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STUDY OF DIETARY FACTORS FOR BODY WEIGHT CONTROL THROUGH GUT BRAIN-AXIS

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Chapter 1. General introduction

Noncommunicable chronic diseases (NCDs) are not passed from person to person, they are of long duration and generally slow progression. The four main types of NCDs are cardiovascular diseases (CVD),including heart attacks and stroke, some type of cancers, chronic respiratory diseases and type 2 diabetes. NCDs kill 38 million people each year with the first four groups of diseases accounting for 82% of all NCD deaths (WHO, 2014). In fact, cardiovascular diseases account for most NCD deaths, or 17.5 million people annually, followed by cancers (8.2 million), respiratory diseases (4 million), and diabetes (1.5 million) (WHO, 2014).

Obesity is strongly correlated with all of these four NCDs: obesity increases the likelihood of diabetes, hypertension, coronary, heart disease, stroke, certain cancers and obstructive sleep apnoea (WHO, 2014). Overweight and obesity, characterized by body mass index (BMI) $\geq$25 kg/m^2 and $\geq$30 kg/m^2, respectively, were estimated to account for 3.4 million deaths in 2010 (Lim, 2010). Obesity has been increasing in all countries. In 2014, 39% of adults aged 18 years and older (38% of men and 40% of women) were overweight. The worldwide prevalence of obesity nearly doubled between 1980 and 2014. In 2014, 11% of men and 15% of women worldwide were obese. Thus, more than half a billion adults worldwide are classed as obese. Age-standardized estimates on prevalence of obesity in males and females, aged 18 years and over are shown in Figure. 1.1 and Figure 1.2, respectively. The prevalence of overweight and obesity increases with the income level of countries. The prevalence of obesity in high-income and upper-middle-income countries is double than that of low- income countries. Even worse, the prevalence of childhood overweight is increasing worldwide too. It is estimated that the prevalence of overweight in children aged under 5 years will rise to 11% worldwide by 2025 if current trends continue (UNICEF-WHO, 2014). Therefore, WHO included the reduction of obesity as one of the nine global target to reduce NCDs.

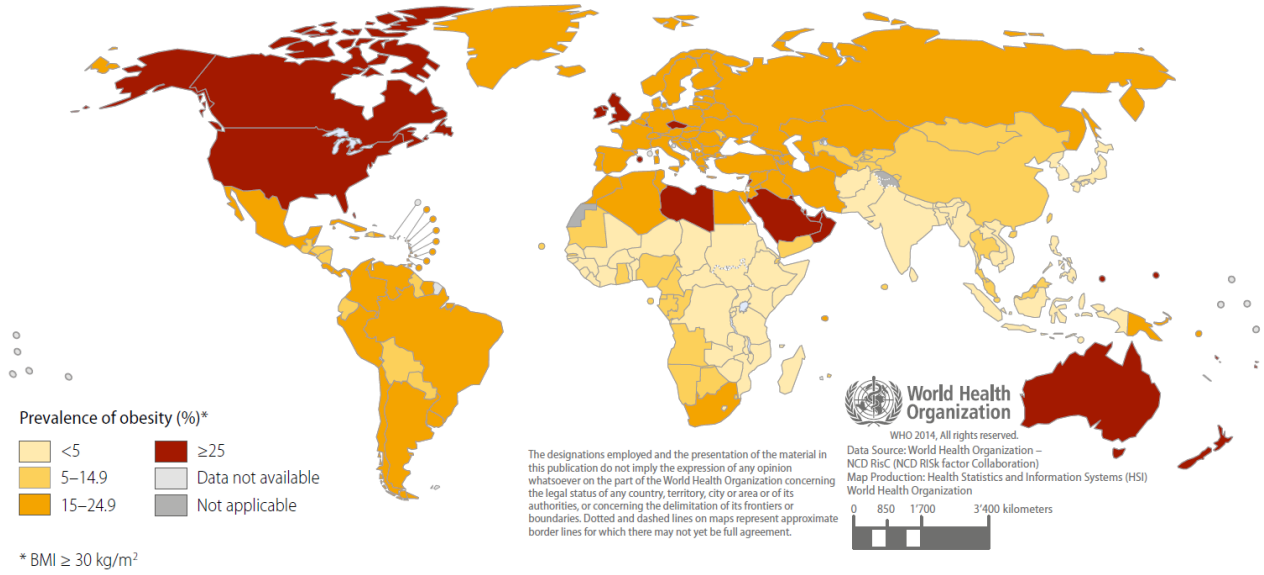

Fig. 1 Age-standardized prevalence of obesity in men aged 18 years and over (BMI ≥30 kg/m^2; WHO, 2014).

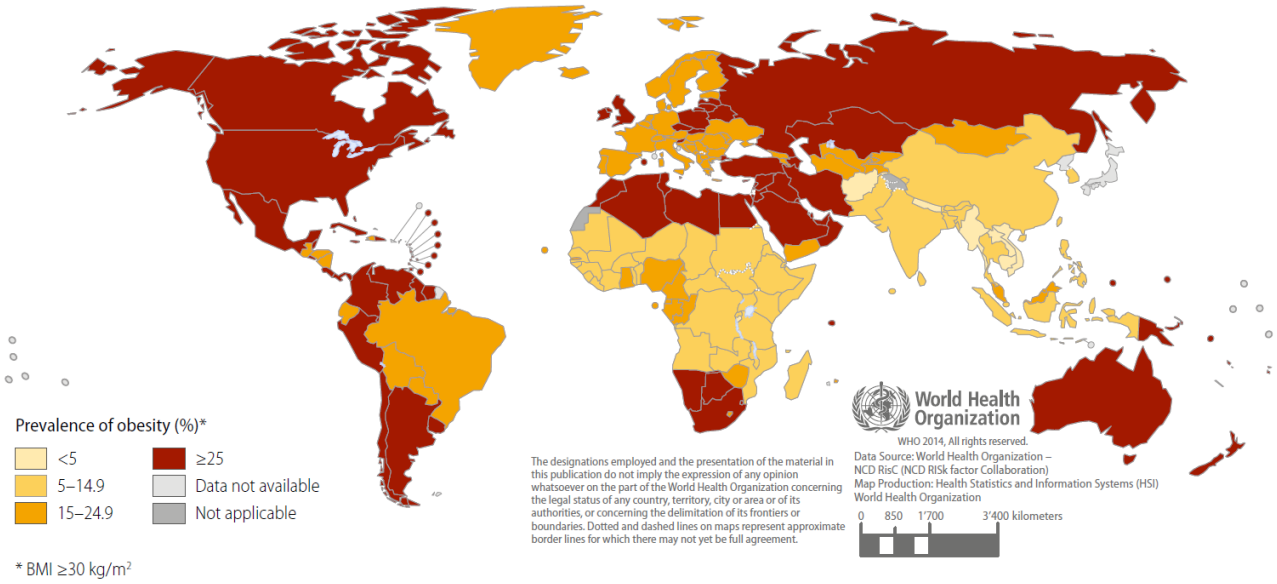

Fig. 2 Age-standardized prevalence of obesity in women aged 18 years and over (BMI ≥30 kg/m^2; WHO, 2014).

No government can ignore the rising burden of NCDs. In the absence of evidence-based actions, the human, social and economic costs of NCDs will continue to grow and overwhelm the capacity of countries to address them. To lessen the impact of NCDs on individuals and society, a comprehensive approach is needed that requires all sectors, including health, finance, foreign

affairs, education, agriculture, planning and others, to work together to reduce the risks associated with NCDs, as well as promote the interventions to prevent and control them.

In this context, food industry plays a main role. In the nineties technological innovations and marketing techniques have modified dietary preferences led to major changes in the composition of diet. There was a shift towards high fat, refined carbohydrate and low-fibre diet (Popkin, 1998). On the contrary, nowadays, the countries policies to tackle obesity together with the greater trust in technology to produce functional foods stimulates the demand of healthier foods such as non-refined grains products and foods for appetite control, weight control and weight loss (Niva et al, 2005). In particular, whole grains have received increased attention for their potential role in body weight control and reduction of CVD risk. Epidemiological evidence shows that higher whole grain intake, when compared with lower whole grain intake, is associated with lower BMI, body weight, and abdominal adiposity, smaller waist circumference and smaller weight gain over time. However, the evidence from randomized controlled studies is less consistent (Thielecke et al, 2014)

Gut brain axis in appetite control

Almost 30 years ago Blundell, Rogers and Hill (Blundell et al, 1987) proposed the concept of "Satiety Cascade" describing a framework to assess the mechanisms influencing satiation (processes that bring an eating episode to an end, or intra-meal satiety) and satiety (processes that inhibit further eating in the postprandial period until the next meal, or inter-meal satiety). The physiological systems underlying the control of satiation and satiety involve associations between peripheral physiology (stomach emptying and gastrointestinal peptides) and metabolism (glucose homeostasis and adiposity), which in turn are linked to various brain processes. All hormonal messengers released from enteroendocrine cells in the gut mucosa can inform the brain either through the circulation or via primary afferent neurons or both (Berthoud, 2008).

Gastrointestinal peptides in appetite control

The role of several peptides is well recognized in the regulation of the satiety cascade**.**

Ghrelin is a 28-amino acid peptide elaborated and secreted mainly from the stomach and proximal small intestine. Ghrelin is the only known circulating orexigenic peptide, and is secreted pre-prandially and suppressed by food intake (Kojima et al, 1999; Cummings et al, 2001). The ghrelin receptor can be found in the hypothalamus, heart, lung, pancreas, adipose tissue and intestine. Together with the fact that ghrelin stimulates hunger contractions, registered as phase III of the

migrating motor complex (Tack et al, 2006), this peptide stands out as a major regulator for the timing of food intake and eating frequency.

Cholecystokinin (CCK) is produced in I-cells of the upper small intestine and exists in several different molecular forms (CCK-58, CCK-37, CCK-33, CCK-22 and CCK-8). They all are present in the circulation (Rehfeld et al, 2007). CCK acts on two related G-protein coupled receptors, the CCK-1 and CCK-2 receptors. The CCK-1 receptor mediates the delay in gastric emptying as seen after administration of CCK, and the CCK-2 receptor is present in the central nervous system (CNS). CCK is released into the circulation by lipids and protein. It is not clear if CCK mediates any effect of ingested carbohydrates. Gut sweet taste receptors (T1R2/T1R3) are present on I-cells only in small numbers, in contrast to a high presence on L-cells (that secrete GLP-1 and PYY). Inhibition of these gut sweet taste receptors results in reduced secretion of GLP-1 and PYY but not CCK (Rehfeld, 2011; Dockray, 2012), suggesting that CCK has a limited role in mediating effects of ingested carbohydrates.

Glucagon-like peptide-1 (GLP-1) belongs to the glucagon peptide family. GLP-1 is released into the circulation after a meal in proportion to the amount of calories ingested (Ørskov et al, 1994). The release is bi-phasic and the first peak occurs before nutrients reach the distal gut. This peak is increased with the ingestion of carbohydrates, which is why it has been suggested that the first peak of release is due to absorption of carbohydrates in the proximal intestine, suggesting an indirect neural mechanism of GLP-1 release in addition to a direct action on the L-cells. The second peak is thought to be mediated by lipids in the intestinal lumen (Elliott et al, 1993; Edfalk et al, 2008; Smeets et al, 2008). Studies on the effect of protein on GLP-1 secretion have given inconsistent results. The half-life in the circulation is short (2 min) where the peptide is degraded by the enzyme dipeptidyl peptidase IV (DPP-IV).

Peptide YY (PYY) is a member of the pancreatic polypeptide family, comprising pancreatic polypeptide (PP) and neuropeptide Y (NPY). PP, PYY and NPY bind to the Y receptor family. ProPYY is cleaved in the L-cells to PYY 1–36 and once released into the circulation PYY 1–36 is further cleaved to PYY 3–36by DPP-IV (Grandt et al, 1994). Plasma PYY concentrations are elevated already 15 min after a meal, and remain elevated for several hours (Adrian et al, 1985). As the half-life of PYY is about 8 min, this speaks in favour of a continued release over prolonged periods of time after food intake. As seen with GLP-1, plasma PYY concentrations are elevated in proportion to the caloric load. PYY release is mediated by both direct nutrient stimulation of the L-cells and indirect duodenal mechanisms. Duodenal lipids contribute to the early phase of PYY

release and are most probably mediated by hormonal and neuronal mechanisms. The administration of CCK increases plasma PYY concentrations (Moran et al, 2011).

Role of adipokines in body weight regulation

Adipokines include classic pro-inflammatory proteins such as TNF-a and IL-6, both secreted by adipocytes, but synthesized also by immune cells infiltrating WAT such as macrophages (Hotamisligil et al, 1993; Lower et al, 2003; Harden et al, 2006). These pro-inflammatory adipokines appear to significantly contribute to the so-called low grade inflammation of obese subjects, a condition associated with increased risk of cancer, type 2 diabetes, cardiovascular complications, autoimmune, and inflammatory diseases (Trayhurn et al, 2006). Other adipokines have an essential roles in the control of glucose and lipid metabolism.

Leptin, the product of the ob gene, is a 16-kDa secreted protein mainly produced by adipocytes (Zhang et al; 1994). The secretion of leptin is proportional to the amount of adipose tissue, and its plasma concentrations are markedly increased in obesity (Considine et al; 1996). Leptin plays a key role in the control of body weight through central and peripheral mechanisms (Gautron et al; 2011). On binding its receptors (OB-R) in the hypothalamic neurons containing POMC and CART, leptin induces a decrease in food intake and, a consequent reduction in adiposity and body weight (Harvey et al; 2003). Furthermore, leptin increases energy expenditure through the activation of sympathetic nerve activity and the turnover of norepinephrine in BAT (Scarpace et al, 1997). Moreover, leptin appears to play a crucial role in brown adipogenesis, since leptin-deficient ob/ob mice show a 'white-like' appearance of BAT (large unilocular lipid droplets instead of the characteristic small multilocular lipid droplets in brown adipocytes) (Becerril et al, 2010). In addition to its central function as a satiety factor, many peripheral effects of leptin on glucose, lipid and protein metabolism have been described (Frühbeck 2006). Leptin improves insulin sensitivity by stimulating insulin induced glucose uptake through GLUT4 in skeletal muscle (Sáinz et al; 2012). Moreover, leptin stimulates FFA oxidation via the activation of the α 2 catalytic subunit of AMPK in skeletal muscle (Unger; 2004). Leptin also stimulates muscle protein synthesis reducing the expression of the ubiquitin-ligases MAFbx and MuRF1 (Sáinz et al; 2009).

Adiponectin, also known as Acrp30, AdipoQ, amp-1 or GBP28, is a 30-kDa hormone produced by adipocytes, with insulin-sensitizing, anti-inflammatory and anti-atherogenic properties (Ouchi et al; 1999). Adiponectin exists in various forms in plasma such as trimer, hexamer, and high molecular weight (HMW), as well as a proteolytically cleaved form, globular adiponectin. Adiponectin improves insulin sensitivity by increasing glucose uptake in the muscle, decreasing hepatic

gluconeogenesis as well as increasing fatty acid oxidation, leading to a reduction in lipid content in liver and skeletal muscle (Kadowaki et al; 2006). In spite of the beneficial effects of adiponectin on insulin sensitivity, there is a paradoxical decrease of adiponectin concentrations in obesity and type 2 diabetes. These low circulating concentrations of adiponectin are associated with higher percentage of body fat, hyperinsulinaemia and hyperglycaemia (Weyer et al; 2001). Obesity and type 2 diabetes are associated not only with hypoadiponectinaemia, but also with an impairment of adiponectin-induced AMPK signalling in liver and skeletal muscle, leading to an increased lipid deposition in these insulin-sensitive tissues (Mullen et al; 2009)

Brain in appetite control

The central regulation of energy homeostasis is located in the hypothalamus, which integrates the humoral and neural signals involved in the control of food intake. The arcuate nucleus (ARC) in the ventral hypothalamus participates in the control of food intake through the secretion of neuropeptides implicated in central nervous system-dependent anabolic and catabolic pathways (Gao et al; 2013). There are two subtypes of neurons in the ARC regulating the eating behaviour: (i) neurons containing the orexigenic peptides NPY and agouti-related peptide (AgRP); (ii) neurons containing anorexigenic peptides such as proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) and, to a lesser extent, neurotensin. These hypothalamic circuits are regulated by energy status and several circulating hormones. In these nutrient-sensing neurons, nutrients act as signalling molecules to engage a complex set of neurochemical and neurophysiological responses, thereby regulating energy intake, the release of stored nutrients, and nutrient utilization in most tissues, thus compensating for increased energy availability (Gao et al; 2013). Numerous peripheral signals, which can be classified as short- and long-term signals, control the feeding behaviour and body weight. Short-term signals (i.e. nutrients, neural signals and hormones) influence the size of a single meal and either initiate or terminate a meal. These signals are generated by the liver, pancreas, skeletal muscle or gastrointestinal tract as either afferent sensory relays (vagal, splanchnic or spinal) or nutrients or hormones that reach the central nervous system through the blood–brain barrier. Long-term signals (adiposity) provide information to the brain about the energy stores and induce adaptive responses to maintain energy homeostasis. The short- and long-term signals need to operate in concert to integrate energy intake and energy expenditure to ensure that energy balance is maintained. Also cognitive, hedonic and emotional neural processes play important roles in energy intake and expenditure and the resulting energy balance. Eating behavior is not limited to the act of eating, but consists of preparatory, consummatory, and post-consummatory phases. Hedonic evaluation and reward processing is

carried out in each of these three phases of eating behavior and critically determines their outcome (Berthoud et al, 2011). Food reward process can be divided in three main sub-factors: "liking" (hedonic impact), "wanting" (incentive motivation), and "learning" (associations and predictions; Berridge et al, 2009). All occur together, but the three psychological components have separable brain systems that permit dissociation among them in some conditions (Berthoud et al, 2011).

Endocannabinoids in gut-brain communication

Endocannabinoids (ECs) are a family of biologically active lipids that bind to and activate G protein-coupled CB1- and CB2-cannabinoid receptors (DiPatrizio et al, 2011). Endogenous ligands to CB1 such as anandamide (AEA) and 2-arachidonoylglycerol (2-AG) have been shown to play a major role in the regulation of energy balance and body composition affecting both the sensing of energy deficiency/abundance and gastric load (homoeostatic mechanisms), and the salience as well as the incentive/motivational value of food (hedonic aspects; Di Marzo et al, 2009). The hypothalamus is the key brain structure involved in energy homeostasis by integrating the action of central orexigenic and anorexigenic neuropeptides with that of peripheral signals coming from the sympathetic system and peripheral organs deputed to sensing the status of energy stores. Although hypothalamic CB1 expression is among the lowest in the brain, activation of the receptors shows high efficiency and leads to profound effects on the crosstalk between the hypothalamic nuclei and peripheral organs. Compelling evidence shows that ECs act as "gatekeepers" of the hypothalamic–pituitary–adrenal axis (HPA) constraining the activity of the latter under stressful conditions by lowering glucocorticoid levels (Di Marzo et al, 2009). The CB1 receptors are present in the mesolimbic system and, in particular, in the nucleus accumbens shell (NAcS) and ventral tegmental area (VTA), wherein they play a role in the circuits involved in motivation and reward animals. Exposure to foods with high salience and incentive properties stimulate an EC tone to induce dopamine release in this limbic area. This latter event, in turn, might lead to both increased motivation to consume palatable foods and heightened rewarding effects after the consumption of such foods. Regarding the gut control of food intake by ECs, AEA and 2-AG showed orexigenic properties in rodents as they dose-dependently increased food intake by peripheral administration. In contrast, oral or intraperitoneal administration of the endocannabinoid related N-acylethanolamine, oleyl ethanolamide (OEA), as well as its duodenal increase, determined a decrease of food intake in mice and rats (Piomelli, 2013). In humans Joosten and co-workers found that fasting and non-fasting plasma ECs concentrations were positively associated with both serum total free fatty acids and their specific fatty acid precursors. Moreover, it was demonstrated that the

oleic acid content of a meal increase the post-prandial response of circulating OEA and to reduce energy intake at subsequent meals (Mennella et al 2015).

Macronutrients and satiety

The impact of individual macronutrients on satiety is typically measured in experimental studies using a preload design. Participants consume preloads differing in energy density (the caloric content of a given weight of food) or in the amount of carbohydrate, protein, or fat, and their energy intakes at the same meal are observed. A food that is reported to have high satiety tends to produce a longer intermeal period (a period of time between eating episodes during which an individual does not experience hunger). Alternatively, foods that are reported to have lower satiety tend to produce a shorter intermeal period. Satiety and satiation are distinct but interrelated factors that influence both the type and amount of food consumed. Foods, and more specifically macronutrients, with the same caloric content exert different effects on satiation and satiety independently from their caloric value. In other words, not all calories are treated equally by the body. In Stubbs and colleagues' review of the energy density of foods (calories/g), they noted that under normal circumstances in which fat contributes disproportionately to energy density, protein, carbohydrate, and fat exert hierarchical effects on satiety in the order of proteins > carbohydrates > fats (Stubbs et al, 2000). However, one study suggests that this effect is mediated almost exclusively by energy density (Raben et al, 2003). Although most research has suggested that the macronutrient protein has the most potent action on satiety, there is less clear consensus regarding the relative satiety values of carbohydrates and fats. The relative satiety values of these macronutrients (carbohydrates and fats) tend to vary depending on whether the macronutrients are studied in isolation or in foods (Gerstein et al, 2004).

Protein is an essential part of the diet necessary for body growth and maintenance, and can also serve as a fuel source. Proteins are broken down in the stomach during digestion by enzymes into smaller polypeptides to provide amino acids for the body, including the essential amino acids (those that cannot be biosynthesised by the body itself). Proteins are more satiating than carbohydrates and fats in the short term, over 24 h and in the medium term (Veldhorst et al, 2008). The speed of absorption of dietary amino acids by the gut varies according to the type of ingested dietary protein, and since amino acids are potent modulators of protein synthesis, breakdown, and oxidation, different patterns of postprandial aminoacidemia might well result to influence satiety or satiation. Evidences support the conclusion that meals higher in proteins tend to increase satiety when compared to meals lower in proteins, at least in the short term. In long term studies conducted over

a few days, the higher post-absorptive satiety and thermogenesis are sustained irrespective of the protein source (Halton et al, 2004). There is no clear consensus that one type of proteins is more satiating than other. Overall, the weight of evidence suggests that higher protein intakes cause a decreased subsequent energy intake, although the results are not entirely consistent. It appears that the closer the methodology is to real life situations (real food vs liquid, sense of taste unaltered, free living vs whole body calorimeter), the more likely it is for protein to exert a significant decrease in subsequent energy intake. Dietary proteins and amino acids, including glutamate, generate signals involved in the control of gastric and intestinal motility, pancreatic secretion, and food intake. Protein reduces gastric motility and stimulates pancreatic secretions. Protein-induced satiety coincides with a relatively high glucagon-like peptide-1 (GLP-1) release, stimulated by the carbohydrate content of the diet, peptide YY (PYY) release, while ghrelin does not seem to be especially affected, and little information is available on cholecystokinin (CCK). Energy expenditure and glucose are probably involved as metabolic signals in protein-induced satiety. Protein induced satiety appears to be of vital importance for weight loss and weight maintenance. With respect to PYY responses, Batterham et al. (2003) observed significantly higher plasma PYY responses to an high protein meal in both lean and obese subjects. At the brain level, two afferent pathways are involved in protein and amino acid monitoring: the indirect neural (mainly vagus-mediated) pathway and the direct humoral pathway. The neural pathways transfer pre-absorptive and visceral information through the vagus nerve innervating part of the orosensory zone (stomach, duodenum and liver). Localised in the brainstem, the nucleus of the solitary tract is the main projection site of the vagus nerve, and integrates sensory information of oropharyngeal, intestinal, and visceral origins. Ingestion of proteins also activates satiety pathways in the arcuate nucleus, which is characterised by an up-regulation of the melanocortin pathway (alpha-melanocyte-stimulating, hormone-containing neurons) and a down-regulation of the neuropeptide Y pathway (Tomé et al; 2009).

Fat is an important macronutrient in a normal western diet; in the so-called Standard American Diet, fat intake is responsible for 35% of total caloric intake, mostly in the form of triacylglycerols (TAG). Apart from delivering calories, fat also delivers essential fatty acids (FA) and may increase palatability of food products. The release of gut peptides after fat infusion occurs in response to the sensing of fat by small intestinal receptors. Studies where the lipase inhibitor Orlistat was used demonstrated that inhibition of lipase activity abolishes the satiating effects of fat infusion into the small intestine (Feinle et al; 2003). This suggested that, hydrolysis of fat to FAs is necessary to induce satiety in the intestine and that the sensing of fat is the result of the interaction between a fatty acid and a small intestinal receptor. On gut epithelial cells, a wide range of receptors involved

in fatty acid sensing are expressed, which are mainly of the G-protein-coupled receptor-type. Furthermore, a number of these receptors have been found in the oral cavity. *In vivo* experiments in mice showed that GPR120 is expressed by endocrine L-cells that produce glucagon-like peptide 1 (GLP-1) and peptide YY (PYY) in the large intestine (Hirasawa et al, 2005). *In vitro* studies in enteroendocrine cell lines (STC-1 cell line) suggest an important role of GPR120 in the secretion of both cholecystokinin (CCK) and GLP-1 (Hirasawa et al, 2005). Moreover, CD36, which is expressed on enterocytes and which is involved in the process of fat absorption in the small intestine, is essential in the production of oleoylethanolamine (OEA). This lipid messenger is produced from oleic acid in response to intestinal exposure to fat. Its mobilization from the mucosa leads to suppression of food intake via the activation of the peroxisome-proliferator-activated receptor-alpha (PPAR-alpha) (Schwartz et al, 2008). Ingestion of fat-containing foods induces the start of the cephalic phase of food digestion. Studies using the modified sham feeding (MSF) technique, in which food is chewed but not swallowed, have shown that this includes the stimulation of gastric lipase and insulin secretion, the stimulation of pancreatic polypeptide and the suppression of ghrelin, which is an appetite-stimulating peptide arising from the stomach (Little et al, 2011; Page et al, 2012). Oral sensing of fat is partly induced by texture and olfactory signals (Rolls, 2012). However, a number of receptors on taste receptor cells (TRC) in the oral cavity have been recently identified that directly interact with FAs (Stewart et al, 2011). These include CD36 (formerly known as fatty acid transporter,FAT) and a series of G-protein-coupled receptors (GPR), including GPR40,GPR41, GPR43 and GPR120. These receptors have a specificity for different FA chain lengths. The role of these receptors remains uncertain, but there is some evidence that they play an important role in determining fat taste and preference. In both animal and human studies, substantial inter-individual differences in the ability to detect FAs in the oral cavity have been reported and associated with marked differences in fat intake, which may have consequences for body weight regulation and obesity (Little et al, 2011). However, whether this is a cause for, or a consequence of, a preference for high-fat food remains to be established.

The role of *carbohydrates* (CHOs) in weight loss has long been controversial and much of this debate continues and clearly the effect of CHOs on appetite and food intake is central to this debate. CHOs provide a large percentage of our daily energy and are consumed in a wide variety of forms. CHO can be divided into the available CHOs, which are digested and absorbed in the small intestine (SI), and the unavailable CHOs, which pass through the SI into the large bowel where they may provide a nutrient source for the resident microbiota producing by fermentation short chain fatty acids (SCFA).

