A TECHNOLOGICAL AND PHYSIOLOGICAL INTEGRATED APPROACH FOR APPETITE CONTROL.

FROM IDENTIFICATION OF NOVEL BIOMARKERS TO DEVELOPMENT OF NEW FUNCTIONAL INGREDIENTS.

Ilario Mennella

Thesis committee

Promotor
Prof. Dr V. Fogliano
Professor of Food Quality and Design
Wageningen University

Co-promotor
Prof. Dr P. Vitaglione
Associate Professor of Human nutrition, Department of Agriculture and Food Science
University of Naples, Italy

Other members
Prof. Dr R.F. Witkamp, Wageningen University
Dr G. Jager, Wageningen University
Prof. M. Yeomans, University of Sussex, Brighton, United Kingdom
Dr D. Mela, Unilever R&D Vlaardingen

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Ilario Mennella
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“As we penetrate into matter, nature does not show us any isolated building blocks, but rather appears as a complicated web of relations between the various parts of the whole”.

F. Capra
The Tao of Physics

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Chapter 1. General introduction

Life is not possible without the maintenance of homeostatic processes (Schofield et al, 2014) defined as stationary states of non-equilibrium. In fact, biological systems reach a state of thermal and mechanical equilibrium with the external environment only when dead (Recordati et al, 2003). In these terms, energy homeostasis is the time dependent oscillatory pattern given by the total energy balance (the difference between the energy intake and the energy expenditure). Apart from a small proportion lost in the feces and urine, energy expenditure may be attributed to three main factors: physiological needs such as growth, pregnancy or lactation; physical activity, which can range from less than half to two times the basal energy expenditure (Pinheiro et al, 2011); and metabolic processes (Tseng et al, 2010). In the short term, even high fluctuations may be very well tolerated without cause any physiological impairment. On the one hand, body has mechanisms such that a low energy intake is followed by an increase in calorific efficiency which means a reduction of the energy expenditure (Padwal et al, 2007, Redman et al, 2009). On the other hand, excessive calorie intake is dissipated through the diet-induced thermogenesis that is a physiological mechanism allowing individuals to eat without gaining weight (Ravussin, 2002). On the contrary, long term unbalance between energy intake and energy expenditure result in malnutrition or overweight, which can have serious consequences on individual health.

According to World Health Organization (WHO, 2012), over the past three decades, the prevalence of obesity and overweight has increased substantially. In fact, this rapid prevalence increase and the health consequences related to obesity have turned it into one of the most serious health challenges of the last years. It was estimated that a total of 1.7 billion people are overweight while 310 millions are obese with an obesity growing rate in the poor countries three times bigger than 20 years ago (Demerath, 2012). Obesity and overweight pose a major risk for serious diet-related chronic diseases, including type-2 diabetes, cardiovascular disease, hypertension and stroke, and certain forms of cancer. The health consequences range from debilitating problems associated such as respiratory difficulties, chronic musculoskeletal problems, skin problems and infertility to shorter life expectancy, and to serious chronic conditions that reduce the overall quality of life (WHO, 2003). The WHO established category of underweight, overweight and obesity in adults using the body mass index (BMI), defined as the weight in kilograms divided by the square of the height in metres (kg/m2) (table 1). Besides the related health issue, individuals with a BMI greater than or equal to 30 accrued medical costs approximately 30% higher than those with BMIs lower than 25 (Withrow and Alter, 2011). For instance, in the United States approximately 300,000 people die annually from obesity-related causes and the associated morbidities account for a substantial amount of the national healthcare expenditure in the country (Samaranayake et al, 2012). Around

70% of type 2 diabetes appears to be related to a BMI higher than 25 kg/m². Hence, diabetes mellitus type 2 appears to be the most common obesity-related comorbidity and the one that is likely to cause the greatest health burden (Avenell et al, 2004).

Table 1. WHO classification of adult underweight, overweight and obesity according to BMI

Classification	BMI(kg/m²)	
	Principal cut-off points	Additional cut-off points
Underweight	**<18.50**	**<18.50**
Severe thinness	<16.00	<16.00
Moderate thinness	16.00 - 16.99	16.00 - 16.99
Mild thinness	17.00 - 18.49	17.00 - 18.49
Normal range	**18.50 - 24.99**	**18.50 - 22.99**
		23.00 - 24.99
Overweight	**≥25.00**	**≥25.00**
Pre-obese	25.00 - 29.99	25.00 - 27.49
		27.50 - 29.99
Obese	**≥30.00**	**≥30.00**
Obese class I	30.00 - 34.99	30.00 - 32.49
		32.50 - 34.99
Obese class II	35.00 - 39.99	35.00 - 37.49
		37.50 - 39.99
Obese class III	≥40.00	≥40.00

Source: Adapted from WHO, 1995, WHO, 2000 and WHO 2004.

An "obesogenic environment" can be characterized as comprising the ready availability and consequent high consumption of high-energy foods in a globalized economy, coupled with increasing sedentary lifestyles (WHO, 2007). The increased availability of high calorie-foods coupled to the diffusion of sedentary activities in Western Countries, where people were historically habituated to restrictive dietary conditions and to a very active life, have conferred a great burden of overconsumption and obesity (Shapira, 2013).

Diet and weight regain

As the laws of thermodynamics must be obeyed, all the treatments for body weight control and body weight loss may be ultimately grouped in three main groups: treatments for reduction of total

energy intake, treatments to increase energy expenditure or treatments which can combine both (Tseng et al, 2010).

Weight loss is easily achieved with calorie restrictive short term dietary regimes. Body weight management over time and after weight loss is much more difficult to be achieved. According to different definitions of weight maintenance the percentage of people who successfully maintain weight loss varies from 3% up to 28% (Kramer et al, 1989; Christiansen et al, 2007). Several physiological changes which accompany diet-induced weight loss can explain the difficulties in maintaining body weight after weight loss. Mainly, a reduction of body weight entails changes in total energy expenditure and substrate metabolism. The total energy expenditure is lowered by change in resting and non-resting energy expenditure, whereas regarding fuel utilization there is a reduction of fat oxidation and a concomitantly increase of carbohydrate oxidation (Leibel et al, 1995; MacLean et al, 2004). Other physiological changes involve the suppression of the hypothalamic–pituitary–thyroid axis, which is responsible to boost adaptive thermogenesis, and the alteration of gastrointestinal hormones, which collectively promote weight regain and restoration of energy balance (Mitchell et al, 2010; Sumithran et al, 2013).

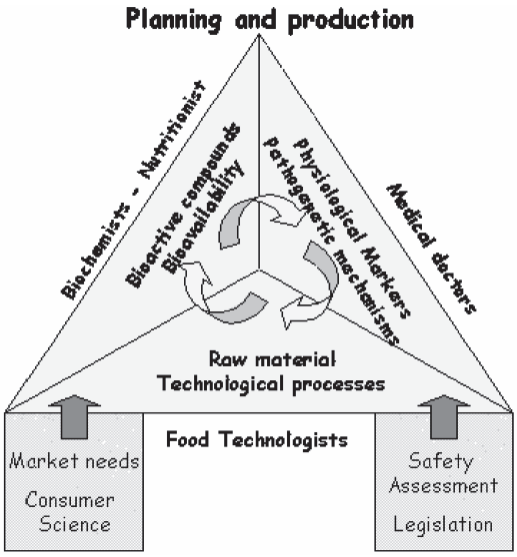
Even though a sedentary lifestyle is an important cause of the obesity epidemic, it is not so obvious that a moderate physical activity may help body weight control. In fact, there are good evidences that long term exercise alone produces only a modest weight loss and in combination with diet does not result always in an higher weight reduction (Catenacci et al, 2007). Moreover, a moderate physical activity cannot limit a weight regain during weight maintenance (Martins et al, 2008). This evidence can be completely understood only taking into account the three main factors that regulate the eating behavior: restraint, disinhibition and hunger. Restraint is the control of food intake by thought or cognitive restraint to eating, disinhibition is the inability to resist food cues and hunger is the feeling to general hunger triggered by internal and external cues (Stunkard et al, 1985). In 1995 Hill and co-workers described a model where the overall energy balance was driven by the restraint and disinhibition traits. Energy expenditure due to physical activity would be completely compensated in unrestrained lean subjects, but not in unrestrained overweight. Restrained individuals with a low levels of disinhibition would not compensate energy expenditure from exercise with an higher energy intake, but restrained individuals with high levels of disinhibition would (Hill et al, 1995). The eating behavior is a key factor even during weight loss and weight maintenance. Subjects who increase their restraint levels obtain better results in either weight loss during a diet or during the weight maintenance (Vogels et al, 2005).

EFSA guidelines for health claims related to appetite control

The increasing population with excessive body weight and the ineffectiveness of dietary regimes to avoid weight cycling and relapse of body weight stimulates the demand of product for appetite control, weight control and weight loss.

