactive compounds are present in foods and may be effective in the control of appetite, intestinal inflammation and glycaemia. In this thesis the potential of food bioactive peptides contained in milk proteins, casein and soy hydrolysates towards appetite, inflammation and glycaemia was tested and described in the human study in Chapter 4, and in *in vitro* studies of Chapter 5 and 6, respectively.

In **Chapter 4** two beverages, one enriched with milk proteins and one with carbohydrates (control) were developed and a human study with women consuming the beverage after an exercise session was carried out aiming at evaluating the effect of the beverages on the energy intake at the subsequent dinner. Data showed that the milk protein-enriched beverage was able to influence hunger sensation and reduce energy intake at subsequent meal only in women having a strong cognitive control of eating behaviour.

In **Chapter 5** casein and soy hydrolysates were tested in Caco-2 cells cultivated in a normal condition and in the presence of TNF-α to induce intestinal inflammation. Data showed that digested casein and soy protein hydrolysates positively influenced cells viability, particularly in inflamed condition. Data about monolayer integrity (TEER) showed an absence of irreversible damage at the normal and inflamed cell monolayer when it was treated with digested samples.

In **Chapter 6** casein (CH) and soy (SH) protein hydrolysates were tested as functional ingredients for glycaemia control, through inhibition of Dipeptidyl peptidase-IV (DPP-IV) activity. This study showed that when used in a model food CH and SH loosed their inhibitory activity against DPP-IV possibly because of other food components that could hide the bioactivity of CH and SH at the doses used in the model food.

All in all, the findings of this thesis showed that:

- Despite the attention and the knowledge on dietary factors influencing etiology and symptomatology of FGIDs is increased, there is a need of large nutrition surveys with a prospective design in order to provide information on dietary habits of FGID subjects and clarify whether dietary preferences reflect the subconscious exclusion of foods to which subjects are intolerant.

- Celiac subjects after more than 1 year on a gluten free diet did not recover a complete functionality of the intestine and this might determine an adaptive hormone postprandial response that could influence appetite sensations and insulin resistance over long period.
- A functional beverage enriched with milk proteins could modify daily eating habits by enhancing satiety in women with a stronger cognitive control of eating behaviour.
- Simulated gastrointestinal digestion of the dietary protein hydrolysates increases the bioaccessibility of bioactive peptides and amino acids, enhancing the chance of these compounds to be absorbed and to exert a biological effect, regulating the intestinal epithelial barrier structure and functions and restoring the properties (and functions) of intestinal epithelial cells.
- Casein (CH) and soy hydrolysates (SH) may be functional ingredients for glycaemia control through inhibition of DPP-IV activity. A food matrix effect could hide the bioactivity of those ingredients at the doses used in the biscuits during *in vitro* enzymatic digestion. *In vivo* studies are necessary to evaluate the effectiveness of CH and SH-enriched food for glycaemia control.

This scenario highlights the importance of validation of efficacy of new foods/whole meals both *in vitro* to assess the potential functionalities before performing human studies as well as *in vivo* in a well-characterized population. Indeed, growing evidence shows that the effect of food on wellness and dietary behaviour may be also influenced by psychological attitude of people as well as by gut microbiota reactivity to a food/diet.

In the context of new foods/diets to tackle obesity and FGIDs the gut-brain interconnection is central and should be better targeted both at the step of the food design and validation.