Available CHOs typically comprise the mono- and disaccharide 'simple sugars' and the starch polysaccharides, whilst unavailable CHOs comprise resistant starch (RS), non-starch polysaccharide (NSP) 'fibres' and other more minor components. The available CHOs may in turn be slowly or rapidly digested and absorbed in the SI, resulting in different postprandial glycaemic responses and possibly different appetitive responses. 'Slow' CHOs lower glycaemia relative to rapidly digested 'fast' CHOs, and this effect on circulating glucose has been proposed to suppress appetite (Thomas et al, 2007) although it is a far from universal finding (Sands et al, 2009). The leading proponent of the 'glycogenostatic' theory of energy and body weight control was Professor J.P. Flatt, who hypothesised that depletion of the body's relatively small glycogen storage pool through either prolonged exercise or dietary CHO restriction would stimulate hunger and drive EI as the body recognises and attempts to replenish the depleted stores (Flatt, 1996). Epidemiological studies show dietary fibre and whole grains to be associated with a lower risk of overweight or obesity (Williams et al, 2008) and early studies showed high fibre foods to enhance satiety (Gustafsson et al, 1994). Fibre can be defined as two main forms, soluble and insoluble. Soluble fibre absorbs water in the GI tract to become at different extent gelatinous, viscous substance and undergoes fermentation by bacteria in the large bowel to generate SCFAs. Many of the soluble fibres are also defined as 'viscous' as they induce thickening when mixed with liquids. These include the gums, pectins, aliginates and β-glucans. Soluble fibres may alter satiety by a number of mechanisms including lowering the ED of foods, since fibre is not absorbed in the SI and hence contributes less energy per gram than available CHOs. Viscous soluble fibres absorb large amounts of water from the GI tract and as a result increase in volume and may act as a 'bulking' agent within the gut. It has been proposed that these fibres may increase gastric distension (De Graaf et al, 2004) and possibly retard gastric emptying, both of which may alter appetitive responses. They may also prolong transit time within the SI, the absorption rate of nutrients, and in turn the release of appetite-suppressing GI peptides such as cholecystokinin (CCK) from the proximal and GLP-1 and PYY from the distal SI (Maljaars et al, 2008). Conversely, insoluble fibre acts as a bulking agent in the colon (Willis et al, 2010). Wholegrain foods have been suggested as an important constituent of the diet, due to their high fibre content, low ED and increased volume and particle size; however, outcomes are mixed for effects on weight loss. Some observational studies have shown an association with lower risk of weight gain (Bazzano et al, 2005), but whether satiety or food intake is altered is unknown. Wholegrain foods are those in which the starchy endosperm, germ and bran (intact, ground, cracked or flaked) are present in the product as they are in the original intact plant. Studies have shown that post-prandial hunger and fullness may be altered by some wholegrain products such as wholemeal wheat bread (Kristensen et al, 2010) and some barley foods (Schroeder

et al, 2009) but longer term studies have failed to find effects on EI or body weight (Tighe et al, 2010). Growing and intriguing evidence that gut microbiota resident within the large bowel may differ between lean and obese individuals has led to the microbiome being proposed as an environmental factor responsible for both weight gain and altered energy metabolism of obesity (Turnbaugh et al, 2006). The microbiota, which includes viruses, archaea and some unicellular eukaryotes as well as bacteria, is present throughout the human body but in greatest numbers (~10-12 microorganisms/mL) in the large bowel. Unavailable CHOs undergo fermentation by the host microbiota to generate SCFAs. These in turn have been termed 'bacterial dietary metabolites' and hypothesised to have biological activities which may regulate various host functions, including suppression of food intake (Harris et al, 2012). One proposed anorectic mechanism is the enhancement by SCFA of colonic 'satiety' peptide GLP-1 released from enteroendocrine L-cells of the large bowel (Freeland et al, 2010). Exogenous administration of GLP-1 analogues such as liraglutide (Astrup et al, 2009) clearly do suppress appetite and EI, although whether sufficiently high concentrations can be achieved through dietary manipulation such as increased prebiotic substrate is yet to be demonstrated (Mars et al, 2012). An increase in large bowel Bifidobacteria numbers in particular has been associated with enhanced intestinal health (Roberfroid et al, 2010), but whether this association can be extrapolated to obesity is not known. Human obesity has been associated with both a low and high abundance of bacteria from the phylum Bacteroidetes relative to Firmicutes, but other studies show no parallel relationship or find no difference between phyla at all (Turnbaugh et al, 2006; Jumpertz et al, 2011). The role that prebiotic CHOs, which stimulate the growth or activity of the gut bacteria, may have in this relationship is equally unclear.

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Chapter 2. Bioavailability of polyphenols from whole grain is enhanced by resistant starch in Zucker Diabetic Fatty rats

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The present chapter is going to be submitted as a research article for publication.

Abstract

Recent studies showed that whole grain (WG) consumption can reduce the sub-clinical inflammation status in healthy overweight/obese subjects. This effect is related to the bioavailability of bound polyphenolic compounds that, as a consequence of dietary fibre fermentation, are released from WG matrix and absorbed into the bloodstream. This study was conducted to clarify the role of gut-microbiota and the mechanisms behind WG polyphenols bioavailability. To this aim 4groups of Zucker Diabetic Fatty (ZDF) were fed for 11 weeks semi-purified diets made with either an isolated digestible control starch, a WG control flour with 6.9% resistant-starch (RS), an isolated RS-rich starch with 25% RS, or a WG corn flour with 25% RS. Phenolic compounds were extracted from serum and analysed by HPLC–tandem mass spectrometry (MS/MS). Data showed that both groups fed WG diet had higher polyphenols concentration in serum compared to the control and RS diet. Moreover, WG corn flour with 25% RS diet resulted in higher serum polyphenols concentration compared to WG control flour with 6.9% RS.

In conclusion, data demonstrated that bound phenolic acids from WG are bioavailable in an animal model of obesity, insulin resistance and hyperglycemia, such as ZDF rats, and that the combination of RS and WG in the diet increase the bioavailability of WG polyphenols.

Introduction

Resistant starch (RS) is a prebiotic dietary fiber. According to the source and the process it's possible to distinguish five different categories of RS: RS1, starch granules present in indigestible plant material (whole grain, WG) therefore inaccessible to amylolytic and digestive enzymes; RS2, native granular starch not digestible by enzymes due to its conformation or structure (such as in potato or in green bananas); RS3, retrograded amylose starch formed during cooling of gelatinized starch (cooked and cooled starchy foods); RS4, chemically modified starch (i.e. starches esterified or cross-bonded with chemicals to decrease their digestibility); RS5, amylose complex with lipids (1).

In humans, the consumption of RS showed an improvement of insulin sensitivity in healthy individuals, in patients with metabolic syndrome and those with type 2 diabetes (T2D) (2-4).

Series of beneficial properties on prevention and treatment of obesity-related diseases due to its prebiotic effect were also shown in animal models.

Rodents fed with RS exhibited higher fermentation, reduced fat accumulation, enhanced insulin sensitivity, have a better control of glucose homeostasis and lipid metabolism. These effects seems to be mediated by gut microbiota production of SCFA which in turn stimulate the secretion of incretins GLP-1 and PYY by L-cells (5-7).

Gut microbiota play a crucial role in modulate the healthy effects of RS: when the fermentation is inhibited RS failed to show its effects on glucose and fat metabolism (8).

During the fermentation by gut microbiota also phenolic compounds (PC) bound to dietary fibres are released in the gastro-intestinal tract. In cereals, hydroxycinnamic acids are the most abundant PC bound to cell wall polysaccharides through ester bonds. The action of gut microbiota esterases release in a slow and continuous way the PC in the intestine where they can be adsorbed, pass to the bloodstream and exert they beneficial effect on the whole body (9). The PC concentration in cereals varies depending on cereal variety and milling procedure: in refined cereals deprived of the germ and bran the concentration is much lower than WG cereals (10). Therefore, it was hypothesized that the WG effect shown in epidemiological studies to lower the risk of chronic diseases, such as cardiovascular disease, diabetes, and cancer, and to help body weight control may be due to their high fiber and phytovchemicals content (11-13). However, the food source is a fundamental factor to determine the bioefficacy of the WG, because the chemical and physical structure of the food matrix strongly influence the bioaccessibility and final bioavailability of PC (14).

Zucker Diabetic Fatty (ZDF) rat were derived from a mutation occurred in a colony of outbred Zucker rats. At six/seven weeks of age they start suffering of hyperinsulinemia, hyperglycaemia, insulin resistance and obesity beginning thus being a good animal model of T2D (15). .

In the present study, we compared four diets with different source and amount of RS and WG to investigate if RS consumption may have any beneficial effect on the bioavailability of PC from WG in an animal model of T2D. A secondary aim was to assess if the ingestion of RS and WG had an effect on food intake and body weight.

Materials and Methods

Chemicals and reagents

Water, acetonitrile and methanol were HPLC grade (Merck, Darmstadt, Germany). Formic acid was purchased from Fluka (Milano, Italy). Caffeic acid, ferulic acid (FA), chlorogenic acid, hippuric acid, vanillic acid, protocatechuic acid, coumaric acid, homovanillic acid standards were purchased from Sigma (Italy). 3-(4-hydroxyphenyl)propionic acid (HPP), 3-hydroxyphenylacetic acid **(**HPA), (3,4-dihydroxyphenyl)acetic acid (DHPA) were obtained from Aldrich (Italy). Oasis cartridge HLB 1cc (30 mg): were from Waters (U.S.A).

Rats and diets

A total of 45 male Zucker Diabetic Fatty rats (Charles River Laboratories) were used. ZDF rats four-week old were maintained in quarantine for a period of one week and acclimated to powder control diet for 2 weeks. The ZDF rats, at 7 weeks of age, with an average body weight of 250.5 ± 17.5 g,

were randomly divided into 4 groups fed isocaloric diets (3.2 Kcal/g) containing: AC (amioca control) (n=12), DWGC (Whole Grain) (n=11), HM260 (resistant starch) (n=11), HMWG (Whole Grain + resistant starch) (n=11).

AC group diet was characterized by starch amioca that is a starch derived from waxy maize, rich in amylopectin (starch highly digestible). Group DWGC received a diet characterized by corn flour integral (whole grains). Corn flour with a high amylose starch (resistant starch) was characteristic of HM260 diet group. Group HMWG was given the corn flour high in amylose (resistant starch) and corn flour with whole meal enriched corn meal high in amylose (grains + resistant starch). Concentration of resistant starch in the different diets was: AC 0%, DWGC 6.9%, HM260 25%, WGRS 25%. The nutritional composition and the phenolic acid content (including total, free and bound to dietary fiber) of each specific diet are reported in *table 1*.

Table 1. Nutritional composition and phenolic acid profile of the diets used in the study.

Parameter	Diets			
Macroutrients, g/kg	***AC***	***DWGC***	***HM260***	***HMWG***
Amioca	533.6	83.7	61.7	169.4
HM260	0	0	619	0
Whole Grain	0	0	0	576
Dent Whole Grain	0	550	0	0
Sucrose	100	100	100	100
Caseine	136.6	93.7	135.1	84.6
Cellulose	140	108	0	15.8
Soybean oil	40	14.8	34.4	4.4
Micronutrients, g/kg				
Mineral mix	35	3	35	35
Viatmin mix	10	10	10	10
Choline	3	3	3	3
L-Cysteine	1.8	1.8	1.8	1.8
Phenolic compounds, mg/kg				
Ferulic acid (total)	6.31	626.25	3.56	607.31
Free	1.02	0.75	0.43	0.81
Bound	5.29	625.50	3.14	606.50
Diferulic acid (total)	12.32	763.24	4.78	821.20
Free	–	–	–	–
Bound	12.32	763.24	4.78	821.20
Coumaric acid (total)	4.45	63.96	1.46	56.55
Free	2.99	0.81	0.86	0.88
Bound	1.46	63.15	0.60	55.68
Vanillic acid (total)	3.20	7.71	1.16	9.58
Free	3.20	2.06	1.16	3.10
Bound	–	–	–	–
Caffeic acid (total)	0.45	0.58	0.52	0.28
Free	0.45	0.29	0.52	0.22
Bound	–	0.28	–	0.07
Salycilic acid (total)	0.11	0.22	0.07	0.14
Free	0.07	0.08	0.04	0.09
Bound	0.04	0.14	0.03	0.04
Total phenolics	26.84	1461.96	11.55	1495.05
Free	7.73	4.00	3.01	5.09
Bound	19.11	1457.96	8.54	1489.96

The study lasted 11 weeks and during this period body weight and food intake were monitored twice per week weighing the ration for each rat at the entrance and exit of the cage, after being fed, considering the animal dejections. At the end of dietary treatment the rats were sacrificed in a state of non-fasting and serum separated by centrifugation was collected and frozen at −80 ◦C.

Biochemical analysis

Phenolic compounds from serum were extracted and purified according to the method described by Guerrero et al. (16) slightly modified. Seven hundred fifty microliters of 0.2% acetic acid were added to 250 µL of serum to obtain a final volume of 1 mL. Samples were then centrifuged at 16800 g for 5 min at 4°C. Oasis HLB 1 cc 30 mg cartridges were preconditioned using 1 mL of methanol and 1 mL of H2O. Samples were loaded onto the cartridges and at a flow rate of approximately 1 mL/min. The cartridges were washed with 1 mL of H2O and 1 mL of 0.2% acetic acid. Phenols were eluted with 1 mL of methanol 0.2% acetic acid. The eluate was dried under a stream of nitrogen, dissolved again in 100µL of MeOH/H2O (70:30, v/v) solution, centrifuged at 16800 g for 5 min at 4°C and directly used for LC/MS/MS analysis. For each rat the extraction was performed in duplicate from two different aliquots.

Phenols compounds were analyzed using liquid chromatography coupled to tandem mass spectrometry (LC-MS-MS). Chromatographic separation was performed using an HPLC apparatus equipped with two micro-pumps Series 200 (Perkin Elmer, Norwalk, CT, USA) and a Gemini 5u C18 110 Å, 150 x2 mm, column (Phenomenex, USA). The solvent system consisted of the following mobile phases: (A) water 0.1% formic acid, (B) acetonitrile 0.1% formic acid. The gradient program was as follows: 10% B (1 min), 10–90% B (7 min), 90 % B (2 min), 90–10% B (2 min), at a constant flow of 0.2 mL/min. Injection volume was 20 µL. MS/MS analyses of phenols were performed on an API 3000 triple quadruple mass spectrometer (Applied Biosystems, Canada) equipped with a TurboIonSpray source. The MS parameters declustering potential (DP), focus potential (FP) and collision energy (CE) were the same reported by Vitaglione et al. (17) *(table 2)*. Analysis was performed in the negative ion mode in MRM (Multiple Reaction Monitoring). When analytical standards were not available, compounds were identified comparing molecular weight and fragmentation patterns with those reported in the literature, (17,18).

Table 2. Analyzed compounds, mass spectrometry parameters, limits of detection and quantification, linearity range of calibration curves.

Compound	[M-H]-	Product ions [M-H]-	CE	DP	LD ng/mL	LQ ng/mL	Linearity range ppb
Chlorogenic acid	353	191	21	35	0.5	1	5-250
Ferulic acid (FA)	193	134; 178	22; 17	40	0.5	1	10-1000
Vanillic acid	167	152; 108	20; 26	45	2.5	5	10-250
Protocatechuic acid	153	109	21	45	0.5	1	5-250
Coumaric acid	163	119	23	40	2.5	5	5-500
Caffeic acid	179	135	21	49	0.5	1	5-250
Hippuric acid	178	134; 77	13; 20	35	2.5	5	5-250
Dihydroferulic acid (DHFA)	195	136	21	40	2.5	5	
Dihydrocaffeic acid;	181,1	137; 109	10; 18	30	25	100	100-2500
DHPA	167	123	18	30	0.5	1	100-10000
3-HPA	151	107; 77	13	35	3	5	100-10000
HPP	165	121; 105.9; 76.7	10;20;10	25	25	100	100-10000
5(3',4'-dihydroxyphenyl-ɣ-valerolactone) DHPV	207	163; 122	25; 25	35			
Hydroxybenzoic acid	137	93	25	50			
Ferulic acid glucuronide	369	193	30	40			
Ferulic acid sulfate	273	193	30	40			
Furuloylglycine	250	191; 206, 177	30	40			

Statistical analysis

All values are reported as means ± SEMs. The means of the different groups in total PC and for each PC s were compared for the analysis of variance (ANOVA). When the ANOVA analysis indicated a difference among the groups, a post-hoc Tukey's test was applied to identify any significative difference between two groups.

Statistical analyses were performed by using Statistical Package for Social Sciences (version 21.0; SPSS, Inc., Chicago, IL, USA).

Results

Phenolic compounds

PC retrieved in serum samples were ferulic acid, dihydroferulic acid, coumaric acid, protocatechuic acid, hippuric acid, caffeic acid, chlorogenic acid, HPP, 3-HPA and DHPA. The groups fed WG had higher serum concentrations of total polyphenols compounds compared to the control and to the group fed resistant starch. Specifically, the highest concentration of total PC were found in the HMWG group (6453±549 nmol/L) with values more than 11 times higher than AC and HM260 and

2.4 times higher than DWGC ($p<0.001$). In the DWGC group the total PC were also significantly higher than AC and HM260 ($p<0.01$) *(figure 1, panel A)*.

In all of the treatments the most abundant phenol was the hippuric acid, but at a significant different concentration among groups being in the DWGC group higher than AC and HM260 (respectively 1522±290nmol/L, 312±47nmol/L and 392±79nmol/L; $p<0.001$) and in the HMWG group higher vs all the other groups (2856±399nmol/L; $p<0.001$) *(figure 1, panel B)*.

In the HMWG group, the serum concentration of dihydroferulic acid (DHFA) was similar to that of hippuric acid (2726±287nmol/L) and it was significantly higher compared to that found in AC, DWGC and HM260 rats ($p<0.001$). On the contrary DHFA in rats fed with DWGC and HM260 did not change compared to those fed with AC *(figure 1, panel C)*.

A similar trend was observed for the ferulic acid (FA). It ranged from 2.8±2nmol/L in the AC group up to 157±27nmol/L in the HMWG group which was significantly higher compared to the other groups ($p<0.001$). No significant difference was observed among the other groups *(figure 1, panel D)*. For both compounds DHFA and FA a high variability among rats was found in the DWGC group (serum concentration ranged from 0 to 258 nmol/L and from 0 to 66 nmol/L for DHFA and FA, respectively).

In terms of abundance, another important PC found in serum was the hydroxyphenyl propionic acid (HPP). Treatments with WG resulted in a significantly higher HPP serum concentration: in fact, both DWGC and HMWG had higher values compared to AC and HM260 (respectively 879±122nmol/L, 543±81nmol/L, 107±56nmol/L, 36±11nmol/L; $p<0.001$). In turn, DWGC had higher values than HMWG ($p<0.05$) *(figure 1, panel E)*.

The WG diets also significantly increased the cumaric acid serum concentration, since it was 101±10 nmol/L and 82±8 nmol/L in HMWG and DWGC respectively vs 37±9 nmol/L and 29±3nmol/L in AC and HM260 respectively ($p<0.001$) *(figure 1, panel F)*.

The treatments with high amount of RS (HM260 and HMWG) also resulted in higher serum concentration of 3-hydroxyphenilpropionic acid (3-HPA) vs AC and DWGC ($p<0.05$). However the serum concentration was very low for all the treatments (0.61±0.06; 0.63±0.07; 0.98±0.10; 0.83±0.11; for AC; DWGC; HM260 and HMWG respectively).

No differences were found regarding the others PC retrieved in serum among treatments.

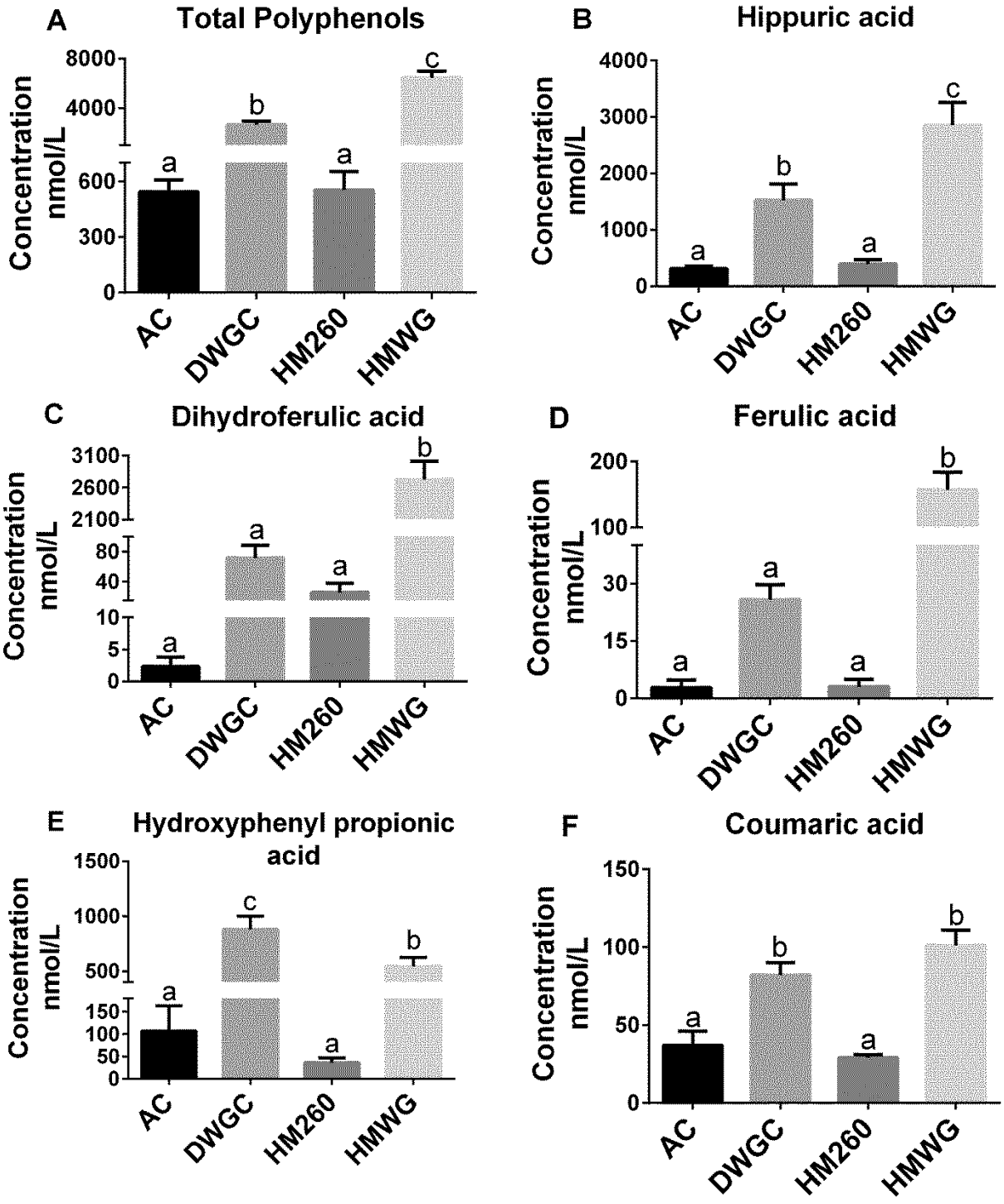

Figure 1. Polyphenols serum concentration in ZDF rats following 11week of diet. Values are expressed as mean±SEM. Various letters indicate values significantly different ($p<0.05$) on the base of ANOVA and Tukey post-hoc test

Food intake and body weight

Food intake was monitored twice a week and results were expressed as the average of food amount consumed per week (g/wk). Despite the foods supplemented were isocaloric (3.2 kcal/g) animals fed DWGC, HM260 and HMWG ate more than control ($p<0.05$). Moreover, tukey's test revealed both treatments with 25% RS resulted in a significantly higher food intakes compared to DWGC ($p<0.05$) (*figure 2*).

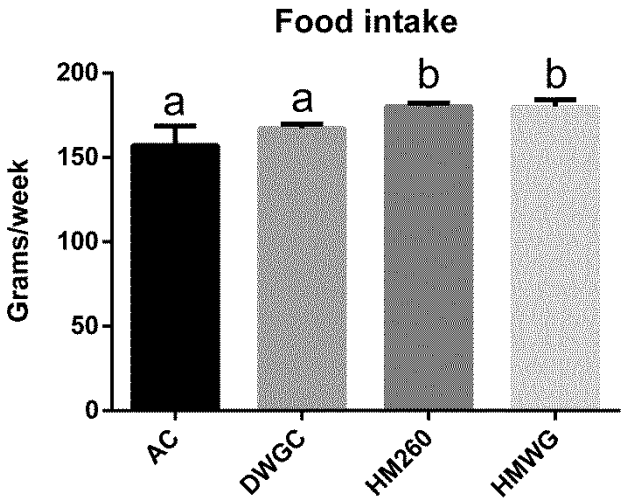

Figure 2. Food intake of each group of rats per week. Values are expressed as mean±SEM. Different letters on the bars indicated significantdifferent values ($p<0.05$; ANOVA and Tukey post-hoc test).

Body weight did not differ among groups neither at the beginning of the experiment nor after 11 weeks *(figure 3).*

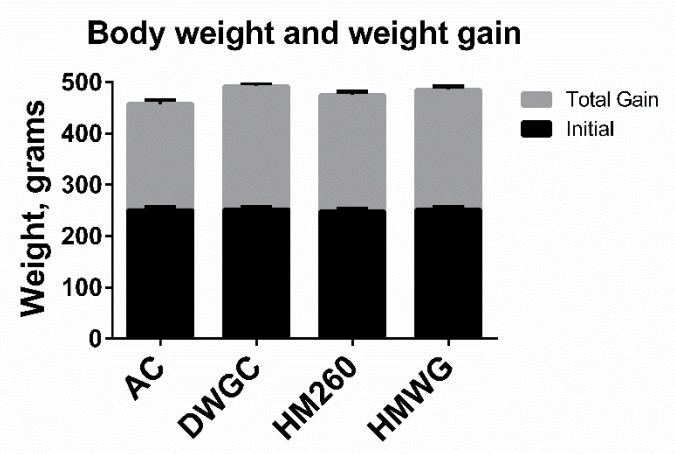

Figure 3. Initial and total weight gain of rats at the beginning and after the 11weeks of diet. Values are expressed as mean±SEM.

Discussions

The present study was designed to assess the bioavailability of PC from WG with or without the addition of RS to the diet in an animal model of diabetes. The effect of the dietary treatments on body weight was also investigated. To this purposes an amylopectin corn starch (AC group), WG dent corn flour, with a moderate (7%) amount of RS (DWGC group), an isolated source of RS from corn starch, which only provided RS type 2 (HM260 group), and a WG high amylose corn flour, which provided both RS type 1 and 2 (HMWG group), were used. ZDF rats were chosen since they represent a model of T2D with high insulin resistance and hyperglycemia associated to obesity. Moreover, two previous studies found that ZDF rats have a low fermentation response when fed with a prebiotic oligofructose dietary fiber suggesting that they can also have altered gastrointestinal microbiota (19,20). WG cereals contain a high amount of total PC, but they are almost totally bound to dietary fiber (*table2*), therefore, to obtain a healthy effect from their release into the bloodstream the breakdown of fiber due to fermentation by gut microbiota plays a fundamental role(17). Our finding demonstrated that ZDF rats fed WG diet had higher serum concentration of total PC compared to a control diet and a high RS diet. On the best of our knowledge, this is the first time that the bioavailability of PC from WG cereals were investigated in a long term study in rats. Previous studies in rats investigated the short-term pharmacokinetic of PC from cereals. They showed that the supplementation of bran cereals causes a slower but longer, up to 24h, release of PC compared to the administration of pure PC (21,22). However, a single dose PC supplementation not always result in a greater protection against oxidation (21). Moreover, even middle-term dietary intervention with WG cereals failed to show beneficial effects on conventional oxidative stress markers probably due to the low antioxidant concentrations or to poor bioavailability of PC (23). Therefore, to obtain a significant increase in serum PC concentration that can result in a whole body health effect the length of the supplementation and the amount of the total PC must be considered. To this regard in a dietary treatment with WG cereals the characterization of the phenolic fractions should be always carried out.

In humans, it was already demonstrated that the replacement of refined carbohydrates with WG cereals ameliorate the inflammatory markers in healthy overweight subject with sub-optimal nutritional status in humans (24). In a meta-analysis Aune and colleagues (25) found evidences of a nonlinear inverse association between WG consumption and type 2 diabetes, with most of the reduction observed when increasing the intake up to 2 servings per day. A combination of several mechanism probably modulate the healthy effect of WG on T2D. In fact, WG are an important source of cereal fiber, phytochemicals, vitamins and minerals. Of course the phytochemicals component can be responsible for the protection against oxidative stress. In fact, the consumption of

cereal varieties, particularly rich in PC, such as red or black rice, was reported to affect some oxidative stress biomarkers (26).. In addition, WG intake may reduce risk of T2D by reducing the serum concentration of proteins correlated to an inflammatory status such as plasminogen activator inhibitor type 1 and C-reactive protein, liver gamma-glutamyltransferase and aspartate aminotransferase (27-31), but increasing the serum concentration of anti-inflammatory cytokines such as adiponectin (28, 32). Therefore the increase in total PC serum concentration after the treatments with WG may explain the results found in previous studies where a role for the PC were hypothesized, but the PC serum concentration were not investigated.

