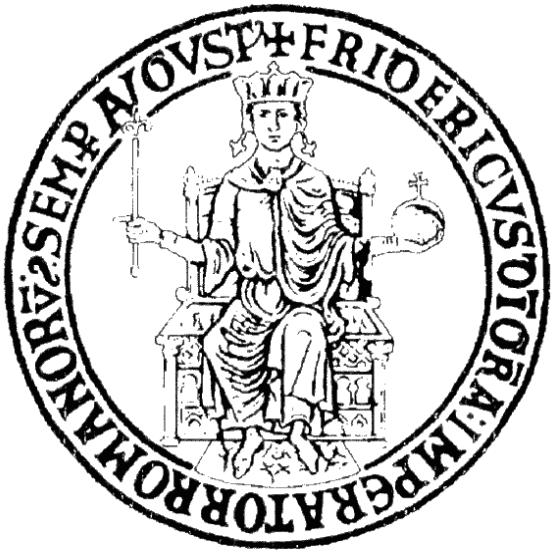

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Influence of dietary fibre-enriched foods and of blueberry-rich diet on saliva composition in humans

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to my beloved parents

谨以此论文献给我的父亲母亲

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Abstract

The main objective of the thesis is to explore the possible "cross-talk" between the brain, intestine and specific foods components (i.e. dietary fibres and blueberry polyphenols). This objective will be achieved through a short-term study and a long-term intervention in humans, with the non-invasive analysis of saliva samples and breath compositions.

The short-term study aimed at evaluating the impact of dietary fibres on salivary NAEs during mastication and in the post-prandial phase. Three types of biscuits enriched with 3% barley β-glucan (βGB) or whole-wheat bran (WWBB) or without dietary fibre (control, CB) were developed. A crossover randomized human study with eighteen healthy and fasting participants was carried out. Saliva samples from subjects in a resting condition, upon mastication of parafilm and one of the three biscuits were collected. Subsequently, the amount of biscuits consumed in an ad-libitum breakfast was measured and post-prandial saliva samples, blood glucose, and questionnaires of appetite and food liking were collected over the following two hours. Data demonstrated that salivary NAEs concentration increased only upon food mastication instead of mastication itself, independently from dietary fibre composition of the food. The type of biscuits did not influence individual appetite nor post-prandial blood glucose; on the contrary it influenced the persistence of NAEs in saliva over 30 min after consumption. Future studies will clarify the mechanisms behind this finding and the role of salivary NAEs in food liking and appetite cues after food consumption.

The aim of long-term intervention study was to test the influence of a one week-consumption of blueberries on the volatile organic compounds (VOCs) of breath and saliva in humans. Fourteen healthy volunteers participated in this three-week single blind study with a two-week cross over design. After a one-week of baseline period with a low polyphenol-diet (BL), subjects were grouped randomly to continue the same diet (control diet, CT) or to add 200 g/day of fresh blueberries (intervention diet, INT) for one week. In the following week they switched to the other arm. At the end of each week, fasting subjects reached the laboratory to collect saliva samples and to have on-line analysis of breath by PTR-ToF-MS. After INT and CT, difference was found neither in VOC fingerprints nor in single VOC of breath and saliva samples. Nevertheless, a significant correlation was shown between saliva and breath composition for methanol, formaldehyde, ethanol, acetone and propanol. Numerous previous studies focused on VOCs composition of breath of patients and of healthy subjects, but very few studies focused on salivary VOCs. Therefore the link found in this study between saliva

and breath VOCs may open a new research path to clarify the mechanisms behind the metabolic effect of the dietary intervention in humans.

Chapter 1. General introduction

1. Brain-gut axis: gastrointestinal metabolism and reward system in appetite control

Clinicians and researchers have long recognized the link between gastrointestinal function and the central nervous system (CNS) (Bercik et al., 2012). Since the original description of a gut-brain axis related to the modulation of cholecystokinin secretion by bombesin (Banks, 1980), the concept actually refers to any interaction between the gastrointestinal (GI) tract and the CNS.

The 'gut-brain' or 'brain-gut' axis refers to a bidirectional communication system between the gut and the brain, depending on whether we emphasize bottom-up or top-bottom pathways. It is comprised of humoral pathways, which include immune mediators such as cytokines, gut hormones, and neuropeptides as signalling molecules, and neural pathways, such as the enteric nervous system (ENS), vagus, sympathetic and spinal nerves (Bercik et al., 2012; Holzer et al., 2012). These mediators transmit information from the gut to the brain, while autonomic neurons and neuroendocrine factors carry outputs from the brain to the gut.

The GI tract is a highly specialized sensory organ and represents the first site of interaction between ingested nutrients and the host, initiating crucial negative feedback systems aimed at controlling food intake and regulating energy balance partly via a gut–brain axis (Figure 1) (Duca et al., 2012; Sam et al., 2012). Specifically, enteroendocrine cells are located throughout the GI tract, where they can sense and respond to specific nutrients, releasing gut peptides that act in a paracrine, autocrine or endocrine fashion to regulate energy balance, thus controlling both food intake and possibly energy expenditure. For example, the release of gut hormones such as PYY, GLP-1, and oxyntomodulin (OXM) is stimulated by distension of the stomach and interactions between nutrients and the luminal wall of the intestine(Adrian et al., 1985; Sam et al., 2012).

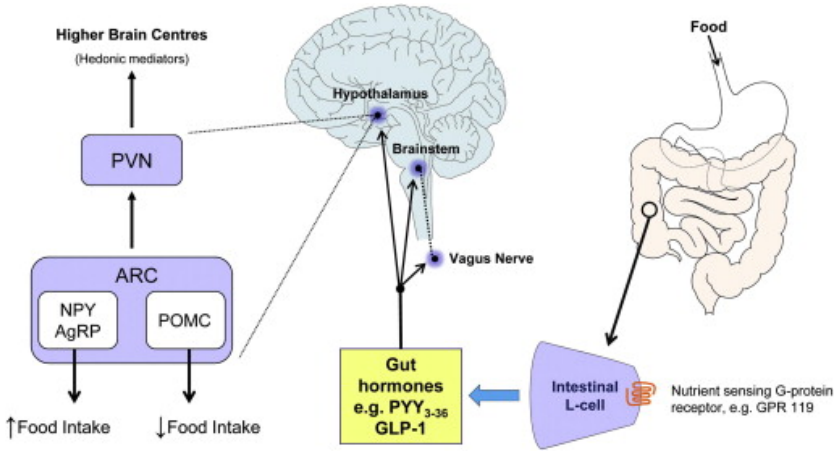

Figure 1. Gut-brain axis: modulation of food ingestion. Nutrients deriving from the food digestion are suggested to activate G-protein coupled receptors on the intestinal enteroendocrine cells. This triggers the release of gut hormones and regulate energy intake at three sites, including brainstem, hypothalamus and the vagus nerve. Within the hypothalamus arcuate nucleus, the orexigenic neuropeptide Y/ agouti related peptide (NPY/AgRP) neurons and the anorexigenic propiomelanocortin (POMC) neurons are taken as critical conduits where peripheral signals are collected to change the drive to eat. Further links between hypothalamic nuclei and higher brain centres may regulate the hedonic aspects of food ingestion. Other abbreviations: arcuate nucleus (ARC), glucagon like peptide-1 (GLP-1), para-ventricular nucleus (PVN) and peptide YY (PYY) (Sam et al., 2012).

It is believed that gut hormones contribute to the short-term feelings of satiety and hunger (Badman and Flier, 2005; Sam et al., 2012). They may reduce food intake by decreasing hypothalamic orexigenic signalling, and increasing anorectic signalling (Batterham et al., 2003; Jobst et al., 2004; Sam et al., 2012). Another effect of these peptides is to mediate inhibitory feedback mechanisms on intestinal transit, contributing to prolonged gastric distension, and increased satiety between meals. Therefore, the combined CNS effects and 'intestinal brake' mechanisms mediated by the gut peptides cholecystokinin (CCK), peptide YY (PYY) and oxyntomodulin (OXM) can facilitate the control of food intake and post-prandial transit through the GI tract (Lin et al., 1996; Sam et al., 2012; Wen et al., 1995).

Meanwhile, general visceral afferent signals in the vagus excite neurons that receive a host of gastrointestinal (GI) mechano- and chemosensory information (Travagli and Rogers, 2001) . The vascular supply of nerves is composed of fenestrated capillaries, thus large blood-borne proteins and peptides (e.g. hormones, serum albumin, IgG, the complement system) have direct access to the neural pathways (Broadwell and Sofroniew, 1993; Rogers et al., 1996; Travagli and Rogers, 2001).

Although gut hormones can modulate GI function by acting on vagal afferents (Travagli and Rogers, 2001), physiological studies of the central nervous system (CNS) effects of insulin,

pancreatic polypeptide (PP), and peptide YY (PYY) suggest that gut hormones can also exert control over digestion by acting directly on neurons (Rogers et al., 1996). For instance, PP released exclusively from specialized pancreatic islet cells after meals can regulate pancreatic secretion and gastric motility by acting directly on neurons to control vagal efferent outflow to the viscera (Rogers et al., 1996). As it happens, "hormones" produced by the immune system can also dramatically affect the function of neurons that comprise the gastric vagovagal reflex control circuit (Travagli and Rogers, 2001).

Moreover, recent evidence suggests that the gut microbiota, strongly participate in the gut-brain communication, playing a key role in host physiology and metabolism, and controlling energy balance, at both the level of intestinal nutrient-sensing mechanisms, and, potentially, at the sites of integration in the central nervous system (CNS) (Duca and Lam, 2014). The intestinal microbiota consists of a community of bacteria that colonize the gastrointestinal tract after birth and persist throughout the adult life along with 'transient' bacteria, such as probiotic bacteria, which are temporarily acquired during ingestion of certain foods. The composition of the intestinal microbiota is established during the first few years of life and is likely shaped by multiple factors including maternal vertical transmission, the genetic make-up of the individual, diet, medications including antibiotics, gastrointestinal infections and stress (**Figure 2**) (Dumas et al., 2006; Gronlund et al., 1999; Lewis and Cochrane, 2007; Zoetendal et al., 1998). Indeed, a well-balanced gut microbiota composition is fundamental for individual health status and well-being (Etxeberria et al., 2013a). In addition, to the known effects of intestinal microbiota and specific probiotics on mucosal and epithelial barrier function, there is experimental evidence to support the effects of microbes on muscle function (Banks, 1980; Eckburg et al., 2005; Hooper and Macpherson, 2010; Mayer, 2011; O'Mahony et al., 2011) and ENS function (Backhed, 2005; Hooper and Gordon, 2001; Husebye et al., 2001; Verdu and Collins, 2004). The information is relayed to the CNS via the neural pathway (Dumas et al., 2006; Gronlund et al., 1999; Zoetendal et al., 1998)or humoral pathways (Hooper and Gordon, 2001).

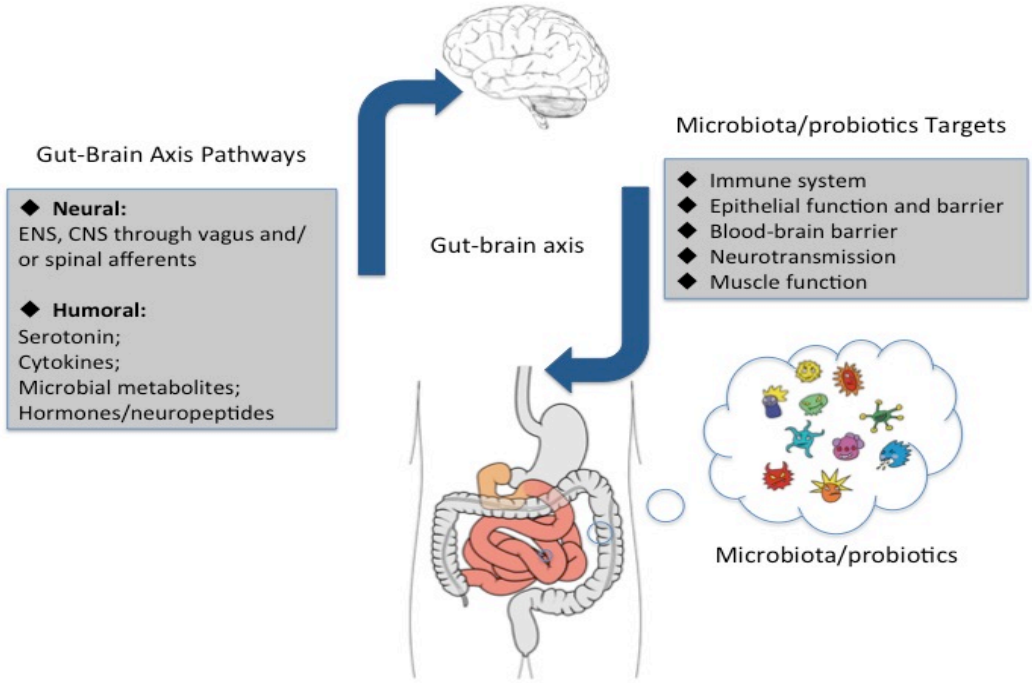

Figure 2. Communication pathways and microbiota targets upon gut-brain axis (Bercik et al., 2012).

Recent evidence, mainly arising from animal models, supports the idea that microbes in the GI tract use signalling components in the gut-brain axis to manipulate host's eating behaviour and to increase their fitness, sometimes at the expense of the host's fitness. Microbes may do this through two potential strategies (Alcock et al., 2014):

(i) Inducing cravings for foods which are utilised for enhanced colony health or, conversely, foods that suppress their competitors;

(ii) Generating dysphoria until people intake foods to improve their fitness.

Potential mechanisms for microbial control upon eating behaviour (**Figure 3**) includes: (1) microbial influence on reward and satiety pathways; (2) production of toxins that alter mood; (3) changes to receptors including taste receptors; (4) and hijacking of the vagus nerve, the neural axis between the gut and the brain (Alcock et al., 2014).