Several drugs have been developed to reduce energy intake such as the Phentermine which should increase satiety (Kaplan, 2005) or Orlistat, acting as enzymatic inhibitor of lipase and thus reducing dietary fat absorption. Unfortunately, drugs may have also some side effects on human health. A striking example is the case of the Sibutramine, marketed until 2010 as an anorexic drug, prescribed for obesity in association to diet and exercise. Cause of cardiovascular events and strokes (Zhou et al, 2012), it was finally withdrawn from the market in several countries including China and the European Union.

On the contrary, the health potential of foods is well perceived by European consumers. The development of functional foods for the prevention and/or treatment of obesity represents the direct answer that food companies could give to this request of "health by food" for body weight control. (Fogliano et al, 2005; Trigueros et al, 2013.) The Functional Food Science in Europe (FUFOSE) proposed that a food to be defined as functional must combine basic nutritional properties to beneficial effects on one or more functions on human organism thus improving the general and physical conditions or/and decreasing the risk of diseases. Moreover, it must not be in the form of pill or capsule but in a normal food form (Siro et al, 2008). Therefore, in order to the design an innovative and effective functional food it is necessary to combine expertise from different areas such as food technology, physiology, marketing. **Figure 1.**



Source: from Fogliano V, Vitaglione P 2005 . Mol. Nutr. Food Res. 2005, 49, 256 – 262

Figure 1. Disciplines and professional figures involved in functional food planning and development.

In this context the market of food products for body weight control represents a huge opportunity for the food industry, since the value of this market is enormous and growing (Marketdata Enterprises, 2010; Market Research.com, 2009). The number of the products available within national markets is enormous and the offer on internet is immeasurable. Unfortunately, the evidence of effectiveness of these products for the claimed activity are often very weak. It often happens that the claims stated on the labels are not supported by scientific data or the evidences are limited to *in vitro* or animal studies.

However, since in 2006 the European Commission adopted the regulation 1924/2006 on the use of "nutrition and health claims made on foods", the European market of functional foods is systematically evaluated and regulated (EFSA, 2006).

The first aim of this regulation is to ensure a high level of protection for consumers, and to facilitate their choice among the increasing number of foods having nutrition or health claims. To this purpose, the principle is that products put on the market, including imported products, should be safe and adequately labelled.

A secondary objective is to make the use of nutrition and health claims across the single market inboard the Community more homogeneous. Each claim should be scientifically substantiated by taking into account the totality of the available scientific data, and by weighing the evidence. Two different categories of claims were identified: *function claims*, for which the beneficial physiological effect may relate to maintenance or improvement of a function, and *reduction of disease risk claims*, for which the beneficial effect refers to a reduction (or beneficial alteration) of a risk factor for the development of a human disease (not a general reduction of the risk of disease).

By June 2011 the European Food Safety Authority (EFSA) evaluated almost 3000 claims, but the vast majority of them were rejected (Halford et al, 2012). This data, pose a major issue in the formulation of healthy foods: more quality and innovation needed in the design of products in order to have an health or a nutrition claim approved by the EC.

EFSA identified some common elements of weakness in the presentation of an healthy claim. First of all it underlined that to develop a food which can be approved for an healthy claim, a special attention has to be given to the population group (the target) for which health claims are intended. Often, candidate products lack in effectiveness because the target group is not well defined. A functional food may be developed for the general (healthy) population or specific subgroups thereof, for example, elderly people, physically active subjects, or pregnant women. For instance, where a health claim relates to a function which may be associated with a disease risk, patients with that disease are not the target for the claim. Therefore, the principle is that a food cannot have a therapeutic effect, but it can only be used to improve a physiological function.

Another key factor to develop a successful functional food is to characterize properly the constituent from which the claim comes from. Characterization should also be sufficient to allow definition of appropriate conditions of use. It may be necessary to distinguish between a specific formulation or a specific constituent/combination of constituents.

Finally, in assessing each specific food/health relationship, a cause and effect relationship has to be established between the consumption of the food/constituent and the claimed effect. The quantity and the frequency of food consumption required to obtain the claimed effect should reasonably be consumed within a balanced diet and realistically reflect its consumption pattern. For instance an enriched milk may be consumed daily, whereas an entrée may be consumed less frequently (EFSA, 2011).

In order to further assist applicants in preparing and submitting their applications for the authorization of health claims, EFSA developed guidance documents about the scientific requirements for the substantiation of health claims in selected areas. Specifically to the substantiation of health claims related to appetite ratings and the weight management, EFSA published a guidance in 2012 with two key issues: claimed effects which are considered to be beneficial physiological effects; studies-outcome measures which are considered to be appropriate for the substantiation of health claims (EFSA, 2012). However, the same document clarifies that it does not provide an exhaustive list of the beneficial effects and studies/outcome measures which are acceptable, but others considerations can be accepted.

Taking into account the complexity of a health claim, it is remarked that each claim will be considered in the context of a specific application. This concept is extremely important as it highlights the uniqueness of each functional food: the ingredient from which the functionality is expected, a clearly defined population group and the food matrix have to be evaluated all together for the claimed beneficial effect. Regarding the studies and outcomes, several aspects has to be considered in order to assess whether the provided studies are properly conducted and pertinent. The study must be carried out with the compounds for which the claim is made, with a good design and appropriate outcomes measures and in a study group representative of the population group for which the claim is intended.

In the context of body weight reduction, claims on appetite ratings (increase in satiety/reduction in hunger) have been proposed. The scientific evidence for an effect on appetite ratings have to be obtained from human intervention studies whereas animal or *in vitro* studies may be used as support for the human studies. They are central for the substantiation of the claim; if there are no human studies which are pertinent to the claim a cause and relation effect between the food constituent and the benefic effect cannot be established. According to the EFSA hierarchy of evidence, randomized controlled trials are the most robust studies which can be conducted to support the substantiation of the claim (EFSA, 2007). Moreover, the measurement of the behavioral response has to be done using methods with appropriate validity and precision: the golden standards for measuring the effects of food properties on appetite are ratings made on visual analogue scales (VAS). However, the reduction of appetite ratings cannot be considered as beneficial physiological effects per se: eating motivations have to show a relationship with the subsequent energy intake. The use of clearly recognized biochemical markers may be used only as a supportive materials of a satiety claim in the context of behavioral assessment. Finally, to be suitable for an health claim for appetite control, a food has to show sustainable and reproducible effects. The term sustainability has been sometimes

interpreted as the persistence of the effect after discontinuation of the food. Instead, EFSA intends the absence of any adaptation, through compensatory mechanisms or attrition, which entail the maintenance of the effect over time during continuous consumption of the food. The reproducibility of the effect is indicated by the consistency of results between studies. With regard to appetite control, a food has to demonstrate a continuous effect during repeated dosing over a minimum of 28 days (Halford et al, 2012).

Designing an effective food for appetite control

The homeostatic physiological mechanisms that evolved with human history, allowing the survival of the species during periods of famine, are now crucial to cope with a modern, westernized diet and lifestyle. Significant cognitive processes are required to consciously control our energy balance reducing energy intake through a reduced-calorie diet and increasing our energy expenditure through physical exercise. However, this conscious control fails in most individuals such as unrestrained people (Vogels 2005). Therefore, it is necessary to regulate the mechanisms that control our feeding habits, such as hunger, satiation, and satiety to obtain an unconscious food intake control. To achieve this objective novel food processing methods to modify food structure have been developed.

For example, aeration of foods have been demonstrated an effective technology to reduce energy intakes decreasing the energy content per volume of food ingested (Rolls et al. 2000; Osterholt et al. 2007). Gastric stretch receptors detect the distension of the stomach stimulating neuronal pathways to trigger satiation and reduce hunger. Therefore, the incorporation of air into food provides a valuable strategy to reduce energy intake especially from energy-dense foods.

A popular approach to slow down the gastric emptying and prolong feeling of fullness for as long as possible is to increase the viscosity of food ingested. Several food fibres are used to this aim such as β-glucan, lupin kernel fiber, rye bran, whole grain rye. Their effectiveness to enhance satiety was shown in more than one publication (Clark et al. 2013). Unfortunately, fibre-rich products are not appealing in terms of taste and therefore they may lead to a subsequent food overconsumption.

Among promising targets "ileal brake" received great attention in the last decade. The presence of nutrients in the distal ileum stimulates the production of satiating peptides such as PYY and GLP-1. An effective strategy to induce feelings of satiety and reduce energy intake is to slow the macronutrients breakdown and hydrolysis (Wilde 2009). Micro and nano-encapsulation have been demonstrated a reliable technology to slow the nutrients digestion and to deliver nutrient directly in

the distal ileum (Kuang et al. 2010). Microencapsulation is described as a process of enclosing micron-sized particles of solids or droplets of liquids or gasses in an inert shell, which in turn isolates and protects them from the external environment (Ghosh 2006). It was developed by the pharmaceutical industry to improve the bioavailability of drugs, to control drug release kinetics, to minimize drug side effects, and to mask the bitter taste of drug substances. Then microencapsulation has been extended to the food industry also to protect some compounds/ingredients unstable to processing (Kuang et al. 2010).

In the perspective of developing ingredients to target the ileal brake, a key to the success is to improve our understanding of the food digestion and food structures in order to develop food ingredients that can prolong digestion and reduce appetite. The behaviour of micro-particles per se or in foods during digestion can be tested using in vitro models in order to predict the satiating power of novel ingredients (Logan 2015).