The most interesting result from our study was that the consumption of WG cereals high in RS increase the total PC serum concentration compared to WG cereals with a low percentage of RS.

This effect may be ascribed to the prebiotic properties of the RS (33).

In fact, RS is resistant to the gastric acidity, to the hydrolysis by mammalian enzymes and to the gastrointestinal absorption; it can be fermented and be utilized by gut microbiota and selectively stimulate activity and the growth of a limited number of gut bacteria that contribute to host health and well being (34). It was demonstrated that mice fed with diets containing high amylose RS2 were colonized by higher levels of Bacteroidetes and Bifidobacterium, Akkermansia and Allobactum species (35). Another nutritional study exhibited that RS is able to induce a 10-fold increase of the gut bifidobacteria (36).

Therefore, since the gut microbiota activity is essential to release the PC bonded to dietary fiber in WG cereals, it can be hypothesized that the gut microbiota enhanced in activity and growth by RS is able to make the PC from WG more bioavailable. This results in a synergetic effect between RS and WG and as a consequence in a higher serum concentration of PC compared to WG alone.

The analysis of retrieved PC also indicated a higher activity of gut microbiota in groups fed high RS diet: it is noteworthy to underline that the 3-HPA serum concentration, a well know microbial metabolite (37), were higher in both treatments with RS. The synergetic effect between RS and WG is also confirmed by the DHFA which could be released by the food matrix, but in part also produced by the gut microbiota fermentation (38,39).

It was present in a comparable amount in DWGC and HMWG cereals, but was retrieved in a significantly higher concentration only in the group fed HMWG.

A secondary aim of the present work was to assess if supplementation with RS results in a lower food intake and in a reduction of body weight. We found that food intake was higher in rats fed high RS amount and this didn't not result in a significant difference among groups in term of total body weight. Several studies were conducted using different rodent models. They indicated that RS may affect body weight in a way dependent of the proportion of RS in fodder, of the baseline nutritional

status and of the animal access to fodder, being *ad libitum* access instead of fodder restriction more advantageous (40-43). In our study all these three parameters were settled in the best condition as indicated by previous studies. A role for GLP-1 and PYY was suggested to explain the mechanism of RS action on body weight. In fact, it was demonstrated that RS fermentation led to an increase in GLP-1 and PYY plasma levels (6,7; 44). However, ZDF rats have a mutation in the leptin receptor gene leading to production of a truncated protein. As a consequence Zucker rats required much higher doses of leptin than normal rats to produce a similar effect (45). Since leptin and GLP-1 were proved to act in concert to control the activity of feeding centres (46), it can be hypothesized that the leptin signalling is fundamental to promote a beneficial effect of RS on appetite and body weight.

In conclusion results from this study demonstrated that PC from WG are bioavailable in ZDF and the addition of 25% RS to the diet increase the bioavailability. This resulted in higher PC serum concentration which can explain the WG capacity to ameliorate the inflammatory status in T2D. Further study should investigate the effect of a long term WG supplementation together with RS in diabetic humans.

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Chapter 3. Wholegrain wheat consumption reduces inflammation in a randomized controlled trial on overweight and obese subjects with unhealthy dietary and lifestyle behaviors: role of polyphenols bound to cereal dietary fiber

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Abstract

Background: Epidemiology associates wholegrain (WG) consumption with several health benefits. Mounting evidence suggests that WG wheat polyphenols play a role in mechanisms underlying health benefits.

Objectives: To assess circulating concentration, excretion and the physiological role of WG wheat polyphenols in subjects with suboptimal dietary and lifestyle behaviors.

Design: A placebo-controlled parallel-group randomized trial with 80 healthy overweight/obese subjects with low intake of fruits and vegetables and sedentary lifestyle was performed. Participants replaced precise portions of refined wheat (RW) with fixed amount of selected WG wheat or RW products for 8 weeks. At baseline and every 4 weeks, blood, urine, feces, and anthropometric and body composition measures were collected. Profiles of phenolic acids in biological samples, plasma markers of metabolic disease and inflammation, and fecal microbiota composition were assessed.

Results: WG consumption for 4-8 weeks determined a 4 fold increase of serum dihydroferulic acid (DHFA), and a 2-fold increase of fecal ferulic acid (FA) compared to RW consumption (no changes). Similarly, urinary FA at 8 weeks doubled the baseline concentration only in WG subjects. Concomitant reduction of plasma tumor necrosis factor-α (TNF-α) after 8 weeks and increased interleukin-10 only after 4 weeks with WG vs RW ($p=0.04$) were observed. No significant change of plasma metabolic disease markers over the study period but a trend towards lower plasma plasminogen activator inhibitor-1 with higher excretion of FA and DHFA in WG group was found. Fecal FA was associated with baseline low Bifidobacteriales and Bacteroidetes abundances whereas after WG consumption it correlated with increased Bacteroidetes and Firmicutes, but reduced *Clostridium*. TNF-α reduction correlated with increased *Bacteroides* and *Lactobacillus*. No effect of dietary interventions on anthropometry and body composition was found.

Conclusions: WG wheat consumption significantly increased excreted FA and circulating DHFA. Bacterial communities influenced fecal FA and were modified by WG wheat consumption.

Introduction

Epidemiological evidence indicates that wholegrain (WG) substantially lowers the risk of chronic diseases such as cardiovascular disease (CVD), diabetes, and cancer, and plays a role in body weight management and digestive health. Dietary guidelines worldwide recommended to increase WG consumption by replacing refined grains (1, 2).

Recently, some doubts on the epidemiological links between WG consumption and disease prevention arose (3) and intervention studies about subclinical inflammation and body weight showed discrepant findings (4, 5). However, convincing evidence to support beneficial effects of WG intake on vascular disease prevention were provided (6).

There is still a knowledge gap on the mechanisms underpinning WG health benefits. It is known that WG physical structures help in reducing glucose and lipid absorption, dietary fiber can contribute to improve several gut functions, and many bran and germ phytochemicals may exert antioxidant and anti-inflammatory properties (7, 8). The bifidogenic effect of WG found in some studies suggested a role of the microbiota in triggering amelioration of gut and systemic inflammation, explaining some of the metabolic benefits attributed to WG consumption (9-15). The interplay between microbiota and the polyphenols bound to WG fibre might explain some of WG health benefits (16). WGs are a rich source of phenolic compounds, mainly hydroxycinnamic acids (17-20) being ferulic acid (FA) the most abundant. The FA concentration varies depending on cereal variety and milling procedure (21) and in WG wheat it is in the range of 4.5-1270 mg/kg (22). From the chemical point of view FA and 95% of the grain phenolic compounds are covalently bound to arabinoxylan chains of cell wall polysaccharides through ester bonds (23). WG fiber can deliver phenolic compounds into the lower gut and that the slow and continuous release of FA by the action of gut microbiota metabolism may increase circulating FA and its metabolites thus providing an amelioration of subclinical inflammation and the long-term benefits associated to WG consumption (16).

However, to the best of our knowledge no intervention study was performed to determine the bioavailability of WG polyphenols and to ascertain their role in prevention of chronic disease over long-term consumption.

Here an 8-week double-arm randomized controlled trial in 80 healthy overweight/obese subjects sharing suboptimal dietary and lifestyle behaviors was performed by daily replacing exact amounts of specific refined wheat products with a WG wheat product (WG group) or selected refined wheat products (control group). The metabolic profiles of phenolic acids in

blood, urine and feces were obtained, and the concomitant change in fecal microbiota composition and obesity-related inflammation and chronic disease risk were determined.

Subjects and methods

Food products

A 100% WG wheat product was used in this study ("Shredded wheat", Cereal Partners Worldwide, Switzerland). It was selected among several commercial products for its WG wheat content (100% WG) and for the amount of polyphenols bound to dietary fiber. Two refined wheat products were selected from the market to guarantee a nutritionally well balanced placebo for the whole grain product ("Magretti" crackers, Galbusera, Cosio Valtellino, Italy and "Mulino Bianco" toasted sliced breads, Barilla, Parma, Italy).

Subjects

Recruitment was performed at the Department of Agricultural and Food Science of University of Naples. Subjects were recruited into the study by public announcements on local newspaper, social networks, and among students and staff of the Department. The selection was carried out by interview on health status and dietary and behavioral lifestyle factors, collection of anthropometric data, and a 7-day food diary recall. Men and women aged > 18 years, with a $25 \leq BMI \leq 35$ kg/m^2, habitual diet characterized by absence of WG cereals and cereal bran containing products, probiotics, vitamins/minerals supplements or complementary and alternative medicines, intake of fruit and vegetables ≤ 3 servings/day (300 g/day), and low level of physical activity (Total physical activity < 500 MET-minutes/week), were eligible to participate.

Subjects having any type of disease (functional or metabolic disease including hyperlipidemia, diabetes and metabolic syndrome) or food allergy, dieting or who were under a controlled dietary regime over the previous three months, being under drug therapy of any type or who were using drugs over the previous three months, participating in other trials or who were pregnant or lactating, were excluded.

Eligible subjects who agreed to participate entered into the study by signing a written informed consent.

Study Design

It was an 8 weeks placebo controlled randomized trial with a double arm parallel design. Once enrolled by the study nutritionist and medical doctor, subjects were randomly assigned by the dietician to the WG or the control (CTR) group on the basis of a randomization sequence that was previously generated by the statistician with the use of a computer-generated permuted blocks (n = 5) randomization scheme.

The dietary intervention was tailored on each subject and consisted of isocaloric replacement of specific amount of some refined wheat products (mainly bread, pasta or sliced toasted bread) habitually consumed by subjects with the selected food products. WG subjects included for 8 weeks in their diet 70 g/day (3 biscuits/day) of WG product, while CTR subjects included 1 package (33g) of crackers and 3 sliced toasted bread (~27 g). In addition all subjects were instructed to consume with experimental foods the same amount of seasonings they usually ate (if any) with the replaced foods in order to maintain unchanged overall nutritional composition of their diets and to maintain unchanged the amount of consumed fruits and vegetables and the level of physical activity.

The nutritional composition and the phenolic acid content (including total, free and bound to dietary fiber amount of each compound) of WG and refined wheat portions daily consumed by volunteers was reported in **Table 1**.

111 **TABLE 1:** Nutritional composition and phenolic acid profile of a daily portion of
112 wholegrain (WG) wheat (70 g) and refined wheat products (60 g, cumulative of two
113 products) consumed in this study by WG and control subjects, respectively.
114

	WG wheat product	**Refined wheat products**
Proteins (g)	7.8	6.5
Carbohydrates (g)	45.8	45.7
- sugars	0.6	1.2
Fats (g)	1.7	2.2
- saturated	0.3	0.3
Dietary fiber (g)	8.0	2.2
Energy (kcal, kJ)	229.5, 960.2	222.4, 930.5
Phenolic compounds (mg)		
- Ferulic acid (total)	96.7	2.6
free	*0.3*	*2.6*
bound	*96.4*	---
- Sinapic acid (total)	26.5	---
free	*0.2*	---
bound	*26.3*	---
- Coumaric acid (total)	9.4	---
free	*traces*	---
bound	*9.4*	---
- Gallic acid (total)	1.9	---
free	*0.1*	---
bound	*1.8*	---
- Syringic acid (total)	1.8	traces
free	*0.3*	*traces*
bound	*1.5*	---
- Vanillic acid (total)	1.6	traces
free	*0.2*	*traces*
bound	*1.4*	---
- Salycilic acid (total)	0.5	---
free	*0.1*	---
bound	*0.4*	---
- Caffeic acid (total)	0.3	---
free	*traces*	---
bound	*0.3*	---
Total phenolics	**138.7**	**2.6**
free	*1.2*	*2.6*
bound	*137.5*	---

115

Study protocol

The study protocol was approved by Ethics Committee of University of Naples and it was illustrated in **Figure 1**.

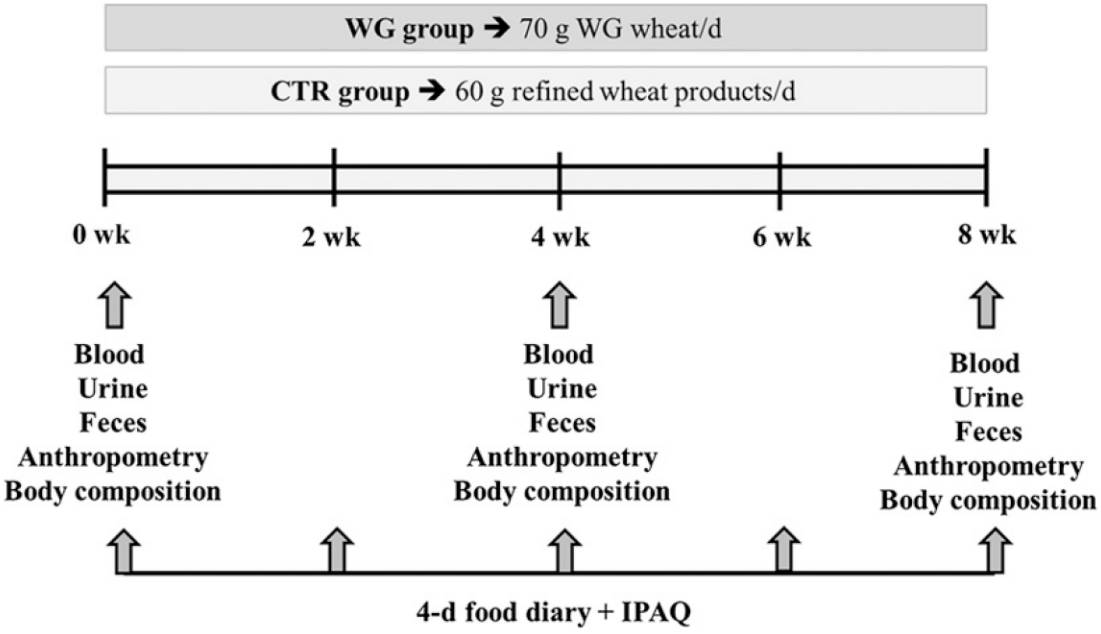

Figure 1: Schematic outline of the study protocol. WG, wholegrain; CTR, control; IPAQ, International Physical Activity Questionnaire

Food products were supplied at baseline and after 4 weeks at the Department of Agricultural and Food Science of University of Naples. Compliance to the dietary treatments was assessed every 2 weeks by self-recorded 4-day (3 working days and 1 weekend day) food diaries and also every 4 weeks by weighing the uneaten foods returned by subjects; moreover, phone call interviews at 2 and 6 weeks were done by an expert dietician to monitor the compliance to the protocol and the physical activity level by International Physical Activity Questionnaire (IPAQ) (24). At baseline and every 4 weeks of treatment, fasting participants reached the laboratory to collect blood and urine samples, anthropometric and body composition data. During those occasions they also delivered a fecal sample (collected the day before and stored at -20°C until arrival) and food diaries filled at weeks 2-4 or 6-8. Biological samples were collected, treated and analyzed as required for the specific procedures by personnel who was blinded after assignment of interventions.

Determination of phenolic compounds in serum, urine and feces

Blood samples were collected in serum tubes for gel separation and immediately centrifuged at 2600 g for 10 min at 4°C. Urine samples were immediately treated with 0.005% of

butylated hydroxytoluene (BHT). Feces were diluted in the ratio 1:10 (w/v) in PBS (10 mM) containing 0.005% of BHT, vortexed and centrifuged at 2600 g for 15 min at 4°C. Serum, urine and fecal supernatants were stored at -40°C prior to analysis.

PC were extracted and analyzed by HPLC/MS/MS as recently described (25). Briefly 500 µL of serum and 1.5 mL of urine and fecal suspensions were extracted by ethyl acetate (1.5 mL x 2 or x 3 times, respectively); supernatants were dried under nitrogen flow and dissolved in 50 µL methanol–water (70:30); 30 µL were injected into HPLC/MS/MS.

A HPLC system consisting of two micropumps by Perkin Elmer Series 200 (Shelton, Connecticut, USA), coupled with an API 3000 Triple Quadrupole mass spectrometer (Applied Biosystem Sciex, Framingham, Massachusetts, USA) was used and elution was achieved with a Phenomenex Luna 3µ C18(2) 100 A (50 x 2.00 mm) column, using water:acetonitrile:formic acid, 94.9:5:0.1 (by vol.), and acetonitrile:formic acid, 99.9:0.1 (v/v) as mobile phases, a flow rate of 200 µL/min and a linear gradient. Phenolic acids were detected and quantified through electrospray ionisation MS/MS analysis (negative mode ionisation, multiple reaction monitoring mode tracking) using the MS parameters and specific calibration curves (for method details see 25).

To normalize the excretion rate of urinary phenolic compounds 1 mL aliquots of urine were stored at -40° C and urinary creatinine concentration was measured by an automated system based on the buffered Jaffe reaction and analyzed by the COBAS Integra (Roche Diagnostic Ltd, Rotkreuz, Switzerland).

Determination of markers of metabolic and inflammatory disease in plasma

Metabolic disease intermediate markers were determined in duplicate in 12.5 µL of plasma by using Bio-Plex Pro™ human diabetes immunoassays multiplex kit (Bio-Rad, Hercules, California) and by using Luminex Technology (Bio-Plex; Bio-Rad, Hercules, California), according to the manufacturers' instructions. Blood samples were collected into EDTA containing tubes and were immediately added with protease inhibitors, such as dipeptidylpeptidase IV (DPPIV) inhibitor (Millipore's DPPIV inhibitor; St Charles, MO, USA) and phenylmethanesulfonyl fluoride (PMSF, Sigma, St. Louis, MO, USA). They were centrifuged at 2400 g per 10 min at 4 °C, and the supernatants were stored at -40 °C prior to analysis.

The Bio-Plex Pro™ immunoassays kits allowed the simultaneous quantification of the following biomarkers: C-peptide, ghrelin, glucose-dependent insulinotropic peptide (GIP), glucagon-like peptide -1 (GLP-1), glucagon, insulin, leptin, plasminogen activator inhibitor-1 (PAI-1), resistin, visfatin, adiponectin and adipsin, interleukin-6 (IL-6), interleukin-10 (IL-

10), and tumor necrosis factor - α (TNF-α). The sensitivity levels of the assay (in pg/mL) correspond to the following: C-peptide, 14.3; ghrelin, 1.2; GIP (total), 0.8; GLP-1 (active), 5.3; glucagon, 4.8; insulin, 1; leptin, 3.1; PAI-1, 2.2; resistin, 1.3; visfatin, 37.1.

The interassay variation (%CV) was 4%, and the intra-assay variation (%CV) was 5%.

Glycemia

Glycemia was measured immediately before the blood draw by finger pricking and using a bedside glucometer (OneTouch Sure Step; Life Scan Inc., Milpitas, CA, USA). Accuracy of the glucometer was evaluated by the manufacturer using least-squares linear regression analysis and found to be 97% ‘‘clinically accurate’’ when compared with reference (YSI2700) results.

Determination of plasma lipids

Cholesterol and triglycerides were assayed on plasma and HDL by enzymatic colorimetric methods (ABX Diagnostics, Montpellier, France; Roche Molecular Biochemicals, Mannheim, Germany; Wako Chemicals GmbH, Neuss, Germany, respectively) on a Cobas Mira autoanalyzer (ABX Diagnostics, Montpellier, France). HDL were isolated from plasma by a precipitation method with a sodium phosphotungstate and magnesium chloride solution.

Determination of the fecal microbiota by 16S rRNA gene sequencing and data analysis

Microbial DNA extraction was carried out using the PowerSoil® DNA isolation kit (MoBIO Laboratories, Inc. Carlsbad, CA) using 250 mg of fecal samples collected at baseline and at the end of intervention (8 weeks). The V4 region of the 16S rRNA gene (515F-806R) was amplified using the Earth Microbiome Project barcoded primer set. PCR conditions and library preparation were as described previously (26, 27). Sequencing was carried out using on the Illumina MiSeq platform (Argonne Core Sequencing Facility).

Sequence data processing and analyses were performed with scripts from the Quantitative Insights into Microbial Ecology (QIIME) software package, version 1.5.0 (28), using default parameters. Raw sequence files were quality filtered and demultiplexed using the split_libraries_fastq.py script in QIIME, with default settings (28). 1,615,683 sequences remained after demultiplexing. The pick subsampled reference otus through otu table.py script was used to generate 97% Operational Taxonomic Unit (OTU) clusters (open reference OTU picking), an OTU table (singletons removed), a representative sequence file (based on cluster centroids), an alignment of the representative sequences, and a phylogenetic tree based on the alignment. Sequence alignments were carried out using PyNAST (28). The above OTU picking workflow has been renamed pick_open_reference_otus.py in the latest version of

QIIME (v 1.8.0). The February 4, 2011, release of Greengenes was used as the reference database for OTU picking (29). In the final OTU table, there were 22,019 non-singleton OTUs, and the number of sequences per sample varied from 3,743 to 55,750 (median = 16,018; excluding 3 samples that failed to sequence properly). Therefore all samples were rarified to 3,740 sequences per sample prior to downstream analyses. Statistical tests were run using the otu_category_significance.py (ANOVA) and compare_categories.py (ADONIS, ANOSIM, and MRPP) scripts in QIIME as previously reported (27). Weighted and unweighted UniFrac (30) distance matrices were used for constructing Principal Coordinate Analysis (PCoA) plots.

Determination of anthropometric measurements and body composition

All measurements were performed by the same operator following standard procedures.

Height of subjects was measured during the selection phase to the nearest 0.5 cm with a stadiometer (Seca Mod. 213, Germany). Weight was measured, after voiding, with subjects wearing light clothing to the nearest 0.1 kg on a digital scale (Seca Mod. 703, Germany).

Waist circumference was measured on undressed subjects at the midpoint between the lower margin of the last palpable rib and the top of the iliac crest. Hip circumference was measured around the widest portion of the buttocks, with the tape parallel to the floor.

Body composition was determined by conventional BIA with a single frequency 50 kHz bioelectrical impedance analyzer (BIA 101 RJL, Akern Bioresearch, Firenze, Italy) in the post absorptive state, at an ambient temperature of 22–24 °C, after voiding and after being in the supine position for 20 min.

Body composition was calculated from bioelectrical measurements and anthropometric data by applying the software provided by the manufacturer using validated predictive equations for total body water (TBW), fat mass (FM), and free fat mass (FFM).

Statistical analysis

The sample size needed to detect an effect of WG treatment on primary outcome (FA bioavailability) and secondary outcome (metabolic and inflammatory markers) was defined on the basis of previous studies. From post-hoc analysis of data collected by (9) it was calculated that 25 participants in each treatment group would give sufficient power (alpha-error 0.05; 80% power, and 2-sided testing) to detect a 50% change in plasma FA. In addition, considering an alpha-error of 0.05, a power of 0.80 and 2-sided testing, a sample size of 28 participants was estimated to be adequate for detection of a 10% change in fasting total-cholesterol using variation in accordance with (31-33) and for detection of a 30% change of

circulating IL-6 using variation in accordance with (12), respectively; 30 participants was estimated to be adequate for detection of a 15% change in fasting TNF-α using variation in accordance with (32) and (34). The participant number was increased to 40 per group considering possible drop-outs.

All values were reported as means ± SEM. Kolmogorov-Smirnov and Shapiro test were used to evaluate the normality of distribution of all monitored variables and logarithmic transformation was applied to non-normally distributed data. Differences of variables between baseline and over times within and between interventions were tested by two-way analysis of variance (ANOVA) with repeated measures on one factor in combination with Tukey's post-hoc tests; $p<0.05$ was considered statistically significant. Pearson's correlation coefficients were calculated to assess bivariate associations between data sets ($p<0.05$ was considered significant).

Statistical analyses were performed using Statistical Package for Social Sciences (version 16.0; SPSS, Inc., Chicago, IL, USA). The microbiota composition and the relative statistical associations were determined by using specific scripts from of the QIIME software (28) as above described.

Results

Compliance to the treatment

The study recruitment and follow-up started on January 2011and March 2011, respectively; while the study was completed on May 2013. No adverse events were identified in WG and CTR group over the study period. Twelve subjects (4 from WG and 8 from CTR group) dropped out of the study during the second and third week for personal reasons unrelated to the intervention. The reasons included the need of taking antibiotics for 3 subjects and particular personal and familial events that voluntary and involuntary constricted volunteers to change their dietary habits and behavior such as change/loss of job for 4 subjects, a mourning for 2 subjects, health conditions of parents/son for 3 subjects. Sixty-eight subjects (36 in WG group and 32 in CTR group) completed the study and were included in the analyses (**Figure 2**).

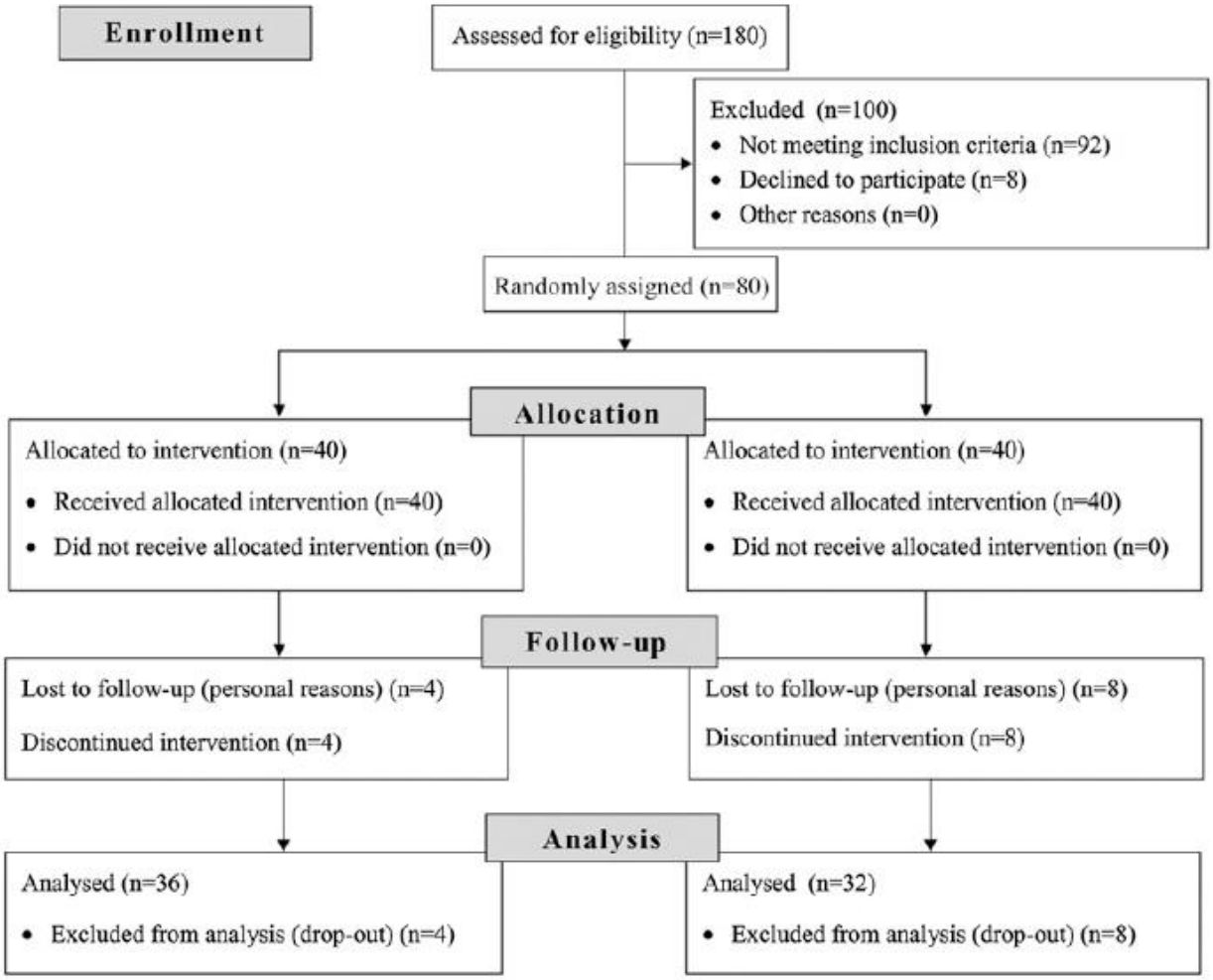

Figure 2: Participant flow over the study period.