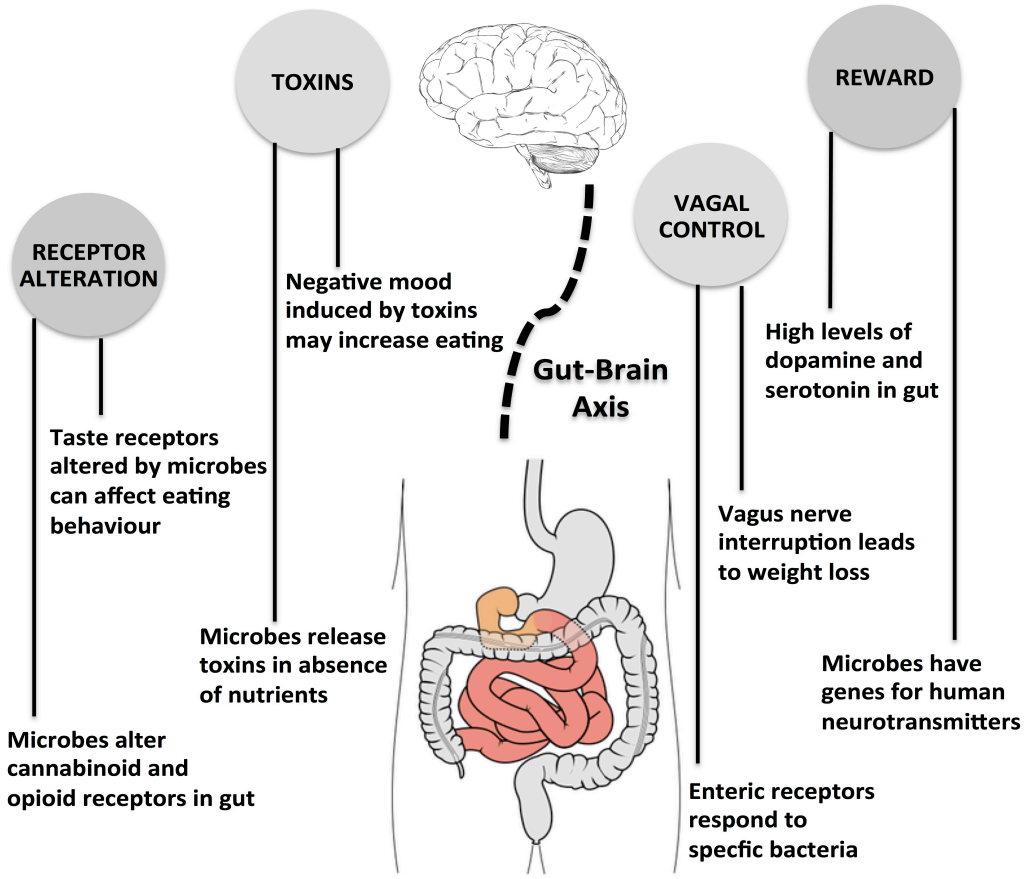

Figure 3. Potential mechanisms: the pathways of which gut microbes may manipulate hosts' eating behaviour (Alcock et al., 2014; Amaral et al., 2008; Baraldi et al., 2009; Camilleri et al., 2008; Chen et al., 2011; Chiu et al., 2013; Clarke et al., 2014; Duca et al., 2012; Eisenhofer et al., 1997; Hill et al., 1991; Kim and Camilleri, 2000; Kortman et al., 2012; Medzhitov et al., 2012; Miras and le Roux, 2013; Njoroge and Sperandio, 2012; Njoroge et al., 2012; Raybould, 2010; Roth et al., 1985; Rousseaux et al., 2007; Sarr et al., 2012).

Mounting evidences indicate that microbiota composition is modulated by prebiotics, probiotics, antibiotics, faecal transplants, and dietary changes. So, altering our microbiota offers a fine approach to counteract obesity and unhealthy eating behaviour (Alcock et al., 2014). Therefore, a better understanding of the intricate host energy-regulating mechanisms working in the short-term and long-term period, is crucial for uncovering the possibilities responsible for appetite and body weight control and for potential therapeutic strategies of dietary intervention upon GI tract metabolism.

2. Biological markers of gut metabolism and reward

The gastrointestinal tract releases more than 20 different regulatory peptides working as hormones and modulating many physiological processes underpinning food intake (Sam et al., 2012). These peptide are considered as biological markers of gut metabolism and reward system. Among them the most studied molecules are ghrelin, CCK, PYY3-36 and OXM:

(i) Ghrelin is secreted from the stomach and is the only orexigenic gut hormone (Bewick et al., 2005; Sam et al., 2012). It can increase food intake as well as weight gain in rodents following administration in peripheral and in central (Lawrence et al., 2002; Tschop et al., 2000; Wren et al., 2000);

(ii) CCK is a hormone produced by mucosal endocrine cells in the upper small intestine (Murphy and Bloom, 2006; Small and Bloom, 2004). It is released post-prandial in response to saturated fats, long chain fatty acids, amino acids and small peptides that would normally result from protein digestion (Liddle et al., 1985; Rehfeld et al., 2003). Data suggest that CCK reduces food intake and increases the perception of fullness and it may play a role in acute rather than long-term energy homeostasis (Lieverse et al., 1995; Sam et al., 2012);

(iii) PYY_{3-36}, an endogenous form of another gut peptide PYY, has anorectic effects on satiety and central control of appetite and inhibits food intake in humans (Batterham et al., 2003).

(iv) OXM is also a product of post-translational processing of preproglucagon in the intestine and the CNS (Holst, 1997; Small and Bloom, 2004). In addition, OXM delays gastric emptying and decreases gastric acid secretion (Schjoldager et al., 1989), and reduces food intake (Cohen et al., 2003; Sam et al., 2012).

A number of other gut-derived peptides have been found to control food intake, however, the physiological role of them in the regulation of food intake and energy homeostasis still remains unclear.

Besides gut peptides, another class of compounds that was recently shown to influence food intake is that of endocannobinoids (eCBs) and endocannabinoid-like (eCB-like) compounds N-acylethanolamines (NAEs). ECs are endogenous compounds that bind to the cannabinoid receptors CB1 and CB2. The major and most studied endocannabinoids are anandamide (N-arachidonoylethanolamine, AEA) and 2-arachidonoylglycerol (2-AG), which are widely expressed in human and mammalian tissues. They control energy balance (i.e. pancreas, muscle, gut, adipose tissue, liver, and hypothalamus) and have a broad range of physiological effects, including regulating feeding behaviours and metabolism (Geurts et al., 2014; Kleberg et al., 2014; Matias and Di Marzo, 2007). The role of the eCB system in normal conditions is to facilitate energy intake and storage, which can promote obesity in pathological situations (Geurts et al., 2014; Pagotto et al., 2006). Together with other pharmacological effects, such as relaxation and analgesia, the eCBs intersect with many areas of biochemical and medicinal

research, in particular for obesity, pain and drug addiction (Kleberg et al., 2014; Makriyannis et al., 2005).

Besides those 'real' eCBs, some other endogenous NAEs, such as oleoylethanolamide (OEA), palmitoylethanolamide (PEA), and linoleoylethanolamide (LEA), are widely distributed in animals, but the concentrations differ between tissues and species (Hansen, 2013; Kleberg et al., 2014). The structural resemblance of the NAEs to the eCBs makes them players in the eCB system, where they can interfere with the actions of the true eCBs. Structural analogues in several cases engage the same synthesizing and degrading enzymes and indirectly interfere with the eCB system, through an entourage effect. Moreover, NAEs have pharmacological actions through noncannabinoid receptors, which are particularly interesting in a nutritional and metabolic context. At present, NAEs are generally believed to have a signalling function in the small intestine, where they have been associated with a regulatory role in the control of food intake linking intestinal responses to nutrients with the appetite centre of the brain; a process mediated via activation of the transcription factor peroxisome proliferator activated receptor alpha (PPARα) (Artmann et al., 2008; Diep et al., 2011; Fu et al., 2003; Lo Verme et al., 2005). Additionally, NAEs are also activators of other receptors, for example, GPR119 (Kleberg et al., 2014; Overton et al., 2006), a receptor found in both the intestine and pancreas (Kleberg et al., 2014; Odori et al., 2013), as well as GPR55, and the vanilloid receptor (Geurts et al., 2014; Ho et al., 2008; Hoareau et al., 2009; Kleberg et al., 2014; Muccioli et al., 2010; Ryberg et al., 2007). Furthermore, the concentrations of eCBs and NAEs in plasma could be impacted by food palatability during the cephalic phase response (CPR), thus 2-AG and pancreatic polypeptide can be taken as biomarkers of food liking (one type of the food reward in eating behaviour) (Mennella et al., 2015a). In general, eCBs and NAEs are associated with food wanting (referring to appetite, i.e. the disposition to eat) and food liking (referring to palatability, i.e. the pleasure derived from eating a provided food) (Havermans et al., 2009). It is worth of note that chewing behaviour is linked to food liking, therefore oral processing can affect the sensory sensations in humans (Jeltema et al., 2015). For instance, mastication could enhance specific sensations through changing the intensity of flavours released from foods (de Wijk et al., 2006; de Wijk et al., 2008). However, very few investigations have been done analysing the impacts of dietary fibre on mastication and post-prandial salivary response, which may correlate to both food liking and appetite.

In addition to recent data showing the impact of the eCB system on gut barrier regulation, numerous reports have demonstrated that the altered eCB system homeostasis observed in the

gut and adipose tissue may be directly associated with specific changes in the composition of the gut microbiota (Bluher et al., 2006; Geurts et al., 2014; Muccioli et al., 2010). For instance, it was recently suggested that the regulation of the gut microbiota or the blocking of CB1 with an antagonist, impacted the gut barrier integrity and could reduce low-grade inflammation (Muccioli et al., 2010).

Certain gas species are produced during the lifetime of the microbiome as by-products of their metabolic activities, as shown in figure 4 (Audrain et al., 2015; Nicholson et al., 2012)(Audrain et al., 2015; Nicholson et al., 2012). It is becoming increasingly understood that these gas species affect both directly and indirectly the status of the gut and subsequently the health of the human. These gas species may be used as markers to assess gastrointestinal functions and the treatment of gastrointestinal-related diseases as well as many other illnesses that are affected by gut states (Carbonero et al., 2012; Ou et al., 2015). Consequently, examining the metabolites of gut microbiota, such as volatile organic compounds (VOCs), could be considered as non-invasive options for assessing the modulations of gut microbiome and arose many interests in medical and health researches.

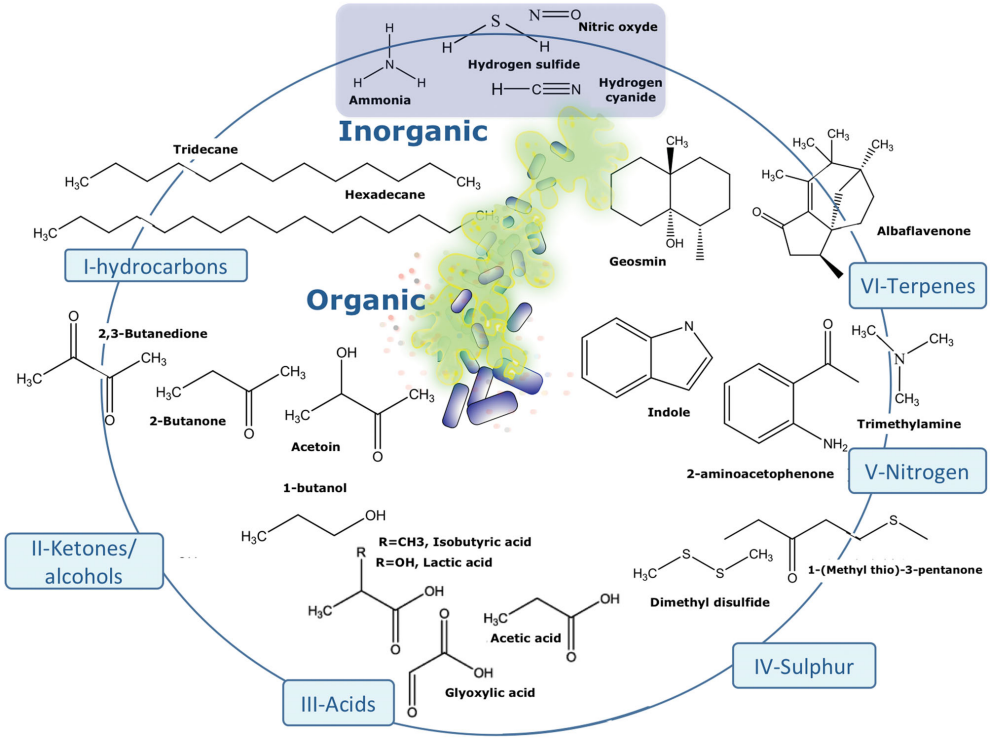

Figure 4. The volatile organic compounds (VOCs) released by bacteria fit six chemical classes, including hydrocarbons (I), ketones/alcohols (II), acids (III), sulfur compounds (IV), nitrogen-containing compounds (V) and terpenes (VI). Inorganic compounds are grouped in the grey box at the top of the diagram. Synthetic representation of VOCs could impact bacterial behaviour (Audrain et al., 2015).

Many microorganisms in the GI tract satisfy their energy needs predominantly through fermentation of undigested carbohydrates (e.g. dietary fibres, polyphenols and melanoidins), with the subsequent production of short-chain fatty acids and certain gas species including carbon dioxide, hydrogen, and methane (Nicholson et al., 2012; Tagliazucchi and Bellesia, 2015; Vitaglione et al., 2012). Aromatic, nitrogen-containing, and other VOCs, such as methanethiol and dimethyl sulfide, are also generated by certain microorganisms in the gut, deriving from the fermentation of peptides and amino acids (Smith and Macfarlane; Thorn and Greenman, 2012). A proportion of the gas produced from microbial fermentation is absorbed into the systemic blood circulation and eventually excreted by the lungs (Shin, 2014). The intestinal gas concentrations in the excreted breath are low and depend on many physiological parameters, resulting in inconsistencies between the detected composition of breath gases and those generated in the gastrointestinal tract (Ou et al., 2015; Sahakian et al., 2010).

In fact besides breath, a new approach may be to follow the metabolism of the intestinal microbiota by salivary VOCs analysis. Human saliva is a clear, slightly acidic (pH = 6.0 - 7.0) biological fluid containing a mixture of secretions from multiple salivary glands, including the parotid, submandibular, sublingual and other minor glands beneath the oral mucosa as well as gingival crevice fluid (Spielmann and Wong, 2011). Like blood, saliva is a complex fluid, containing approximately 99% water with minerals, nucleic acids, electrolytes, mucus and proteins such as enzymes, cytokines, immunoglobulins, mucins and other glycoproteins (De Almeida et al., 2008; Rehak et al., 2000; Zelles et al., 1995). These salivary constituents can be broadly classified into two groups: (1) constituents that enter saliva from the plasma and (2) constituents that are produced locally by the salivary glands. Reliable measurement of blood-borne constituents assumes a constant saliva/plasma ratio (SPR), which implies that the concentration in saliva truthfully follows intra- and inter-individual variations in plasma (Bosch, 2014). As one of the most complex, versatile, and important body fluids, it supplies a wide range of physiological needs and it is also called the “mirror of the body” or “a window on health status” (Wang et al., 2014).

3. Foods in gut-brain axis mechanisms: the effect of dietary fibre and polyphenols

Visceral information and gustatory could be taken as chemical senses during a meal (i.e. in short term). Thus they are cues to recognize food intake and to determine the nutrients stimulation on sensory organs. On one hand, the brain realizes food ingestion and predicts efficient digestion, to regulate each nutrient concentration in blood and in brain as well as to

maintain their levels within normal limits. Moreover, visceral senses for each nutrient from the digested products in the alimentary tract can also be used as intake signals of nutrients to notify the brain through inner body sensory organs (Nakamura et al. 2013; Torii et al., 2013).