Food intake: biochemichal control and assessment

Appetite sensations are a bridge between energy intake and expenditure. Hunger and satiety are the main psycho-physiological states which elicit or inhibit food intake determining meal by meal eating behavior. Hunger is defined as the motivation to seek and consume food initiating a feeding episode. It cannot be directly measurable, but can be inferred from modifications occurring in some organs, such as stomach or head, and from feelings such a weakness. (Blundell et al, 2010). On the opposite, satiety is defined as the feeling of fullness after a meal has finished. It leads to inhibition of further eating and therefore regulates the inter-meal food intake affecting the period of time between eating occasions (Benelam, 2009). Conversely, the intra-meal satiety, also called satiation, leads one to the termination of a meal thus affecting the meal size (Allirot et al, 2014). Since hunger, satiety and satiation are subjective constructs, it is not possible to obtain a direct measurement. Three common methods frequently used are: the measurement of subjective disposition to eat, measurement of food intake and measurement of biomarkers (Mattes et al, 2005).

The measure of appetite sensations through self-report scale, such as Visual Analogue Scales (VAS), before and in response to meals, are a reliable and a valid method to establish the subjective states of motivation to eat (Flint et al, 2000). They consist of questions that assess subjective appetite related sensations in a controlled setting. Normally, they are asked right before and after consuming a preload or a test meal, and then at regular time intervals and the answers are collected

on a 100 mm horizontal line, anchored at each end with opposing extremes of a specific scale (Blundell et al, 2009). (table 2). The study participants are instructed to make a mark on the line which corresponds to their feelings. The subjective sensation is quantified by measuring the distance from the left end of the line to the mark. They are noninvasive to participants and easy to use and found to be sensitive to experimental manipulation, reproducible in repeated protocols where the effect of different treatments are compared under similar circumstances and exhibit a good degree of within subject reliability (Livingstone et al, 2000).

Table 2. Appetite sensations. Questions and scale anchors.

Scale	Questions	Anchors	
		Low	High
Hunger	How hungry are you?	Not at all hungry	Extremely hungry
Fullness	How full are you?	Not at all full	Extremely full
Desire to eat	How strong is your desire to eat?	Not at all strong	Extremely strong
Prospective Food Consumption	How much do you think you could eat?	None at all	A large amount

Source: Adapted from Blundell et al. 2009 in Allison DB and Baskin ML.

However, according to the EFSA, appetite VAS needed to be used together with a measure of food intake. In appetite research, a gold standard method does not exist and a compromise needs to be found between the precision and the naturalness. Laboratory studies have a high internal validity since they are sensitive and reproducible over the intervention and the outcome measures. In this case, the test meal has typically been either a buffet style meal or a single meal preselected by the participant from a list of foods offered by the experimenter (Blundell et al, 2010). The macronutrient composition and the amount of each course eaten from the volunteers is known in order to determine the kcal consumed during the experiment. The other possibility to determine the energy intake is to use a free living study. On the one hand, this choice allows subjects to be in a natural environment, but on the other hand the internal validity is low. In fact, the precision in data collection is low due to errors in the registration of consumed foods that may occur (Livingstone et al, 2003). All in all, both laboratory and free-living studies have advantages and problems therefore the preference of one instead of the other depends from the study design and outcomes.

In agreement with EFSA the use of biomarkers may reinforce the evidence and explain the biological mechanism effect in humans. Therefore, their analysis in an experiment to assess the effect on appetite control is suitable and recommended. Food intake is regulated by two

complementary systems: the homeostatic and the hedonic pathways. The homeostatic pathway controls energy balance by increasing the motivation to eat as a consequence of energy depletion stores while hedonic or reward-based regulation incentives eating in absence of metabolic needs. Under some circumstances, such as an environment rich in appetizing foods, the reward system can override the homeostatic increasing the desire to consume foods that are highly palatable (Lutter et al, 2009).

All the appetite sensations involve a complex interaction between cognitive, sensory, post-ingestive and post-absorptive signals generated by the ingested food or drink, and are conceptualized in the notion of a "satiety cascade" in which the relative importance of the different cue types varies over time from the point at which food is ingested (Yeomans, 2010). The satiety cascade involves pre-prandial events which stimulate food intake, prandial ones which are the processes triggered by the ingestion of food and they terminate intake and those post-prandial ones which occur right after the termination of eating (Harrold et al, 2012). According to Blundell et al (2012), the physiology of appetite regulation comprises episodic signals arising from the mouth and GI tract in response to the periodic consumption of food. The role of several peptides is well recognized in the regulation of the satiety cascade.

Leptin is an adipose tissue hormone which acts as an afferent signal in a negative feedback loop and which maintains homeostatic control of adipose tissue mass and reduces food intake. Circulating leptin concentrations are directly proportional to body fat stores. Its receptors are expressed in dopaminergic and GABAergic neurons in the ventral tegmental area (VTA), but also occur in peripheral organs, such as adipose tissues, skeletal muscles, pancreatic beta cells, and the liver, indicating endocrine, autocrine, and paracrine roles of leptin in energy regulation. Activation of hypothalamic leptin signaling results in reduced food intake and enhanced energy expenditure. Moreover, leptin produced in the gastric epithelium locally amplifies gut satiation signals such as cholecystokinin (CCK) (Domingos et al, 2011; Feng et al, 2012; Yu & Kim, 2012).

Ghrelin is a 28-amino-acid peptide which is produced predominately by the stomach and, to a lesser extent, by the duodenum. It is the only circulating hunger-inducing hormone known so far in humans. It acts stimulating the gastric motility and is involved in central stimulation of appetite and energy. Circulatory levels of ghrelin increase before meals and decrease after food intake in humans (Reinehr et al, 2014). There is also strong evidence for the role of endogenous cholecystokinin (CCK), glucagon-like-peptide (GLP-1) and peptide YY (PYY) in the regulation of the satiety enhancing effects of macronutrients. Food consumption (mainly protein and fat) stimulates the

release of CCK while GLP-1 is released from the gut into the blood stream in response to intestinal carbohydrate and fat, and PYY in response to intestinal fat and other nutrients (Halford et al, 2008).

Another class of compounds which are recently demonstrated to influence food intake are the endocannabinoids (ECs). They are a family of biologically active lipids that bind to and activate G protein-coupled CB1- and CB2-cannabinoid receptors (DiPatrizio et al, 2011). Endogenous ligands to CB1 such as anandamide (AEA) and 2-arachidonoylglycerol (2-AG) have been shown to play a major role in the regulation of energy balance and body composition. Two cannabinoid receptor-inactive and biosynthetically related congeners of AEA, oleoylethanolamide (OEA) and palmitoylethanolamide(PEA), have been suggested to activate the peroxisome proliferator-activated receptor-α (PPAR-α) and, therefore, to control hyperlipidemia (Côtè et al, 2007). In general, the stimulation of the ECs system has an anabolic effect promoting pleasure and motivation to eat, on the contrary its suppression has an anorexigenic result (Cristino et al, 2014).

Mounting evidence pointed out that the brain endocannabinoid system controls food intake at two levels. First, possibly by interacting with the mesolimbic pathways involved in reward mechanisms, which tonically reinforces the motivation to find and consume foods with a high incentive value. Secondly, it is activated on demand in the hypothalamus after short-term food deprivation and then transiently regulates the levels and/or action of other orexigenic and anorectic mediators to induce appetite (Di Marzo et al, 2005).

In addition to the gut peptides and endocannabinoids, recent studies focused on the potential role of saliva in food intake regulation. In fact, salivary composition influence food perception in the mouth mainly affecting the flavor release and initiating the breakdown of food (Engelen et al, 2007). In some kind of food, such as custards, alpha-amylase induced breakdown of starch is the main determinant of the viscosity-related sensations and could also boost high desirable food sensation as perceived creaminess. (de Wijk et al, 2004). Since the presence in the mouth of specific receptors for fatty acids responsible for the “fat taste” was hypothesized, also salivary lipase may play a role in this context. The role of salivary lipase may be the digestion in the mouth of food triglycerides to enhance the fat taste. Some recent experiments found a correlation between the individual salivary lipolytic activities and sensitivities for triglycerides (Voigt et al, 2014). Therefore it was suggested that the salivary enzymatic activity, influencing the oral starch viscosity and fat perception, in turn may contribute significantly to individual differences in dietary starch and fat intake and consequently, to overall nutritional status (Mandel et al, 2010; Neyraud et al, 2011).

All-in-all, appetite control is a dynamic and very complex system. In fact, as recently highlighted by Mars et al. (2012) it is unlikely that a single peptide can contribute to a difference in satiating capacity of foods, but it is more likely that a combination of biomarkers can affect the post-prandial appetite response. Therefore, in developing a food for appetite control and in testing its effectiveness a multidimensional approach is strongly recommended.