Their general characteristics were reported in **Table 2**.

TABLE 2: General characteristics of participants at baseline [1].

	WG (n=36)		CTR (n=32)	
	mean± SEM	Range	mean± SEM	Range
Subjects (n)	36		32	
Gender (M/F)	11/25		12/20	
Age (y)	40±2	19-67	37±2	21-62
BMI (kg/m^2)	30.0±0.5	25.0-34.9	29.5±0.4	25.6-34.9
Total-cholesterol (mg/dL)	176.8±5.6	116-195	179.7±4.8	112-190
HDL-cholesterol (mg/dL)	49.5±2.4	24-79	48.9±1.9	32-74
Triglycerides (mg/dL)	95.2±8.2	43-145	87.6±5.9	51-144
Glycemia (mg/dL)	93.9±2.1	56-121	95.9±1.7	72-114
Waist circumference (cm)	100.0±1.9	76-119	98.6±2.2	75-125
Hip circumference (cm)	110.5±1.0	99-125	108.5±1.1	96-126
Free Fat Mass (%)	63.2±1.2	54-75	61.3±2.8	28.3-73
Fat Mass (%)	36.8±1.2	25-46	33.3±1.6	18.8-43
Total PA (MET-min/week)	287.5±17.3	220-357	317.5±15.0	260-375

[1] WG, wholegrain group; CTR, control group; PA, physical activity. There are no statistical differences between the groups at baseline.

The analysis of food diaries and the weight of foods returned by subjects over the study period showed a good compliance of subjects to the treatments (**Supplemental Table 1**). No significant difference between groups in energy intake and macronutrient composition of diets over time was found (**Table 3**).

WG wheat consumption resulted in a significant increase in total dietary fiber in WG subjects at 4 and 8 weeks compared to baseline and compared to CTR subjects. Over the study period WG subjects consumed a mean of 61±1.5 g/day (~2.5 biscuits) of WG product (out of the assigned 70 g, 3 biscuits). This WG wheat provided an amount of ~7.1 g/day of cereal dietary fiber, which well matched the increased intake of total dietary fiber in this group. No differences in dietary fiber from any other source except WG or refined wheat products were found over the study period (**Figure 3**).

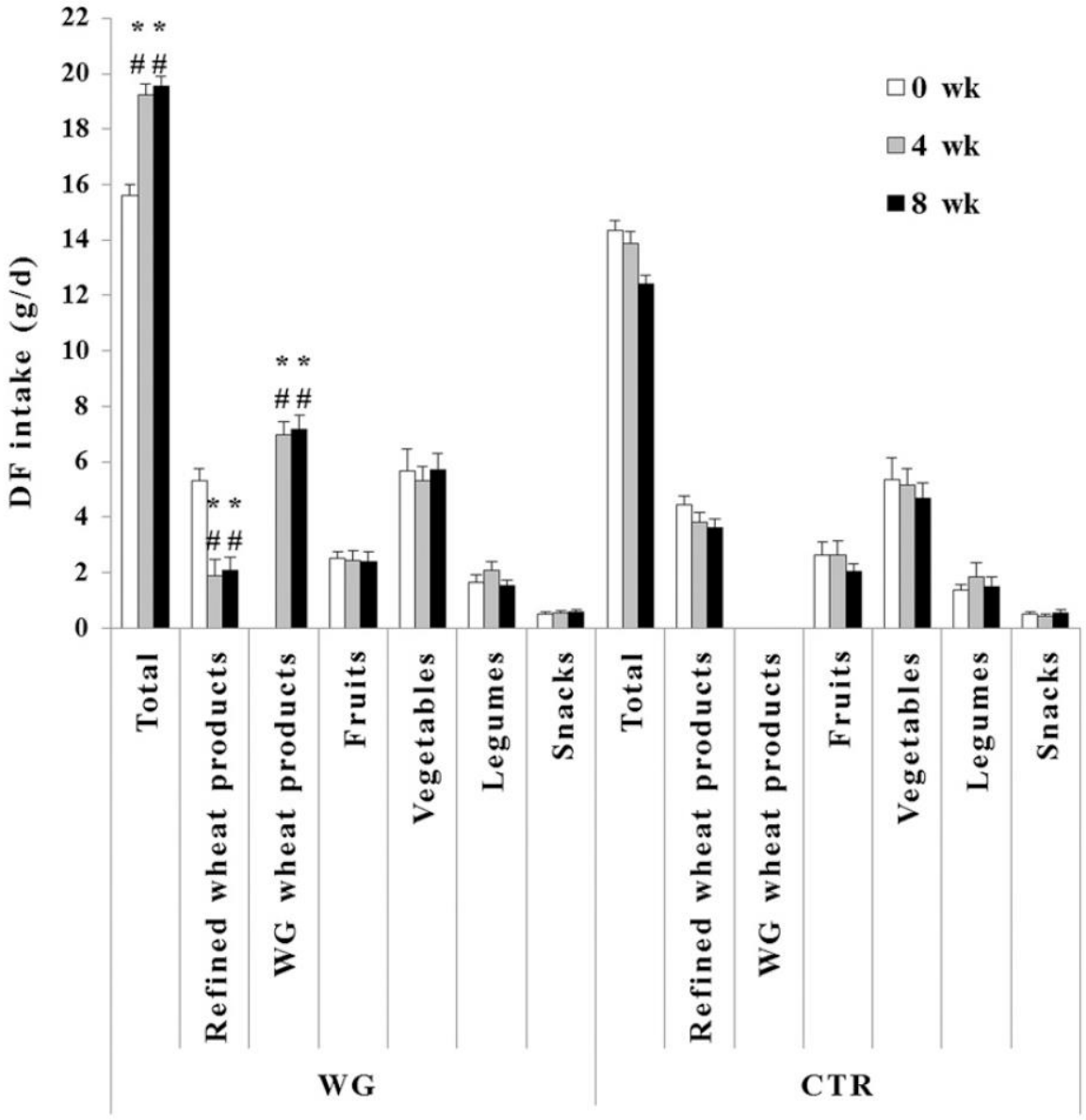

Figure 3: Daily intakes (g/day, mean±SEM) of dietary fiber (DF), total and from each dietary source, over the study period in WG (n=36) and CTR (n=32). From ANOVA and Tukey's post hoc test: *, $p<0.05$ for the difference between a given wk and baseline values within treatments; #, $p<0.05$ for the difference between treatments at a given wk.

TABLE 3: Energy intake and macronutrient composition of individual diets over the study period [1].

	WG (n=36)			CTR (n=32)		
	0 wk	**4 wk**	**8 wk**	**0 wk**	**4 wk**	**8 wk**
Energy (Kcal)	1600.4±95.4	1622.3±87.8	1553.7±78.7	1615.6±87.5	1570.7±69.4	1561.5±85.6
Carbohydrates						
Total (g)	198.0±13.1	195.2±10.1	189.4±10.0	186.9±11.3	183.2±10.2	181.0±11.0
Dietary fiber (g)	15.6±1.2	19.2*#±1.0	19.5*#±0.9	14.2±1.0	13.9±0.9	12.4±0.9
% Energy	47.5±1.0	46.0±1.3	46.2±1.1	45.0±1.4	44.7±1.4	44.9±1.2
Proteins						
g	66.3±3.9	71.0±3.7	70.2±3.2	69.5±5.0	69.4±3.6	69.1±4.2
% Energy	16.8±0.4	17.8±0.5	18.5±0.5	17.0±0.6	17.9±0.7	17.7±0.5
Fats						
Total (g)	60.0±3.7	62.7±4.4	58.4±3.7	65.3±4.1	61.1±3.4	61.9±4.1
Saturated (g)	22.8±2.5	25.7±3.3	24.2±3.0	23.1±3.2	21.6±2.3	22.5±2.4
Monounsaturated (g)	25.7±1.7	25.9±1.8	23.7±1.7	28.9±1.9	26.2±1.5	26.3±1.9
Polyunsaturated (g)	12.3±1.6	10.2±1.1	9.7±1.2	12.5±1.8	10.7±1.1	9.8±1.1
Total (% Energy)	34.0±0.9	34.4±0.9	33.5±0.8	36.2±1.2	35.1±1.1	35.8±1.2
Alcohol						
g	4.7±1.8	5.0±1.4	4.7±1.7	4.1±1.2	5.2±1.8	3.7±1.5
% Energy	1.7±0.6	1.8±0.5	1.8±0.6	1.8±0.6	2.3±0.9	1.6±0.7

[1] All values are means±SEM; WG, wholegrain group; CTR, control group; wk, week. From ANOVA and Tukey's post hoc test: *, $p<0.05$ for the difference between a given wk and baseline values within treatments; #, $p<0.05$ for the difference between treatments at a given wk. No significant differences between the groups at baseline were present.

Anthropometry, body composition, glycemia and plasma lipids

No significant variations of anthropometric data, body composition, plasma lipids, and glycemia were found over the study period within and between groups (**Supplemental Table 2**).

Phenolic acids in serum, urine and feces

Supplemental Table 3 reported concentrations of phenolic acids retrieved in biological samples monitored over the study period. As expected, a greater number of phenolic acid compounds were detected in urine and feces than in serum samples (13 and 14 vs 6, respectively). No significant difference was found among baseline concentrations of single and total phenolic acids in biological samples from WG and CTR subjects. As expected, no significant variation over the study period for any of monitored compounds was found in CTR subjects. On the contrary, WG consumption resulted in a significant 4.2 and 5 fold increase in serum dihydroferulic acid (DHFA) concentration, and a 1.3 and 0.8 fold increase in fecal FA concentration, after 4 and 8 weeks, respectively, and a 0.8 fold increase in FA urinary excretion after 8 weeks compared to baseline within and between groups (**Figure 4)**.

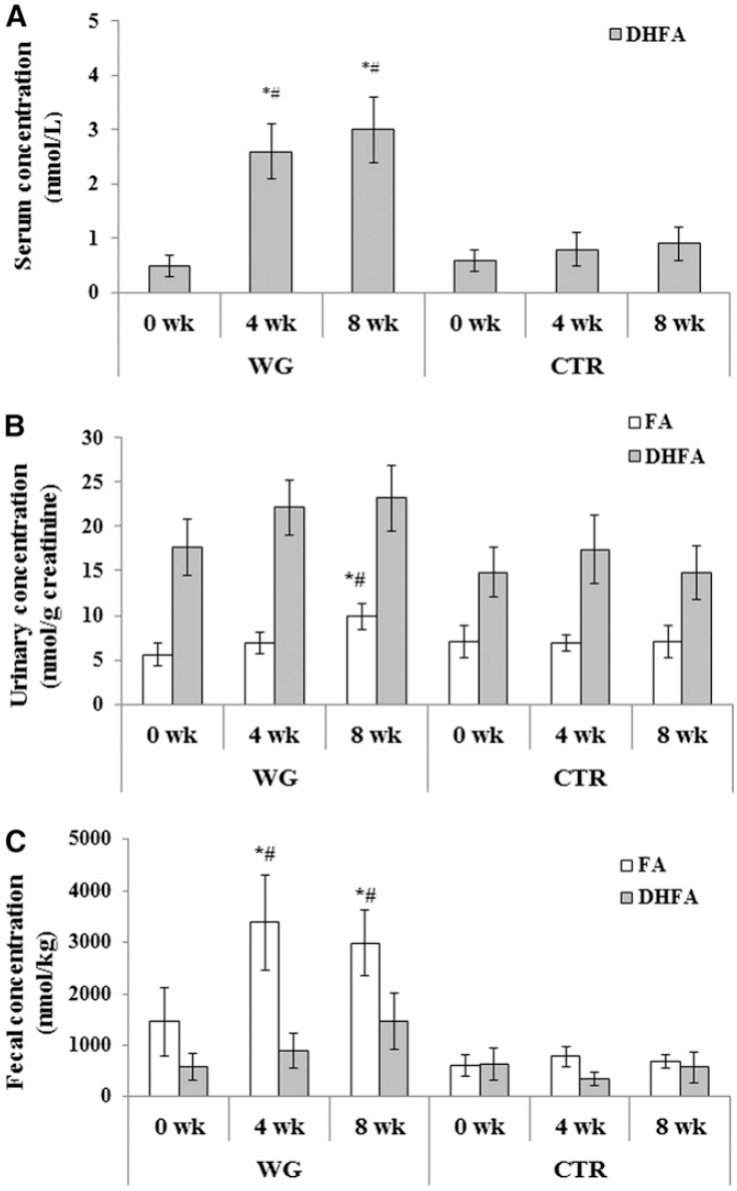

Figure 4: Concentration of ferulic acid (FA) and dihydroferulic acid (DHFA) in serum (A), urine (B) and feces (C) over the study period in WG (n=36) and CTR (n=32). From ANOVA showing significant ($p<0.05$) treatment x time interaction and Tukey's post hoc test on serum DHFA and urinary and fecal FA concentrations: *, $p<0.05$ for the difference between a given wk and baseline values within treatments; #, $p<0.05$ for the difference between treatments at a given wk.

In WG group a trend of increased urinary DHFA after 4 weeks ($p=0.08$) and 8 weeks ($p=0.09$) compared to baseline and also compared to CTR group ($p=0.06$) after 8 weeks was observed.

In WG subjects (but not in CTR subjects) FA serum concentrations at 4 and 8 weeks significantly correlated with serum DHFA (Pearson's; $r=0.734$, $p<0.001$, $n=36$ and $r=0.684$, $p<0.001$, $n=36$), whereas urinary FA correlated with fecal ($r=0.331$, $p=0.004$, $n=36$ and $r=0.431$, $p=0.002$, $n=36$) and urinary ($r=0.231$, $p=0.002$, $n=36$ and $r=0.411$, $p=0.001$, $n=36$)

DHFA; interestingly, in subjects experiencing increased urinary FA also a positive variation of fecal FA was found (r =0.618, p=0.032, n=29) after 8 week intervention.

Metabolic disease and inflammatory markers in plasma

No difference at baseline and no variation over the study period were found for diabetes and obesity markers within and between groups (**Supplemental Table 4**). Plasma concentrations of inflammatory status markers (**Table 4**) were similar at baseline of both groups. However, significant modifications over the study period were found between groups and within WG group. In WG group, there was a significant reduction in inflammatory TNF-a after 8 weeks compared to baseline and compared to CTR group and a significant increase in anti-inflammatory IL-10 after 4 weeks compared to baseline and to CTR, but not compared to 8 week data in either group. Moreover, a trend of reduction in IL-6 at 8 weeks vs 4 weeks in the WG group compared to CTR was found (p = 0.06).

The urinary excretion of FA and DHFA over WG treatment (after 4 and 8 weeks of intervention) tended to be negatively correlated with plasma PAI-1 concentrations (Pearson's; r= -0.264, p=0.075, n=36 and r= -0.341, p=0.061, n=36 for FA; r= -0.271, p=0.071, n=36 and r= -0.302, p=0.059, n=36 for DHFA).

TABLE 4: Plasma concentration of inflammatory status markers over the study period [1].

	WG (n=36)			CTR (n=32)			WG vs CTR, p^4		
pg/mL	**0 wk**	**4 wk**	**8 wk**	**0 wk**	**4 wk**	**8 wk**	Δ_{4-0}	Δ_{8-0}	Δ_{8-4}
IL-6	57.5±7.5	69.5±11.2	46.9±4.0	65.5±11.4	56.3±7.5	60.2±7.2	---	---	---
IL-10	26.9±3.0	41.7±2.8[2]	26.8±3.2[3]	28.8±5.1	27.5±4.3	27.9±3.89	**0.04**	0.29	**0.03**
TNF-α	341.9±25.5	370.1±30.5	243.0±26.0[2,3]	321.9±52.1	314.9±50.3	329.8±50.6	0.15	**0.04**	0.20

[1] All values are means±SEM; WG, wholegrain group; CTR, control group; wk, week; data were log transformed prior to analysis; [2] $p<0.05$ vs baseline, ANOVA and Tukey's post hoc test; [3] $p<0.05$ vs 4 wk, ANOVA and Tukey's post hoc test; [4] *p* values for the difference between WG and CTR groups with respect to the pairwise time point differences (Δ) – they were calculated when a significant treatment x time interaction was found; no significant differences between the groups at baseline were present.

Microbiota composition: effect of dietary treatments and impact on circulating and excreted FA and inflammatory/metabolic markers

Microbial community data was analysed by comparing OTUs composition between subjects, with treatment group, age and gender as independent variables. Data showed that fecal microbial community structure was significantly different between men and women ($p<0.05$), while no significant variation was found in relation to dietary treatments or age. Weighted and unweighted UniFrac phylogenetic metrics (measures of overall community composition) clearly showed that the microbial community structure of WG and CTR subjects was not significantly different; in fact, the different categories of individuals did not form discrete clusters in the PCoA plot suggesting the overall microbiota to be similar (**Figure 5**).

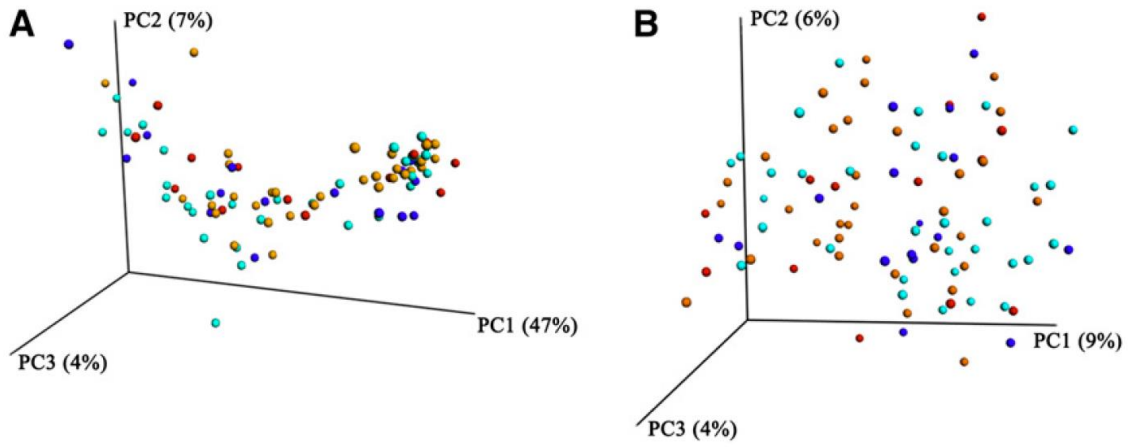

Figure 5: Principal Coordinates Analysis of A weighted and B unweighted UniFrac distances for 16S rRNA gene sequence data from WG subjects before (orange dots) and after 8 weeks of intervention (green dots), CTR subjects before (red dots) and after 8 weeks of intervention (blue dots).

In addition, no difference was observed between WG subjects at time zero and after the treatment (Figure 5). However, individual bacterial taxa showed significant variation in relative abundance in relation to diet and gender. Specifically, *Prevotella* sp. significantly increased from 1.8% to 3.5%, while other taxa were significantly reduced in WG subjects ($p<0.05$) i.e. *Dialister* sp. (from 2.5% to 0.6%), *Bifidobacterium* sp. (from 6.6% to 5.3%), *Blautia* sp. (from 9.7% to 6.7%) and *Collinsella* sp. (from 1.8% to 0.9%).

Pearson's correlations were used to evaluate the potential interplay between baseline microbiota composition and circulating FA as well as that between fecal FA and microbiota over WG treatment. Results showed that in WG subjects a lower baseline relative abundance of Bifidobacteriales (Actinobacteria) of 5.0% ($r=-0.74$; $p=0.014$; $n=34$) and Bacteroidetes of 9.6% ($r = -0.66$; $p=0.02$; $n=29$) was associated with an increased release of FA in the gut and

urinary excretion, respectively. After the WG treatment, fecal FA was associated with an increase in the relative abundances of Bacteroidetes from 9.6% to 14.5% (r =0.76; p=0.01; n=34) and Firmicutes from 75.3% to 79.7% (r =0.64; p=0.04; n=34); while a reduction of *Clostridium* from 3.1% to 1.6 % (r = -0.72; p=0.02; n=34) was registered.

No significant correlation was found between any OTU at baseline and specific variation of any metabolic or inflammatory marker in both treatment groups. Interestingly, the reduction of TNF-α after 8 weeks of WG consumption correlated with an increased abundance of fecal *Bacteroides* from 9.9% to 14.7% (r = -0.637, p=0.002, n=31) and *Lactobacillus* from 0.03% to 0.12% (r = -0.572, p=0.021, n=31).

Discussion

In this study the phenolic profile of serum, urine and feces upon WG wheat consumption, their effect on metabolic and inflammatory parameters and the correlations with changes in the fecal microbiota were assessed. Overweight/obese subjects with suboptimal lifestyle factors (such as limited fruit and vegetable intake and low physical activity), were considered in this study as they were suitable subjects to verify: i) the distribution of WG polyphenols among the main biological fluids by reducing the interference of other major dietary sources of polyphenols (35); ii) the hypothesis that WG polyphenols might prevent the development of some pathophysiological pathways which are possibly unbalanced in these subjects (although they were still healthy) (16); iii) the interplay among circulating and excreted WG polyphenols, gut microbial community composition, and health benefits possibly induced by WG wheat consumption in a population at risk of developing chronic diseases (14,36-40).

Biochemical data showed that among 15 phenolic acids monitored in serum, urine and feces, an 8-week consumption of WG resulted in a significant increase in urinary and fecal FA and serum DHFA concentrations. The observation that FA concentration can increase in the blood upon WG wheat consumption was conceptually in agreement with a previous study conducted in healthy normal weight subjects (9), while in a recent study in overweight healthy subjects a 4 week-consumption of bread and cereals enriched with an aleurone fraction failed to increase serum FA (34). Interestingly, in the present study WG consumption significantly increased also serum DHFA concentration, which is positively correlated with serum FA; while excreted DHFA correlated with urinary FA. DHFA is a well-known microbial metabolite derived from FA and chlorogenic acid, absorbable through the colon and retrievable in serum and urine (14, 41-46). In this study, WG wheat represented the unique dietary source of FA (~97 mg/day) differentiating WG from CTR group therefore these findings indicated that FA can be absorbed from WG wheat, it is released in the gut and, it is mainly converted to DHFA by microbiota.

Moreover, the study of gut microbial communities showed that FA was majorly retrieved in the blood and excreted in urines in subjects harbouring a low relative abundance of Bacteroidetes (phylum) and Bifidobacteriales (order) at the baseline. After 8 weeks these subjects experienced an increase of Bacteroidetes and total Firmicutes, although, within Firmicutes, a reduction of *Clostridium* relative abundance took place.

Previous *in vitro* studies showed that the release of FA in the colon might be associated with wheat bran polysaccharide fermentation and sustained by the action of bacterial extracellular xylanase and FA esterase (47,48). These enzymes are mainly synthetized by bacterial species

belonging to the genus *Lactobacillus* and *Roseburia* (Firmicutes), *Bifidobacterium* (Actinobacteria), *Bacteroides* and *Prevotella* (Bacteroidetes) in presence of arabinoxylans with esterified FA (49-53). Thus it can be hypothesized that in overweight/obese subjects who showed a low abundance of Bacteroidetes and Bifidobacteriales, Firmicutes were the main responsible for the fermentation of WG polysaccharides and the released FA once WG wheat was introduced in the diets.

The contemporary observation of an increase in the relative abundance of *Prevotella* and a significant positive correlation between fecal FA and the abundance of the whole Bacteroidetes (although not *Prevotella* alone) in WG subjects, suggests that also B*acteroides* have a role in intestinal release of WG FA. These findings were in agreement with Lappi and co-workers (13) who found a trend to reduced *Bacteroides* and *Prevotella* and increased *Clostridium* in Finnish subjects with metabolic syndrome replacing rye bread with white wheat bread for 12 weeks (13). However, those authors concluded that dietary fats explained *Bacteroides* changes better than WG, while in the present study fats did not affect WG-microbiota interplay.

All together these findings suggested that in this study the intestinal release of WG FA might be activated by Firmicutes and sustained over time with the contribution of Bacteroidetes. Moreover, the reduction of *Clostridium* in subjects experimenting higher FA release might be due to the competition with other species or to a direct antimicrobial effect of FA towards clostridia (54).

Data on inflammatory markers showed a significant reduction of inflammatory TNF-α and a trend toward reduced IL-6 after 8 weeks as well as an increase of the anti-inflammatory IL-10 after 4 weeks of WG consumption. Two previous intervention trials demonstrated the ability of WG consumption to ameliorate subclinical inflammation (12,32), while many others studies failed to find such a positive association (31,33,55,56). In the study by Katcher and co-workers (32) the reduction of inflammation followed the inclusion of WG in hypocaloric and healthy diets, and in the study by Martinez and co-workers (12) the nutritional composition of diets were not controlled.

The strong point of the present study is that the amelioration of individual inflammatory status was found in the context of a controlled energy and nutritionally balanced replacement of refined wheat with WG wheat and coherently it was not accompanied by any modification of body weight.

Moreover, the correlation between a reduced TNF-α and an increased abundance of *Bacteroides* (as observed in subjects with a higher bioaccessibility of FA) and *Lactobacillus*

(known for releasing feruloyl-esterase activity in the gut as discussed above) provided a further potential link between the increase of serum FA and the amelioration of inflammation in our subjects. In addition, the trend towards an inverse correlation found between urinary FA and DHFA with PAI-1 concentration suggested a role for WG FA and its gut metabolite in triggering mechanisms that may result in a reduced risk of CVD, diabetes and others pathologies associated with obesity and low-grade inflammatory status (57, 58).

PAI-1 is a well-known biomarker of cardiovascular risk, metabolic syndrome (59), non-alcoholic fatty liver disease (60) and cancers (61). An inverse association between WG consumption and PAI-1 was found in a recent observational study (62), but previous intervention studies failed to find a significant effect of WG consumption on this parameter (31, 63). The trend found in this study might suggest that WG's effects on metabolic diseases may be better observed in subjects having the ability of releasing and metabolizing the FA bound to dietary fiber; this may explain the conflicting evidence found thus far. According to this hypothesis, the benefits of WG wheat polyphenols are mediated by the metabolic activity of the gut microbiota, as already observed for soybean or ellagitannin rich foods, whose health benefits are linked to the ability of individual bacterial taxa to convert genistein into equol and ellagitannins/ellagic acid into urolithins, respectively (64, 65). On the other hand, it cannot be excluded that other bioactive components in WG such as resistant starch, betaine, and some minerals might have contributed together with FA (directly or through their microbiota metabolites, but in absence of a prebiotic effect) to ameliorate inflammation (7).

In conclusion, in this study it was demonstrated for the first time that WG wheat FA is released and absorbed in the gut, it is likely metabolized by gut microbiota, and DHFA is the most abundant circulating metabolite in overweight/obese subjects. Even though WG wheat did not cause significant modification of microbial community composition or structure, there were significant relationships between FA release in the gut and relative abundance of Firmicutes at baseline and Bacteroidetes following WG consumption. The increased abundance of these bacteria together with *Lactobacillus* was associated with the ameliorated inflammatory status of subjects upon WG treatment, which may suggest that WG FA may play a role in reducing the risk of pathologies associated with subclinical inflammation. This was also supported by evidence that a greater excretion of FA and DHFA in urine, reflecting a better release, metabolism and absorption of the compounds, was associated with a trend towards lower PAI-1 plasma concentrations.