On the other hand, changes of glucose and free fatty acid concentration in the blood and related hormones secreted from specific endocrine tissues can also primarily be monitored by chemical senses (i.e. stimulation by nutrients) for the control of appetite and satiety as a major marker for energy balance in the body (**Table 1**, Orr and Davy, 2005). For example, metabolism of polysaccharides results in the production of short-chain fatty acids, such as butyrate and propionate, which provide an important source of nutrients to the gut microbiota as well as regulatory control of the host digestive system. This impact upon host metabolism is also seen in the ability of the prebiotic inulin to influence production of relevant hormones such as glucagon-like peptide-1, peptide YY, ghrelin, and leptin (Di Mauro et al., 2013). However, these chemosensory mechanisms remain poorly understood. Previous studies suggested that taste signalling mechanisms known from the oral epithelium also operate in the mucosal epithelium. Several nutrient-responsive G-protein coupled receptors (GPCRs) have been identified in enteroendocrine cells (EEC) including the sweet-taste responsive T1R2/T1R3 heterodimer or GPR120, responsive to free fatty acids (FFAs) (Steinert and Beglinger, 2011).

Table 1. Peripheral hormones regulating energy intake, impact on energy intake, and influence of diet.

Hormone	Predominant secretion site	Effect on energy intake	Impact of dietary macronutrient composition
Ghrelin	Stomach	Increase	Decrease with fibre (psyllium) supplement [a] Decrease with dietary carbohydrate consumption [b]
Peptide YY3-36 (PYY_{3-36})	Distal GI tract	Decrease	Increase with dietary fat ingestion and increase with dietary protein relative to other macronutrients [c]
Cholecystokinin (CCK)	Upper GI tract	Decrease	Increase with dietary fibre intake [d]

[a]: (Nedvídková et al., 2003); [b]: (Erdmann et al., 2003; Greenman et al., 2004; Monteleone et al., 2003; Weigle et al., 2003); [c]: (Adrian et al., 1985; Pedersen-Bjergaard et al., 1996); [d]: (Heini et al., 1998).

Recent information suggested that sense of taste profile adapts to modulate the intake of particular nutrient groups and forms a boundary concentration to feel specific basic tastes such as sweetness for energy, saltiness for electrolytes, and the umami taste for protein. But foods without smell and/or taste are hard to either stimulate appetite or to reach satiety. Therefore, some food components, with their ability of influencing the tastes and overall palatability, may be very interesting to develop new types of foods.

Interestingly, dietary fibres were shown to provide health benefits (e.g. cholesterol lowering

effect) and have an impact on food structure. The texture of food is one of the strongest drivers of food aversion and could impact oral processing consequently influencing food liking (Jeltema et al., 2015). For instance, soluble fibres (e.g. β-glucan) can form gels and increase the viscosity of the contents in the gastrointestinal tract (Mudgil and Barak, 2013a). Its gel-forming ability could be used to change food structures, which might provide different oral senses and stimulations through changes in the viscosity of bolus or the tastes during mastication.

Similarly, polyphenols have increasingly elicited interest in the scientific community due to their proposed health benefits, mostly focused on their bioavailability in foods. Polyphenols are present in a broad range of plant foods (e.g. fruits, vegetables, herbs, seeds and cereals) as well as beverages (e.g. coffee, tea and wine) (Dueñas et al., 2015; Vinson et al., 2001). In particular, compared to many other fruits, blueberries are rich in polyphenols, for example, every kilo of fresh blueberry could provide 250 ~ 5000 mg of anthocyanins (Manach et al., 2004). Passing through the small intestine without being absorbed, most polyphenols (about 70% of the ingested dose) encounter the gut microbiota in the colon (Scalbert and Williamson, 2000). Therefore, the two-way mutual reaction (i.e. in long term) between the biotransformation of polyphenols into their metabolites by gut microbiota and the modulation of gut microbiota composition by polyphenols contributes to positive health outcomes. Thus some polyphenols may act as a prebiotic metabolite and enrich the beneficial bacteria (Lee et al., 2006). Although there are many studies on the *in vivo* bioavailability of polyphenols, the mutual relationship between polyphenols and gut microbiota is not fully understood (Ozdal et al., 2016). Furthermore, the wide variety of polyphenol compounds and their food sources, as well as their coexistence with other bioactive compounds within a normal diet make it difficult in understanding of the interactions between dietary polyphenols and gut microbes (Valdés et al., 2015).

4.Current research

Saliva is now considered as an excellent diagnostic tool has it contains a large array of metabolites and low molecular weight compounds such as VOCs, eCBs, NAEs, selected pesticides, and some specific trace elements (Michalke et al., 2014). Salivary diagnostics contribute a non-invasive, fast, safe and inexpensive approach for disease detection. In addition, discovery of salivary biomarkers that could be used to scrutinize health and disease surveillance has advanced its diagnostic value for clinical applications. Availability of emerging metabolomic techniques gives optimism that saliva can eventually be reliably used

for non-invasive clinical analysis (Zhang et al., 2012). Hence comprehensive salivary metabolome will be an important resource for researchers who are studying metabolite chemistry, such as eCBs, NAEs and VOCs, etc.

Individual members of the microbiota, and consortia of those microbes, have been shown to be highly dependent on the nutrient composition of the diet (Alcock et al., 2014). Many foods commonly found in the normal diet are rich in polyphenols or in dietary fibre. However, little is known about the impact of these ingested polyphenols and dietary fibres upon the human intestinal microbiota. Studies involving humans enable the direct analysis of the interactions between food and microbiota, but *in vivo* intervention trials have practical and ethical limitations (Hervert-Hernández and Goñi, 2011). Up to now, most of the studies have focused on single polyphenol molecules and selected bacterial populations while only a few studies have examined the *in vivo* impact of dietary polyphenols on the human gut microbiota (Dueñas et al., 2015; Vitaglione et al., 2015).

To the best of our knowledge, very few studies have been performed in view of taking VOCs as metabolites to investigate effects of rich polyphenols diet on human health, although many have claimed that the positive impact through evaluating biomarkers in blood, faeces and urine. Furthermore, it makes sense to discovery VOCs biomarkers derived from intervention of polyphenols as well as to discuss any possible correlations among VOCs in saliva and breath, gut microbiota and human physiological metabolism. Therefore, it is a very promising direction and deserves a better understanding.

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Chapter 2. Salivary concentration of N-acylethanolamines upon food mastication and after meal consumption: influence of food dietary fibre

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Abstract

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

Three types of biscuits enriched with 3% barley β-glucan (βGB) or whole-wheat bran (WWBB) or without dietary fibre (control, CB) were developed. A crossover randomized human study with 18 healthy and fasting participants collecting saliva samples in a resting condition, upon mastication of parafilm and one of the three biscuits was carried out. Subsequently, the amount of biscuits consumed in an *ad-libitum* breakfast was measured and post-prandial saliva samples, blood glucose, appetite, and food liking were collected over the following two hours.

The concentration of oleoylethanolamide (OEA) in saliva collected upon food mastication was ~222 and ~138 folds higher than that present in saliva collected during parafilm mastication or in a resting condition, respectively. Subjects consumed always 75g of biscuits at breakfast. Salivary OEA and linoleoylethanolamide (LEA) peaked at 15 min only after CB and WWBB and returned to baseline within one hour after breakfast. No difference of biscuit type on post prandial blood glucose was recorded.

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

Keywords: N-acylethanolamines, oleoylethanolamide, saliva, dietary fibres, β-glucan, appetite

1. Introduction

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

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

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

In this context we have recently demonstrated that tasting of a food (before swallowing) influences plasma levels of the endocannabinoids (eCBs) - 2-acylglicerol (2-AG) and anandamide (AEA) -, of the congeners N-aceylethanolamines (NAEs) - oleoylethanolamide (OEA), linoleoylethanolamide (LEA) and palmitoyelethanolamide (PEA) – and of gut peptides in a manner that is dependent from the individual liking of the tasted food (Mennella et al., 2015a). This finding was in line with the well-known role of gustatory system on the cephalic phase of eating and suggested that some mediators could act during mastication and elicit the plasma response.

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

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

To the best of our knowledge, no studies are present in the literature about NAEs presence in saliva during mastication.

In this study the hypothesis that NAEs could be formed in the mouth during food mastication and might influence satiation upon eating biscuits with different content and types of dietary fibre was tested. Moreover, the appetite sensations and liking of the different biscuits over two hours after biscuit consumption was monitored together with salivary NAEs and blood glucose.

To this purpose, biscuits enriched with 3% barley beta-glucan (βGB), 3% whole-wheat bran (WWBB) and without dietary fibre (control biscuits, CB) were developed and a crossover randomized design protocol was performed in healthy normal weight subjects.

2. Materials and methods

2.1 Foods

Three types of biscuits containing 3.0% barley β-glucan (βGB), 3.0% whole-wheat bran fibre (WWBB), or without dietary fibre (control, CB) were developed using a traditional recipe for biscuits. All the biscuits were prepared with the following ingredients purchased by local supermarket: flour (Divella, Bari, Italy), sugar (Eridania, Bologna, Italy), margarine (Vallé, Milan, Italy), yeast (PaneAngeli, Brescia, Italy). To produce βGB, 3% wheat flour were replaced by a barley β-glucan concentrate (Glucagel^{TM}, containing more than 77.5% dietary fibre) purchased from DKSH (Miribel Cedex, France); whereas for WWBB, a whole-wheat bran concentrate (VITACEL, containing 97% dietary fibre) purchased from ITALI (Reggio Emilia, Italy) was used.

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

2.2 Subjects selection

The recruitment was performed among the students of the Department of Agricultural Sciences University of Naples, who were interviewed about their medical status, subjective

eating habits and food preferences (100 recipes, scores from 1 to 9). The selected subjects were healthy, they were not undergoing any medication or drug therapy, they usually had breakfast, they were not on a restrictive diet and had a normal eating behaviour as assessed by the Three Factor Eating Questionnaire (TFEQ) (Stunkard and Messick, 1985).

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

2.3 Study design

The study design and protocol was approved by the Ethics Committee of University of Naples.

The protocol had a crossover, single blind, randomized design. It was characterized by three treatments per each subject that were conducted on separate days with a 1-week washout period from each other (**Figure 1**). Each subject participated to three tests.

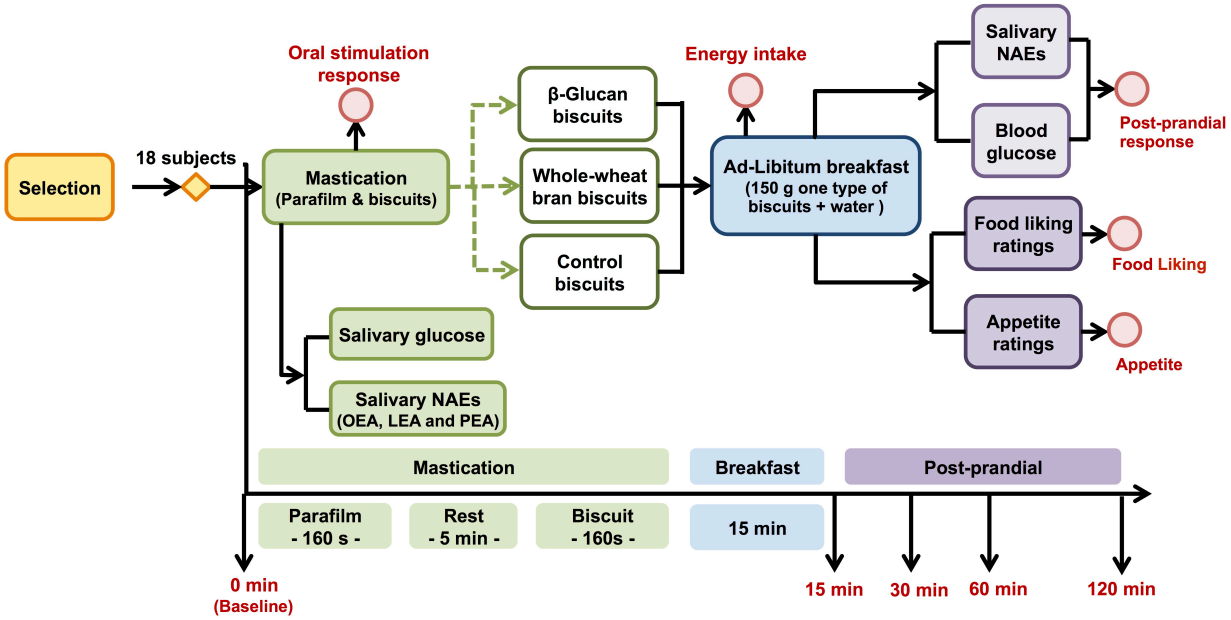

Figure 5. Study design

The subjects were instructed to consume a standardized dinner in the evening before the experimental days within the 22:00 h. On the experimental days fasting subjects reached the nutritional laboratory of the Department of Agricultural sciences at 08:30 h and after 10 minutes of rest, baseline blood glucose was measured and they were asked to collect baseline non-stimulated and mechanically stimulated (by parafilm and by food) saliva samples. Immediately after, participants were offered a breakfast comprising 150 g of the same type of

biscuit they had just masticated and were asked to eat biscuits until they felt satisfied within 15 min. A glass of water (125 mL) was also offered. The remaining biscuits in subject plates were weighted and energy intake consumed was calculated. After breakfast and 15, 30, 60 and 120 min after breakfast, subjects were asked to collect saliva for 5 min, and to rate appetite feelings and actual liking of the biscuit on VAS questionnaires. Blood glucose at the same time points was also measured.

2.4 Saliva sample collection and preparation for analysis

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

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

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

2.5 Salivary N-acylethanolamines measurement

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

μL) for HPLC/MS/MS analysis. To estimate the extraction efficiency, peak areas obtained for deuterated internal standards extracted from saliva were compared with non-extracted controls in 100 μL acetonitrile:water (50 : 50) (Lam et al., 2010).

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

2.6 Appetite and food liking questionnaires

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

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

2.7 Blood and salivary glucose

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

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

2.8 Statistical analysis

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

By the analysis of variance (ANOVA) for repeated measures the subjective appetite and hedonic sensations recorded after the consumption of the three types of biscuit and the response curves of salivary glucose and NAEs were compared and tested for the effect of treatment and of time as factors. For all the 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 and standard errors.

3. Results and discussion

3.1 Foods

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

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

Table 1: Nutritional composition of 100 g biscuits

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

3.2 Subjects

Eighteen volunteers (7 M/11 F), with mean age of 27 ± 1 years (range 20 – 37) and with mean body mass index (BMI) of 23.3 ± 0.58 kg/m^2 (range 19.5-28.0 kg/m^2), participated in this study. Among them, 8 subjects were overweight with BMI ≥ 25 kg/m^2.

3.3 Salivary NAEs upon mastication

The concentration of NAEs in saliva samples collected upon mastication is reported in **Figure 2**.