References

1. Allirot X, Saulais L, Disse E, Nazare JA, Cazal C, Laville M. *Integrating behavioral measurements in physiological approaches of satiety.* Food Quality and Preference. 2014; 31,181–189
2. Avenell A, Broom J, Brown TJ, Poobalan A, Aucott L, Stearns SC, Smith WC, Jung RT, Campbell MK, Grant AM. *Systematic review of the long-term effects and economic consequences of treatments for obesity and implications for health improvement*. Health Technology Assessment. 2004; 8,(21), iii-182.
3. Benelam B. Satiation, satiety and their effects on eating behaviour. Nutrition Bulletin. 2009; 34,126–173.
4. Blundell J, de Graaf C, Hulshof T, Jebb S, Livingstone B, Lluch A, Mela D, Salah S, Schuring E, van der Knaap H, et al. *Appetite control: methodological aspects of the evaluation of foods*. Obes Rev. 2010; Mar;11(3):251-70. doi: 10.1111/j.1467-789X.2010.00714.x.
5. Blundell JE, Caudwell P, Gibbons C, Hopkins M, Naslund E, King N, Finlayson G. *Role of resting metabolic rate and energy expenditure in hunger and appetite control: a new formulation*. Dis Model Mech. 2012; Sep;5(5):608-13. doi: 10.1242/dmm.009837.
6. Blundell JE, de Graaf K, Finlayson G, Halford JCG, Hetherington M, King N, Stubbs J. *Measuring food intake, hunger, satiety, and satiation in the laboratory.* Chapter 8, Allison DB and Baskin ML (eds). Handbook of assessment methods for eating behaviors and weight related problems: measures, theory and research. 2 nd edition. 2009 SAGE Publications Inc. 283325.
7. Catenacci VA, Wyatt HR. *The role of physical activity in producing and maintaining weight loss.* Nat Clin Pract Endocrinol Metab. 2007; 3:518–529.
8. Christiansen T, Bruun JM, Madsen EL, Richelsen B. *Weight loss maintenance in severely obese adults after an intensive lifestyle intervention: 2- to 4-year follow-up*. Obesity. 2007; 15,413–420.
9. Clark MJ, Slavin JL. The effect of fiber on satiety and food intake: a systematic review. J Am Coll Nutr. 2013;32(3):200-11. doi: 10.1080/07315724.2013.791194.
10. Côté M, Matias I, Lemieux I, Petrosino S, Alméras N, Després JP, Di Marzo V. *Circulating endocannabinoid levels, abdominal adiposity and related cardiometabolic risk factors in obese men.* Int. J. Obesity. 2007; 31, 692–699.
11. Cristino L, Becker T, Di Marzo V. *Endocannabinoids and energy homeostasis: an update.* Biofactors. 2014; Jul-Aug;40(4):389-97. doi: 10.1002/biof.1168.
12. de Wijk RA, Prinz JF, Engelen L, Weenen H. *The role of alpha-amylase in the perception of oral texture and flavour in custards.* Physiol Behav. 2004; Oct 30;83(1):81-91.
13. Demerath EW. *The genetics of obesity in transition*. Coll Antropol, 36(4),1161-8.
14. Di Marzo V, Matias I. *Endocannabinoid control of food intake and energy balance*. Nat Neurosci. 2005; 8,585–589.
15. DiPatrizio NV, Astarita G, Schwartz G, Li X, Piomelli D. *Endocannabinoid signal in the gut controls dietary fat intake.* Proc Natl Acad Sci U S A. 2011; Aug2;108(31):12904-8. doi: 10.1073/pnas.1104675108.

16. Domingos AI, Vaynshteyn J, Voss HU, Ren X, Gradinaru V, Zang F, Deisseroth K, de Araujo IE, Friedman J. *Leptin regulates the reward value of nutrient.* Nat Neurosci. 2011; 14,1562–8.

17. EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA). *General guidance for stakeholders on the evaluation of Article 13.1, 13.5 and 14 health claims.* EFSA Journal. 2011; 9(4):2135

18. EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA). *Guidance on the scientific requirements for health claims related to appetite ratings, weight management, and blood glucose concentrations.* EFSA Journal. 2012; 10(3):2604.

19. EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA). *Scientific and technical guidance for the preparation and presentation of the application for authorisation of a health claim.* The EFSA Journal. 2007; 530,1-44.

20. Engelen L, van den Keybus PA, de Wijk RA, Veerman EC, Amerongen AV, Bosman F, Prinz JF, van der Bilt A. *The effect of saliva composition on texture perception of semi-solids*. Arch Oral Biol. 2007; Jun;52(6):518-25.

21. Feng H, Zheng L, Feng Z, Zhao Y, Zhang N. *The role of leptin in obesity and the potential for leptin replacement therapy*. Endocrine. 2013; Aug;44(1):33-9. doi: 10.1007/s12020-012-9865-y.

22. Flint A, Raben A, Blundell JE, Astrup A. *Reproducibility, power and validity of visual analogue scales in assessment of appetite sensations in single test meal studies*. Int J Obes Relat Metab Disord. *2000*; Jan;24(1):38-48.

23. Fogliano V, Vitaglione P. *Functional foods: planning and development*. Mol Nutr Food Res. 2005; Mar;49(3):256-62.

24. Ghosh, S.K. Functional coatings and microencapsulation: A general perspective. In Functional Coating. Ed.,Wiley-VCH, Germany. 2006; pp. 1-26.

25. Halford JC, Harrold JA. *Neuropharmacology of human appetite expression*. Dev Disabil Res Rev. 2008; 14: 158 – 164.

26. Halford JC, Harrold JA. *Satiety-enhancing products for appetite control: science and regulation of functional foods for weight management.* Proc Nutr Soc. 2012; May;71(2):350-62. doi: 10.1017/S0029665112000134.

27. Harrold JA, Dovey TM, Blundell JE, Halford JC. *CNS regulation of appetite.* Neuropharmacology. 2012; 63(1),3-17.

28. Hill AJ, Melby C, Johnson SL, Peters JC. *Physical activity and energy requirements.* Am J Clin Nutr. 1995; 62(Suppl):1059S–1066S.

29. Kaplan LM. *Pharmacological therapies for obesity*. Gastroenterol Clin North Am. 2005; 34,91–104.

30. Kramer, FM, Jeffery RW, Forster JL, Snell MK. *Long-term follow-up of behavioral treatment for obesity: patterns of weight regain among men and women*. Int. J. Obes. 1989; 13,123–136.

31. Kuang SS, Oliveira JC, Crean AM. *Microencapsulation as a tool for incorporating bioactive ingredients into food.* Crit Rev Food Sci Nutr. 2010 Nov;50(10):951-68. doi: 10.1080/10408390903044222.

32. Leibel RL, Rosenbaum M, Hirsch J. *Changes in energy expenditure resulting from altered body weight.* N. Engl. J. Med. 1995; 332,621–628.

33. Livingstone MBE, Black AE. *Markers of the validity of reported energy intake*. J Nutr. 2003; 133: 895S–920S.

34. Livingstone MBE, Robson PJ, Welch RW, Burns AA, Burrows MS, McCormack C. *Methodological issues in the assessment of satiety*. Scandinavian Journal of Nutrition/Näringsforskning. 2000; Vol. 44 No. 3 pp. 98-103.

35. Logan K, Wright AJ, Goff HD. Correlating the structure and in vitro digestion viscosities of different pectin fibers to in vivo human satiety. Food Funct. 2015 Jan;6(1):63-71. doi: 10.1039/c4fo00543k. Epub 2014 Oct 22.

36. Lutter M, Nestler EJ. *Homeostatic and hedonic signals interact in the regulation of food intake*. J Nutr. 2009; Mar;139(3):629-32. doi: 10.3945/jn.108.097618..

37. MacLean PS, Higgins JA, Johnson GC, Fleming-Elder BK, Peters JC, Hill JO. *Metabolic adjustments with the development, treatment, and recurrence of obesity in obesity-prone rats*. Am. J. Physiol. Regul. Integr. Comp. Physiol. 2004; 287,R288–R297.

38. Mandel AL, Peyrot des Gachons C, Plank KL, Alarcon S, Breslin PA. *Individual differences in AMY1 gene copy number, salivary α-amylase levels, and the perception of oral starch*. PLoS One. 2010; Oct 13;5(10):e13352. doi: 10.1371/journal.pone.0013352.

39. Market Research. *Global Weight Loss and Diet Management*. 2009–2014. Pub ID: MKMK2382487

40. Mars M, Stafleu A, de Graaf C. *Use of satiety peptides in assessing the satiating capacity of foods*. Physiol Behav. 2012; Jan 18;105(2):483-8. doi: 10.1016/j.physbeh.2011.08.033.

41. Martins C, Morgan L, Truby H. *A review of the effects of exercise on appetite regulation: an obesity perspective*. International Journal of Obesity. 2008; 32,1337–1347.

42. Mattes RD, Hollis J, Hayes D, Stunkard AJ. *Appetite: measurement and manipulation misgivings*. Journal of the American Dietetic Association. *2005*; 105,S87–S97.

43. Mitchell CS, Savage DB, Dufour S, Schoenmakers N, Murgatroyd P, Befroy D, Halsall D, Northcott S, Raymond-Barker P, Curran S, et al. *Resistance to thyroid hormone is associated with raised energy expenditure, muscle mitochondrial uncoupling, and hyperphagia*. J. Clin. Invest. 2010; 120,1345–1354.