Since no specific correction was made for multiple comparisons, possibly leading to some false positive findings, some results of the study should be cautiously taken into account and

493 further more detailed study may be warranted. The application of a completers analysis
494 instead of an intention-to-treat analysis of data might be also seen as a study limitation.
495 However, it was preferred because dropouts in both groups left the study for personal reasons
496 within the first 3 weeks and no data were available after baseline and because the power of the
497 study was unaffected by the exclusion from analysis of those few dropouts (66).
498 In addition, in this study un-blinded participants might have led to possible biases in
499 psychological response and compliance to the dietary interventions, whereas the blinded
500 outcome assessors guaranteed unbiased interaction with participants and data collection.
501 However, from the viewpoint of public health and optimal personalized nutrition it can be
502 concluded that in subjects at high risk to develop chronic diseases (because of obesity and
503 unhealthy lifestyle) the modification of dietary habits alone, through an isocaloric dietary
504 replacement of refined wheat products with 70 g WG wheat, can boost a positive immune
505 response possibly reducing the risk to develop obesity-related diseases over the long term.
506

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509 ***Authors' contributions to manuscript:*** PV and VF designed research; PV, IM, AAR, RG,
510 MAG conducted the experiments and collected data; FT, SJ provided WG; PV and RF
511 analyzed bioavailability data; PV and IM analyzed data of inflammatory and metabolic
512 disease markers; IM and LS analyzed anthropometry data; ALS, DE, SMG, JAG and PV
513 performed microbiological analyses and analyzed data; PV wrote the paper; PV and VF have
514 primary responsibility for final content; all authors read and approved the final manuscript.

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SUPPLEMENTAL TABLE 1: Food intake for each dietary group [1]

	WG (n=36)			CTR (n=32)		
g/day	**0 wk**	**4 wk**	**8 wk**	**0 wk**	**4 wk**	**8 wk**
Fruits	111.6 ± 12.7	116.8 ± 17.8	133.8 ± 20.9	112.1 ± 15.6	108.5 ± 15.1	113.3 ± 13.3
Vegetables	238.2 ± 24.5	252.5 ± 17.5	275.3 ± 21.4	247.9 ± 36.0	255.9 ± 21.0	223.7 ± 18.8
Cereal products	191.2 ± 15.5	167.3 ± 10.4	161.0 ± 8.7	177.4 ± 11.3	156.6 ± 10.4	157.8 ± 12.6
Wholegrain wheat	0	60.0* ± 1.7	62.0* ± 1.3	0	0	0
Animal products	95.3 ± 9.3	100.8 ± 8.6	98.5 ± 8.1	109.0 ± 11.5	102.4 ± 8.9	99.5 ± 8.7
Fishery products	37.7 ± 5.9	43.7 ± 6.2	42.8 ± 4.8	37.1 ± 7.3	42.3 ± 8.5	53.3 ± 9.1
Dairy products	150.3 ± 19.9	154.0 ± 17.2	164.3 ± 19.8	141.4 ± 16.4	157.2 ± 18.1	163.0 ± 22.9
Seasonings	19.0 ± 1.9	20.1 ± 2.0	16.9 ± 1.7	20.5 ± 1.9	18.8 ± 1.9	18.4 ± 2.4
Snacks	76.7 ± 8.1	89.6 ± 8.4	84.8 ± 9.2	71.7 ± 8.3	83.9 ± 8.7	84.4 ± 9.3
Beverages	135.3 ± 24.2	155.2 ± 27.1	128.1 ± 20.4	162.9 ± 26.9	152.5 ± 23.9	127.1 ± 23.8

[1] All values are means±SEM; WG, wholegrain group; CTR, control group; wk, week. From ANOVA and Tukey's post hoc test: *, $p<0.05$ for the difference between a given wk and baseline values within and between treatments.

SUPPLEMENTAL TABLE 2: Body weight, blood lipids, glycemia and anthropometry over the study period [1]

	WG (n=36)			CTR (n=32)		
	0 wk	**4 wk**	**8 wk**	**0 wk**	**4 wk**	**8 wk**
Weight (kg)	82.0 ± 2.1	81.1 ± 2.1	81.0 ± 2.1	80.0 ± 2.3	79.5 ± 2.4	77.4 ± 1.9
BMI (kg/m^2)	30.0 ± 0.5	29.9 ± 0.5	29.8 ± 0.4	29.5 ± 0.4	29.4 ± 0.5	29.0 ± 0.4
Tot-chol (mg/dL)	176.8 ± 5.6	172.4 ± 5.6	176.8 ± 7.0	179.7 ± 4.8	178.1 ± 5.4	182.2 ± 4.8
HDL-chol (mg/dL)	49.5 ± 2.4	49.4 ± 2.0	48.5 ± 2.3	48.9 ± 1.9	48.1 ± 1.9	49.4 ± 2.0
TG (mg/dL)	95.2 ± 8.2	95.4 ± 9.1	99.4 ± 9.9	87.6 ± 5.9	87.0 ± 6.7	87.8 ± 6.8
Glycemia (mg/dL)	93.9 ± 2.1	93.7 ± 2.1	99.9 ± 2.2	95.9 ± 1.7	91.1 ± 1.6	94.3 ± 1.8
Waist (cm)	100.0 ± 1.9	99.5 ± 2.0	98.9 ± 2.0	98.6 ± 2.2	99.8 ± 2.2	97.8 ± 2.4
Hip (cm)	110.5 ± 1.0	109.9 ± 1.1	106.2 ± 3.2	108.5 ± 1.1	108.6 ± 1.2	108.2 ± 1.1
FFM (%)	63.2 ± 1.2	62.4 ± 0.9	63.3 ± 1.0	66.7 ± 2.8	67.2 ± 3.4	67.5 ± 2.1
FM (%)	36.8 ± 1.2	37.7 ± 0.9	36.7 ± 1.0	33.3 ± 1.6	32.8 ± 1.9	32.5 ± 2.1

[1] All values are means±SEM; WG, wholegrain group; CTR, control group; wk, week; chol, cholesterol; TG, triglycerides; FFM, free fat mass; FM, fat mass. No significant difference between change values of any variable at baseline and at all week within and between treatments was found (ANOVA and post hoc Tukey's test).

SUPPLEMENTAL TABLE 3: Phenolic acid concentrations in biological samples collected over the study period [1].

	WG (n=36)			CTR (n=32)			WG vs CTR, p^3		
	0 wk	**4 wk**	**8 wk**	**0 wk**	**4 wk**	**8 wk**	**Δ_{4-0}**	**Δ_{8-0}**	**Δ_{8-4}**
SERUM (nmol/L)									
CA	2.7 ± 0.8	2.9 ± 0.8	3.0 ± 0.9	6.2 ± 2.2	5.5 ± 1.4	3.7 ± 1.2			
FA	n.d	0.3 ± 0.3	0.3 ± 0.3	0.3 ± 0.3	0.1 ± 0.1	n.d.			
DHFA	0.5 ± 0.2	2.6 ± 0.5[2]	3.0 ± 0.6[2]	0.6 ± 0.2	0.8 ± 0.3	0.9 ± 0.3	***0.04***	***0.04***	*0.97*
HPVal	4.3 ± 1.0	4.5 ± 0.6	4.4 ± 0.8	4.3 ± 1.0	3.4 ± 0.8	4.6 ± 1.4			
HA	0.5 ± 0.2	3.1 ± 2.2	2.0 ± 0.9	0.3 ± 0.2	0.4 ± 0.2	8.9 ± 5.6			
HBA	32.2 ± 6.0	33.5 ± 4.9	36.9 ± 5.5	21.5 ± 5.0	26.0 ± 6.2	28.4 ± 7.0			
TOTAL	40.2 ± 8.2	46.9 ± 8.8	49.6 ± 8.4	33.1 ± 8.9	36.1 ± 9.1	46.4 ± 15.4			
URINE (nmol/g creatinine)									
CA	0.5 ± 0.2	0.9 ± 0.4	0.7 ± 0.2	0.6 ± 0.2	0.3 ± 0.1	0.6 ± 0.2			
FA	5.6 ± 1.3	6.9 ± 1.2	9.9 ± 1.7[2]	7.1 ± 1.8	6.9 ± 0.9	7.1 ± 1.8	*0.37*	***0.03***	*0.49*
DHFA	17.6 ± 3.2	22.1 ± 3.1	23.2 ± 3.7	14.8 ± 2.8	17.4 ± 3.8	14.8 ± 3.0			
VA	13.7 ± 2.0	17.0 ± 2.4	14.7 ± 3.2	17.2 ± 2.6	18.5 ± 2.7	17.7 ± 3.2			
di HPA	19.8 ± 3.0	22.7 ± 3.1	28.3 ± 4.5	24.5 ± 5.3	24.7 ± 5.7	33.3 ± 6.7			
HPA	8042.9 ± 874.5	9704.5 ± 934.7	8665.9 ± 1108.9	12692.9 ± 2828.3	9693.3 ± 1248.3	10150.4 ± 1536.7			
PCA	26.5 ± 5.4	36.1 ± 6.7	26.8 ± 4.7	29.1 ± 8.2	22.2 ± 4.8	20.2 ± 3.3			
HBA	3.2 ± 0.6	4.2 ± 0.9	5.4 ± 1.3	3.8 ± 1.0	3.3 ± 0.5	4.6 ± 1.3			
HPVal	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.5 ± 0.3			
CaA	2.4 ± 0.4	3.3 ± 1.2	2.0 ± 0.4	2.7 ± 0.7	3.1 ± 0.7	1.9 ± 0.5			
CuA	2.1 ± 0.3	2.0 ± 0.3	1.9 ± 0.3	2.2 ± 0.3	2.6 ± 0.3	2.6 ± 0.4			
HA	1463.9 ± 209.8	2294.9 ± 844.9	1321.7 ± 202.5	1191.5 ± 185.2	1411.7 ± 237.1	1631.4 ± 227.5			
HVA	589.7 ± 79.6	708.6 ± 99.8	668.5 ± 100.4	602.4 ± 74.6	633.3 ± 117.8	905.4 ± 269.5			
TOTAL	10188.3 ± 1177.3	12823.6 ± 1895.7	10746.2 ± 1428.2	14588.8 ± 3111.1	11837.5 ± 1623.0	12790.6 ± 2054.3			

FECES *(nmol/kg)*									
CA	3.7 ± 0.9	17.8 ± 13.4	4.6 ± 1.1	9.7 ± 3.5	9.1 ± 5.0	9.7 ± 5.4			
FA	1460.1 ± 666.4	3389.3 ± 928.1[2]	2989.5 ± 630.1[2]	610.2 ± 217.9	787.0 ± 195.2	680.6 ± 134.3	***0.02***	***0.04***	*0.40*
DHFA	587.6 ± 252.0	905.4 ± 340.3	1476.7 ± 550.4	643.7 ± 312.7	344.7 ± 136.2	575.9 ± 295.6			
VA	1181.8 ± 309.5	843.8 ± 114.5	2058.4 ± 958.5	1945.5 ± 966.1	1581.0 ± 689.6	1264.7 ± 412.0			
di HPA	228.4 ± 78.0	374.4 ± 147.0	590.3 ± 255.3	103.0 ± 46.6	212.4 ± 124.3	149.9 ± 103.7			
HPA	218.6 ± 56.6	159.7 ± 41.4	201.1 ± 40.0	254.2 ± 13.2	179.6 ± 73.1	186.8 ± 25.1			
HPP	3597.7 ± 1427.1	4215.0 ± 1199.8	4978.3 ± 1221.4	5609.4 ± 2484.6	7899.9 ± 3396.2	2152.0 ± 794.7			
PCA	104.3 ± 34.0	99.1 ± 20.0	138.0 ± 48.2	50.7 ± 21.5	95.7 ± 38.7	76.6 ± 26.9			
CuA	171.6 ± 47.0	203.3 ± 49.6	289.1 ± 70.1	82.2 ± 18.7	208.1 ± 65.9	275.2 ± 107.1			
HPVal	1.4 ± 1.2	0.3 ± 0.2	1.8 ± 1.0	19.2 ± 15.3	1.4 ± 1.2	0.0 ± 0.0			
CaA	212.0 ± 42.9	311.5 ± 67.3	263.1 ± 43.0	235.9 ± 142.0	220.5 ± 89.9	221.7 ± 67.4			
HA	81.4 ± 65.7	58.0 ± 35.4	121.7 ± 48.4	53.5 ± 28.3	89.5 ± 43.8	80.2 ± 40.6			
HVA	13049.2 ± 3784.5	22307.3 ± 7343.3	17681.6 ± 6905.3	7330.8 ± 2284.6	9221.4 ± 2623.3	10222.7 ± 4166.2			
HBA	102.7 ± 33.8	131.5 ± 32.6	109.9 ± 19.1	56.8 ± 8.3	41.7 ± 6.6	121.5 ± 75.1			
TOTAL	21009.5 ± 6799.5	33016.6 ± 10533.0	30603.9 ± 10791.9	17004.8 ± 6563.3	20892.0 ± 7489.0	16387.5 ± 6654.2			

[1] All values are means±SEM; WG, wholegrain group; CTR, control group; wk, week; [2] $p<0.05$ vs baseline, ANOVA and Tukey's post hoc test; [3] *p* values for the difference between WG and CTR groups with respect to pairwise time point differences (Δ) – they were calculated when a significant treatment x time interaction was found; no significant differences between the groups at baseline were present. CA, chlorogenic acid; FA, ferulic acid; DHFA, dihydroferulic acid; HPVal, hydroxyl-phenyl-valerolactone; HA, hippuric acid; HBA, hydroxybenzoic acid; VA, vanillic acid; di HPA, di-hydroxy-phenyl-acetic acid; HPA, hydroxyphenyl acetic acid; PCA, protocatechuic acid; CaA, caffeic acid; CuA, cumaric acid; HVA, homovanillic acid; n.d., not detected. P values from ANOVA and Tukey's post hoc test for variations at given weeks within and between groups; $p<0.05$ indicates significant differences.

SUPPLEMENTAL TABLE 4: Plasma concentration of metabolic disease markers over the study period [1].

	WG (n=36)			CTR (n=32)		
pg/mL	**0 wk**	**4 wk**	**8 wk**	**0 wk**	**4 wk**	**8 wk**
C-PEPTIDE	662.1 ±58.7	686.7±64.2	616.0±37.6	607.2±58.5	562.2±40.6	520.0±44.5
GHRELIN	482.6±54.4	521.3±63.8	433.8±31.6	478.1±38.6	429.2±33.2	484.2±39.8
GIP	116.6±41.7	88.3±19.9	87.4±13.7	80.5±8.7	69.9±9.8	86.7±13.9
GLP-1	52.6±9.5	58.2±11.5	52.0±5.5	50.5±5.9	45.1±5.4	46.5±5.5
GLUCAGON	321.8±24.7	343.2±36.4	307.4±13.0	311.5±16.9	294.9±20.5	304.4±15.8
INSULIN	111.4±13.7	120.5±17.4	110.1±7.8	100.5±7.1	92.4±6.8	96.2±5.6
LEPTIN	3327.1±355.5	3876.9±447.5	3258.2±332.7	3146.8±407.1	3461.9±579.9	3461.8±616.5
PAI-1	2913.3±185.4	3326.8±148.1	2892.4±186.4	2964.7±212.4	2765.8±198.0	2690.5±209.3
RESISTIN	1472.6±121.9	1369.5±102.7	1474.8±98.0	1551.3±166.9	1497.6±208.1	1426.1±171.7
VISFATIN	1507.2±282.3	1667.6±392.5	1352.2±163.8	1387.8±183.1	1241.3±161.3	1307.1±160.8

[1] All values are means±SEM; WG, wholegrain group; CTR, control group; wk, week. No significant differences both within and between treatments at baseline and any time point were found.

Chapter 4. Curcumin bioavailability from enriched-bread: the effect of microencapsulated ingredients

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Abstract

Human bioavailability of curcumin from breads enriched with 1 g/portion of free curcumin (FCB), encapsulated curcumin (ECB) or encapsulated curcumin plus other polyphenols (ECBB), was evaluated. Parental and metabolized curcuminoids and phenolic acids were quantified by HPLC/MS/MS in blood, urine and feces collected over 24h. The concentrations of serum curcuminoids were always below 4 nmol/L and those of glucuronides ten folds less. Encapsulation delayed and increased curcuminoid absorption compared to the free ingredient. Serum and urinary concentrations of ferulic and vanillic acid were between 2 and 1000 folds higher than those of curcuminoids: ECBB eliciting the highest amounts. Fecal curcuminoids were 6 folds more abundant after ECB than FCB while phenolic acids after ECBB quadruplicated those after ECB.

Curcuminoid encapsulation increases bioavailability from enriched bread probably preventing their biotransformation: combined compounds slightly reducing this effect. Phenolic acids are the major metabolites of curcuminoids and may contribute to their biological properties.

Introduction

Curcumin is commonly used in food products, mainly as colouring agent. Several biological properties have been attributed to this compound mainly related to the ability to inhibit NF-kB activation [1]. Curcumin has been proposed as potential therapeutic agent against several non communicable chronic diseases having an inflammatory origin such as neurodegenerative diseases (Alzheimer's and Parkinson's disease, multiple sclerosis, epilepsy), CVD, diabetes, obesity, allergies and certain types of cancer [2]

Although clinical studies in humans proved that curcumin is safe and well tolerated even at very high doses (8-12 g/die) its use as therapeutic agent is limited by its low bioavailability, poor absorption, rapid metabolism and systemic clearance [3,4].

Drug delivery systems such as nanoparticles, liposomes, microemulsions, and polymeric implantable devices are emerging as viable alternatives that can be used to deliver therapeutic concentrations of various chemopreventive agents such as curcumin, ellagic acid, green tea polyphenols, and resveratrol into the systemic circulation [5].

Several absorption enhancers have also been used to improve curcumin bioavailability. Piperine enhanced the bioavailability both in preclinical studies and in studies on human volunteers [6]. This was attributed to the ability of piperine in reducing first-pass metabolism [6]. Animal studies also demonstrated that inclusion of curcumin into nanoparticles caused at least 9-fold increase in oral bioavailability when compared to curcumin administered alone or with piperine [7]. On the other hand interactions among bioactive compounds which may positively influence oral bioavailability of individual molecules are known for genistein towards epigallocatechingallate [8] as well as for several natural bioactive compounds (quercetin, hesperitin, curcumin, piperin and naringenin) with P-glycoprotein-inhibiting activity, towards some anticancer drugs [9, 10].

Encapsulation may confer new properties and potentials to bioactive compounds trough modification of physical and nutritional properties [5]. This may be of particular interest in the formulation of functional foods, where technological and nutritional aspects must be strictly considered [11]. In this respect, selecting suitable coating materials can increase water solubility of bioactive compounds and/or permit their controlled delivery into gastrointestinal tract [5].

In this framework, the aim of this study was to evaluate the bioavailability of curcumin from different types of bread containing curcumin in different form: free and microencapsulated in a cellulose derivative coating containing curcumin alone or in combination with a mixture of three bioactive compounds including piperine, quercetin and genistein. A cross-over, randomized, single blind study in healthy subjects was performed. Curcuminoid bioavailability over 24 hours following consumption of the breads was assessed by HPLC/MS/MS determining blood, urine and fecal

concentrations of curcuminoids, their metabolites (glucuronides, sulphated and reduced compounds) and several phenolic acids.

Materials and methods

Standards and reagents

All chemicals and reagents were of analytical grade. Methanol, water, acetonitrile were from Merck (Darmstadt, Germany); ethyl acetate, glacial acetic acid and hydrochloric acid from Clean Consult International (Lodi, Italy); formic acid with (98% purity) was obtained by Sigma (St. Louis, MO). All analytical standards chlorogenic acid (95%), ferulic acid (99%), 4-hydroxyphenylacetic acid (HPA, 98%), 3-(4-hydroxyphenyl)propionic acid (HPP, 98%), vanillic acid (97%), and curcumin (≥ 80%) were purchased from Sigma (St. Louis, MO).

Curcumin ingredients and breads

Three types of curcumin containing ingredients were used, namely free curcumin (FC), encapsulated curcumin (EC) and encapsulated curcumin plus three bioactive compounds i.e. piperine, quercetin and genistein (EC+B).

FC was a 95% pure curcuminoid extract from turmeric and was constituted by 79% curcumin, 19% desmethoxycurcumin and 2% bisdesmethoxycurcumin. EC and EC+B were obtained by fluidized bed spray coating, followed by bottom spray. Curcumin was encapsulated by double coating, whereas the inner coating material of microcapsules was constituted by cellulose derivative (Ethocel 100, Dow Chemicals) as a first layer, and hydrogenated vegetable oil (HVO) as an external layer. Ethocel 100 (88% Ethocel 100 and 12% liquid castor oil, as emulsifier) was dissolved in 80% acetone and 20% methanol to get a 4%w/w solution for coating and hydrogenated vegetable oil was melted by heating to 95^0C prior to coating. The particles of curcumin were placed at the bottom of the chamber and blown upward by hot air. The coating polymer solution (Ethocel 100) and HVO were sprayed upward in the same direction, one by one. In this way, curcumin particles pass though a simultaneous coating (drying) environment upward by reaching the top of the chamber, the partially coated particles move downward and undergo further drying until the desired coat thickness is reached.

Finally EC and EC+B contained 72.7% and 66.5% of curcuminoids, respectively; in EC+B, piperine, quercetin and genistein, 1.0% of each were also present (**Table 1**).

Table 1: Composition of the functional ingredients included in the different type of bread.

	Core				Coating		
	Curcuminoids	**Piperine**	**Quercetin**	**Genistein**	**Cellulose derivative (Ethocell 100)**	**Castor Oil**	**HVO**
FC (Free Curcumin)	95%	---	---	---	---	---	---
EC (Encapsulated Curcumin)	72.68%	---	---	---	7.48%	1.02%	15%
EC+B (Encapsulated Curcumin + other polyphenols)	66.5%	1.0%	1.0%	1.0%	6.84%	0.93%	13.73%

All combined compounds were encapsulated one by one with single layer of polymer solution (Ethocel 100) by different amount of coatings (0 – 20% coat), mixed together in order to achieve controlled release mechanism.

Each ingredient was included in a classical bread recipe and three types of bread containing 1g curcuminoids in a 100g portion were formulated. The bread with EC+B also contained 0.01g piperine, 0.01 g quercetin and 0.01 g genistein . Depending from the ingredient used the breads will be hereinafter indicated as: FC bread (FCB), EC bread (ECB) and EC+B bread (ECBB). They were produced in laboratory scale and curcumin bioavailability upon their consumption was studied. By consuming one portion of bread, subjects ingested: 0.8 g (2.1 mmol) curcumin, 0.2 g (473.4 µmol) desmethoxycurcumin and 0.08 g (259.7 nmol) bisdemethoxycurcumin (2.6 mmol of total curcuminoids).

Subjects and treatment

The protocol of the study was approved by the Ethics Committee of "Federico II" University of Naples (Approval Number: 37/10)

Ten healthy subjects, age of 31±2 years, BMI of 23.5±1.2 kg/m^2, were enrolled. Subjects with gastrointestinal pathologies and/or metabolic disease, those taking anti-inflammatory drugs, or under controlled diet in the previous six months were excluded from the study. Volunteers signed a written informed consent before starting the experimental protocol.

Study design is schematized in **Figure 1**. Volunteers were asked to follow a polyphenol-free diet for three days before and over the experiment days. Thus they were recommended to exclude from their diet all polyphenol-rich foods and beverages such as fruits, vegetables, chocolate, tea, coffee, wine, beer, supplements, herbal extracts, and whole grains-based foods. Assumption of FANS and anti-inflammatory drugs was also avoided during 1 week before the study. On the experiment day, at

08.00 h, 12 hours-fasted subjects reached the laboratory and were randomized to receive one of the three experimental breads, that was consumed within 15 minutes. Before consumption of bread and after 30 min, 1h, 2h, 4h, 6h, blood drawings was performed. Urine volume was measured over 24h and 10 mL samples were collected before and at 0-2h, 2-4h, 4-6h, 6-8h, 8-10h, 10-24h time intervals post-bread ingestion.

After 6 h from breakfast ingestion, subjects left the research centre and consumed their lunch (always choosing among allowed foods). A further bread portion was consumed at dinner (within 10.00 h pm). The day after the experiment 12 hours-fasted participants returned to the laboratory, they were submitted to a blood drawing (24h from the first bread consumption) and let the faecal sample collected on the experiment day.

After a 1 week wash-out period during which subjects returned to consume their own habitual diet, participants were crossed-over to receive a new treatment with a different experimental bread. All subjects completed the study receiving all 3 treatments.

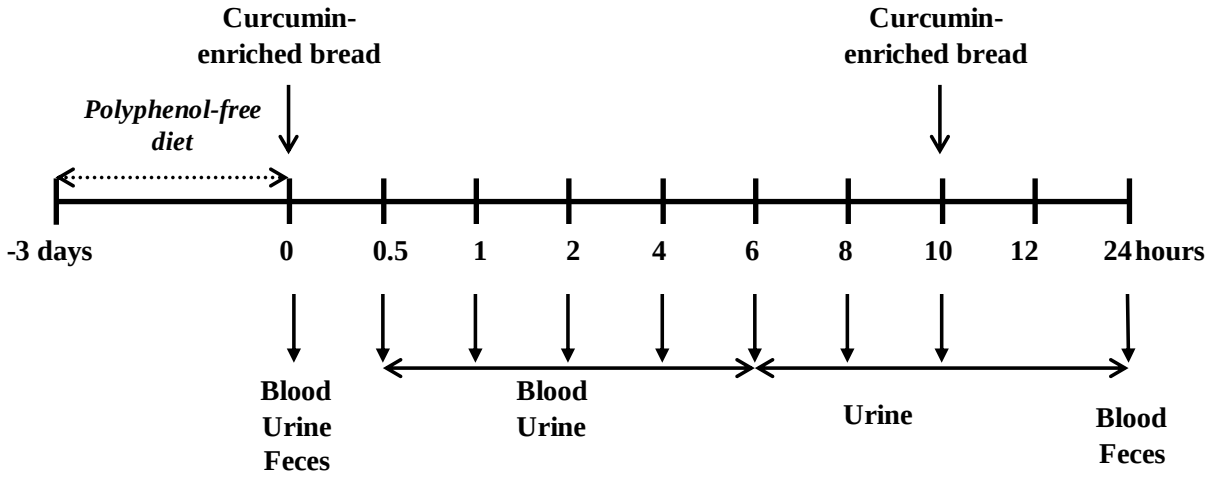


Figure 1: Study design. Each subject followed this time schedule for each type of curcumin-enriched bread by a cross-over randomized design.

Biological sample treatment

Blood samples were collected in Vacutainer tube for gel separation, and immediately centrifuged at 4000 rpm for 10 min at 4°C. Urine samples were immediately treated with 0.005% of BHT. Feces were diluted 1:10 (W/V) in PBS 10 mM, containing 0.005% of BHT, vortexed and centrifuged at 4000 rpm for 15 minutes at 4°C. Serum, urine and fecal supernatants were stored at -40°C until the analysis.

Five-hundred microliter of serum and 1.5 mL of urine and fecal samples were extracted, by 3 mL and 4.5 mL of ethyl acetate respectively. The supernatants were dried under nitrogen flow and dissolved in 50 µL of methanol/water (70:30). Thirty microliters were used for HPLC/MS/MS analysis. Each sample was extracted in duplicated.

HPLC/MS/MS analysis

Chromatographic separation of curcumin and metabolites was performed with a HPLC system consisting of 2 micropumps by Perkin Elmer (USA) Series 200. Elution was achieved with a Phenomenex Luna 3 µ C18(2) 100 A (50x2.00mm) column and by using the following mobile phases: A = H_2O/acetonitril/formic acid 94.9:5:0.1 (v/v/v), and B = acetonitrile/formic acid 99.9:0.1; (v/v); the flow rate was 200 µl/min. A linear gradient was applied as follows: 0–1min,4–40% B; 1–3min,40–100% B; 3–5min,100%B; 6–10 min, 4% B. Analysis was performed using an API 3000 Triple Quadrupole mass spectrometer (Applied Biosystem Sciex). For identification and quantification of compounds, ionization in negative mode was used and a multiple reaction monitoring (MRM) analysis was employed tracking the transition indicative of parent and product ion specific for each compound. Previous direct infusion experiments were performed to optimize following parameters: capillary voltage, focusing potential, entrance potential, declustering potential, and collision energy. After performing infusion following parameters were fixed: dwell time, 100 ms; nebulizer gas, 10; curtain gas, 12; auxiliary gas temperature, 400 °C; auxiliary gas flow rate, 6,000 cm3/min; capillary voltage, −3,700 V; entrance potential, −10 V. Detailed transitions for parent molecules and product ions and MS parameters are listed in **Table 2**.