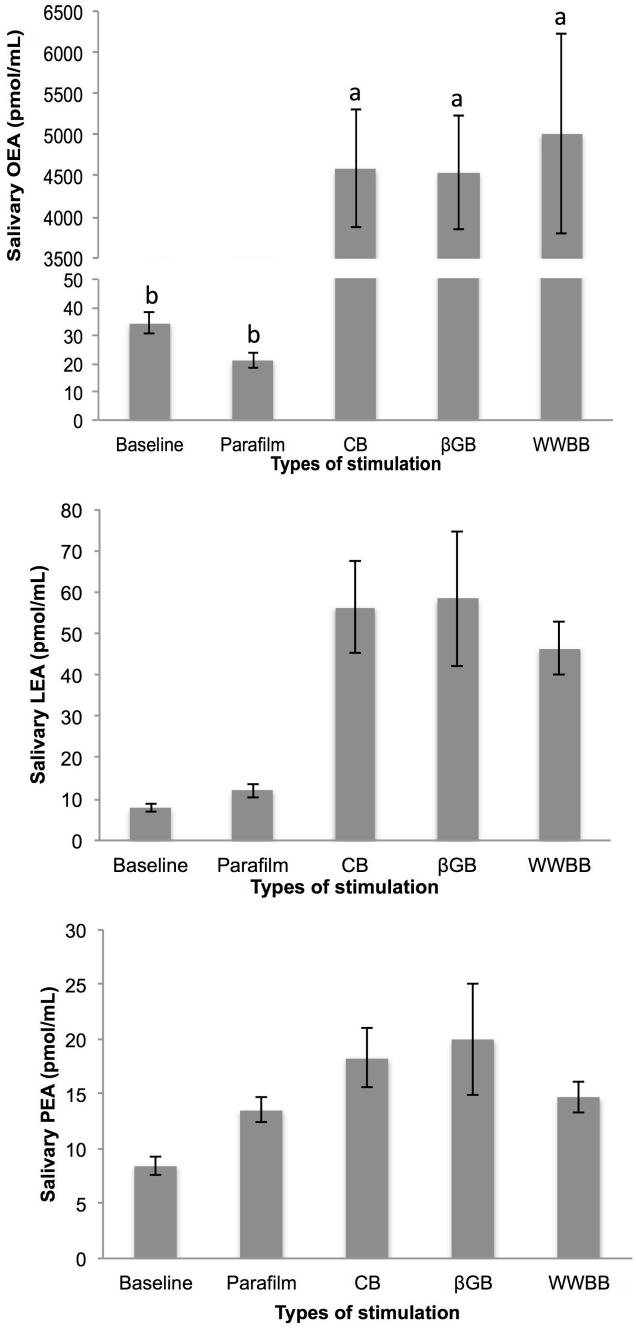

Figure 6. Concentration of salivary NAEs from fasting subjects at baseline (resting condition) and upon mastication of parafilm or control (CB), β-glucan-enriched (βGB) or whole wheat bran-enriched (WWBB) biscuits. Different letters on the bars indicate $p<0.05$.

The concentration of OEA in saliva samples collected upon food mastication was significantly higher than in non-stimulated (baseline) and parafilm-stimulated saliva samples, whereas no difference between the last two was found. On the contrary, the concentration of LEA and PEA did not vary significantly among the saliva samples.

3.4 Salivary glucose upon mastication

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

Interestingly, a significant positive correlation between the concentrations of OEA and glucose in saliva from chewed CB and WWBB was found (**Figure 3**).

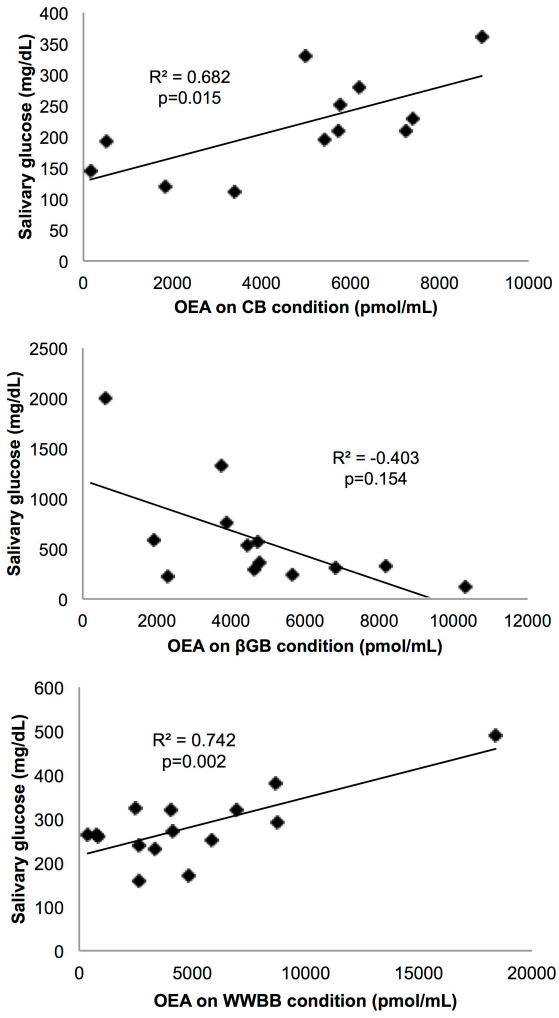

Figure 3. Correlations between salivary glucose and OEA in chewed biscuits.

3.5 Food intake

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

3.6 Postprandial salivary NAEs

The variation from baseline of salivary NAEs concentration over 2 hours following biscuit consumption and the area under the curve (AUC) of each NAE are reported in **Figure 4**.

Salivary concentration of all NAEs, except PEA following βGB, peaked at 15 minutes post-breakfast compared to baseline, this increase being significant for OEA after all types of biscuits and for LEA only after WWBB. From 15 min to 60 min, a general trend towards reduction of mean concentrations following consumption of all three types of biscuit was found. At 60 min, concentrations of LEA and OEA returned to the baseline values (**Figure 4**).

At 15 min, significant difference of OEA between βGB and WWBB was found ($p = 0.017$). Similarly, significant difference for lower LEA ($p = 0.001$) and PEA ($p = 0.038$) after βGB compared to WWBB was observed. The AUCs of NAEs did not significantly change after the consumption of the different biscuits (although the mean magnitude was always in the order WWBB > CB > βGB) (**Figure 4**).

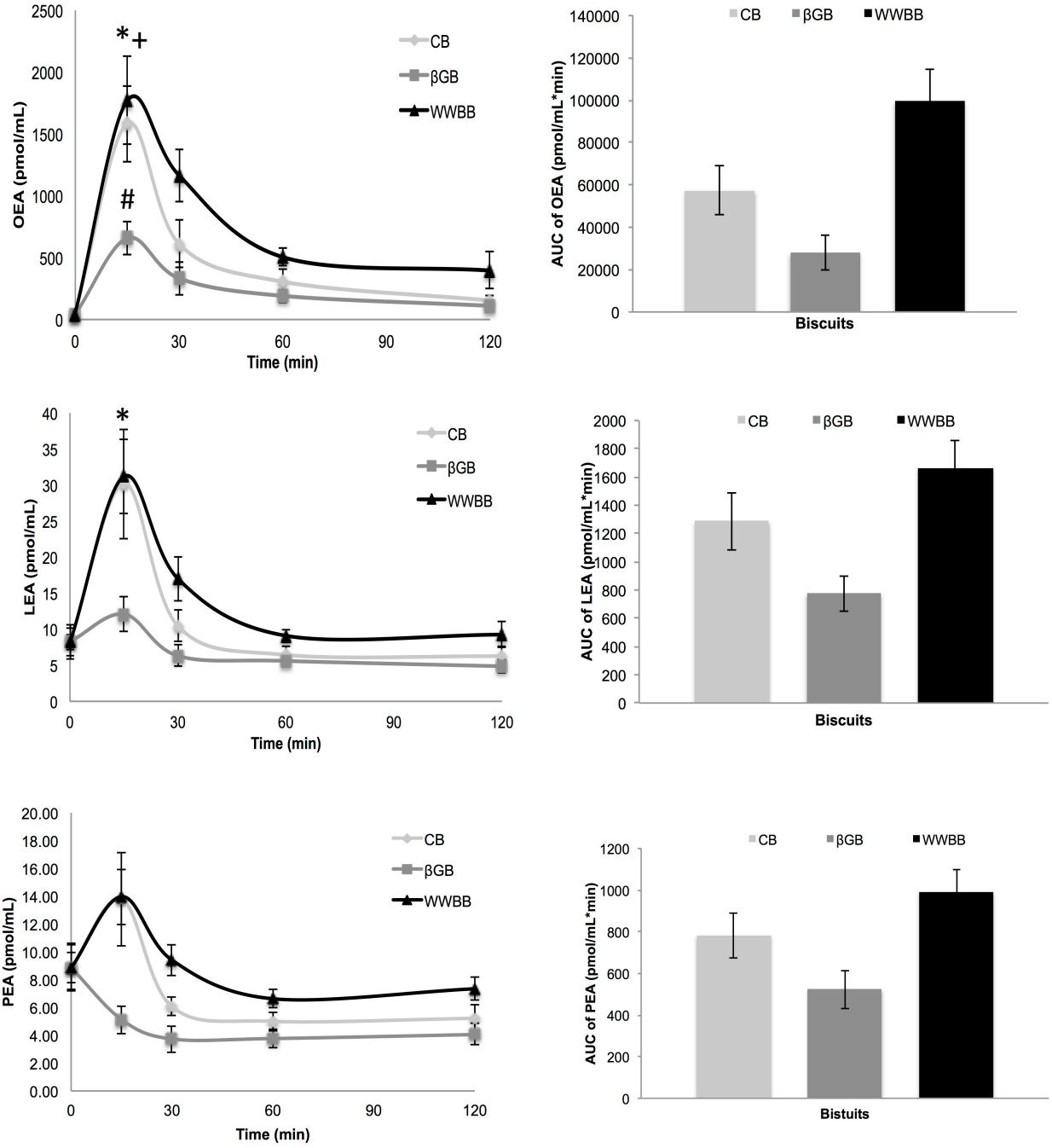

Figure 4. Concentration-time curves and $AUC_{(0\text{-}120)}$ of postprandial salivary NAEs. $^{*, +, \#}p < 0.05$ *vs* baseline.

3.7 Postprandial appetite and food liking

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

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

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

Table 2: Appetite and hedonic scores (means ± SEM) collected over 120 min after consumption of the three types of biscuits.

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

3.8 Blood glucose response

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

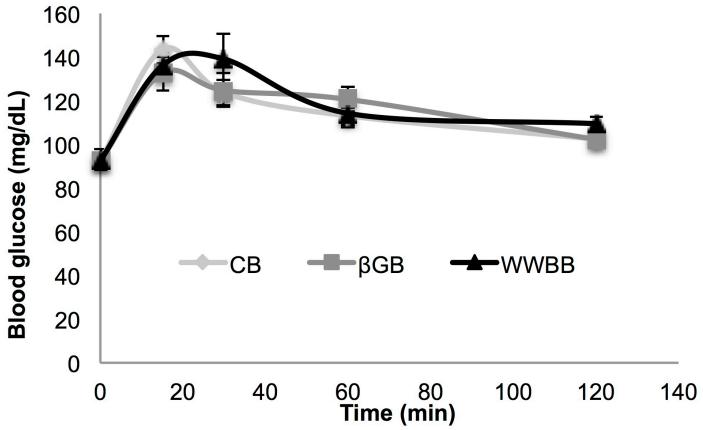

Figure 5. Concentrations (Means ± SEM) of blood glucose over 2h following consumption of CB, βGB or WWBB.

The AUC of blood glucose was 13,835 ± 546 mg min/dL, 14,231 ± 604 mg min/dL and 13,824 ± 372 mg min/dL after βGB, WWBB and CB, respectively and no significant difference among the types of biscuit was found (glycaemia × treatment interaction; $p > 0.05$). Pearson's analysis showed a negative correlation between salivary OEA concentration and blood glucose at 30 min (-0.480, $p = 0.044$) and a tendency at 15 min (-0.456, $p = 0.066$).

4. Discussion

For the first time in this study, the concentration of NAEs was measured in stimulated saliva samples and it was demonstrated that mastication itself did not cause variation of these compounds compared to baseline resting saliva in healthy normal weight subjects. Indeed the salivary concentrations of all NAEs meanly increased in saliva only upon biscuit mastication, independently from the type of biscuit, compared to non-food (parafilm) condition mastication.

In particular data showed that OEA increased more (~138 folds) than LEA (~7 folds) and PEA (~2 folds) and was the only NAE to reach a significant increase upon food mastication.

All in all these findings strongly suggested that NAEs directly derived from the food or they

were biotransformation products of their NAPE precursors during mastication.

In fact, although it is known that NAEs can be endogenously formed in the intestine (Artmann et al., 2008; Hansen, 2014; Hansen and Diep, 2009; Petersen et al., 2006; Sarro-Ramirez, 2013), brain (Artmann et al., 2008; Hansen, 2014; Simon and Cravatt, 2010; Tsuboi et al., 2013), liver (Artmann et al., 2008) and other mammalian tissues (Hansen & Diep, 2009; Hansen, 2014 (Hansen, 2014; Hansen and Diep, 2009)**,** they are also largely diffused together with their natural precursors N-acylphospatidylethanolamines (NAPEs) in both plant and animal kingdoms being found in plants, yeast, slime molds, insects, and mammals (Coulon et al., 2012; Fezza et al., 2003; Hansen, 2014; Hansen and Diep, 2009; Hayes et al., 2013; Muccioli et al., 2009). So both NAEs and NAPE are present in wheat flour (Bomstein, 1965) and, as a consequence, they can be found in biscuits. From the biscuits NAEs could be directly delivered in the mouth due to the mechanical and salivary enzyme disruption of the food matrix upon mastication as well as be formed by the NAPEs upon the action in the mouth of NAPE-PLD (Matias et al., 2012).

In this view, the lacking differences of salivary NAEs upon mastication of different types of biscuits (in the case of OEA) or even under different stimuli (in the case of LEA and PEA) suggested that the substitution of 3% wheat flour with dietary fibres in the enriched biscuits did not significantly influence the content of NAEs or NAPEs in the biscuits as well as the oral factors that might underpin the release of NAEs in the mouth. A further study to clarify the mechanisms and individual factors behind salivary NAEs formation should be performed.

The fixed rate of bites during mastication protocol excluded that individual way of masticating food might have influenced NAEs formation as well as glucose release in the mouth upon different food conditions. The individual mastication speed was established on the basis of preliminary experiments (*data not shown*) where participants' behaviour and possibility to chew the biscuits and spit the chewed food without swallowing were evaluated. In other words, the mastication protocol was suited on the participants and the best conditions to collect all chewed food avoiding any loss due to unwanted swallowing were used. So, it was assumed that individual way of eating biscuits during the protocol to assess satiation at breakfast was similar to that adopted during mastication protocol and caused similar concentrations of NAEs in the food bolus that, in that occasion, were ingested. Data showed that in parallel with the lacking differences of salivary OEA during different biscuits mastication, no effects of the different types of biscuits on satiation was present.

Interestingly, the types of dietary fibres in the biscuits influenced immediate post-prandial salivary concentration of NAEs. Moreover, salivary OEA was associated with fullness and satiety sensations as well as actual liking of the biscuits for sweetness.