44. Neyraud E, Palicki O, Schwartz C, Nicklaus S, Feron G. *Variability of human saliva composition: possible relationships with fat perception and liking.* Arch Oral Biol. 2012; May;57(5):556-66. doi: 10.1016/j.archoralbio.2011.09.016.

45. Osterholt KM, Roe LS, Rolls BJ. *Incorporation of air into a snack food reduces energy intake.* Appetite. 2007;48(3):351–8.

46. Padwal RS, Majumdar SR. *Drug treatments for obesity: orlistat, sibutramine, and rimonabant.* Lancet. 2007; 369,71–77.

47. Pinheiro Volp AC, Esteves de Oliveira FC, Duarte Moreira Alves R, Esteves EA, Bressan J. *Energy expenditure: components and evaluation methods.* Nutr Hosp. 2011; 26(3):430-40. doi: 10.1590/S0212-16112011000300002.

48. Ravussin E. *Cellular sensors of feast and famine*. J. Clin. Invest. 2002; 109:1537–1540.

49. Recordati G, Bellini TG. *A definition of internal constancy and homeostasis in the context of non-equilibrium thermodynamics*. Exp Physiol. 2004; Jan;89(1):27-38.

50. Redman LM, Heilbronn LK, Martin CK, de Jonge L, Williamson DA, Delany JP, Ravussin E; Pennington CALERIE Team. *Metabolic and behavioral compensations in response to*

caloric restriction: implications for the maintenance of weight loss. PLoS ONE. 2009; 4,e4377.

51. *Regulation (EC) No 1924/2006 of the European Parliament and of the Council of 20 December 2006 on nutrition and health claims made on foods*. OJ L 404, 30.12.2006, p. 9–25.
52. Rolls BJ, Bell EA, Waugh BA. *Increasing the volume of a food by incorporating air affects satiety in men*. Am J Clin Nutr. 2000;72(2):361–8.
53. Samaranayake NR, Ong KL, Leung RY, Cheung BM. *Management of Obesity in the National Health and Nutrition Examination Survey (NHANES*), 2007–2008. Annals of Epidemiology. 2012; 22(5),349-353.
54. Schofield GM, Urch CE, Stebbing J, Giamas G. *When does a human being die?* QJ Medicine. 2014. doi:10.1093/qjmed/hcu239.
55. Shapira N. *Women's higher health risks in the obesogenic environment: a gender nutrition approach to metabolic dimorphism with predictive, preventive, and personalised medicine*. EPMA J. 2013; 12; 4(1):1.
56. Siró I, Kápolna E, Kápolna B, Lugasi A. *Functional food. Product development, marketing and consumer acceptance. A review*. Appetite. 2008; Nov;51(3):456-67. doi: 10.1016/j.appet.2008.05.060.
57. Stunkard A, Messick S. *The three-factor eating questionnaire to measure dietary restraint, disinhibition and hunger*. Journal of Psychosomolic Research. 1985; Vol.29, No. I, pp. 71-83.
58. Sumithran P, Proietto J. *The defence of body weight: a physiological basis for weight regain after weight loss*. Clinical Science. 2013; 124, 231–241 doi: 10.1042/CS20120223.
59. Tampa FL. *Weight Loss & Diet Control Market*. 10th ed., USA: Market data Enterprises. 2010.
60. Thomas Reinehr, Christian L. Roth. *The gut sensor as regulator of body weight*. Endocrine. 2015; May;49(1):35-50. doi: 10.1007/s12020-014-0518-1.
61. Trigueros L, Peña S, Ugidos AV, Sayas-Barberá E, Pérez-Álvarez JA, Sendra E. *Food ingredients as anti-obesity agents: a review*. Crit Rev Food Sci Nutr. 2013; 53(9):929-42. doi: 10.1080/10408398.2011.574215.
62. Tseng YH, Cypess AM, Kahn CR. *Cellular bioenergetics as a target for obesity therapy*. Nat Rev Drug Discov. 2010; Jun;9(6):465-82. doi: 10.1038/nrd3138.
63. Vogels N, Westerterp-Plantenga MS. *Categorical strategies based on subject characteristics of dietary restraint and physical activity, for weight maintenance*. Int J Obes (Lond). 2005; Jul;29(7):849-57.
64. Voigt N, Stein J, Galindo MM, Dunkel A, Raguse JD, Meyerhof W, Hofmann T, Behrens M. *The role of lipolysis in human orosensory fat perception.* J Lipid Res. 2014; May,55(5):870-82. doi: 10.1194/jlr.M046029.
65. WHO. *Childhood obesity*. 2012
66. WHO. *European ministerial conference on counteracting obesity - Conference Report.* [pdf] Available at < www.euro.who.int/document/e90143.pdf > 2007.
67. WHO.*Obesity and overweight*. www.who.int/dietphysicalactivity/media/en/gsfs_obesity.pdf 2003.

68. Wilde PJ. Eating for life: designing foods for appetite control. J Diabetes Sci Technol. 2009 Mar 1;3(2):366-70.

69. Withrow D, Alter DA. *The economic burden of obesity worldwide: A systematic review of the direct costs of obesity*. Obesity Reviews. 2011; 12,131-141.

70. Yeomans MR. *Psychological Approaches to Understanding Satiation &Satiety*. AgroFood HiTech. 2010; 21(4):16-19.

71. Yu, J.H., Kim, M.S. *Molecular mechanisms of appetite regulation*. Diabetes Metab J. 2012; 36, 391-398.

72. Zhou YH, Ma XQ, Wu C, Lu J, Zhang SS, Guo J, Wu SQ, Ye XF, Xu JF, He J. *Effect of anti-obesity drug on cardiovascular risk factors: a systematic review and meta-analysis of randomized controlled trials*. PLoS One. 2012; 7(6):e39062. doi: 10.1371/journal.pone.0039062. Epub 2012 Jun 20.

Chapter 2. Salivary lipase and α-amylase activities are higher in overweight than in normal weight subjects: Influences on dietary behavior

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Abstract

Mounting evidence shows that hedonic eating, leading to overeating just for pleasure, can be driven by orosensory factors through the activation of reward processing and learning in the brain. Foods rich in sugars and fats are potent rewards and saliva composition influences oral taste, texture and aroma perception. A role for salivary α-amylase and lipase in the gustatory systemand a link between salivary α-amylase activity and dietary habits were recently hypothesized.

The objective of this studywas to verify the relationship among salivary lipase and α-amylase activities as well as zinc concentration with food preference and choice of people with different body mass indices. Forty-two (23 normal weight and 19 overweight) healthy subjects participated in the study. Data showed that α-amylase and lipase were 1.8 and 2.4 folds higher in overweight than in normal weight subjects, respectively. On the other hand, overweight subjects showed a 33% reduced salivary zinc concentration compared to normal weight subjects. Only lipase activity positively correlated with individual preference for high-fat foods and with fat content of the diets.

All in all data suggested that high salivary lipase activity in overweight subjects could be an adaptive response to the lowfat-taste perception related to the reduced zinc concentration. It cannot be ruled out that other factors but diet might influence salivary α-amylase activity in overweight subjects.

Introduction

Obesity is dramatically increasing worldwide (Nguyen et al, 2012). Its etiology is very complex involving genetic, environmental and behavioral factors, and ultimately resulting from the imbalance between energy intake and expenditure. Eating can be triggered by metabolic needs, hedonic drive, or an interaction between the two (Berthoud, 2011). In this respect, nutritive properties of foods satisfy metabolic needs while orosensory properties may be fundamental in hedonic eating since they can drive neurotransmitter responses activating reward processing and learning (Hammond et al, 2012). Particularly, foods rich in sugars and fats are potent rewards (Lenoir et al, 2007; Toepel et al, 2010) in fact, it is known that sugars are able to stimulate dopamine production (Wise, 2004), and that oro-sensory pleasantness to stimuli with different fat content and texture differently activates the brain lateral hypothalamus and amygdala (Grabenhorst et al, 2010). Moreover, the very recent discovery that the long chain fatty acid receptors CD36 and GPR120 are present on the surface of the circumvallate and foliate taste buds cells in humans (Simons et al, 2011; Stratford et al, 2009; Galindo et al, 2012) corroborated the idea that free fatty acids released by the lingual lipase action on dietary triglycerides, may play a role in satiety system also acting as primary taste (Khan et al, 2009; Smeets et al, 2006). These findings supported the hypothesis that the efficiency of the "taste of fat" might influence the food choice and, as consequence, energy balance. The actual knowledge on the taste of fat and on the regulatory mechanisms in mammals was recently reviewed by Passilly-Degrace et al (2014).