Table 2: Protonated molecules and product ions of compounds analyzed by LC-MS/MS, and MS parameters

Compound	M-H	Product ions			DP	FP	CE			CXP	
		1	*2*	*3*			*1*	*2*	*3*	*1*	*2*
Curcumin	367.1	217.1	148.9		-46	-400	-16	-25		-10	
Desmethoxycurcumin	337	217			-38	-400	-17			-10	
Bisdesmethoxycurcumin	307	217			-46	-400	-49			-6	
Curcumin glucuronide	543.1	367			-60	-375	-30			-7	
Curcumin sulphate	447	367			-60	-375	-25			-7	
Tetrahydrocurcumin glucuronide	547	135			-60	-375	-25			-7	
Hesahydrocurcumin	373	179			-60	-375	-25			-7	
Hesahydrocurcumin glucuronide	549	373			-60	-375	-30			-7	
Vanillic acid	167	152	108	123	-45	-250	-22	-26		-9	-11
Ferulic acid	192.8	133.9	177.9		-35	-250	-22	-17		-10	
Chlorogenic acid	353	191			-35	-250	-21			-8	
Hydroxyphenylvalerolactone	207	163	122		-35	-250	-29			-7	
3,4-dihydroxyphenylacetic acid (diHPA)	167	123.1			-30	-250	-11			-7	
4-hydroxyphenylacetic acid (HPA)	151	107	78.9		-35	-250	-16	-25		-7	
3-(4-hydroxyphenyl)propionic acid (HPP)	164.9	121	105.9	76.7	-25	-250	-10	-20	-10	-7	

Statistical analysis

The number of subjects was based on power calculations derived from our previous study [12]. We calculated that, at α = 0.05 with a power of 80%, 8 subjects would allow us to detect a 20% difference in serum and urinary concentrations of parental compounds, glucuronides and phenolic acids.

Statistical analysis was performed using the statistical package SPSS for Windows (version15). The results from HPLC/MS/MS analysis of curcumin and metabolites were analyzed and expressed as the absolute changes from the baseline to reduce possible effects of inter-subject fasting variability. The area under the curve (AUC) for each compound from baseline over 6h after first bread portion consumption in the case of serum samples and over 0-10 h and 10h-24h for urine samples were estimated using the linear trapezoidal rule. By the analysis of variance (ANOVA) for repeated measures the subjective time curves for all measured compounds were compared and tested for the effect of treatment and of time as factors. For all tests, following a significant main effect in the ANOVA, individual means were compared using the Bonferroni test ($p < 0.05$). Results were considered significant at $p < 0.05$. All values were reported as means ± SEM.

Results

Serum

The serum mean concentration-time curves of total curcuminoids, curcuminoid conjugated compounds and phenolic acids over 24h following consumption of FCB, ECB and ECBB are reported in **Figure 2**.

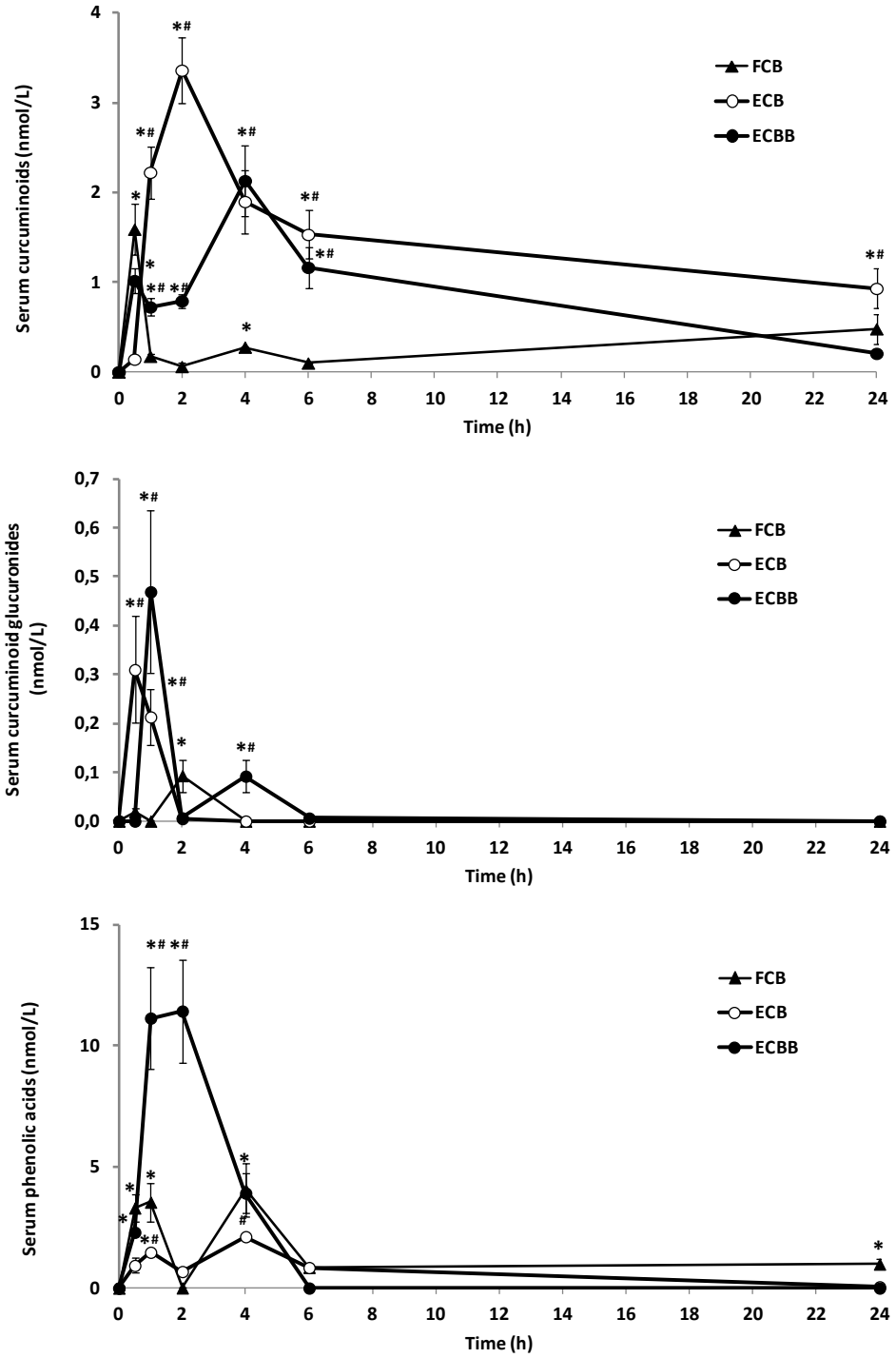


Figure 2: Serum concentration-time curves of total curcuminoids, curcuminoid glucuronides and phenolic acids following consumption of FCB, ECB and ECBB. *:p<0.05 vs baseline; #: p<0.05 vs FCB.

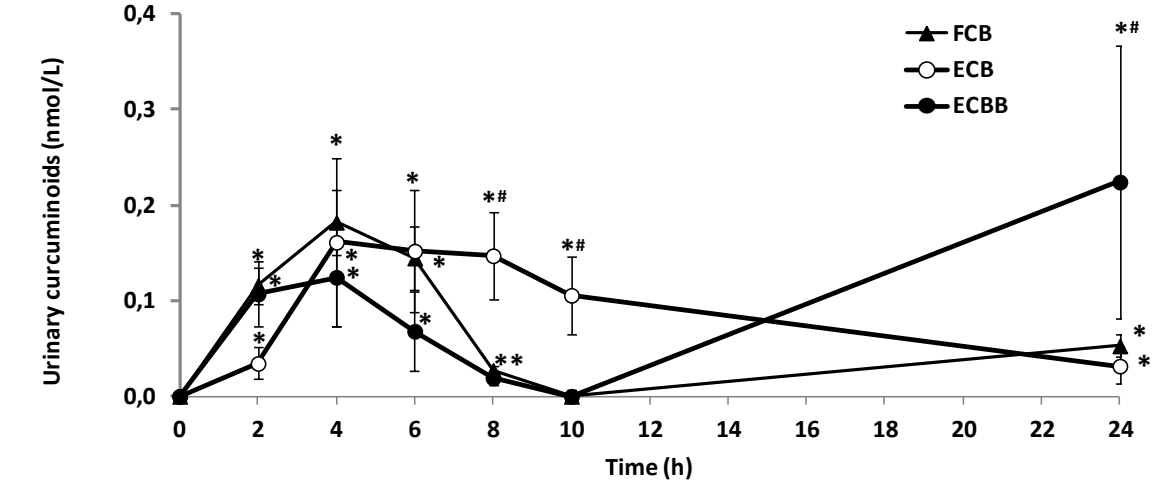
Modification of the amount and of the time course of curcuminoids absorption after consumption of ECB and ECBB compared to FCB were found. Following FCB, curcuminoids peaked at 30 min with a C_{max} of 1.59 ± 0.28 nmol/L, while following ECB and ECBB t_{max} of 2h and 4h, with C_{max} of 3.36 ± 0.36 nmol/L and 2.13 ± 0.39 nmol/L, respectively, were recorded. Serum concentrations of curcuminoids over 6 hours after consumption of bread with encapsulated ingredients were always higher than after consumption of bread with the free ingredient. ECB consumption determined at 1h and 2h serum curcuminoid concentrations higher than ECBB ($p<0.05$ for ECB and ECBB *vs* FCB and for ECB *vs* ECBB). The consumption of the second portion of bread at 10 h guaranteed at 24h (after 14h) a serum concentration of curcuminoids higher than baseline. Following ECB that concentration was higher than after FCB (0.93 ± 0.22 nmol/L *vs* 0.48 ± 0.17 nmol/L, $p<0.05$).

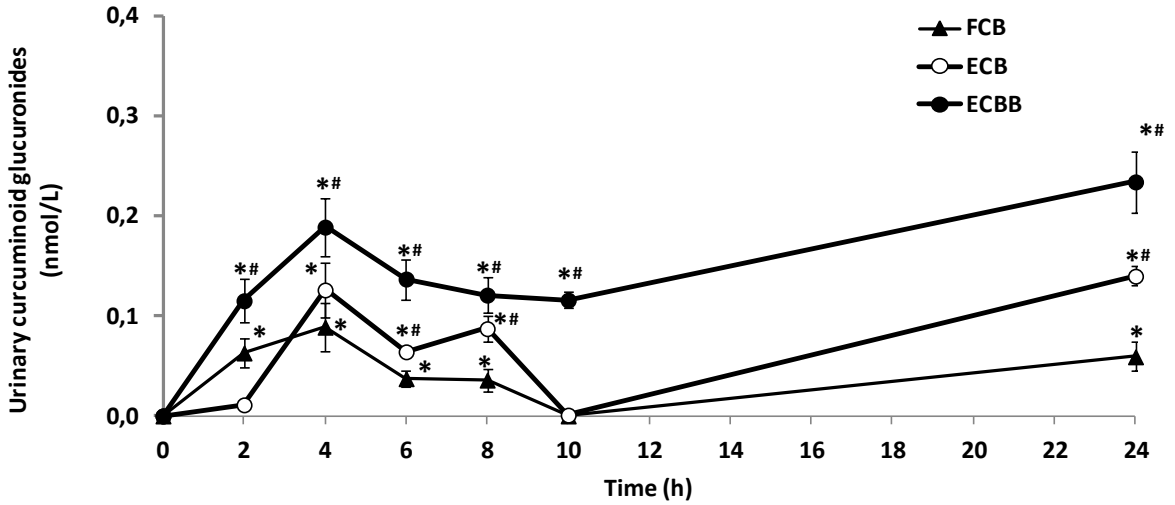
The curcumin conjugated metabolites found in serum, curcumin glucuronide and esahydroxycurcumin glucuronide, were at concentrations ten folds lower than parental curcuminoids. Interestingly, after consumption of encapsulated ingredients, the concentration peaks were anticipated compared to the free ingredient (30 min and 1h *vs* 2h). In accordance with curcuminoids also AUC_{0-6} of conjugated compounds were higher after ECBB or ECB than after the FCB (0.32 ± 0.20 nmol·h/L and 0.55 ± 0.39 nmol·h/L *vs* 0.15 ± 0.06 nmol·h/L , respectively). As expected no glucuronides were found in serum at 24 h.

Following the consumption of all types of bread the phenolic acids retrieved in serum were ferulic and chlorogenic acid; while vanillic acid was found only after ECBB. Their appearance in serum was already at 30 min and all peaked between 30min – 2h; after FCB a double peak at 4h was recorded. Surprisingly, C_{max} after ECBB was almost 3-fold and 10-fold higher than that after FCB and ECB, respectively (11.43 ± 2.13 nmol/L vs 4.06 ± 1.11 nmol/L and 1.27 ± 0.10 nmol/L, respectively; $p<0.05$). Measure of AUC_{0-6} of total phenolic compounds demonstrated that amount of phenolic acids in the bloodstream following ECBB consumption was almost double compared to that after FCB and even 7-fold higher than following ECB (23.3 ± 5.0 nmol·h/L *vs* 13.3 ± 2.5 nmol·h/L and 3.3 ± 0.54 nmol·h/L, respectively). In all cases, ferulic acid was the most abundant phenolic acid retrieved in serum always contributing by ~ 75% of total. The repeated consumption of FCB and not that of the two encapsulated bread determined a 24h serum concentration of phenolic compounds significantly higher than baseline (1.0 ± 0.2 nmol/L).

Urines

Figure 3 reports urinary mean concentration-time curves of total curcuminoids, curcuminoid conjugated compounds and phenolic acids over 24h following consumption of FCB, ECB and ECBB.





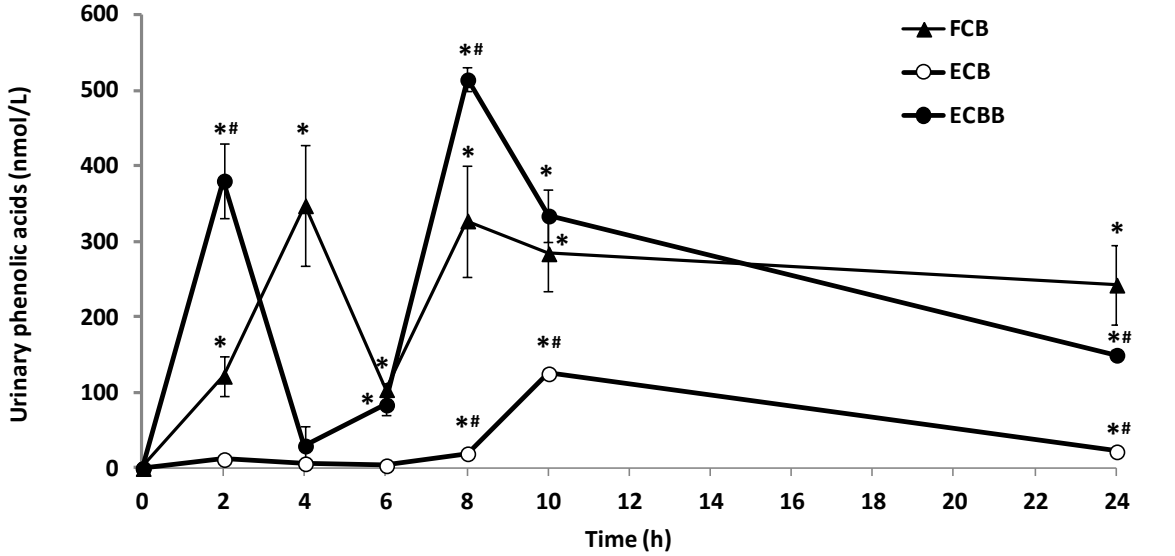


Figure 3: Urine concentration-time curves of total curcuminoids, curcuminoid glucuronides and phenolic acids following consumption of FCB, ECB and ECBB. *:p<0.05 vs baseline; #: p<0.05 vs FCB.

Urinary excretion of curcuminoids began 2h after consumption of all types of bread. Although AUC_{0-10} of urinary curcuminoids were not significantly different (0.98 ± 0.55 nmol·h/L, 1.10 ± 0.47 nmol·h/L and 0.50 ± 0.27 nmol·h/L after FCB, ECB and ECBB, respectively) the excretion kinetics showed a different trend among treatments. Curcuminoid concentration peaked within 4h and was absent 10h after FCB and ECBB consumption while it reached a maximum concentration at 4h and maintained this plateau concentration up to 10 h after ECB. Concentrations higher than baseline were still found 14h after consumption of the second portion of each type of bread.

Curcumin-glucuronides and esahydroxycurcumin-glucuronides were the conjugated compounds retrieved in urines (the same compounds found in serum). In the urines conjugated compounds and parental curcuminoids were present at the same concentration range. Similarly to parent compounds, glucuronides peaked at 4h after all types of bread, but they were excreted within 10h only after FCB and ECB. Over 10h from consumption of ECBB, glucuronides concentration was always significantly higher than those found after FCB and ECB (AUC_{0-10} being 1.24 ± 0.41 nmol·h/L vs 0.45 ± 0.24 nmol·h/L and 0.58 ± 0.17 nmol·h/L, respectively, $p<0.05$). The consumption of the second portions of breads enriched with encapsulated ingredients determined an overnight excretion significantly higher than that found with FCB (AUC_{10-24} being 0.98 ± 0.02 nmol·h/L after ECB and 2.45 ± 0.48 nmol·h/L after ECBB vs 0.41 ± 0.20 nmol·h/L for FCB).

Phenolic acids found in urines after consumption of all types of bread were ferulic and vanillic acid, diHPA, HPP and HPA were also found after ECB and ECBB, respectively. The concentrations of phenolic acids were 1000 fold higher than the other urinary metabolites and 50-100 fold higher than serum phenolic acids. In accordance to serum data, ECB determined the lowest urinary excretions of phenolic acids: they appeared in urines 6h after consumption peaking at 10h with a C_{max} of 125.6 ± 6.3 nmol/L. On the contrary, phenolic acid excretion after ECBB paralleled that after FCB: a first excretion peak within 4h after consumption of bread and another peak over the next 4 h, with C_{max} ranging between 347.7 ± 79.6 nmol/L and 514.4 ± 16.0 nmol/L. AUC_{0-10} of phenolic acids following ECB (0.21 ± 0.059 µmol·h/L) was 10 folds lower than those measured upon consumption of FCB (2.1 ± 1.3 µmol·h/L) and ECBB (2.4± 1.0 µmol·h/L). Interestingly, individual phenolic acids differently contributed to total amount excreted upon each experimental condition. After FCB, vanillic acid was the most abundant phenolic acid being 73% of total phenolic acids while ferulic acid contributed by 16% and diHPA by 11%. Encapsulation deeply modified the pattern of phenolic acids retrieved eliciting a significant amount of HPP: after ECB consumption the percentages excretion of ferulic acid (24%) and diHPA (8%) were, respectively, slight higher and lower to those found after FCB while, the remaining part was almost equally represented by vanillic acid (38%) and HPP (30%). The presence of piperine, quercetin and genistein in the encapsulated material

further modified the pattern: in fact, after ECBB, compared to ECB, an increase of vanillic acid (56%) *vs* a reduction of ferulic acid (9.2%) and diHPA (0.6%) as well as the excretion of HPA (34%) instead of HPP were found.

Feces

Concentrations of monitored compounds in fecal samples collected the day after consumption of each type of bread is reported in **Table 3**.

Table 3: Concentrations of curcuminoids, curcuminoid glucuronides and phenolic acids found in fecal samples collected the day after consumption of each type of bread. Data are means ± S.E. pmol/g.

	FCB	**ECB**	**ECBB**
Total curcuminoids	**0.59 ±0.38**	**3.49 ±2.13**	**0.01 ±0.01**
Curcumin	0.29 ±*0.18*	0.83 ±*0.46*	---
Desmetoxycurcumin	0.30 ±*0.19*	2.65 ±*1.67*	0.01 ±*0.01*
Bisdesmetoxycurcumin	---	0.01 ±*0.01*	---
Total glucuronides	---	---	---
Curcumin-glucuronide	---	---	---
Esahydroxycurcumin-glucuronide	---	---	---
Total phenolic acids	**0.73 ±0.47**	**0.49 ±0.32**	**2.04 ±1.27**
Chlorogenic acid	---	---	---
Ferulic acid	---	0.01 ±*0.00*	0.01 ±*0.01*
Vanillic acid	0.69 ±*0.45*	---	2.03 ±*1.26*
di-HPA	---	0.02 ±*0.01*	---
HPA	---	0.46 ±*0.30*	---
HPP	0.03 ±*0.02*	---	---

Data showed that ECB consumption increased about 6 folds the amount of curcuminoids in the feces respect to FCB. On the contrary following ECBB consumption traces amount of curcuminoids and the highest amount of phenolic acids were found. The latter being even 4 fold higher than after ECB.

Discussion

Previous bioavailability studies reported that following consumption of 2-12 g of curcumin administered in form of capsules as pharmaceutical preparations, serum C_{max} was never below 136 nmol/L in healthy subjects [4, 13, 14], while concentration around 10 nmol/L were found in patients ingesting 3.6 g/die of curcumin [15]. Our data showing curcuminoid serum concentrations always lower than 4 nmol/L, suggested that curcumin is less bioaccessible when it is included in bread probably due to a strong interaction of the compound with the processed food matrix. Encapsulation increased the bioavailability of curcuminoids from bread both upon consumption of a single and a double portion while co-encapsulation of curcuminoids with piperine, quercetin and genistein, slightly reduced this effect. In fact, over 10h from the first consumption of ECB and ECBB, total curcuminoids were, respectively, 7.3 and 4.6 folds higher than after FCB; ECB leading to a 63% higher serum amount of curcuminoids than ECBB (ECB>ECBB>>FCB) (**Table 4**).

The *in vivo* formation of several phenolic acids following consumption of curcuminoids was demonstrated for the first time in this study. They might derive both by curcumin chemical instability and *in vivo* metabolism. Curcumin degradation is pH-dependent (faster at neutral-basic conditions), due to oxidative mechanisms and leading to formation of trans-6-(4'-hydroxy-3'-methoxyphenyl)-2,4-dioxo-5-hexenal, vanillin, ferulic acid, and feruloyl methane [16-19]. In particular, it has been shown that when curcumin was incubated in 0.1 M phosphate buffer (pH 7.2, 37°C) about 90% decomposed within 30 min while 20% decomposed within 1 h by incubation in cell culture or in human blood leading to increase of vanillin [17]. Thus it can be hypothesized that in the intestine, most part of curcumin may decompose at neutral pH before absorption and a minor part may be even degraded in intestinal mucosa and in the bloodstream. When curcuminoids are microencapsulated they are protected from intestinal degradation thus increasing their amount in blood in the original chemical form. On the other hand, concomitant presence in the intestine of curcuminoids and the three bioactive compounds as in ECBB, did not influence intestinal and hepatic glucuronidation of curcumin, but it promoted phenolic acid formation. This might be caused by the instauration of a competitive absorption between curcumin and the other compounds at level of intestinal mucosa leading to a delayed curcumin absorption and a consequent increased degradation rate in the intestinal lumen. This hypothesis is consistent with a recent study showing a faster and a more efficient absorption of piperine than curcumin in rats [20]. Moreover, recent studies highlight the influence of dietary piperine, quercetin and genistein on drug absorption by several mechanisms (i.e. modification of absorptive sites on mucosa or interaction with P-glycoproteins) [21-23], other than modification of first pass metabolism [6, 10].

Table 4: Amount (nmol) of total curcuminoids, curcuminoid glucuronides and phenolic acids found in serum, urines and feces following consumption of one portion of each type of bread or over 24 (following consumption of two portions of each type of bread).

	Bioavailability upon single portion					Bioavailability upon double portion				
	total curcuminoids (nmol)	**total glucuronides** (nmol)	**phenolic acids** (nmol)	**total** (nmol)	**% dose ingested**	**total curcuminoids** (nmol)	**total glucuronides** (nmol)	**phenolic acids** (nmol)	**total** (nmol)	**% dose ingested**
FCB										
serum	5.00	0.45	39.85							
urines	1.15	0.33	2465.88	**2512.66**	**0.10**	1.45	0.64	5416.15	**5419.56**	**0.10**
feces						0.59		0.73		
sum	***6.15***	***0.78***	***2505.73***			***2.04***	***0.64***	***5416.88***		
ECB										
serum	*36.34*	*0.97*	*9.95*							
urines	*1.65*	*0.75*	*907.88*	**957.54**	**0.04**	2.45	1.28	1166.10	**1173.33**	**0.02**
feces						3.49		0.01		
sum	***37.99***	***1.72***	***917.83***			***5.94***	***1.28***	***1166.11***		
ECBB										
serum	23.00	1.65	103.40							
urines	0.73	1.31	4518.11	**4648.20**	**0.18**	2.18	2.83	6204.10	**6211.64**	**0.12**
feces						0.49		2.04		
sum	***23.74***	***2.96***	***4621.51***			***2.67***	***2.83***	***6206.14***		

Thus, in the case of ECBB, we hypothesize that phenolic acids deriving from curcumin and from co-ingested piperine, quercetin and genistein biotransformation [25], might be also absorbed more efficiently.

On the other hand, the prevalence of ferulic acid in serum was clearly explainable from degradation of curcumin, while vanillic acid might form in liver by aldehyde oxidase-operated oxidation of vanillin [26]. The metabolism by gut microflora of not absorbed curcuminoids [18] and of compounds formed in the upper intestine and successive absorption of metabolites through the colon could explain the presence of phenolic acids in biological samples collected at 24h. The active role of intestinal microbiota on metabolism of curcuminoids was consistent with a recent study where a microbial enzyme isolated from human feces able to convert curcumin in dihydrocurcumin and tetrahydrocurcumin was isolated [27].

In conclusion, in this study the bioavailability and biotransformation of curcuminoids present in free and microencapsulated form in a processed food such as bread was elucidated. The concentration of curcuminoids in serum following consumption of the new types of bread was lower than that reported from supplements. Data demonstrated that ingesting encapsulated ingredients can protect curcumin by *in vivo* biotransformation thus increasing its circulating concentration compared to the free ingredient. On the other hand, co-ingestion of piperine, quercetin and genistein did not increase curcuminoid bioavailability in their original form but increased their biotransformation in phenolic acids. It was demonstrated for the first time that phenolic acids (mainly ferulic acid and vanillic acid) are the major metabolites following consumption of curcuminoids thus envisaging their potential contribution to the biological properties recognized to curcuminoids despite their generally low bioavailability.

Abbreviations used

Free Curcumin Bread (FCB); Encapsulated Curcumin Bread (ECB), Encapsulated Curcumin plus other polyphenols Bread (ECBB); 4-hydroxyphenylacetic acid (HPA); 3,4-dihydroxyphenylacetic acid (diHPA); 3-(4-hydroxyphenyl)propionic acid (HPP).

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Chapter 5. Fat taste and appetite: a combined physiological and sensory approach

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The present chapter is going to be submitted as a research article for publication.

Abstract

During the cephalic phase of eating, sensory perception provides information about the food nutrients allowing digestive tract organs to prepare for its reception and digestion. In this phase, even before swallowing, food liking was demonstrated to modulate some endocannabinoids (ECs) and N-acylethanolamines (NAEs) in human plasma (Mennella et al., 2015). This study aimed to evaluate whether salivary ECs and NAEs concentrations upon mastication were associated with sensory perception of fat taste, the food palatability, the appetite and reward scores in humans. To this purpose, a fat-enriched (FEP) and a free-fat control pudding (CP) were developed and used in a randomized cross over study. The experimental procedure was based on a modified sham-feeding protocol (MSF) combined with multiple-sip Temporal Dominance of Sensations (TDS) method. In particular, while masticating (without swallowing) for 3 minutes multiple sips of the pudding subjects selected the dominant sensations among a list of sensory attributes previously defined. Nineteen healthy volunteers participated in the study. Saliva samples, appetite and food liking scores were collected at baseline, immediately after the MSF of one pudding and every 5 min up to 20 minutes after the MSF. A different modulation of ECs and NAEs during MSF of FEP as well as over the next 20 min compared to CP was found. Attribute "fatty taste" was rated as significantly different in FEP compared to the CP during MSF. Only the MSF of FEP increased individual fullness and satiety and reduced hunger. In conclusion, in this study for the first time the combination of MSF and multiple-sip TDS to study the physiological and sensory mechanisms underlying appetite and food liking was used. Data indicated an association between dietary fats, individual sensory perceptions, appetite and salivary ECs and NAEs concentrations during cephalic phase of eating.