To the best of our knowledge no one before us studied saliva composition in the immediate post-prandial phase. Matias and co-workers (2012) collected saliva after 1h from meal consumption and demonstrated that eCBs and NAEs (AEA, 2-AG, OEA and PEA) did not change in the post-prandial compared to the pre-prandial phase. On the contrary, data of the present study clearly showed that in the immediate post-prandial phase NAEs formed during mastication was still present in the mouth and did not reduce equally after eating the different types of biscuits. In particular, a faster and dramatic rebound of all salivary NAEs after βGB consumption than CB or WWBB was observed. This finding might depend from the physical properties of the βGB bolus. In fact, it could be hypothesized that the βGB bolus, thanks to its content of barley β-glucan having a gel-forming capacity and binding ability (Hughes and Swanson, 1989; Mudgil and Barak, 2013b), could better entrap compounds and let the mouth free from NAEs upon swallowing determining a better effect of mouth cleaning compared to WWBB and CB. This hypothesis would take into account the food origin of NAEs in saliva and it is in accordance with the recommendation to study saliva composition by avoiding eating before the collection because residues of foods present in the mouth can influence results (Kaufman and Lamster, 2002; Yoshizawa et al., 2013). However, it should be noticed that together with biscuits, participants had a glass of water they partly drank after eating causing a rinsing of their mouths that was supported by the fact that the post-prandial saliva samples appeared free from evident food residues at all the time points. Another hypothesis might be that β-glucan in the βGB might differently influence the activity of oral NAPE-PLD or FAAH, the enzyme degrading NAEs (Matias et al., 2012), compared to WWBB and CB, and this effect could be evident in presence of few food residue that might remain in the mouths even after drinking. On the other hand an effect of the gel-forming beta-glucan on salivary enzyme activities (including α-amylase) could be also suggested from the lacking association between salivary OEA and glucose in the mastication study, despite the positive associations found in the cases of WWBB and CB. Further studies should test these hypotheses and clarify the mechanisms behind the present findings.

Worth of notice were some associations found between post-prandial (at 15 min and 30 min) salivary OEA and liking of biscuits (sweetness and overall palatability) as well as fullness and satiety sensations. These findings suggested that salivary OEA might be implicated in the

sensory mechanisms occurring in the post-ingestive phase and influencing the flavor-consequence learning that underpin individual food preference and choice (Yeomans, 2006). In this study fasting subjects were asked to eat the biscuits until they felt comfortably satisfied and no other food was available. However, in a normal life condition it may also be possible that the salivary OEA may have a role on the amount or type of foods people can eat when exposed to different food cues, as it happens for example in a buffet-style meal. Appropriate studies should be performed to test these hypotheses.

Finally, in this study no effect of βGB consumption on the postprandial blood glucose was found compared to WWBB and CB. The amount of biscuits eaten by subjects (75g on average) could explain this result. In fact considering the amount of biscuit consumed and the 3% enrichment with β-glucan, βGB provided 2g of barley β-glucan corresponding to a 3.9% β-glucan by available carbohydrates. In these conditions the lacking effect of βGB in modulation of blood glucose was in line with a previous study from our research group showing that a meal providing 3g β-glucan (2.3% by available carbohydrates) did not influence blood glucose response compared to the control meal (Barone Lumaga et al., 2012). On the contrary meals providing 3g of β-glucan (5.2% by available carbohydrates) (Vitaglione et al., 2009) or 6.2g - 7.3g β-glucan (12.4% or 14.6% by available carbohydrates) (Jenkins et al., 2002) showed the hypoglycaemic effect of the β-glucan-enriched meal compared to the control.

5. Conclusions

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

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Chapter 3. Impact of blueberry consumption on volatile organic compounds of human breath and saliva: a pilot study

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Abstract

Blueberry is a rich dietary source of polymerized polyphenols. Previous studies showed that these compounds may behave as dietary fibre and have a prebiotic effect.

The aim of this study was to test the influence of a one week-consumption of blueberries on the volatile organic compounds (VOCs) of breath and saliva in humans.

Fourteen healthy volunteers (7 M/7 F; mean age of 31.2 years and BMI 22.5 ± 2.0 kg/m^2) participated in this three-week single blind study with a two-week cross over design. After a one-week of baseline period (BL) where all subjects had a low polyphenol diet, in a randomized manner subjects continued with this diet (control, CT) or added 200 g/day of fresh blueberries for one week (intervention, INT) switching to the other arm for the following week. Before and after each week fasting subjects reached the laboratory to collect saliva samples and to have on-line analysis of breath by Proton Transfer Reaction Time-of-Flight Mass Spectrometry (PTR-ToF-MS). Saliva samples were analysed by PTR-ToF-MS and Headspace Gas Chromatography Mass Spectrometry (Headspace GC-MS).

No difference in VOC profiles of breath and saliva after INT and CT was found. Interesting correlations between saliva and breath composition especially for methanol, formaldehyde, ethanol, acetone and propanol were shown.

Keywords: VOCs, saliva, breath, blueberry polyphenols

1. Introduction

The relationship between breath composition and health is well known since many centuries. As early as 2500 years ago in Greece, physicians considered that breath odor could be associated with diseases and health status (Ajibola et al., 2013)as a non-invasive approach, nowadays analysis of volatile organic compounds (VOCs) in breath, not only endogenously generated biomarkers but also derived from exogenous substrates or drugs for patients, holds great promise as a potential new diagnostic tool.

Actually, VOCs can be determined not only in breath but also in faeces, urine, blood, saliva and skin (de Lacy Costello et al., 2014; Kusano et al., 2011; Kusano et al., 2013). De Lacy Costello (2014) and co-workers recently listed a total of 1840 VOCs in several biological samples and fluids including breath (872), saliva (359), blood (154), milk (256), skin secretions (532) urine (279), and faeces (381) from apparently healthy individuals. In the literature studies VOCs were studied as metabolites deriving from physiological processes, as in the case of isoprene that can be generated during cholesterol biosynthesis (Stone et al., 1993a), or as diseases biomarkers (e.g. lung cancer and hepatocirrhosis) (Fernández del Río et al., 2015; Li et al., 2016; Matsumura et al., 2010; Morisco et al., 2013).

Moreover, Nagler et al. (2002) found high correlation between the salivary and blood concentrations of relevant components. Therefore saliva, to some extent, reflects the biochemical and metabolic information in blood, which could be considered as a potential way to study human physiological and pathological conditions (Al-Kateb et al., 2013). Indeed, metabolic by-products of bacterial species in the oral cavity, as well as environmental organic chemicals exposure, such as consumption and ingestion of contaminated food, could influence salivary composition. However, few studies focused on the correlation of VOCs between breath and saliva under conditions of diet intervention (Soini et al., 2010).

Diet, either altering the gut microbiota or impacting directly on metabolism, was suggested to modify breath composition (Ajibola et al., 2013; El Oufir et al., 1996; Pauling et al., 1971). In particular, plant polysaccharides-rich diets contain resistant starch and oligosaccharides that can modify microbiota composition thus eliciting local and systemic health benefits through the gut. These mechanisms were also suggested to underpin the health benefits of polyphenols-rich diets (Vitaglione et al., 2008; Vitaglione et al., 2012). However, despite many evidence in animal studies, few intervention studies could prove the association

between polyphenol intake and metabolic and disease biomarkers in humans (Vitaglione et al., 2015). Most of studies specifically focused on the impact of dietary polyphenols or specific compounds on the human gut microbiota or selected bacterial populations. Red fruits, including strawberries, cherries, raspberries, blueberries and cranberries are a rich dietary source of polyphenols mainly proanthocyanidins (oligomers of flavan-3-ols). These compounds are known to pass unmodified through the first part of gastro-intestinal tract and to reach the colon where they can be metabolized by, and in turn modify, local microbiota, sometimes exerting a prebiotic effect (Etxeberria et al., 2013b). This effect was demonstrated after blueberry drink consumption for six-weeks and after red wine consumption for twenty days causing the increase of the faecal number of *Bifidobacterium* spp. (Queipo-Ortuño, 2012; Vendrame et al., 2011; Yoshizawa et al., 2013).

As a result of actual knowledge in the field, it might be supposed that red fruit polyphenols, especially the polymerized ones, can influence the intestinal microbiota and initiate gut-derived or metabolic changes of VOCs in breath and saliva.

Numerous publications have reported the capability of instrumental analysis to measure large variety of VOCs, including gas-chromatography MS (GC-MS), selected- ion-flow-tube MS (SIFT-MS), ion-mobility MS (IMS) and proton-transfer- reaction MS (PTR-MS) (Herbig et al., 2009; Schwarz et al., 2009). Due to the high sensitivity for VOCs measurements, the fast response time and the possibility of direct analysis without sample pre-treatment, PTR-MS has been repeatedly applied for both single and real-time measurements of VOCs in breath (Herbig et al., 2009).

The aim of this study was to verify the hypothesis whether a one-week intervention with 200g day of blueberry, providing 440 mg/day of polyphenols (as total anthocyanins), could influence breath and saliva VOCs in healthy humans. A PTR-ToF-MS profile of VOCs in breath and saliva samples was obtained and possible correlations between them were discussed.

2. Subjects and methods

2.1 Subjects

The recruitment was performed among the students and researchers of the Fondazione Edmund Mach (FEM, San Michele All'Adige, Trento, Italy), who were interviewed about their medical status, subjective eating habits and food preferences. The selected subjects were

healthy, without undergoing any medication or drug therapy and with no symptoms of food allergy or intolerance. They were not on a restrictive diet and had normal eating behaviour on the basis of scores obtained filling the Three Factor Eating Questionnaire (TFEQ) (Stunkard and Messick, 1985).

Eligible subjects signed an informed written consent before the enrolment. They were advised not to vary their physical activity during the whole period of the study, and to avoid sport activities the day before the experimental days.

2.2 Study design

Study design and protocol were approved by the Ethics Committee of University of Naples "Federico II" and all participants signed the informed consent before the enrolment. A scheme of the study design is shown in **Figure 1**. The study had a cross-over, single blind, randomized design.

Once enrolled in the study participants were asked to control polyphenols as much as possible in their diets for one week (baseline, BL). In particular, subjects were advised to avoid high polyphenol foods, including fruits, vegetables and drinks, such as apples, pears, grapes, wine and coffee. After the baseline week, subjects were randomized for the intervention (INT) or control (CT) week and they switched on the other arm after one week. During INT subjects added to the low polyphenol diet 200g/day of fresh blueberries that were purchased by a local supermarket and were provided to the participants every working day (i.e. from Monday to Friday); the last two portions for the weekend were provided on Friday evening. During CT subjects were advised to keep their own diets as in BL.

At the end of each week fasting participants reached the research centre at 07:30 h for the analysis of VOCs in the breath, and for collection of saliva samples and dietary data.

For breath and saliva collection in the evening before the experiments participants were instructed to consume a standardized dinner within the 22:00 h and were asked to refrain from eating foods containing garlic, onion, mint. Moreover, in the morning since the measurement, they were not allowed to smoke, eating chewing gum or drinking alcohol and coffee, using mouthwash, brushing teeth.

To analyse VOCs in the breath, subjects were required to rest in the test lab for at least 30 minutes, breathing with the ambient air to balance the differences of air inside and outside. During this period, they were asked to collect saliva in a clean tube (50 mL) for 5 minutes.

Then, after rinsing their mouth with pure water, they performed for three times the on-line breath test by PTR-TOF.

The compliance to the protocol was assessed through dietary questionnaires that were filled before the three test sessions. Each subject was asked to indicate the average portion and the frequency of intake of over 60 foods belonging to the following groups: fruits and vegetables, fish, meat products, eggs, cereals and cereal products, milk and dairy products, snacks and soft drinks and alcohol. The food questionnaires were compiled with the help of photographs and images to calculate the energy and polyphenols intake. The food questionnaires, mainly included two questions ('How many portions do you consume per week?' and 'How many grams is each portion?'), (Morisco et al., 2013).

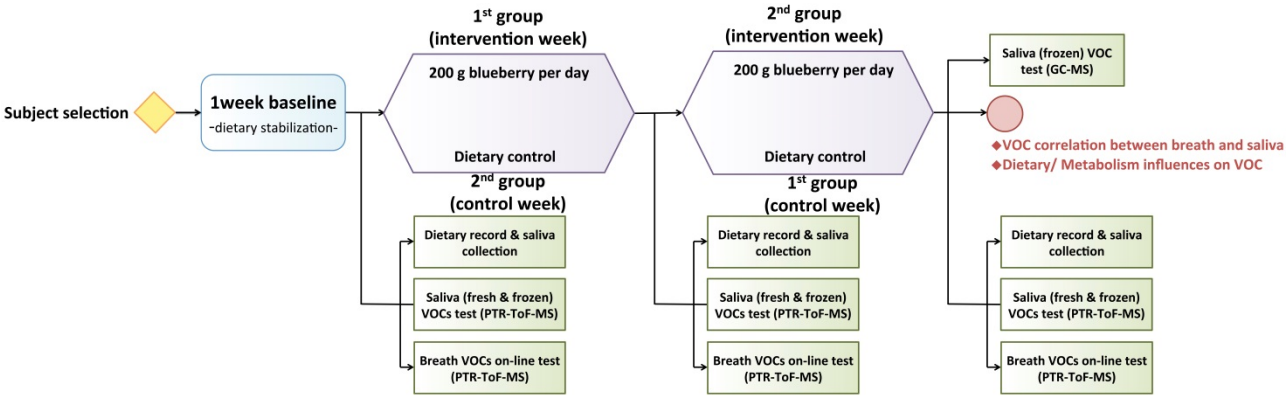


Figure 7. Study design

2.3 Breath analysis by PTR-ToF-MS

Real time breath analysis was performed using a buffered end- tidal (BET) on-line sampler (Herbig et al., 2009) coupled to a Proton Transfer Reaction Time-of-Flight Mass Spectrometer 8000 (PTR-ToF-MS 8000, Ionicon Analytik GmbH, Innsbruck, Austria). The subject was required to sit in front of the interface and to breathe normally room air. After a short time, the subject was given a single exhalation in a disposable mouthpiece, as well as a sputum trap, which were connected to the BET system. With the BET system, collection of the end-tidal fraction of exhaled breath gas was performed, middle part of which is considered as the richest in those molecules derived from exchange at the alveolar-capillary membrane and less affected by inhaled breath air gas. BET system help avoiding the effect of hyperventilation on volatile concentration. Fractions of exhaled gas, collected through the BET system, were drawn directly to the drift tube of the PTR-ToF-MS as an on-line detection, recording the volatile organic compound spectra (Morisco et al., 2013).

Instrumental conditions for the proton transfer reaction were the following: Inlet temperature 110 °C, drift voltage 500 V, drift temperature 80 °C and drift pressure 2.4 mbar affording an E/N value of 120 Townsend (1Td = 10^{-17} cm^2 V^{-1} s^{-1}). Sampling was performed with a flow rate of 43 sccm. The mass resolution (m/Δm) was at least 3500 (Aprea et al., 2012).

Herbig et al. (2009) reported the application of the PTR-MS technique for breath analysis in their research. Briefly, water vapor in the ion source is used to produce primary ions (H_3O^+). In the drift tube, the proton transfer reaction occurs between the formed H_3O^+ and neutral analyte molecules (M): $M + H_3O^+ \rightarrow MH^+ + H_2O$.