On the other hand oral taste and texture perception, as well as aroma, is highly influenced by saliva that dissolves and diffuses substances to the taste receptors (Matsuo, 2000; Salles et al, 2011), and can enhance salty and bitter taste thanks to its hypotonicity and its proteolytic activity, respectively (Humphrey et al, 2001; Dsamou et al, 2012). Among salivary minerals, zinc plays a crucial role in taste perception by activating a zinc-dependent metalloprotein [carbonic anhydrase VI (CA6)], also known as gustin (CA6), whose action as trophic factor promoting the growth and development of taste buds, was also suggested (Henkin et al, 1999; Henkin et al, 1988). Among salivary proteins, α-amylase is the most abundant in humans, accounting for 40 to 50% of the total proteins (Noble, 2000). Its involvement in the perception of a food textural property, such as viscosity, has been recently showed by Mandel and co-workers (Mandel et al, 2010). They suggested that the genetic variability for salivary α-amylase among population might influence food liking and preference and, as a consequence, carbohydrate-related dietary habits. This hypothesis was recently tested, by the same authors in normal weight subjects (Mandel et al, 2012), but no data are present in the literature

about salivary α-amylase activity and dietary habits in overweight subjects (Body Mass Index (BMI)>25 kg/m^2).

On the other hand, a role for human salivary lipase in the human gustatory system can be also hypothesized. Salivary lipase has not a digestive role in adult humans (Hamosh, 1990), and recent studies suggested that it plays a role in fat taste and texture perception. Rats preferred triolein emulsion compared to the same emulsion added with orlistat, a potent lipase inhibitor (Kawai et al, 2003) and, similarly, fat detection threshold increased in humans when triolein emulsions added with orlistat was administered (Pepino et al, 2012). Moreover, very recently Kulkarni et al, (2013) demonstrated that free fatty acids are formed (at range concentration of 20-60 μM) in the mouth by chewing high fat foods thus reinforcing previous evidence by Stewart et al, (2010), that micromolar amounts of free fatty acids produced by the saliva lipolytic activity are sufficient to stimulate oral sensors in humans.

In this framework, the aim of this work was to verify the relationship between salivary lipase and α-amylase activities as well as zinc concentration with food preference and choice of people with different nutritional status, basing on the concept that those parameters may influence gustatory stimuli from dietary fats and sugars as well as overall taste sensitivity. Saliva samples, from normal weight (NW) and overweight (OW) subjects were collected, and enzyme activity and zinc concentration data were analyzed with respect to individual fatty food preference and habitual diet composition.

Materials and methods

Ethics Statement

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Ethics Committee of University of Naples "Federico II. Selected subjects participated in the study after reading and signing an informed consent document.

Subject selection and enrollment

Forty two subjects were selected among students and workers of the Agriculture Faculty of University of Naples (Italy) basing on a medical history interview. Eligible subjects were those who did not have any kind of disease (hyperlipidemia, gastro-intestinal disease, chronic infections, dental diseases, allergies), were nonsmokers, did not take drugs and were not regular consumers of alcohol or energy drinks, were not participating in body weight reducing programs or did not experience body weight variations over the three months before the study. Selected subjects were all Caucasian. Participant descriptive characteristics are reported in **Table 1**. Based on individual BMI the whole sample was divided in two groups: the normal weight (NW) and overweight (OW) groups including subjects with BMI≤24.9 kg/m^2 or with BMI≥25 kg/m^2, respectively. Within the second group only three subjects had BMI >30 kg/m^2.

Table 1: Descriptive characteristics of the subjects participating to this study grouped according to the body mass index (BMI) into normal weight (NW) and overweight (OW). All values are expressed as mean and/or range. BMI is expressed also as mean (range and standard deviation, SD). * p<0.0001 *vs* NW, one-way ANOVA.

	NW	**OW**
Number (n)	23	19
Sex (M/F)	11/12	11/8
Age, mean (range) (years)	27 (25-35)	31 (19-54)
BMI, mean (range, *SD*) (kg/m²)	22.2 (19.4-24.4, *1.7*)	28.5* (25.3-34.8, *2.1*)

Subject treatment and saliva collection

Whole unstimulated saliva was collected by subjects at 10 am after a 2h fasting period following the procedure described by Navazesh et al (2008). Briefly, subjects were instructed to have their usual breakfast at 08 am, to brush their teeth and to avoid eating foods or drinking beverages for the next 2 h before saliva collection; only water was allowed. Moreover, subjects were asked to abstain from doing stressful activities 30 min before saliva collection, so that actual stress level did not influence salivary enzymes secretion and/or activity (Thoma et al, 2012). At 10.00 h subjects came to our laboratory to collect the saliva sample. In order to measure the salivary flow rate saliva samples were obtained using the gold standard method that is the direct and passive salivating from subject mouth for five minutes into pre-weighted tubes. Total salivary flow rate was measured calculating the difference between the post and pre weight and expressed as g/min. Samples were stored at -20°C until the assays were performed. Samples on three consecutive days were collected from each subject and all analyses were performed in triplicate.

Lipase activity by spectrophotometric assay

Lipase activity was measured by the method described by Kurooka et al (1977) slightly modified. Into 1.5 mL centrifuge tubes 0.1 mL saliva were mixed with 1 mL of 100 mM Tris-HCl buffer (pH 8.5) containing 0.3 mM 5-5'-dithio-bis (2-nitrobenzoic acid) (DTNB). Then 20 µL of phenylmethylsulfonyl fluoride (PMSF) 20 mM in ethanol were added as non-specific esterase inhibitor and tubes incubated for 5 minutes at 37°C. The ability of 0.4 mM PMSF to inhibit salivary esterases, without modifying lipase activity as described by Kurooka et al (1977) was demonstrated during the set up of the assay. Finally, 0.1 mL of 20 mM 2,3-dimercapto-1-propanol tributyrate in ethanol were added and samples were incubated for 60 min at 37°C. Salivary lipase released 2,3-dimercapto-1-propanol that reacted with DTNB to release TNB anions. Tubes were then centrifuged at 6500 rpm for 10 min and the absorbance at 412 nm was measured. Color intensity was proportional to the lipase activity that was quantified in saliva samples using a specific calibration curve. It was done using triacylglycerol lipase from Aspergillus Niger (200U/g, purchased by Sigma, St. Louis, MO, USA) as standard. A mother solution of 1U/mL was prepared dissolving the powder in Tris-HCl buffer (pH 8.5) and consecutive dilutions were carried out to obtain a curve ranging from 6.25 to 200 U/L. The intra-assay CVs were 6.7%, 3.3% and 1.1% for the control at 10 U/L (low), 100 U/L (medium) and 200 U/L (high), while the inter-assay CVs values were 8.5% and 3.2% for low and high controls, respectively.

Lipase activity by Gas Chromatography (GC) method

The triacylglycerol lipolytic activity of saliva samples was verified using tripalmitin (Sigma, St. Louis, MO, USA) as specific substrate. Fifty microliter of tripalmitin (12.4 μM in hexane) was added to 300 μL of saliva and the mix was kept at 37°C in agitation for 1 hour. Then the lipids were extracted by hexane and free fatty acids were esterified with born trifluoride (BF3) in methanol (Sigma, St. Louis, MO, USA) as described by Chapman (1979). A blank of each sample (skipping addition of tripalmitin) was prepared using the same procedure. Finally the samples were mixed with heptanoic acid as internal standard and injected into a Shimadzu GC17A gas chromatography (Shimadzu Italia, Milan, Italy) equipped with split/splitless injection port, flame ionization detector and a 60 m fused-silica capillary column (Quadrex Corporation, New Heaven, U.S.A.) coated with cyanopropyl methyl silicone (0.25 μm film thickness). Helium was used as the carrier gas and the flow rate was 1.8 mL min^{-1} at a split ratio of 1:60. The temperature of both the injector and detector was 250 °C. The oven temperature was set at 160°C for 10 min prior to being ramped to 220°C at 10° C/min and hold 10min for GC separation. The total amount of palmitic acid produced in the reaction was calculated subtracting the free fatty acid in the blank from that found in the added sample. The analysis was repeated in duplicate for each sample.

Salivary α-amylase activity

Salivary α-amylase activity was measured using a commercial assay kit purchased by Salimetrics (State College, PA, USA). Samples were brought to room temperature, vortexed and then centrifuged at 1500 x g for 15 minutes. A dilution of 1:200 was performed with the provided diluent. Eight microliters of controls (high and low) or samples and then 320 μL of preheated (37ºC) α-amylase substrate solution were added to each well. Plate reader was set at 405 nm and 37°C and absorbance was read twice at 1 minute and at 3 minutes. For accurate timing, one strip once was performed. The α-amylase activity was calculated following the kit instruction: the one minute readings were subtracted to the three minutes readings and the values were multiplied by the conversion factor. The intra-assay CVs were 2.5% (high control), 6.7% (medium control) and 7.2% (low control) while the inter-assay CV were 3.6% and 5.8% for the high and low control, respectively.