Introduction

Dietary fat is an essential nutrient in human nutrition ensuring appropriate function of hormonal and immune system, thermal protection, functioning as a medium for fat-soluble vitamins and lastly being the most dense source of energy. Excessive energy intake causes body fat accumulation and obesity -related health issues (WHO, 2003, Willet et al 2012). Human orosensory perception, especially during cephalic phase of eating, has a plausible role in providing the information about the consumed nutrient type, thus sending signals to different sites of the body and allowing digestive tract organs to prepare for its reception and digestion upon the nutrient type, which results in different satiating effect (Mattes, 2005). Mechanisms behind fat perception and palatability may be interconnected with the reward system. Some endocannabinoids (ECs) and N-acylethanolamines (NAEs) were demonstrated to be modulated by food liking upon mastication (before swallowing) in humans (Mennella et al., 2015). The cephalic phase of the digestion can be studied in humans using a modified sham feeding protocol (MSF). In MSF experiments, the food is tasted and chewed, but ultimately expectorated. Therefore, all variations found in biochemical parameters during the protocol can be considered as a cephalic effect without any interference of food digestion (Teff et al. 2010). Plenty of research is focusing on the fat taste in order to assess its eligibility as a primary taste. Lack of knowledge still exist about physiological mechanisms behind the fat taste and its role in dietary choices and behavior. In this study a holistic approach considering both physiological response linked to appetite and reward system as well as the sensory description of the fat taste upon mastication will be considered.

Subjects and methods

Subject selection and enrolment

Nineteen subjects were selected among students and employees of Department of Agricultural Sciences of Federico II University of Naples (Italy). Basing on the medical history interview, subjects were eligible if they did not suffer from any disease (hyperlipidaemia, gastro-intestinal disease, chronic infections, dental diseases, general and food allergies), were non-smokers, did not regularly consume alcohol, were not taking any medications, did not undertake a restrictive diet or experienced body weight variations over three months preceding the study. Eating behaviour was assessed for the Restraint, Disinhibition, and Hunger factors using a validated Italian translation of the Three Factor Eating Questionnaire (TFEQ) as described by Stunkard & Messick (1985) and for the preference and the consumption of high fat foods using the “Fat Preference questionnaire”

(Ledikwe et al, 2007). Descriptive characteristics of the participants are reported in **Table 1**. The study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects approved by the Ethics Committee of University of Naples "Federico II". Selected subjects participated in the study after reading and signing an informed consent document.

Table 1. General characteristics of the subjects. Eating behaviour result from TFEQ (Restrain, Disinhibition and Hunger; Stunkar et al,1985) and Fat Preference Questionnaire (Taste, Freq and Diff; Ledikwe 2007). Values are expressed as mean ± standard deviation (SD).

	Male	**Female**	**Total**
Number (n)	8	11	19
Age, mean (years)	23.1	21.7	22.4
BMI, mean ± SD (kg/m2)	26.3±2.7	22.2±2.9	23.9±3.4
Restraint (score)	8.9±4.6	11.7±4.1	10.5±4.4
Disinhibition (score)	6.6±3	6.6±3.1	6.6±3
Hunger (score)	5.4±2.3	4.8±3.7	5±3.1
Taste (%)	56.3±16.7	67.6±17.5	62.8±17.6
Freq (%)	44.7±11.1	49.4±13.1	47.4±12.2
Diff (%)	16.6±11.7	23.1±17.6	20.4±15.4

Foods

Two types of vanilla pudding differing only for the fat content were developed and used in this study. They were a fat-enriched pudding (FEP) containing high-oleic sunflower oil (2.6% w/w) and a control pudding (CP) that did not contain any added fat. The high-oleic sunflower oil was provided by Oleifici Mataluni (Montesarchio, Benevento, Italy). The nutritional composition of the two puddings is reported in **Table 2**.

Table 2. Nutritional composition of the two foods used in the study. FEP (Fat-enriched pudding); CP (Control Pudding). Values are reported in g and as % of total energy coming from each product.

	Energy density (kcal)/100g	**Protein**		**Carbohydrates**		**Fats**	
		g	% E	g	% E	g	% E
FEP	136.6	10.8	32%	12.0	35%	5.0	33%
CP	115.9	11.1	38%	12.4	43%	2.5	19%

Study protocol

The study had a single blind randomized cross-over design with repeated measures. Participants were divided into four groups and each group invited in four occasions to arrive in a fasting condition to the laboratory and were involved in a protocol including MSF and Temporal Dominance of Sensations (TDS). Each experiment was conducted during two days per week, 1 day per each treatment, separated with a 2 day-wash-out period. Next replication was conducted after 1 week break. Before the experimental day, subjects were instructed to consume a standardised dinner on the evening before and to restrain from eating and drinking energy-containing foods and beverages from 22:00h until the experiments and to clean their teeth no later than 1h before the study time. Before experiments, two training sessions were organised. During the first one the control pudding was presented to participant group and a list of most frequently used attributes was created and used later in the TDS procedure (which was explained to volunteers). The selected attributes were: sweetness, creaminess, vanilla, milk, fatty, white chocolate, compactness, watery, gelatinous. The second training included the liking evaluation of the two types of pudding through the use of visual analogue hedonic scale anchored from 1 to 9 (Lim, 2011) and a TDS test training. This made volunteers more comfortable with the procedure and allowed us to correct/reject the volunteers unable to perform the study correctly.

Modified Sham Feeding (MSF) and Temporal Dominance of Sensations (TDS) protocol

On the experimental days, once the fasted subjects arrived to the sensory laboratory of the Department of Agricultural Sciences, they were generally asked about their actual health status and the session started only for subjects who did not report any health issue or psychological discomfort; otherwise the experiment session was postponed for the subject. Each participant was asked to seat in an assigned sensory booth equipped with a computer where the software FIZZ (*Biosystemes, Couternon, France*) designed for the TDS could run. Once the participant was given the pudding samples coded with 3-digits following a William's Latin square design the experiment started. Firstly, participants completed the baseline Visual Analogue Scale questionnaire (VAS Q0) by rating their actual health status and appetite feelings (fullness, satiety, hunger and desire to eat the sample). Volunteers were asked to mark the point corresponding to their sensations on the 100 mm VAS scale anchored at 1-100 with answers depending on the nature of questions. After completing the baseline questionnaire (Q0), during the time of 2 minutes subjects collected a saliva sample (baseline saliva, T0) and then continued the protocol with the MSF procedure. Subjects were asked to take a spoon (6-8 g) of the sample, to chew it for at least 20 seconds and then expectorate the sample into a plastic cup. They were instructed not to

swallow any food and to repeat this procedure with total number of 10 spoons and total time of 3 minutes which is adequate for eliciting a cephalic phase response (Teff et al, 2010). During MSF procedure, volunteers were presented with a TDS questionnaire and during the mastication of the food they had to choose the most dominant attribute from the presented list including *sweetness, creaminess, vanilla, milk, fatty, white chocolate, compactness, watery, gelatinous*. To avoid list order bias, the order of the attributes was different for each assessor, following William's Latin square design. A dominant attribute was defined as the sensation that caught attention at a given time, not necessarily being the most intense. During 20 second- lasting evaluation of each spoon (10 in total) they were able to change the dominance whenever their perception has changed, with no restrictions for the number of chosen attributes (Zorn et al, 2014). A specific TDS Multiple-sip evaluation incorporated in MSF procedure was created aiming at recording a dynamic sensory profile of each product while stimulating the cephalic phase responses. Joint MSF and TDS procedure ended with 2 minutes break which was followed by saliva sample collection that was repeated 4 times every 5 minutes (5, 10, 15 and 20 mins after MSF). Each saliva sample collection was preceded by appetite (fullness, satiety, and desire to eat) and food liking questionnaires using VAS. After the last time point (20 min after MSF) subjects were provided a second coded sample of food and only TDS procedure during mastication was repeated without saliva collection. The procedure was conducted under artificial light and water was provided to rinse the mouth between the samples and to ensure appropriate hydration before and after saliva collection.

Saliva and samples treatment

Saliva samples were collected in 50 mL tubes and immediately after collection they were centrifuged at 4000rpm for 5 minutes and the supernatant was aliquoted in 2 mL Eppendorf tubes and stored at - 40°C until the analyses. One milliliter aliquots from the chewed pudding samples were used for later endocannabinoid analysis. The remaining part from chewed puddings together with not-chewed left-over and reference puddings were freeze-dried and used for the recovery. It was measured basing on their dry-weight compared to the dry-weight of the reference puddings.

Endocannabinoids (ECs) and N-acylethanolamines (NAEs) measurement by LC/MS/MS analysis

All the ECs (2-AG, AEA, AEAd8) and NAEs (OEA, LEA and PEA) standards were purchased from Cayman (Cayman Chemical, Ann Arbor, MI). The extraction, purification and quantification of the ECs and NAEs in saliva were performed as described by Di Marzo (1999). Samples were centrifuged before the analysis (14000 rpm, for 10 minutes). Subsequently, samples containing internal standard were treated with acetone for the protein precipitation and lipid extraction was

done with chloroform: methanol (2:1 v/v/). The organic phase was then dried under nitrogen and the dried residue was re-suspended in 100µL of acetonitrile: water (1:1 v/v) and centrifuged (14000 rpm, 4 °C, 10 min). Supernatant was transferred into glass vials and tandem mass spectrum analysis was performed by API 3000 Triple Quadrupole instrument. The analysis was repeated in duplicate for each sample. The compounds amounts were calculate by isotope dilution using a 5-point calibration curve and expressed as pmol/mL of saliva.

Salivary lipase activity by spectrophotometric assay

Salivary lipase activity at baseline was analysed according to the method described by Mennella et al., 2014. Briefly, 100 µL saliva sample was treated with 0.3 mM 5-5′-dithio-bis (2-nitrobenzoic acid) (DTNB) and 20 mM of phenylmethylsulfonyl fluoride (PMSF) followed by a 5 minutes incubation (37°C). Then, 20 mM of 2,3-Dimetylocapto-1-propanol-tributyrate (BALB) was added and samples were put for the 30 min incubation at 37°C. Preceded by centrifugation (14000 rpm, 4 °C, 10 min) spectrophotometric assay was done measuring the sample absorbance at 412 nm wavelength. Using 4-point calibration curve, sample concentration was calculated basing on the absorbance of the sample colour and expressed in ug/ml. A mother solution of 1 U/mL was prepared dissolving the powder in Tris–HCl buffer (pH 8.5) and consecutive dilutions were carried out to obtain a curve ranging from 6.25 to 100 U/L.

Statistical analysis

Statistical analysis of biochemical data were performed using SPSS® software (IBM, version 21). Salivary ECs and NAEs were analysed and expressed as the absolute change from the baseline to reduce possible effects of the inter-subjects fasting variability. Using analysis of variance (ANOVA) for repeated measures, the subjective appetite sensations recorded before and after MSF of the two different puddings together with the ECs and NAEs response curves were compared and tested for the effect of treatment and time as factors. Influence of the sample type on the overall liking rates for different attributes was analysed and compared. The Pearson's product moment correlation test was employed to analyse possible correlation among the variables. Results were considered as significant at $p<0.05$. As regards the TDS data, the attribute regarded as dominant at every time was recorded for each assessor. For each spoon, dominance rate (%) for each attribute at a given time (every 1s) was determined as the percentage of judgments (assessors x replicates) for which the given attribute was selected as dominant. Dominance rates were plotted against time for each sample to obtain TDS curves describing each spoon. Chance (P0) and significance level (Ps) were calculated and represented on the TDS

curves. Chance level was calculated as the inverse of the total number of attributes (Labbe et al., 2009), whereas significance level was calculated using a binomial test, as recommended by Pineau et al. (2009). Difference curves between fat-enriched and control sample were constructed by subtracting their TDS curves at each time for each spoon. Dominance rate differences were considered significant when they were significantly different from 0 according to a classical test of comparison of binomial proportions (Pineau et al., 2009).

Results

Fat content does not influence individualoverall liking

Control and fat-enriched pudding liking evaluation during the training session revealed that presence of sunflower-oil in fat-enriched pudding significantly influenced appearance ($p=0.036$), colour ($p=0.028$), texture spoon ($p=0.047$) and aftertaste ($p=0.032$) attributes which appeared to be higher for the CP than for FEP. However, the score for the fatty taste attribute did not change between the puddings.

Salivary ECs and NAEs upon MSF

Figure 1 shows the time-concentration curves of salivary ECs and NAEs upon the cephalic phase of eating the CP and FEP. All ECs and NAEs peaked in saliva samples collected during mastication of the two puddings (3 min) compared to baseline saliva concentrations. Significant lower concentrations of all monitored compounds (being $p=0.041$ for AEA, $p=0.032$ for LEA, $p=0.005$ for OEA and $p=0.006$ for PEA) but 2-AG ($p=0.25$) during mastication of FEP than CP were also found. Significant differences between the two puddings for the elicited response of AEA ($p<0.01$ at 10, 15 and 20min), LEA ($p<0.001$ at 10 15 and 20 min), and PEA ($p=0.037$ and $p= 0.008$ at 15 and 20 min, respectively) over the 20 min following the MSF were also observed.

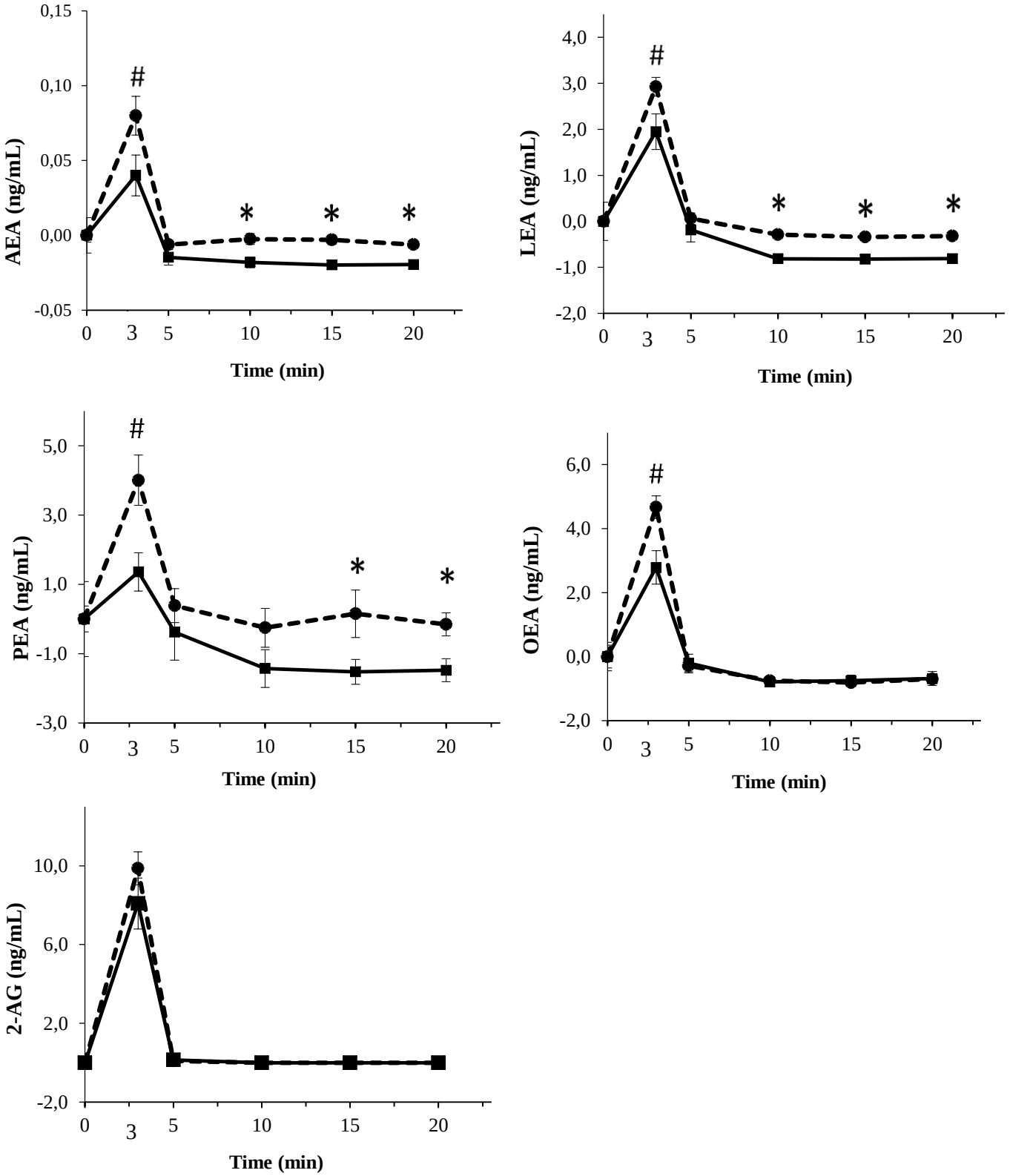


Figure 1. Salivary ECs and NAEs variation vs baseline (T0) time-concentration response. Dotted line indicates the MSF of control; smooth line refers to the fat-enriched pudding MSF. The data point at 3 (T3) min represents ECs and NAEs concentration in chewed food containing saliva collected during MSF. Values are means ± SEM. * $p<0.05$ for CP vs. FEP; # $p<0.05$ for T3 vs T0.

Appetite

The influence of MSF protocol on appetitive responses is expressed as variations from baseline values (**Figure 2**). MSF of fat-enriched pudding (FEP), caused a rapid increase from baseline of fullness (panel A) and satiety (panel B) scores and a reduction of hunger (panel C) that was not found when subjects chewed control pudding. Nonetheless, no significant difference was found in this rapid change from baseline to 20 min. Desire to eat (panel D) scores were not affected by fat content of food stimulus. Liking (panel E) values for both types of food has been similar throughout the time of the study without any significant difference for CP being of 4.98 ± 0.13 and for FEP being 4.91 ± 0.16, $p>0.05$).

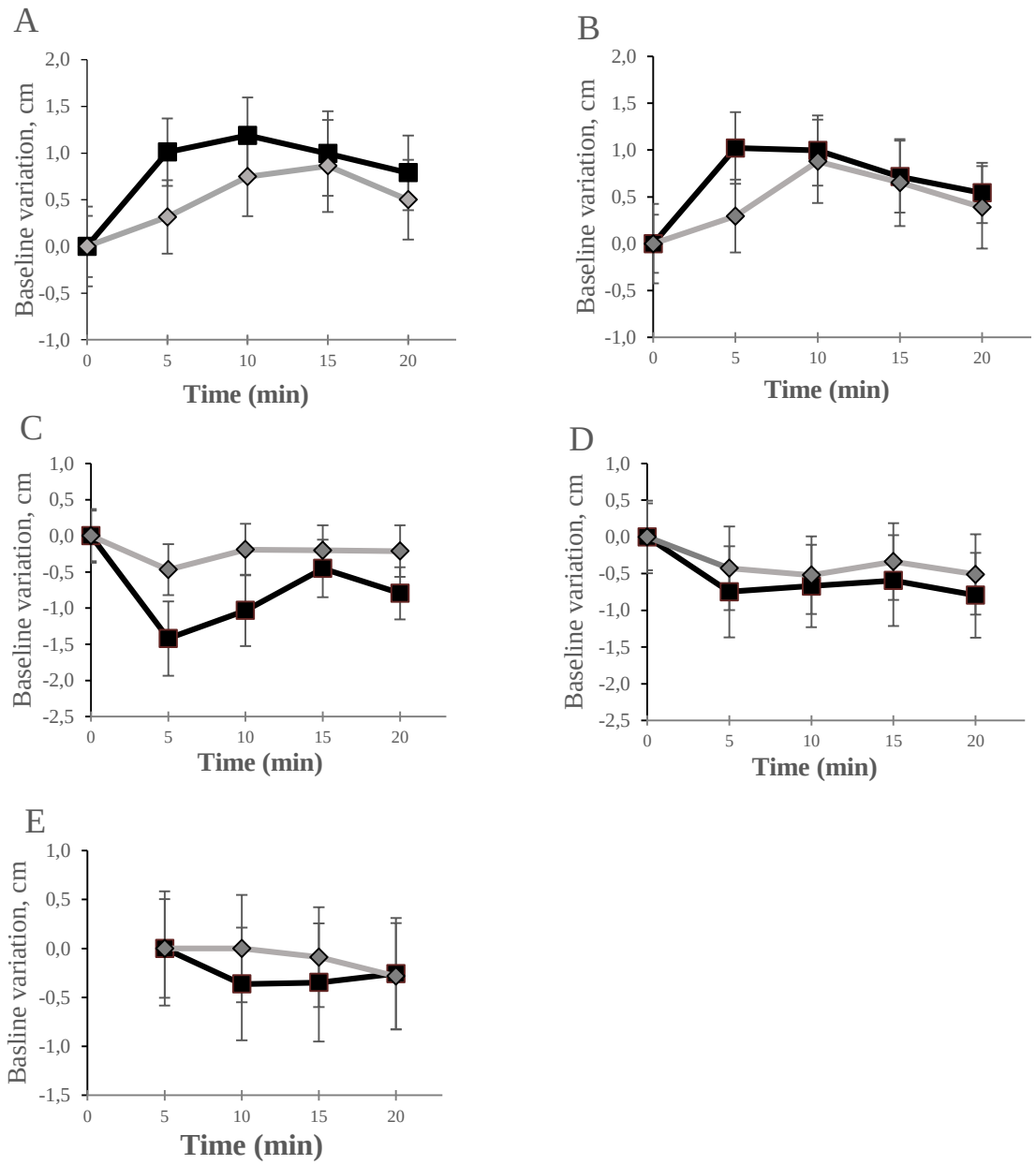


Figure 2. Visual Analogue Scale responses - variation from the baseline for Fullness (A) , Satiety (B), Hunger (C), Desire to eat (D) and Liking (E – scored only after MSF procedure) evaluated before each time point of saliva collection at the baseline and chewing FEP (black line) and CP (grey line). Values are means ± SEM.

Dominant dynamics of fatty taste

Dominance curves has been obtained comparing the % of dominance of each attribute against time during the TDS evaluation of each pudding (**Figure 3**). Fatty taste has been significantly dominant with the 18% of dominance surpassing the significance level (P_s =17.28) level already in the 2st spoon (fig. A). In comparison, second spoon of control pudding (fig. B) is characterised by significantly dominant sensation of watery attribute, elicited at the end of 40th second of the test. Another dominant sensation in fat-enriched sample was recorded at the beginning of the 94th (fig C – 5th spoon) second, during which creaminess was reported as significant (21%), followed by watery attribute (also 21%).Difference between dominance rates of two types of puddings has been plotted for each spoon (**Figure 4,** Panel A and B). Attribute listed as "fatty taste" has been rated as significantly different in fat-enriched pudding compared to the control pudding in almost all spoons (excluding the 8th), with the highest rate for 2nd, 9th and 10th spoon. First spoon of fat-enriched pudding is characterised by slight white chocolate attribute dominance, occurring also in 9th spoon. Difference in dominance of control pudding was more distinguish for the vanilla taste attribute. Compactness in FEP was markedly different in 6th (110 s) and 7th spoon (130s) during which also milk taste attribute was distinguished. Finally, fatty-taste attribute finishes the whole evaluation as the last most significant sensation at the time of 190 seconds. As for sensory attribute differentiation in CP, apart from small differences for sweetness and compactness at the end of the 3rd and 4th spoon respectively, notably different sensations in control pudding finish in the 5th spoon, with a significant occurrence of gelatinous attribute.

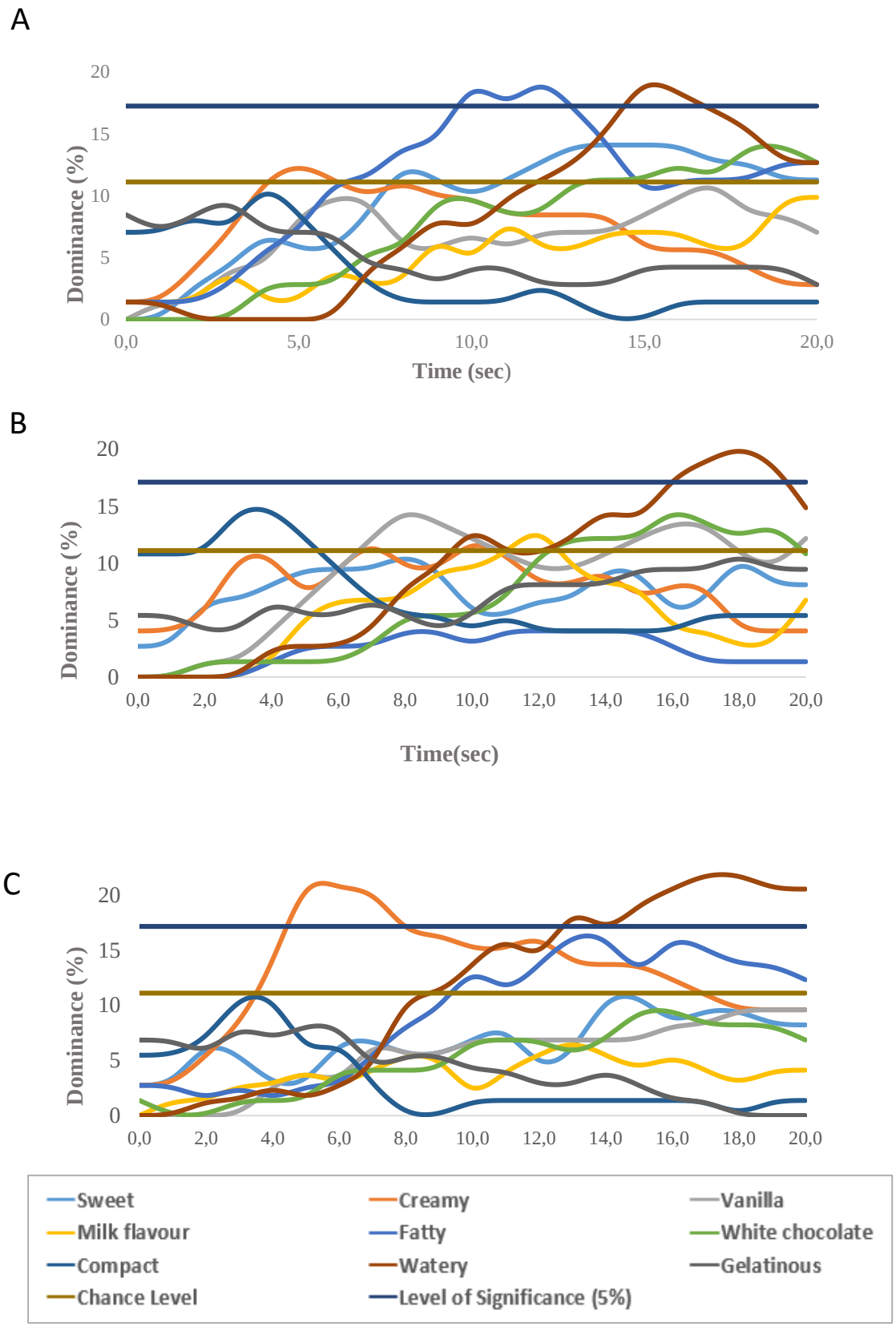
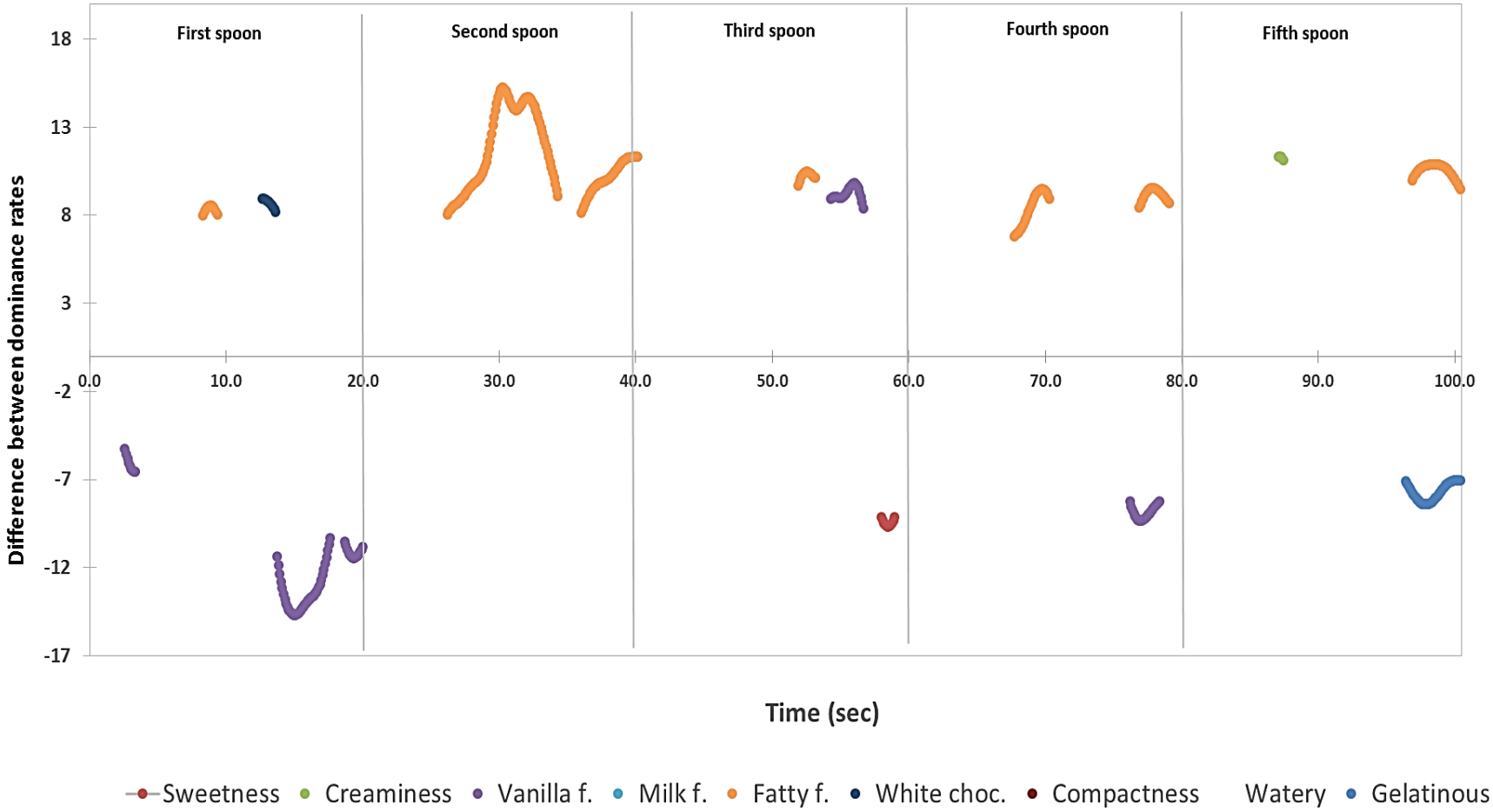


Figure 3. Curves of temporal dominance of 9 attributes lasting 20s each representing different spoons of each sample A) 2nd spoon of fat-enriched pudding B) 2nd spoon of control pudding C) 5th spoon of fat-enriched pudding. Chance and significance level marked as vertical lines.