It was also considered the effect of different reactant species (H_3O^+, NO^+, and O_2^+). In our pilot study, all three ionization reagents were applied as chemical ionization (CI), to analyse breath and saliva. Considering the fragmentation of the VOCs, as well as effects of multi precursor ions, H_3O^+ mode was applied in all tests, and these data were used for further analysis (Trefz et al., 2013).

2.4 Saliva VOCs analysis by PTR-ToF-MS

Saliva VOCs were immediately quantified by HeadSpace PTR-ToF-MS whereas the rest of each sample (~1 mL) was immediately frozen. Part of that was re-analysed by Headspace PTR-ToF-MS after 6 hours of freezing and part was stored until HeadSpace SPME-GC/MS for further VOC identification which is not discussed here. Injections were performed by using a multipurpose GC automatic sampler (Gerstel GmbH, Mulheim am Ruhr, Germany). Each vial was closed with a Teflon/Silicon cap through which during measurements headspace air was sampled via a needle entering the vial and connected to the inlet of PTR-ToF-MS via a peek tube. A second needle provided clean air generated by a gas calibration unit (GCU, Ionicon Analytik GmbH, Innsbruck, Austria), which was used as zero air generator. Test cycle for each vial consisted of flushing the headspace of the sample with clean air for 1 min at 43 sccm, then incubation for 30 min at 37 °C and finally measurement. The analysis order was randomized to avoid possible systematic memory effects. The sample headspace was measured by direct injection into the PTR-ToF-MS drift tube via a heated (110 °C) peek inlet. Each sample was measured for 30 s, at an acquisition rate of one spectrum per second.

2.5 Saliva VOCs analysis by SPME-GC/MS

Frozen saliva samples were also analysed by SPME-GC/MS (Agilent 6890, Agilent Technologies, USA). Collected saliva samples ready for the analysis were accurately handled according to the work of Kusano (2011) and Wang (2009). The extraction of volatile compounds was performed according to Soini et al. (2010), slightly modified. Five hundred microliters of saliva were transferred into a 10 mL vial, previously added with 2 mL of deionised water and 0.84 g of NaCl (Wang and Lu, 2009). The vial was put in an apparatus regulating water temperature and stirring of sample at 200 rpm. The sample was kept for 180 min at 40 °C in the apparatus and the SPME fibre was inserted through the Teflon septum in the vial and exposed to the sample headspace.

The SPME device (Supelco Co., Bellefonte, USA) was equipped with a 75μm thickness carboxen/ polydimethylsiloxane (CAR/PDMS) fibre coated with 1 cm length stationary phase (Wang and Lu, 2009).

VOCs were analysed by GC coupled with a mass spectrometer using a GC/MS 6890N (Agilent Technologies, Palo Alto, CA, USA) equipped with a J&W HP-5MS capillary column (30 m×0.25 mm i.d.×0.25 μm Film Thickness; J&W Scientific, Folsom, CA, USA). Temperature was set at 50 °C for 1 min and increased from 50 to 160 °C at the rate of 5°C/min and from 160 to 200 °C min^{-1} at 3°C min^{-1} and where is kept for 10 min, according to (Kusano et al., 2011). The injector was kept at 250 °C. Helium was used as carrier gas (0.7 mL min-1). VOCs thermal desorption was carried out by exposing SPME fibre in the injector for 10 min. A blank test was performed before each analysis to prevent the release of undesirable compounds. Frozen saliva was firstly held in a thermal bath at 37 °C and shaken in order to dissolve any suspension.

2.6 Statistical analysis

2.6.1 PTR-ToF-MS data analysis

Spectral data acquisition was performed by means of the software TOF-DAQ (Tofwerk AG, Switzerland), with a mass range of 10 - 400 Th and data were stored in HDF5 format for further analysis. Signal distortions derived from detector dead time were corrected before mass calibration, peak detection and area extraction through a cumulative peak fitting, following the procedure described by (Cappellin et al., 2011). Internal calibration was based on two peaks: 1) m/z = 21.0221 ($H_3{}^{18}O^+$) and 2) m/z = 29.9974 (NO^+), which were always present in PTR-MS spectra. Peak intensity, expressed as ppbv, was estimated by the formula in (Lindinger et al., 1998), using a constant value for the reaction rate constant (k = 2.10^{-9} cm^3

s^{-1}). Results were then analysed by repeated non-parametric ANOVA (Tukey test) using RStudio (Version 3, USA) software.

2.6.2 SPME-GC/MS data analysis

Compound identification was performed by comparing retention times and mass spectra obtained by analysing pure reference compounds in the same conditions. Moreover, the identification was confirmed by comparing mass spectra with those of NIST database. Mass spectra were recorded at 70 eV. The source temperature was 230 °C and the interface temperature was 250 °C. Before using it, the fibre was conditioned at 270 °C for 1 h for the analysis. Peak data were processed with the software Chemstation (Agilent Technologies, Palo Alto, USA).

3. Results

3.1 Subjects and dietary records

Fourteen healthy volunteers 7 M/7 F with mean age of 31.2 years (range 15 – 52 years) and with body mass index BMI 22.5 ± 2.0 kg/m^2, participated in this study.

The participation of subjects to all the experimental sessions and the analysis of food diaries showed a high compliance to the protocol by all subjects (**Table 1**). No significant change of diets as regards macronutrient composition as well as intake of other polyphenols but the blueberry polyphenols during INT was found. Data showed that during INT subjects almost doubled their dietary polyphenols mainly including anthocyanins, proanthocyanidins and hydroxycinnamic esters.

Table 1: Nutritional composition of individual daily diets over the study period. Data are expressed as means ± SEM (*P<0.05 vs control week).

	Baseline week (BL)		**Intervention week (INT)**		**Control week (CT)**	
	Means ± SEM	% Energy	Means ± SEM	% Energy	Means ± SEM	% Energy
Energy (kcal)	1472±165		1657±151		1703±217	
Energy (kJ)	5723±805		6440±795		6619±1019	
Carbohydrates (g)	196±22	54±2	220±22	53±2	228±32	53±2
Proteins (g)	53±6	14±1	55±5	13±1	60±7	15±1
Fats (g)	48±7	28±2	56±6	30±2	55±7	29±2
Saturated	17±3		18±2		17±3	
Unsaturated	26±4		32±4		29±5	
Dietary Fibre (g)	13±1	2±0.1	15±2	2±0.2	15±3	1±0.1
Alcohol	3±0.8	2±0.5	3±1	2±0.5	4±1	2±0.6
Polyphenols (mg)						
from blueberries	0		440*		0	
from all foods	578±138		543±140		590±158	

3.2 VOC tentative identification

Through the analysis of PTR-ToF-MS (using $H3O^+$), The breath VOC dataset obtained via PTR-ToF-MS analysis (using H_3O^+ as primary ion) consisted of 724 mass peaks and the saliva VOC dataset generated 574 mass peaks. Among them, finally in total 28 mass peaks were associated with a compound in saliva and breath respectively, based on the SPME-GC/MS analysis of saliva samples and literature (**Table 2**).

Table 2: List of measured peaks and identified VOCs in saliva and breath

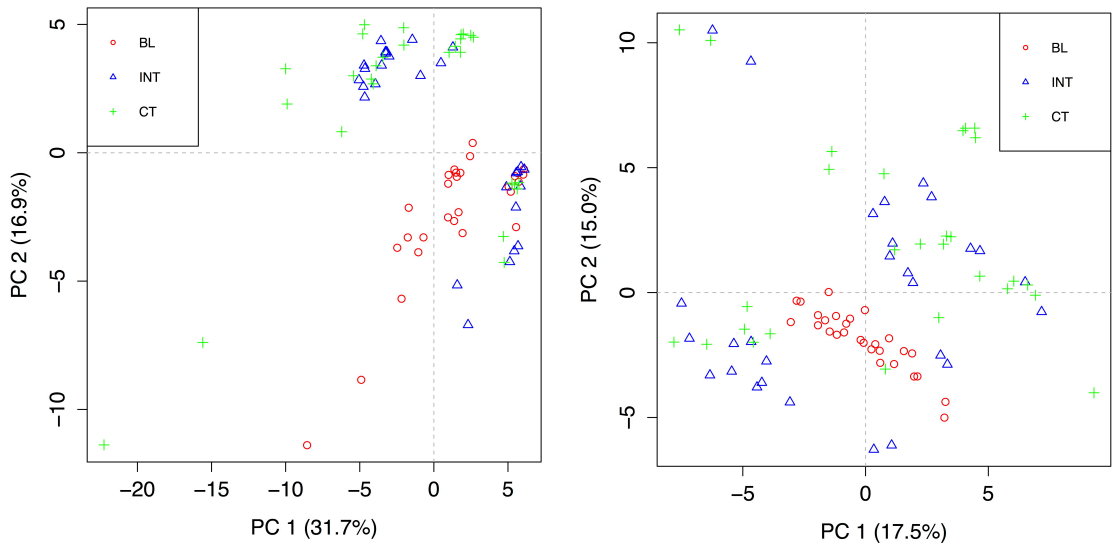
Chemical class	Tentative identification	Protonated Molecular Formula	Theoretical mass (Th)	Saliva		Breath	
				Measured m/z (Th)	Error (mTh)	Measured m/z (Th)	Error (mTh)
Carboxylic acids/ esters	Formic acid	$CH_3O_2^+$	47.0133	47.0130	-0.32	47.0143	0.98
	Acetic acid	$C_2H_5O_2^+$	61.0290	61.0285	-0.47	61.0299	0.93
	Propanoic acid	$C_3H_7O_2^+$	75.0446	75.0445	-0.11	75.0462	1.59
	Butanoic acid/ ethyl acetate	$C_4H_9O_2^+$	89.0603	89.0613	1.03	89.0645	4.23
	Ethyl propanoate/ methyl isobutyrate (t)	$C_5H_{11}O_2^+$	103.0759	103.0780	2.08	103.0799	3.98
Alkene	Ethylene	$C_2H_5^+$	29.0391	29.0400	0.87	29.0364	-2.73
	Isoprene	$C_5H_9^+$	69.0704	69.0704	-0.03	69.0713	0.87
Aldehydes/ ketones/ unsaturated alcohols	Formaldehyde	CH_3O^+	31.0184	31.0182	-0.20	31.0189	0.50
	Acetaldehyde	$C_2H_5O^+$	45.0341	45.0335	-0.55	45.0341	0.05
	Pentanal/ 3-methyl-3-buten-1-ol (t)	$C_5H_{11}O^+$	87.0810	87.0813	0.30	87.0830	2.00
	Hexanal/ 3-methyl-2-pentanone (t)/ 2-hexanone (t)	$C_6H_{13}O^+$	101.0967	101.0982	1.55		
	Heptanal	$C_7H_{15}O^+$	115.1123	115.1146	2.30	115.1168	4.50
	Octanal/ 6-methyl-2-heptanone (t)	$C_8H_{17}O^+$	129.1280	129.1295	1.55		
	Nonanal	$C_9H_{19}O^+$	143.1436	143.1472	3.60		
	Decanal	$C_{10}H_{21}O^+$	157.1593	157.1585	-0.75		
	Acetone	$C_3H_7O^+$	59.0497	59.0490	-0.69	59.0475	-2.19
	2-butanone	$C_4H_9O^+$	73.0654	73.0640	-1.35		
	2,3-butanedione	$C_4H_7O2^+$	87.0446	87.0450	0.39	87.0477	3.09
Alcohols	Methanol	$CH_4O{\cdot}H^+$	33.0341	33.0338	-0.25	33.0342	0.15
	Ethanol	$C_2H_7O^+$	47.0497	47.0492	-0.49	47.0502	0.51
Phenols	Phenol (t)	$C_6H_7O^+$	95.0497	95.0486	-1.09	95.0525	2.81
Nitrogen compounds	Acetonitrile	$CH_3CN{\cdot}H^+$	42.0344	42.0338	-0.57	42.0353	0.93
	Hydrogen cyanide	$HCN{\cdot}H^+$	28.0187	28.0188	0.08	28.0191	0.38
	Indole (t)	$C_8H_8N^+$	118.0657	118.0683	2.63	118.0685	2.83
Sulphur compounds	Methanethiol (Methyl mercaptan)	CH_5S^+	49.0112	49.0103	-0.90	49.0111	-0.10
	Dimethyl disulfide (t)	$C_2H_6S_2{\cdot}H^+$	94.9989	94.9988	-0.11		
	Dimethyl sulphide	$C_2H_6SH^+$	63.0268	63.0268	-0.04	63.0279	1.06
	Hydrogen sulphide	$H_2S{\cdot}H^+$	34.9956	34.9953	-0.25	34.9951	-0.45

3.3 Breath and saliva VOC dataset analysis

3.3.1 Principal components analysis (PCA)

Spectra were analysed by PCA to highlight possible systematic differences between dietary treatments and to check for possible outliers.

A further filtering was performed by VOC concentration above 1 ppbv, which finally recorded only 89 mass peaks in breath dataset and 85 mass peaks in saliva dataset. Then data were analysed by PCA among baseline week (BL), intervention week (INT) and control week (CT) (**Figure 2**). The score plot of PCA components revealed no systematic difference.



(a) (b)

Figure 2. PCA score plot (VOC concentration above 1 ppbv). (a) Saliva VOC. (b) Breath VOC.

3.3.2 Tukey's honest significant difference (HSD) test

Data of VOCs in saliva and breath with concentration above 1 ppbv were filtered by Tukey's HSD test. Upon Bonferroni correction's post hoc comparison test, with respect to its trace level, no significant differences were found among pre-filtered dataset. Setting significance at p=0.05, 33 mass peaks of saliva VOCs and 36 mass peaks of breath were finally selected.

Among the selected peaks, 6 mass peaks in saliva and 6 mass peaks in breath were either identified by GC/MS or referred by literature (**Table 3**). For instance, the one with m/z 33.0338 (p = 0.030) in saliva was identified as methanol (by GC/MS), although its counterpart m/z 33.0342 in breath showed no significant difference (p = 0.235). Boxplot of

salivary methanol during the protocol was shown in **Figure 3**. Data indicated a significant difference of salivary methanol between BL and INT week.

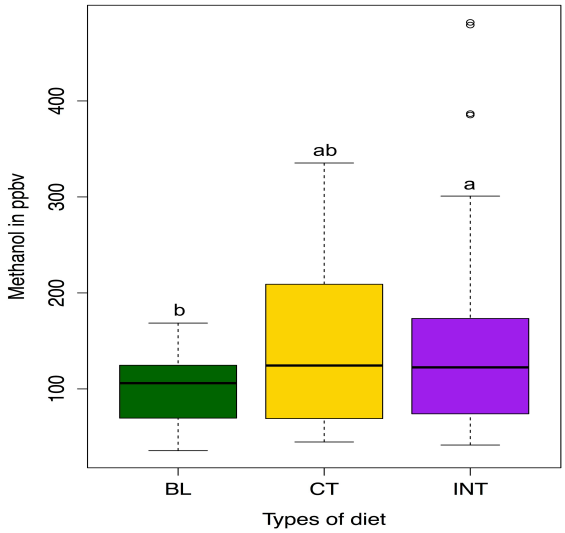


Figure 3. Boxplot of salivary methanol.