Salivary zinc concentration

Ionic zinc concentration was measured with a QuantiChrom zinc-assay kit (Gentaur, Brussels, Belgium) in saliva samples after centrifugation at 1700 g for 10 min to discard cellular debris and after exclusion, by microscopic examination, of food and blood contamination. The last action and the use, upon assay development, of glassware and plasticware that were being soaked for 24 h in 4 M HCl and thoroughly rinsed with metal-free water, were adopted to minimize possible zinc contamination. Reagents were equilibrated at room temperature and then the working solution was prepared mixing the three reagents in the proportion described in the instruction. A calibration curve from 10 to 0 μM was performed using the Zinc standard 50 μM provided with the kit. Two hundreds microliters of samples or samples blank (samples plus EDTA 8 μL 100 mM) were added to 800 μL of working, reagent incubated for 30 minutes in the dark and the absorbance was read at 425 nm. Quantification was performed subtracting blank from sample values and plotting the result against Zn^{2+} standard concentrations.

Fat Preference Questionnaire

A slightly modified version of "The Fat Preference Questionnaire" as described by Ledikwe et al (2007) was used. The questionnaire contained 17 sets of foods comprising of items from a variety of food groups each containing two similar foods only differing for fat content. For each food set, the questionnaire instructed respondents to indicate if they had ever eaten the foods, which food (only differing in fat content) tasted better (TASTE), and which food they ate more often (FREQ). The TASTE and FREQ scores were calculated and expressed as percentage of food sets in which high-fat foods were reported to ''taste better'' and to be ''eaten more often'', respectively. The food sets were considered in the calculation only if both items were eaten by subject at least once. The FREQ score was subtracted from the TASTE score to create a DIFF score: a measure of dietary restraint specific to fat consumption as validated by the Eating Inventory (Stunkard et al, 1985). The questionnaire was completed by all subjects.

Food Frequency Questionnaire (FFQ)

Habitual nutrient and food intake of subjects was measured using a FFQ. All participants were interviewed about the type of foods, recipes used for their preparation (if any) and portion size of

each food consumed over the 24h of the past week (7 days record). The portion sizes were established using a pictorial display of standard meal/food sizes.

Statistical analysis

Statistical analysis was performed using STATISTICA 8 software (StatSoft, Tulsa, OK). The sample size was based on power calculations derived from a preliminary study. We calculated that, at $\alpha = 0.05$ with a power of 80%, 34 subjects would allow us to detect a 25% difference in salivary enzymes activity. Normality and variance homogeneity were systematically checked and it was found for all variables using the K – S normality test. Data were analyzed for differences by one-way analysis of variance (ANOVA). When appropriate, differences between means were tested using the student's *t*-test. The significance level was set at $p<0.05$. Scatter plots, linear regression lines and Pearson correlations were calculated between salivary α-amylase, lipase and BMI.

Results

Salivary flow rate

A high inter individual variability was present and no difference between salivary flow rate of NW and OW was found (0.79 ± 0.14 g/min and 0.80 ± 0.09 g/min, respectively, $p>0.05$).

Salivary enzyme activity

The activity of monitored enzymes showed a large inter individual variability: lipase activity ranged from 8 U/L to 173 U/L, and α-amylase from 11 U/mL to 311 U/mL in the whole sample of subjects. As shown in the scatter plot graph and regression lines for enzyme activities *vs* BMI (**Figure 1**, **panel A**), a significant correlation was present only between lipase activity and BMI ($r=0.67$; $p<0.0001$) while no significant correlation with BMI was found for α-amylase activity ($r=0.29$; $p=0.07$). Moreover, data analysis for subjects grouped based on BMI showed a significant difference between NW and OW for lipase (40.9 ± 1.7 U/L *vs* 100.0 ± 10.1 U/L, respectively, $p<0.0001$). The same trend, although at a lower significance level, was also observed for α-amylase activity (69.3±10.9 U/mL *vs* 126.0±22.6 U/mL, respectively, $p=0.02$) (**Figure 1, panel B**). No differences were found in the salivary enzyme activity when subjects were divided based on the gender.

The measure of lipase activity by the GC method showed that the amount of palmitic acid produced incubating saliva samples with tripalmitin was twice higher ($p=0.0002$) in OW than NW. These data fully confirmed those observed with the previous assay demonstrating that saliva of OW subjects release a double amount of free fatty acids compared to that of NW people.

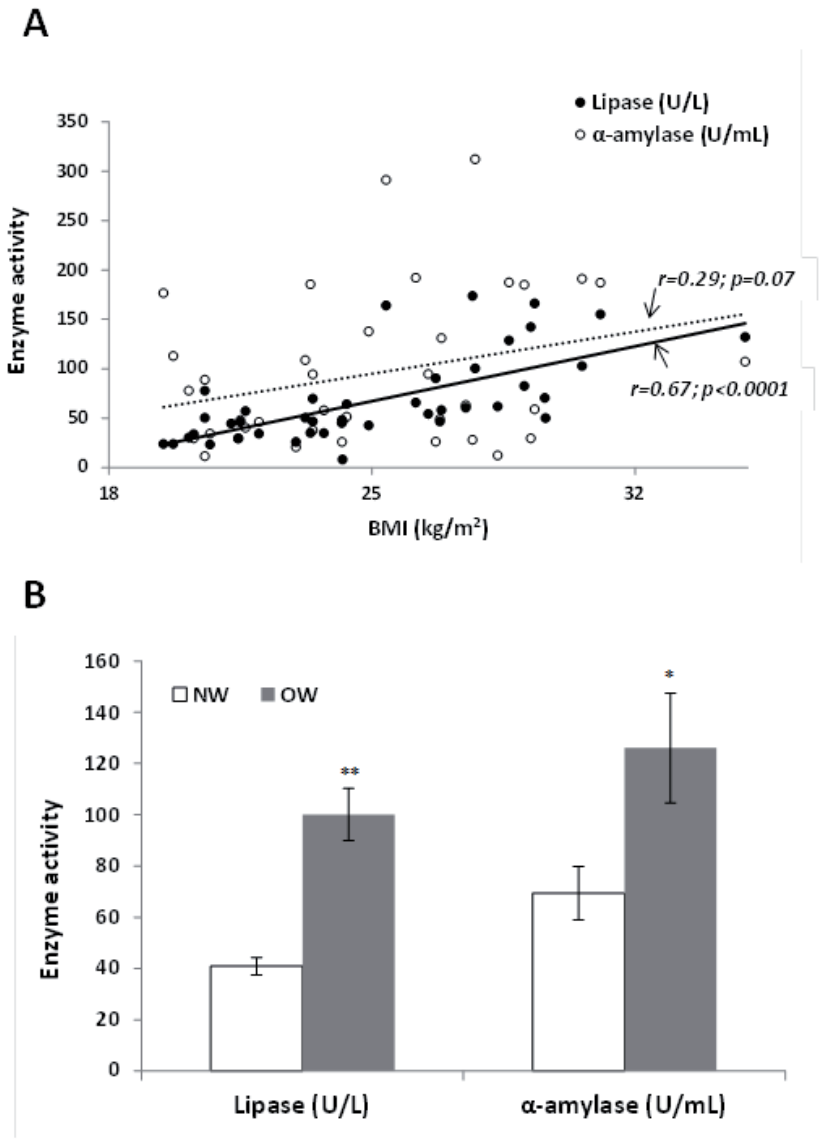

Figure 1: Salivary lipase and α-amylase activities. **A)** Scatter plots and regression lines for mean values of enzyme activities *vs* BMI. Continue line is for lipase while dot line is for α-amylase. Lipase activity was positively correlated with BMI (r=0.67, p<0.0001). **B)** Mean ± SEM enzyme activities in normal weight (NW, *n*=23) and overweight (OW, *n*=19) subjects. Both enzyme activities were higher in OW than in NW (**p<0.0001 for lipase and *p=0.02 for α-amylase activity in OW *vs* NW, one-way ANOVA).

Zinc concentration

Data shown in **Figure 2** indicated that NW had a significant higher salivary zinc concentration than OW (15.35±1.30 µM *vs* 9.94±SEM 1.55 µM, respectively, p=0.01). Interestingly the inter-individual variation on this parameter was relatively low, thus increasing the significance of the difference observed.

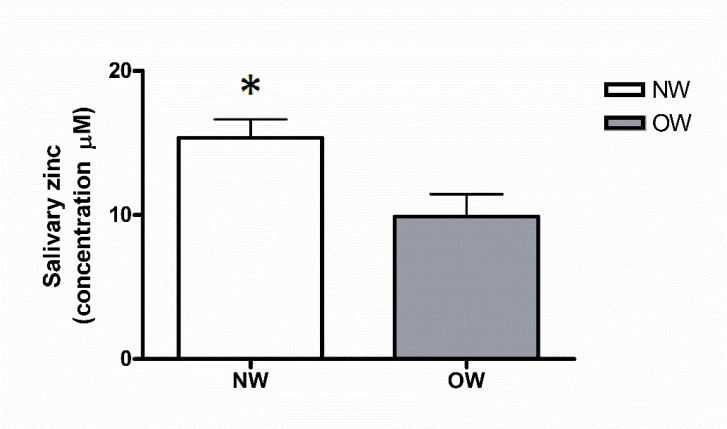

Figure 2: Salivary zinc ion concentration in normal weight (NW) and overweight (OW) subjects. NW showed a significantly higher concentration of zinc than OW (*p=0.01; one-way ANOVA). Data are expressed as mean ± SEM.