A


First spoon
Second spoon
Third spoon
Fourth spoon
Fifth spoon
18
13
8
3
-2
-7
-12
-17
0.0
10.0
20.0
30.0
40.0
50.0
60.0
70.0
80.0
90.0
100.0
Difference between dominance rates
Time (sec)
Sweetness
Creaminess
Vanilla f.
Milk f.
Fatty f.
White choc.
Compactness
Watery
Gelatinous

B

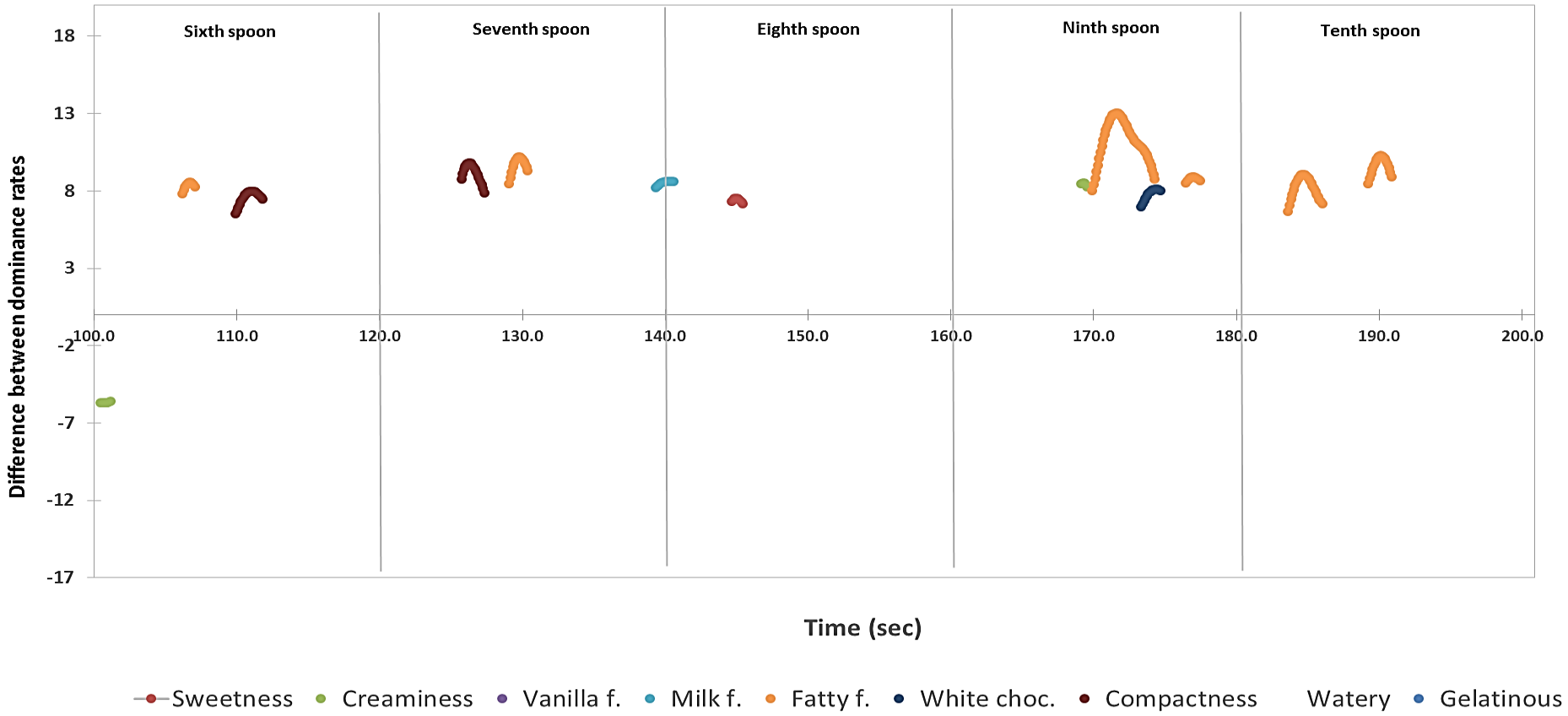


Figure 4. Difference between dominance rates of FEP (above X axis) and CP (below X axis) during 10 spoons of TDS evaluation. Panel A) represents results from the 1st to 5th spoon and Panel B) from the 6th to 10th spoon.

Discussion

Data from previous studies supported the hypothesis that cephalic-positive feedback mechanisms play a key role in the rewarding properties of fat-rich foods (Di Patrizio et al. 2011; Liang et al. 2006; and Kelley et al. 2004). However, they do not address the real, on-time response of ECs and NAEs to fat orosensory stimulation and latter effects on the eating behaviour. In the present study the individual response to fat taste was evaluated using a combined sensory and physiological approach. This allowed us to examine possible interconnection of fat taste perception with the physiological response and appetite. Modified Sham-Feeding (MSF) protocol coupled with a Temporal Dominance of Sensations (TDS) procedure was applied. MSF method, requiring from subjects food mastication without swallowing, allowed to observe effects on appetite sensations specifically linked to the orosensory properties of fat-enriched or control pudding by blunting homeostatic negative feedbacks of food intake (Di Marzo et al, 2008). Incorporation of TDS in the physiological assessment allowed us to evaluate possible dominance of fatty taste and its potential links with the salivary response of ECs and NAEs during mastication. The main finding of the study was that during 3 min of pudding mastication the levels of ECs and NAEs in individual saliva increased, which has not been shown in the literature before. Monteleone et al. (2012) focused only on the post-ingestive reaction and documented elevated levels in plasma 2-AG in eight satiated healthy subjects after *ad libitum* food intake of palatable but no effect after non-palatable food. Di Patrizio et al. (2011) have oriented its investigation particularly on fat palatability. They showed that 30 minutes of sham-feeding with a lipid-based meal stimulated endocannabinoid mobilization in the rat proximal small intestine by altering enzymatic activities that control endocannabinoid metabolism. However, this effect was not observed in other peripheral organs, including tongue tissue or neither assessed in non-invasive matrix as saliva. In the present study salivary ECs and NAEs increased in human saliva during 3 min of oral exposure to food with added dietary fat. Another interesting result of this study is that FEP elicited different responses of AEA, LEA, OEA and PEA compared to CP, independently of the pudding liking. No previous studies are present in the literature to compare this data. Only recent study by Mennella et al. (2015) has demonstrated the effect of sham-feeding palatable sweet pudding has resulted in significantly higher plasma 2-AG and PP levels compared to bitter, not- palatable pudding. Present study has therefore commenced possible future determination of physiological responses for differentiation between two equally palatable products with various fat contents. In this study the fat content differences in the products and the different salivary response between FEP and CP did not influence individual appetite response. Smeets et al (2006) had also investigated effect of oral fat stimulation on appetite rating. It has been noted that apart from the consumed meal, also sham-feeding of high-fat meal

significantly increased feelings of satiety comparing to water. Cephalic and probably vagal stimulation could increase the level of metabolites and induce satiety. Moreover, in another study from Smeets (2009), similar protocol was also applied, using MSF technique and comparing it with consumption and water in the condition of respiration chamber. They found that MSF of a high-fat meal caused an increased energy expenditure, increased insulin levels and increased satiety and fullness ratings comparing to the water consumption. The different results we found might be due to the different protocol used because in the present study subjects were in a fasting condition whereas in both studies from Smeets subjects after a high fat breakfast were involved in the MSF. Moreover, 19 participants took part in our study, the duration of MSF was 3 min and the fat content was of 2.6% while in the study by Smeets and colleagues (2009) there were 36 subjects, the MSF lasted 20 min and the fat-enriched meal provided 35% of fats from energy. Finally, present research has undertaken specific orosensory approach, without the analysis of metabolic hormones responses, known to impact appetite feeling. This information could be also plausible in confronting the findings of the present experiment. Specific TDS multi-sip technique (Pineau et al., 2009) adapted for MSF procedure gave an interesting insight into detailed, spoon by spoon, dominance of the fat-enriched and control pudding, revealing possible differences occurred during the time of mastication. Moreover, as TDS is focused on dominant attributes instead of quantifying attribute intensity, results from this methodology could better explain and more accurately identify the sensations that determine their hedonic perception (Cadena et al., 2014). The sensory approach for hedonic fatty taste perception has shown that it could be distinguished in the dynamic dominance evaluation, throughout time of the experiment. Subjects continuously perceived a significant difference in dominance of fatty taste during chewing the fat–enriched pudding compared to the control one. This sensation had a peak at 30 sec, where fatty sensation difference in FEP comparing to CP has reached 15.2 % of difference. This distinction of fat in product has been also shown in a TDS study from Laguna et al (2013) where dominance rate was shown upon the sensation of complete consumption, with no separation of bites. The study food were cookies, different in fat and fibre content (high-fat, low-fibre; low-fat, high-fibre). Fat presence was shown to be detectable and important for dominance of hedonic attributes important for palatability as crispness, which appeared with high-fat biscuits and crunchiness with low-fat high-fibre ones. In parallel to physiological differentiation shown by significantly different ECs and NAEs response to stimulus differing in fat content, statistical analysis of differences in dominance has revealed an interesting sensory distinction. Fatty taste has been significantly different in dominance in FEP throughout the time of the study when compared to CP. Additionally, creaminess and white chocolate flavour could be significantly distinguished during FEP mastication, possibly associated with the sensory

characteristic of fat. In control pudding no sensations other than vanilla and gelatinous being dominant only in the first and 4th spoon were significantly different.

Conclusions

Mounting evidence indicates a primary role of dietary fat intake and its metabolism on several human health issues. However, a lack of knowledge still exists on the mechanism behind fat perceptions and on its role in dietary behaviour. Previous research focused on separate aspects of fat perception and physiological mechanisms underpinning its intake, thus missing a holistic overview of the issue. Our study has undertaken a two-dimensional approach, aimed at providing physiological and sensory data, which would aid in understanding the human's orosensory response to this highly palatable nutrient. We could conclude that:

- Fat presence causes a rapid increase of ECs and NAEs levels already during food mastication in the mouth;
- Food with a higher fat content evokes a different physiological response than equally palatable control food. This could be a factor to consider in future studies on using physiological biomarkers in assessing the ability to detect of dietary fat in humans.
- A tendency to increased satiety and fullness and reduced hunger was found by mastication of fat-enriched vs control product.
- Fatty taste can be distinguished in dynamic sensory profile evaluation. Moreover it has an effect on products appearance, colour, consistency and aftertaste.

With the use of its dynamic sensory profile and evaluation of physiological response it evokes, this study could contribute to the design of new healthy food with the lowest fat content but preservation of its palatability and increased satiating characteristics compared to the normal food. Further studies should be designed to evaluate the effect of individual nutritional status and eating behaviour on the physiological and sensory responses to dietary fat tasting. Further research in this area is warranted to fully clarify the role of dietary fat in eating behaviour and food choice and to develop new healthy and palatable foods.

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Conclusions

The role of the GI tract as the largest endocrine organ and its secretion of several gut hormones such as ghrelin, cholecystokinin (CCK), peptide YY(PYY), and glucagon like peptide-1 (GLP-1) plays a vital role in maintaining energy balance and body weight regulation. Nowadays, adipose tissue is also a well recognize active endocrine organ secreting several bioactive molecules known as adipokines (Ouchi et al, 2011). This class of molecules comprise a large number of proinflammatory mediators, including tumor necrosis factor (TNF)-α, monocyte chemoattractant protein (MCP)-1, and interleukin (IL)-6, that promote disease progression.

Gut hormones and adipokines interact each other in the control of body weight. In particular, visceral fat accumulation causes chronic low-grade inflammation, which contributes to the initiation and progression of metabolic disorders (More et al, 2011). Chronic low-grade inflammation, caused by high constant release of pro-inflammatory cytokines, disrupt the gut brain axis signalling control of appetite and body weight. It is characterised by raised concentrations of inflammatory markers in the absence of any overt symptoms and is recognized as a risk factor for a number of chronic diseases including cancer, cardiovascular, cerebrovascular and neurodegenerative (Bonaccio et al, 2016). The absence of any definite symptoms make low-grade chronic inflammation underestimate for its incidence and dangerousness. Many studies suggest that low-grade inflammation is mitigated by health-promoting behaviours such as healthy eating patterns, physical activity, body weight maintenance and tobacco smoking cessation (Bonaccio et al, 2016).

Another player in the gut-brain axis (GBA) communication is the gut-microbiota. The entero-endocrine cells form a super-complex ecosystem with the gut microbiota establishing a permanent symbiotic relationship rather than a temporary form of parasitism (Petra 2015). The human intestine is the home for complex plethora of microbes ranging from 10^{13} to 10^{14} microbial cells (Lee 2014; Ghosh 2013). These gut flora have a wide metabolic activity associated with gut and can be truly termed as a virtual organ within an organ. In fact, it influences the secretion of gut hormones by enteroendocrine cells and is able to produce itself several neuropeptides (Oleskin et al, 2016). This relationship with our enteric cells contributes to basic physiological processes, including digestion, growth and self-defense. Recent evidence suggests that gut microbiota influence energy balance and weight (Murphy et al, 2010). Increased energy harvesting from diet, regulation of biologically active fatty acid tissue composition, chronic low-grade endotoxemia, and modulation of gut-derived peptide secretion are some of the proposed routes linking gut microbiota with obesity (Musso et al, 2010).

Chapter 6. Conclusion

In this scenario the experiments described in the present thesis investigated mechanisms involved in both gut and brain regulation of food intake. In the experiments described in the chapters 2 and 3 we tested in animals and in humans the hypothesis that dietary whole grains fibers are able to control body weight through a mechanism involving appetite control and the reduction of inflammatory status. In chapter 4 we described a method to mimic the whole grain slow release of antioxidant compounds using encapsulation. Finally, in chapter 5 we tested the hypothesis that the fat taste can influence the cephalic phase of the digestion.

Table 6.1 . Overview of the experiments reported in the thesis.

Chapter	*Objectives*	*Methods*	*Major findings*
2	to investigate the effect of a diet enriched in WG and/or RS on plasma levels of PC and TBW.	Four groups of ZDF rats were fed with semi-purified diets made with either an isolated digestible control starch, a WG control flour with 6.9% RS, an isolated RS-rich starch with 25% RS, or a WG corn flour with 25% RS. Plasma PC levels and TBW were measured.	WG consumption increased plasma PC levels. Combination of WG plus RS resulted in a higher bioavailability of PC compared to WG alone. No significant effect on TBW was found.
3	to assess circulating concentration, excretion, and the physiologic role of WG wheat polyphenols in subjects with suboptimal dietary and lifestyle behaviors.	A placebo-controlled, parallel-group randomized trial with 80 healthy overweight/obese subjects with low intake of fruits and vegetables and sedentary lifestyle was performed. Participants replaced precise portions of RW with a fixed amount of selected WG wheat or RW products for 8 wk. At baseline and every 4 wk, blood, urine, feces, and anthropometric and body composition measures were collected. Profiles of phenolic acids in biological samples, plasma markers of metabolic disease and inflammation, and fecal microbiota composition were assessed.	WG consumption determined higher urinary and fecal FA and plasmatic FA and DHFA concentration compared to RW. Concomitant reduction of plasma TNF-α and increased interleukin (IL)-10 after WG compared with RW were observed. Fecal FA was associated with baseline low Bifidobacteriales and Bacteroidetes abundances, whereas after WG consumption, it correlated with increased Bacteroidetes and Firmicutes but reduced Clostridium.TNF-a reduction correlated with increased Bacteroides and Lactobacillus. No effect of dietary interventions on anthropometry and body composition was found.
4	Human bioavailability of curcumin from breads enriched with 1 g/portion of free curcumin, encapsulated curcumin, or encapsulated curcumin plus other polyphenols was evaluated.	Ten healthy subjects were enrolled to perform a randomized controlled crossover study. Parental and metabolized curcuminoids and phenolic acids were quantified by HPLC/MS/MS in blood, urine, and feces collected over 24 h.	Encapsulation delayed and increased curcuminoid absorption as compared to the free ingredient. Serum and urinary concentrations of ferulic and vanillic acid were between 2- and 1000-fold higher than those of free curcuminoids. Fecal curcuminoids were 6-fold more abundant after encapsulated curcumin than free curcumin, while phenolic acids after encapsulated curcumin plus other polyphenols quadruplicated those after free curcuminoids. Curcuminoid encapsulation increased their bioavailability from enriched bread, probably preventing their biotransformation.
5	to evaluate human physiological response and the sensory perception to fat taste, the associated palatability and the influence on individual appetite sensations.	Fat-enriched and a control pudding were developed to be used in a randomized controlled crossover human study. The cephalic response to the fat stimuli of salivary endocannabinoids and N-acylethanolamines concentrations by MSF was measured. The sensory approach focused on the profile of sensory perceptions upon food mastication using TDS technique.	Fat presence causes a rapid increase of ECs and NAEs levels already during food mastication in the mouth. AEA (but not LEA or PEA) response upon mastication of fat-enriched product tends to be higher in over-weight than normal-weight subjects. A tendency to increased satiety and fullness and reduced hunger was found by mastication of fat-enriched vs control product.

List of abbreviation. WG: whole grains; RS: resistant starch; PC: phenolic compounds; TBW: total body weight; ZDF: zucker diabetic fatty; RW: refined wheat; FA: ferulic acid; DHFA: dihydroferulic acid; TNF-α: Tumor necrosis factor-α; MSF: modified sham feeding; TDS: Temporal Dominance of Sensations; EC: endocannabinoid; NAE: N-Acylethanolamine; AEA: arachidonoyl ethanolamide; LEA: linoleoyl ethanolamide; PEA palmitoyl ethanolamide.

As already described in the introduction of the present thesis, the role of GBA on the digestive function and appetite control was extensively studied. Since its complexity, it appears clear that a minimum disturbance of the GBA communication can lead to lose the control in the homeostatic and hedonic mechanisms that regulate energy balance and consequently to overweight and subsequently to obesity. GBA is now considered a bidirectional system that uses 4 major information carriers, closely interrelated with each other, for the communication: neural messages, endocrine messages carried by gut hormones, immune messages carried by cytokines and microbial factors that may directly reach the brain via the blood stream but can also interact with the other 3 transmission pathways (Holzer 2014).

From the studies described in the present thesis, it can be concluded that:

1. The consumption of WG in substitution of refined cereals reduce subclinical inflammation and this effect is strengthen when WG are combined with prebiotic fibre.
   In fact, together the consumption of WG resistant starch is able to modulate two main components of the GBA, helping to restabilising an healthier physiological condition: cytokines and gut-microbiota. This effect is mediated by the slow release of bound phenolic compounds which are released from the fibre matrix during the gut microbiota fermentation. Therefore, the addition of a prebiotic fibre able to stimulate the growth and the activity of the gut microbiota increase the release of phenols from WG cereals.

2. Novel ingredients can be designed using encapsulation to obtain a slow release of antioxidant compounds and increase their bioavailability. In fact, a key lesson from the study of phenolic compounds from WG is that the kinetic of their absorption follows a slow but constant pattern: this guarantee a stable antioxidant protection in the bloodstream. In the present thesis, we used curcuminoids as a natural antioxidant -ingredient well-know for their healthy properties and low-grade bioavailability. The same approach can be used for many others antioxidant compounds.

3. Food consumption can modulate the endocannabinoids system which in turn influence eating behaviour.
   During the cephalic phase of the digestion, when food is chewed and even before swallowing, a variation of the ECs in saliva and plasma already appear. This evidence suggest a main role of cephalic phase in the digestive processes related to meal initiation and meal termination.

Further long term studies should investigate if the reduction of low-grade chronic inflammation, which is associated with a disturbance of the GBA, can be also associated with an improvement of the sensitivity of the satiety signals that are lost in a subclinical inflammation condition. This can help the individual body weight management over a long term period. Moreover, the role of endocannabinoids and related species in appetite control through their action in the GBA communication should be extensively investigated together with the food source and food matrix effects on the ECs system.

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Summary

The physiological systems underlying the appetite control involve associations between peripheral physiology and metabolism (glucose homeostasis and adiposity), which in turn are linked to various brain processes. All hormonal messengers released from enteroendocrine cells in the gut mucosa can inform the brain either through the circulation or via primary afferent neurons or both. Gut hormones and adipokines interact each other in the control of body weight. In particular, visceral fat accumulation causes chronic low-grade inflammation, which contributes to the initiation and progression of metabolic disorders. Chronic low-grade inflammation, caused by the constant higher release of pro-inflammatory adipokines from adipose tissue, disrupts the gut hormones signalling at central and peripheral levels in the control of appetite and body weight.

In this scenario the experiments described in the present thesis investigated mechanisms involved in both gut and brain regulation of food intake. In the experiments described in the chapters 2 and 3 we tested in animals and in humans the hypothesis that dietary whole grains (WG) fibers are able to control body weight through a mechanism involving appetite control and the reduction of infiammatory status. In chapter 4 we described a method to mimic the WG slow release of antioxidant compounds using encapsulation. Finally, in chapter 5 we tested the hypothesis that the fat taste can influence the cephalic phase of the digestion.

From the studies described in the present thesis, it can be concluded that:

1. The consumption of WG in substitution of refined cereals reduce subclinical inflammation and this effect is strengthen when WG are combined with prebiotic fibre. In fact, together the consumption of WG resistant starch is able to modulate two main components of the GBA, helping to restabilising an healthier physiological condition: cytokines and gut-microbiota. This effect is mediated by the slow release of bound phenolic compounds which are released from the fibre matrix during the gut microbiota fermentation. Therefore, the addition of a prebiotic fibre able to stimulate the growth and the activity of the gut microbiota increases the release of phenols from WG cereals.

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Sommario

Il comportamento alimentare è modulato da mediatori chimici che, agendo sull'asse intestino-cervello, attraverso meccanismi omeostatici e non omeostatici (sistema di ricompensa) regolano l'appetito nel breve termine e il peso corporeo nel lungo termine. I peptidi rilasciati dalle cellule enteroendocrine nell'intestino, comunicano con le aree del cervello deputate al controllo della fame e della sazietà sia attraverso il sistema circolatorio, sia attraverso il sistema nervoso. Gli ormoni gastrointestinali interagiscono con le adipochine nel controllo del peso corporeo. In particolare, l'accumulo di grasso viscerale causa infiammazione cronica che contribuisce alla generazione e all'avanzamento di disordini metabolici. L'infiammazione cronica, causata dal costante rilascio di citochine pro-infiammatorie dal tessuto adiposo, altera la comunicazione dell'asse intestino-cervello e pertanto danneggia la capacità dell'organismo di controllare il peso corporeo.
In questo scenario, gli esperimenti descritti nella presente tesi hanno studiato i meccanismi dell'asse intestino cervello coinvolti nella regolazione degli introiti alimentari. I capitoli 2 e 3 sono stati riportati due studi in cui è stata testata, su animale e sull'uomo rispettivamente, l'ipotesi che cereali whole grains (WG) siano in grado di mediare il controllo del peso corporeo attraverso un meccanismo che coinvolge la riduzione dell'appetito e dello stato infiammatorio sub-clinico. Nel capitolo 4 è stato descritto un metodo che imita il lento, ma costante rilascio degli antiossidanti da cereali whole grain (WG) realizzato utilizzando un sistema di micro-incapsulazione. Infine, nel capitolo 5 è stata testata l'ipotesi che il "gusto grasso" influenzi la fase cefalica della digestione.

In sintesi dagli studi descritti nella presente tesi può essere concluso che:

1. Il consumo di cereali WG in sostituzione di cereali raffinati riduce l'infiammazione sub-clinica e questo effetto è rafforzato quando i WG sono consumati in combinazione di una fibra prebiotica. Infatti, il consumo di amido resistente insieme con WG è in grado di modulare due componenti principali dell'asse intestino cervello: le citochine e il microbiota intestinale. Questo effetto è mediato dal lento rilascio di composti fenolici, che sono liberati dalla fibra durante la digestione del microbiota. Quindi, l'aggiunta di una fibra prebiotica, in grado di stimolare la crescita e l'attività del microbiota intestinale, aumenta il rilascio di acidi fenolici da cereali WG.
2. Possono essere realizzati nuovi ingredienti funzionali mediante l'incapsulazione, per ottenere un lento rilascio di composti antiossidanti ed aumentare la loro biodisponibilità. La cinetica di assorbimento rappresenta il punto chiave del rilascio degli antiossidanti da WG: questa è caratterizzata da un flusso lento, ma costante che garantisce una protezione antiossidante stabile nel circolo sanguigno. Nella presente tesi, è stata utilizzata curcumina come esempio di composto antiossidante naturale, ben nota per le sue proprietà salutistiche e per la sua scarsa biodisponibilità. Lo stesso approccio potrebbe essere utilizzato per altri composti antiossidanti.
3. Il consumo di alimenti modula il sistema degli endocannabinoidi (ECs) che a sua volta influenza il comportamento alimentare. Durante la fase cefalica della digestione ed in particolare durante la masticazione già vi è un aumento degli ECs salivari. Questa evidenza suggerisce un ruolo primario della fase cefalica nei meccanismi di fame e sazietà.