Table 3: Concentrations (Mean ± SEM, ppbv) of tentatively identified mass peaks in saliva and breath detected by PTR-ToF-MS. Mean concentrations are reported for each sample with Tukey letters ($p < 0.05$) showing the significant difference among dietary treatments. p-values were obtained on the basis of Tukey's HSD.

Sample	Measured mass (m/z)	Tentative identification	BL	INT	CT	p-Value
Saliva VOC	33.0338	Methanol	99.3 ± 9.1^{b}	163.9 ± 34.8^{a}	153.2 ± 25.7^{ab}	0.030
	47.0130	Formic acid	8.89 ± 0.23^{a}	5.79 ± 0.54^{b}	5.07 ± 0.53^{b}	$<10^{-3}$
	59.0490	Acetone	297 ± 52.9^{b}	343 ± 70.3^{b}	775 ± 273.5^{a}	0.008
	69.0703	Isoprene	4.35 ± 0.48^{a}	2.93 ± 0.33^{b}	2.93 ± 0.39^{b}	0.001
	87.0813	Pentanal/ 3-methyl-3-buten-1-ol *(t)*	1.35 ± 0.16^{a}	0.87 ± 0.13^{b}	1.01 ± 0.17^{ab}	0.006
	103.0780	Ethyl propanoate/ Methyl isobutyrate *(t)*	3.83 ± 1.32^{a}	1.02 ± 0.31^{b}	1.57 ± 0.74^{b}	0.005
Breath VOC	31.0189	Formaldehyde	0.69 ± 0.06^{b}	1.17 ± 0.15^{a}	1.48 ± 0.25^{a}	$<10^{-3}$
	47.0143	Formic acid	5.21 ± 0.35^{b}	6.76 ± 0.55^{a}	6.67 ± 0.67^{a}	0.007
	59.0475	Acetone	1497 ± 206^{b}	1439 ± 171^{b}	2896 ± 821^{a}	0.006
	63.0279	Dimethyl sulphide	7.04 ± 0.79^{b}	12.63 ± 2.44^{a}	8.76 ± 1.28^{ab}	0.004
	69.0713	Isoprene	91.1 ± 4.26^{b}	178 ± 31.7^{a}	181 ± 19.4^{a}	$<10^{-3}$
	87.0477	2,3-butanedione	1.32 ± 0.06^{b}	1.56 ± 0.09^{ab}	1.73 ± 0.18^{a}	0.004

3.3.3 Spearman's Rank correlation coefficient

Spearman's Rank Correlation Coefficient analysis was performed among measured mass peaks. Interestingly, three groups of VOC indicated significant correlation.

The first group consisted of the measured mass m/z 33.034, m/z 51.045 and m/z 31.018. They were tentatively identified as methanol, methanol water cluster and formaldehyde,

respectively. In saliva VOC dataset, those three masses showed significant correlation. Similarly, among them, a strong significant correlation was found in breath dataset. Moreover, data analysis revealed significant correlations between VOC in saliva and in breath. For instance, correlation between methanol in saliva and in breath showed a strong link (Rho = 0.537, $p < 10^{-3}$). The correlations were summarized in **Table 4**. The determined concentration of formaldehyde in breath was at the level of its instrumental limit of quantitation (LOQ = 0.96 ppbv), therefore, it was excluded in this analysis.

Table 4: Spearman's Rank Correlation between VOCs in saliva and in breath

VOC		Spearman correlation	Methanol (CH_5O^+)	
			Saliva	Breath
Methanol (CH_5O^+)	Saliva	Rho	——	0.537^{**}
		Sig. (2-code)		$<10^{-3}$
	Breath	Rho	0.537^{**}	——
		Sig. (2-code)	$<10^{-3}$	
Formaldehyde (CH_3O^+)	Saliva	Rho	0.369^{*}	0.21
		Sig. (2-code)	0.02	0.18

* p-value < 0.05.
** p-value < 0.01.

To better understand the correlation between methanol in saliva and methanol in breath, the tentatively identified methanol (m/z 33.034) and methanol water cluster (m/z 51.044), their concentrations were summed for each specimen (i.e. saliva and breath) and it was indicated a strong Spearmen's rank correlation (Rho = 0.546, $p < 10^{-3}$).

The concentrations of ethanol, acetone, propanol and dimethyl sulphide of saliva and breath were significantly correlated (**Table 5**).

Table 5: Spearman's Rank Correlation between VOC in saliva and in breath

VOC	Spearman correlation between VOC in saliva and in breath	
Ethanol	Rho	0.368^{*}
	Sig. (2-code)	0.016
Acetone	Rho	0.746^{**}
	Sig. (2-code)	$<10^{-3}$
Propanol	Rho	0.605^{**}
	Sig. (2-code)	$<10^{-3}$

* p-value < 0.05.
** p-value < 0.01.

Lastly, a group of short chain fatty acids (SCFAs), including acetic acid, propanoic acid and butanoic acid. No links were shown between SCFA in saliva and breath. Neither was a

correlation found among SCFAs in breath. But links were found in saliva dataset (**Table 6**).

Table 6: Spearman's Rank Correlation Coefficient of VOC (short chain fatty acids) in saliva

VOC	Spearman correlation	Acetic acid	Propanoic acid	Butanoic acid
Acetic acid	Rho	——	0.879^{**}	0.865^{**}
	Sig. (2-code)		$<10^{-3}$	$<10^{-3}$
Propanoic acid	Rho	0.879^{**}	——	0.899^{**}
	Sig. (2-code)	$<10^{-3}$		$<10^{-3}$
Butanoic acid	Rho	0.865^{**}	0.899^{**}	——
	Sig. (2-code)	$<10^{-3}$	$<10^{-3}$	

* p-value < 0.05.
** p-value < 0.01.

4. Discussion

To the best of our knowledge, this is the first attempt to investigate the impact of the consumption of blueberry polyphenols on whole VOCs in both saliva and breath.

Data showed that a daily consumption of 200 g blueberries did not modify the VOC fingerprints of saliva and breath, and did not significantly impact any specific single VOC in saliva and breath of the 14 subjects who participated in the study.

Interestingly, data on breath VOCs obtained by PTR-ToF-MS indicated that some VOCs could derive from the metabolism of dietary nutrients. For instance, phenol was the major tyrosine metabolite produced in casein and peptide fermentations, and indole was the sole product of tryptophan metabolism forming from the free amino acid (Smith and Macfarlane, 1997). Moreover polyphenols can be fermented by the primary degrader bacteria resulting in the release of a wide range of SCFAs (mainly acetate, propionate and butyrate) and a number of other metabolites (lactate, pyruvate, ethanol, succinate, soluble oligosaccharides, gases) that are the "fuel" for secondary degrader bacteria (Duda-Chodak et al., 2015). These processes are tightly controlled by environmental factors such as pH and carbohydrate availability, and this ultimately influences the types and amounts of fermentation products that can be formed in different regions of the large bowel. Similarly, dimethyl sulfide was the product from methionine metabolism, which is dependent from intestinal bacteria (Chen et al, 1970; (Ajibola et al., 2013) and isoprene could derive from cholesterol metabolism that is in turn highly impacted by individual exercise, age, haemodialysis and stain therapy (Gelmont et al, 1981; (Karl et al., 2001; Stone et al., 1993b).

Unfortunately, the high inter-individual variability among individual diets during the study (except few dietary indications) did not allow to find associations between these VOCs and the consumption of specific foods.

On the contrary this association was evident for methanol that was found to increase in saliva after the intervention week compared to the baseline week.

Methanol naturally occurs in human blood, urine, saliva and breath, together with formic acid. The two most important sources of body burdens for methanol and formic acid are diet and mainly the intake of fresh fruit, vegetables and fruit juices and metabolic processes. Hence, after consuming blueberries and other fruits, the concentration of methanol in the human body could increase because of the degradation of natural pectin (esterified with methyl alcohol) in the human colon (Lindinger et al., 1997). The metabolism of methanol occurs primarily in the liver, through a series of oxidative steps to formaldehyde, formic acid and carbon dioxide (WHO, 1997; Woods, 1999). This might explain the significant correlations between methanol and formaldehyde in saliva and in breath.

Interestingly in this study, besides methanol and formaldehyde, several other VOCs (such as acetone and propanol) showing strong links between their respective levels in saliva and breath, were found. It is known that metabolites, which derive either from physiological metabolism or from gut microbiota processing, finally diffuse in blood and circulate in human body. So in whole saliva, a number of constituents, including some VOCs, derive from circulating serum (sero-salivary constituents) (Forde et al., 2006). Likewise, VOCs transferred into blood are transported to lung and are exchanged at alveoli into exhalation. Thus to discuss the correlations of VOCs in saliva and in breath, the diffusion of substances from blood into saliva, as well as their solubility in saliva, becomes a key factor. For example, alcohols in serum, including methanol, ethanol and propanol, are unionized, non-protein-bound and lipid soluble, with a low molecular weight (Kaufman and Lamster, 2002; Yoshizawa et al., 2013), allowing them to readily diffuse into saliva and then to volatilize during headspace injection. On the contrary, VOCs with low solubility in saliva, such as isoprene, with much higher concentration in breath (120 ~ 180 ppbv) than that of in saliva (2 ~ 4 ppbv), showed no correlation between two types of specimens.

Furthermore, significant correlations among SCFA (i.e. acetic acid, propanoic acid and butanoic acid) were found only in saliva probably because of saliva buffer capacity, which allows saliva to neutralize large amounts of hydrogen or hydroxyl ions without showing

appreciable changes in the pH (Clark and Kenneth L. Carter, 1927), i.e. to 'accumulate' more acids and then release them out during incubation. In fact, the mean concentrations of SCFA released from saliva are all higher than SCFA in breath.

Large numbers of studies on saliva VOCs have been done in the past decade. Analysis of saliva offers great advantages since its collection is convenient and non-invasive and it is easy to be handled and stored. Therefore, the discovery of correlations of VOCs in saliva and in breath found in this study might provide another approach to assess the influence of dietary compounds on human health.

Another strong point of the design of this study was to align subjects to a low intake of polyphenol-rich foods in the preliminary baseline week and in the control week. This choice reduced the impact of other dietary polyphenols but blueberry polyphenols on the outcomes and might allow us to majorly address the potential findings to the blueberry polyphenols intake. In fact the main difficulty in approaching studies of the effect of polyphenols on health is often due to the wide range of different phenolic compounds in foods (Cheynier, 2005), together with their high variability of bioavailability and bioactivity (Scalbert et al., 2005), as well as the complex relationship established between these compounds and the intestinal microbiota (Etxeberria et al., 2013b) and other food components such as fibres (Cuervo et al., 2014; Valdes et al., 2015).

This study has also some limitations including the quite low number of subjects, the duration of intervention and the absence of a washout period between intervention and control week. These factors due to the high inter-individual variability of VOC profiles in breath and saliva might have influenced the lack of effect found by blueberry consumption (Kemperman et al., 2010). However, it is also worth to notice that increasing the duration of the studies is the main cause of increased dropouts and reduced study compliance by the participants (Queipo-Ortuño, 2012).

5. Conclusions

In this pilot study for the first time the impact of a daily consumption of 200 g of blueberries (providing 440 mg of polyphenols) on VOCs profile of saliva and breath was tested in healthy subjects.

All in all data of this study demonstrated that:

- One week consumption of blueberries did not influence breath and salivary VOCs

profile in fasting subjects;

- Salivary methanol increased after blueberry intervention compared to a low polyphenol diet;
- Five VOCs including methanol, formaldehyde, ethanol, acetone and propanol were significantly correlated between saliva and breath;
- Salivary concentrations of short chain fatty acids including acetic acid, propanoic acid and butanoic acid were significantly associated.

The exact mechanisms of action of polyphenols on the human gut microbial ecosystem have not yet been fully established and further studies with longer intervention period are needed to demonstrate in humans the link between breath and saliva VOCs with gut microbiota composition, human metabolism of polyphenols and individual health status.

Acknowledgement

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Chapter 4. Summary, conclusions and perspectives

1. Summary and conclusions

In this thesis we discussed the possible 'cross-talk' between the brain, intestine and specific foods components (i.e. dietary fibres and blueberry polyphenols) using the analysis of saliva and breath composition as non-invasive methods (**Figure 1**).

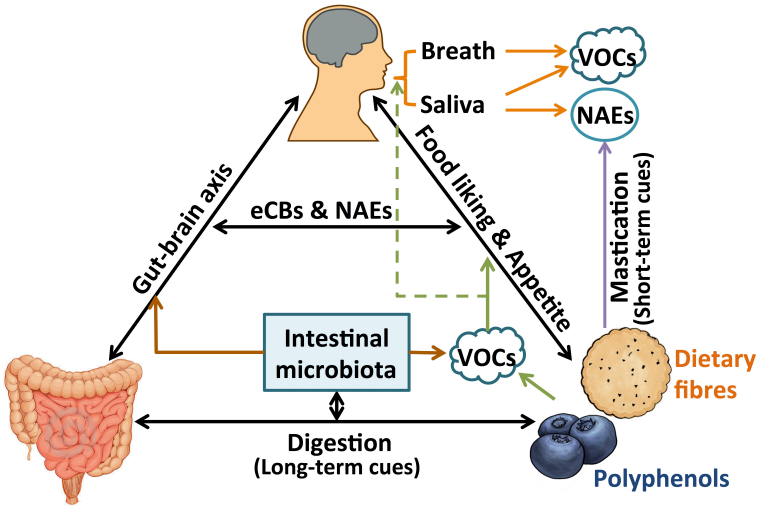

Figure 1. "Cross-talk" among brain, intestine and foods

The thesis starts with a general introduction of the gut-brain axis as well as its research description of its mechanisms and biomarkers (e.g. eCBs and NAEs) together with possible directions to develop new foods and healthy diets for human (**Chapter 1**).

In **Chapter 2**, based on short-term cues in this thesis, the pioneering mastication study investigated the concentration of NAEs in stimulated saliva. To the best of our knowledge, no previous investigation has studied the saliva composition in the immediate post-prandial phase. Interestingly, it was demonstrated that salivary NAEs concentration increased only upon food mastication instead of mastication itself in healthy, normal-weight subjects. Moreover an association between OEA and glucose in chewed food was found in control biscuit (CB) and whole-wheat bran biscuit (WWBB) but not in β-glucan biscuits (βGB) thus allowing us to hypothesize that the enzymatic activity of saliva and the gel-forming ability of β-glucan (βGB) might affect the link between OEA and glucose and, as a consequence, the learning processes behind food liking.

We also discussed the post-prandial NAEs in saliva in **Chapter 2**. Salivary OEA was associated with fullness and satiety sensations as well as the actual liking of the biscuits for sweetness (Matias et al., 2012). It is worth mentioning that some associations were found between the salivary OEA and the liking of the biscuits (sweetness and overall palatability) as

well as between the fullness and satiety sensations in the post-prandial phases (at 15 min and 30 min). Consequently, these links might hint at a possible involvement of salivary OEA in the sensory mechanisms during the post-ingestive phase, as well as its potential role upon food preference and individual choice (Yeomans, 2006).

In addition, compared to CB, no effect of dietary fibre consumption was found in: 1) NAEs oral release during mastication; 2) the satiation in fasting subjects; and 3) the postprandial blood glucose. An overall graphical summary of this chapter was shown in **Figure 2**.

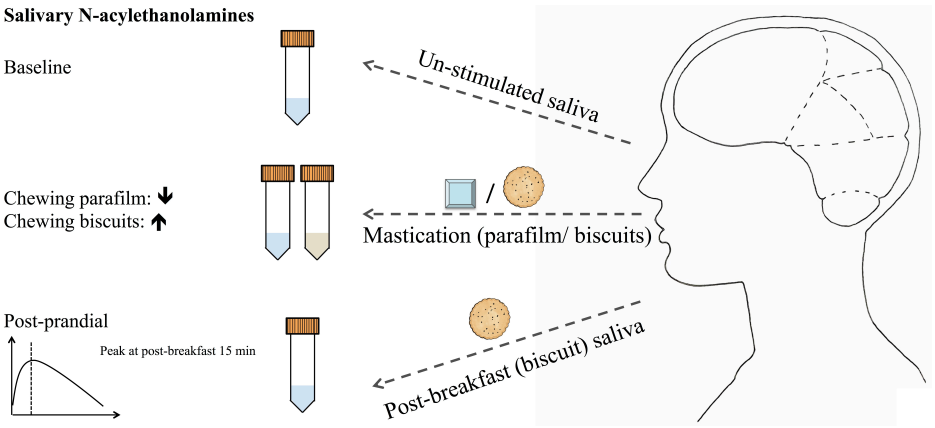

Figure 2. The graphical summary of Chapter 2: salivary NAEs during mastication and post-prandial phase.

Chapter 3 described the first pilot study that was performed to investigate the influence of blueberry polyphenols (long-term cues) on whole VOCs in both saliva and breath among 14 healthy subjects. Data indicated that a daily consumption of 200 g blueberries (providing 440 mg polyphenols) neither modified the VOC fingerprints of saliva and breath nor significantly impacted any single, specific VOC. Nevertheless, salivary methanol increased after blueberry intervention compared to a low polyphenol diet, due to the degradation of natural pectin (esterified with methyl alcohol) in the human colon (Lindinger et al., 1997). Furthermore, intra-individual analysis between the saliva and the breath showed interesting effects between known metabolites.

Worth of notice is that five VOCs including methanol, formaldehyde, ethanol, acetone and propanol were significantly correlated between saliva and breath. Additionally, the salivary concentrations of short chain fatty acids, including acetic acid, propanoic acid and butanoic acid, were significantly correlated. **Figure 3** gives an overall graphical summary of this chapter.

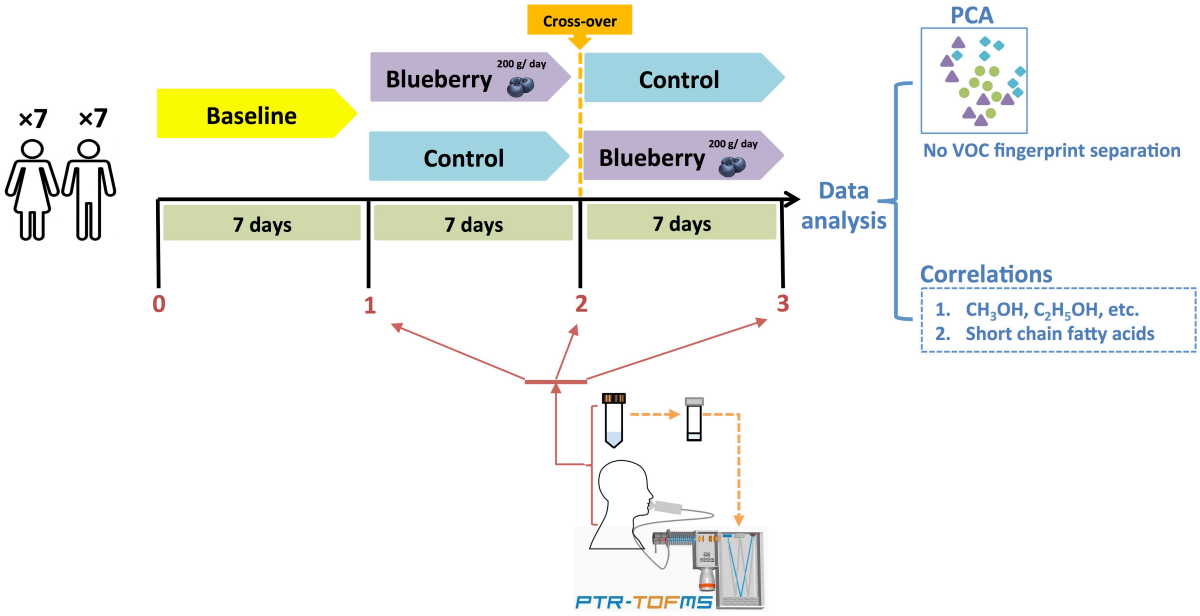

Figure 3. The graphical summary of Chapter 3: Impact of blueberry polyphenols diet (7 days) on VOCs in saliva and in breath.

2. Future perspectives

Both studies described in this thesis aimed to explore the influence of food components (i.e. dietary fibres and blueberry polyphenols) on saliva composition in humans.

In the future, more knowledge of the mechanism of NAEs oral-release can be obtained by further investigating the chewing effect of different types of foods such as biscuits (solid) or puddings (semi-solid) also considering the addition of an aroma. In fact, the flavours were proved to enhance the satiety and this effect could be mediated by eCBs, NAEs and gut hormones (Ruijschop et al., 2009a; Ruijschop et al., 2009b; Ruuschop et al., 2009). In addition, it may be of interest to design some studies to demonstrate the food origin of NAEs in the mouth upon different types of foods and to clarify the mechanisms behind this formation.

In **chapter 3**, we have illustrated that the effect of a one-week intervention with a low daily dose of polyphenols on whole VOCs in saliva and breath. However, despite much evidence from animal studies, only a few human intervention studies could prove the association between polyphenol intake and metabolic biomarkers in humans (Vitaglione et al., 2015).Of note is that most of the studies specifically focused on the impact of dietary polyphenols or specific compounds on the human gut microbiota or selected bacterial populations. Furthermore, little is known about the effective dose during a polyphenol intervention study *in vivo*.

In this respect a longer intervention study with blueberry polyphenols (at least one month with a daily intake of 300 to 400 g blueberry), and a 'wash-out' period (at least one week) between diets-arm are recommended. In addition, a simultaneous evaluation of the gut microbiota and gut hormones is warranted to get a clearer understanding of the effect of polyphenol on intestine microbiota and human metabolism.

Will a prolonged intervention elicit the modulation of intestinal microbiota and their metabolism? Will there be associations between the VOC fingerprints in the saliva, the breath and the regulation of gut flora? What are the principal contributors linked to specific intestine microbiota? These are the import questions for future investigations and their results thereof will most likely affect the health strategy aiming for the guidance of diet, in addition to the concept of non-invasive evaluation of the effects of functional supplements.

3. Final note

High correlations were found between the salivary and blood concentrations of relevant components (Nagler et al., 2015). Saliva, to some extent, 'shares' the biochemical and metabolically active information in the blood and thus could be considered as a potential non-invasive easy-handling choice to study human physiological and pathological conditions (Al-Kateb et al., 2013). In this thesis, we investigated saliva components (NAEs), sensory response (food liking) and satiety, not only upon mastication but also after consumption of dietary fibre enriched biscuits. Additionally in the blueberry polyphenols human intervention study, we evaluated the whole and specific VOC profile in saliva in comparison to VOC in the breath.

We propose that a better mechanism understanding the saliva composition is needed to aid the development of new types of foods and the proposal of a healthy diet. Hopefully, the ensuing emergence of new technologies can enable saliva to be used as a rapid trace-level assessment of dietary intervention both in the short-term and in the long-term.

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Abstract (Italiano)

Il principale obiettivo della tesi è stato quello di esaminare il possibile "*cross-talk*" tra il cervello, l'intestino e componenti specifici degli alimenti (fibre alimentari e polifenoli dei mirtilli). Questo obiettivo potrebbe essere raggiunto attraverso uno studio a breve termine e un intervento a lungo termine nell'uomo, con analisi non-invasive sulla composizione della saliva e del respiro.

Lo studio a breve termine è stato proposto per valutare l'impatto delle fibre alimentari sui NAEs della saliva durante la masticazione e nella fase post-prandiale. A tal scopo, sono stati preparati tre tipi di biscotti: arricchiti con il 3% di β-glucani (βGB) dell'orzo o con crusca di frumento (WWBB) o senza fibra alimentare (controllo, CB).

Uno studio randomizzato nell'uomo è stato condotto su diciotto partecipanti. I campioni di saliva sono stati raccolti da soggetti in condizioni di riposo, subito dopo la masticazione di parafilm e dopo il consumo di uno dei tre biscotti.

Successivamente, è stata misurata la quantità di biscotti consumata in una colazione *ad-libitum.* Nelle due ore successive è stata raccolta la saliva post prandiale ed è stato misurato il livello di glucosio nel sangue, inoltre, attraverso la compilazione di questionari, sono stati valutati l'appetito ed il gradimento per l'alimento.

I dati hanno dimostrato che la concentrazione salivare di NAEs è aumentata solo dopo la masticazione dell'alimento, ciò indipendentemente dalla composizione delle fibre dell'alimento.

Il tipo di biscotto non ha influenzato l'appetito individuale e i livelli di glucosio nel sangue dopo il pasto; al contrario ha influenzato la persistenza dei NAEs nella saliva entro 30 minuti dal consumo. Studi futuri spiegheranno i meccanismi alla base di queste evidenze e il ruolo dei NAEs salivari sul gradimento degli alimenti e sui segnali dell'appetito dopo il consumo di alimenti.

Lo scopo dello studio a lungo termine è stato quello di testare, per una settimana, l'influenza del consumo di mirtilli sui composti volatili organici (VOCs) del respiro e della saliva umana. Quattordici volontari sani hanno partecipato ad uno studio randomizzato controllato a singolo cieco. Questo ha avuto una durata di tre settimane.

Dopo la prima settimana, che corrispondeva al periodo basale (BL), con una dieta a basso contenuto di polifenoli, i soggetti sono stati indirizzati in maniera randomizzata ad un trattamento basato sulla stessa dieta (dieta controllo, CT) oppure basato sulla stessa dieta aggiunta di 200 g di mirtilli freschi al giorno per una settimana (INT). Nella settimana successiva i soggetti venivano sottoposti all'altro ramo di trattamento.

Alla fine di ogni settimana i soggetti a digiuno hanno raggiunto il laboratorio per la raccolta dei campioni di saliva ed è stata effettuata un'analisi on-line del respiro con PTR-ToF-MS.

Dopo le diete INT e CT, non sono state riscontrate differenze nei *fingerprints* dei VOCs e nei singoli VOCs del respiro e della saliva.

Ciò nonostante, è stata mostrata una correlazione significativa tra la composizione della saliva e del respiro per il metanolo, la formaldeide, l'etanolo, l'acetone e il propanolo. Numerosi studi precedenti sono stati incentrati sulla composizione dei VOCs nel respiro di pazienti e di soggetti sani, ma pochi sono stati incentrati sui VOCs della saliva

Pertanto, in questo studio il legame tra i VOCs della saliva e del respiro può aprire una nuova strada di ricerca per chiarire i meccanismi alla base dell'effetto metabolico degli interventi alimentari sull'uomo.

Overview of completed training activities

Discipline specific activities	• 3rd MS Food Day; October 2013; Trento, Italy (Poster presentation) • 7th International Immunonutrition Workshop "Eating for Preventing"; May 2014; Carovigno, Italy (Poster presentation) • Molecular Physics Workshop; July 2015; Caen, France (Poster presentation) • 29th EFFoST International Conference "Food Science Research and Innovation: Delivering sustainable solutions to the global economy and society"; November 2015; Athens, Greece (Poster presentation) • 7th International PTR-MS Conference; February 2016; Innsbruck, Austria (Poster presentation)
General courses and trainings	• PhD course: Progettazione e gestione dei programmi di ricerca, October 2014; University of Naples • PhD course: Data analysis, July, 2014; University of Naples • PhD course: How to write a scientific paper and present experimental results, September 2014; University of Naples • Course in "Writing journal articles and proposals, general principles of scientific writing, and sustainable development communication", April 2014; University of Birmingham and Scriptoria Ltd., UK. • Course in "Basic management skills, effective scientific communications", November 2014; University of Birmingham and Scriptoria Ltd., UK. • Marie Curie PIMMS training in "Hands-on PTR-MS Training Session", October 2013; Ionicon, Innsbruck, Austria. • Marie Curie PIMMS training in "PTR-MS data analysis and multivariate statistics", October 2013; Fondazione Edmund Mach di San Michele all'Adige (FEM), Italy. • Marie Curie PIMMS training in "Instrumental Diagnostics", April 2014; Kore Technology Ltd., Ely, UK. • Marie Curie PIMMD training in "Materials and instruments spare parts design", November 2014; University of Birmingham and Kore Technology Ltd., UK.
Scientific visiting	• Marie Curie PIMMS secondment "PTR-ToF-MS analysis and Blueberry polyphenols intervention project", September to November 2013; Fondazione Edmund Mach di San Michele all'Adige (FEM), Italy. • Marie Curie PIMMS secondment "PTR-MS data analysis training", September to October 2015; Ionicon, Innsbruck, Austria. • Intern with training in "Analysis of food contaminants with GC-MS", August to September 2013; SOFIA GmbH, Berlin, Germany.

List of Publications

- Xianghui Kong, Rosalia Ferracane and Paola Vitaglione. Salivary concentration of N-acylethanolamines upon food mastication and after meal consumption: influence of food dietary fibre. Food Research International. (In submission)

- Xianghui Kong, Iuliia Khomenko, Luca Cappellin, Franco Biasioli, Vincenzo Fogliano, Alessandro Genovese, Paola Vitaglione. Impact of blueberry consumption on volatile organic compounds of human breath and saliva: a pilot study. (In submission)

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Figure 1. People who I would like to appreciate sincerely during my three-years of PhD study and Marie Curie ESR career in Europe. Supervisors: Paola and Vincenzo, who opened the 'gate' and lead me towards becoming a proper researcher; LABS: Colleagues of local laboratory, who gave me countless support and encouragement in work and in life, leaving me unforgettable memories; FEM: Secondment institute in San Michele all'Adige, the team who provided me the platform for my PhD project and numerous kind help on data analysis; PIMMS: Fellowship project, from which I gained well-organised training on career development as well as friendship among ESRs; IONICON: Secondment company in Austria, where I had many training sessions for my PhD project and precious suggestion for my future career; Eurofins SOFIA: Internship laboratory in Berlin, which kindly trained me in instrumental analysis.