Fat food preference and consumption frequency

Figure 3 showed that taste preference and consumption frequency for high fat food items *versus* the low fat counterparts were higher in OW than NW (differences being at a p level of 0.002 for both TASTE and FREQ scores). Moreover, DIFF score was not different (p=0.88) between the two groups, indicating that OW and NW were homogeneous for dietary fat restriction. No significant difference between genders was found for anyone of measured score (p>0.05).

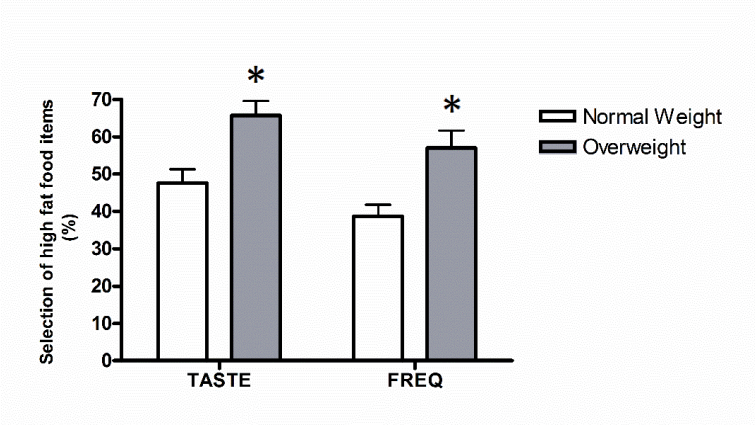

Figure 3: **Preference and consumption frequency for high vs low fat foods in normal weight and overweight.** Overweight (OW) subjects showed a significantly higher taste preference (TASTE) and consumption frequency (FREQ) score than normal weight (NW) (*p=0.002; one-way ANOVA). Data are expressed as mean ± SEM (score, %).

Diet composition

Table 2 reported the total energy intake and macronutrient composition of habitual diets consumed by NW and OW subjects. It showed that the amount of carbohydrates, proteins and fats as well as the energy intake of diets of OW were higher than those of NW ($p<0.0001$). However, no difference was present in the energy repartition of macronutrients in the diets from the two groups. No correlation between gender and energy or macronutrient intake was found. A positive correlation was found between lipase activity and total fat (r= 0.382; $p=0.0125$), protein (r=0.468, $p=0.0018$) or carbohydrate (r=0.484, $p=0.0012$) intakes. No similar correlations were found in the case of α-amylase activity.

Table 2: Daily energy intake (EI, kcal), and macronutrient composition (g and % EI) of habitual diets consumed by normal weight (NW) and overweight (OW) subjects. Data are expressed as mean ± SEM, n=23 NW, n=19 OW. * p<0.001 of OW *vs* NW (one-way ANOVA).

	Amount (g)				**Nutrient composition by EI (%EI)**			
	NW		**OW**		**NW**		**OW**	
	Mean	***SEM***	**Mean**	***SEM***	**Mean**	***SEM***	**Mean**	***SEM***
Energy intake (kcal)	1716.8	*61.7*	2218.3*	*103.1*	---		---	
Proteins	72.0	*2.9*	92.8*	*4.4*	16.9	*0.5*	16.8	*0.4*
Carbohydrates	187.0	*8.3*	245.7*	*12.8*	42.6	*1.3*	44.3	*0.9*
Fats	66.1	*3.9*	85.0*	*4.5*	34.3	*1.0*	34.6	*0.6*
Dietary fibre	16.4	*0.8*	19.2	*1.3*	1.9	*0.1*	1.7	*0.1*
Alcohol	14.7	*0.6*	13.6	*0.4*	6.0	*0.7*	4.3	*0.5*

Discussion

Literature studies reported a high variability of salivary α-amylase and lipase activities among general population (Voho et al, 2006; Neyraud et al, 2012; Thoma et al, 2012). Several genetic, cognitive and environmental factors were indicated to play a role in differentiating people for salivary amylase (Nater et al, 2007; Perry et al, 2007) while a gap of knowledge still exist to explain the role of salivary lipase and its variability among people.

This study was designed to verify the possible link among individual salivary enzyme activities, food preference and dietary habits, and nutritional status. Salivary enzyme activity variability was studied in a group of 42 healthy and nonsmoker subjects, with BMI ranging between 19.4 kg/m² and 34.8 kg/m² and it was correlated to habitual diets. Data showed, for the first time, that salivary α-amylase and lipase activity were significantly higher in OW than in NW subjects, despite similar salivary flow rates between the groups were recorded. Lipase activity significantly correlated with BMI, with the high-fat food preference and consumption frequency as well as with amount of fats in habitual diet. Data had significant relevance since they showed that a double amount of free fatty acids could be released by salivary lipase in OW subjects compared to NW. These findings were in accordance with studies on human behavior indicating that a preference for high fat foods was related to a higher fat consumption (Drewnowski et al, 1999; Pangborn et al, 1985) and with animal

studies demonstrating the ability of high-fat diet to increase salivary enzyme activity (Hamosh et al, 1978; Armand et al, 1990).

Unfortunately OW subjects consumed more of all macronutrients and, as a consequence, an exclusive role of dietary fats in modulating lipase activity level could not be established.

Data of this paper supported the hypothesis that the preference for high fat foods in subjects with high BMI is due to their low fat taste perception. In other words, OW preferred and consumed high-fat foods more frequently than NW because they could not perceive the fat taste in low fat foods thus attributing them a low hedonic value. Tepper et al (1997) demonstrated that subjects with higher BMI could not discriminate fat content in the test food because of a lower density of fungiform taste papillae in their mouths. Moreover, Henkin et al (1999b) showed that gustin, as well as salivary and serum zinc concentration were lower in patients with impaired taste and smell function. In this study, OW were shown to have a 33% salivary zinc concentration lower than NW. This finding was in contrast with a previous work by Padiglia et al (2010) who found an inverse correlation between salivary zinc concentration and BMI. However, it should be noticed that the 75 volunteers involved in that study were all normal weight so probably the correlation with BMI had little physiological relevance in such a narrow range.

Taken together data suggested that, even if the fatty acids delivered by triacylglycerol due to lipase action in saliva of OW is higher than in NW, other oral physic-chemical conditions (namely zinc concentration in saliva) may play a major role in determining a reduced fat taste sensitivity in OW subjects compared to NW. In this framework, the enhanced lipase activity in OW subjects may be an adaptive response to increase the fat taste perception.

Regarding salivary α-amylase activity several studies demonstrated that it is genetically predisposed; it accounts for individual variation in the oral perception of starch viscosity and it is positively associated with improved short-term glycemic homeostasis following starch ingestion in normal weight adults (Mandel et al, 2010; Mandel et al, 2012). In accordance to Mandel et al (2012), who showed no different intake of carbohydrates depending on the salivary amylase activity, our data confirm the lack of a link between the activity of this enzyme and dietary intake of carbohydrates also in overweight subjects.

It is possible that OW subjects presented a high AMY1 gene copy number in their DNA than NW and it cannot be excluded that other factors than genetic, such as psychological stress level, inflammatory status or some metabolic pathways, that can be unbalanced in OW people, might

significantly influence their salivary α-amylase activity (Ali et al, 2012). Future studies integrating neuro-functional, biochemical and behavioral approaches should verify this hypothesis.

Conclusion

In conclusion, in this study it was shown for the first time that OW have higher salivary lipase and α-amylase activities than NW. Data indicated that also in humans the amount of dietary fats usually consumed by subjects may influence lipase activity as previously found in animals. Moreover, it was hypothesized that the higher lipase activity in OW could not ameliorate the sensitivity to fat perception in these subjects who might experiment an altered gustin activity due to a reduced concentration of salivary zinc. This condition might influence the high-fat food taste preference and consumption frequency in OW subjects who need a higher amount of fatty foods to compensate the low oral fat sensitivity. Future studies are needed to confirm our results and to determine a stronger link between salivay lipase activity, eating behavior and overweight.

Conflict of interest

All the authors declare no conflict of interest.

Authors' contributions to manuscript: PV designed research; IM conducted the experiments and analyzed the biochemical and nutritional data; IM, PV and VF wrote the paper; PV has primary responsibility for final content; all authors read and approved the final manuscript.

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References

1. Ali N & Pruessner JC (2012). The salivary alpha amylase over cortisol ratio as a marker to assess dysregulations of the stress systems. Physiology & Behavior 106, 65-72.
2. Armand M, Borel P, Cara L, Senft M, Chautan M, et al. (1990). Adaptation of Lingual Lipase to Dietary Fat in Rats. The Journal of Nutrition. 120, 1148-56.
3. Berthoud HR. (2011). Metabolic and hedonic drives in the neural control of appetite: who is the boss? Current Opinion in Neurobiology 21, 888–896.
4. Chapman GW. (1979). Gas Chromatographic Determination of Free Fatty Acids in Vegetable Oils by a Modified Esterification Procedure. Journal of the American oil chemists'society 59, 77-79.
5. Drewnowski A & Hann C. (1999). Food preferences and reported frequencies of food consumption as predictors of current diet in young women. American Journal of Clinical Nutrition 70, 28–36.
6. Dsamou M, Palicki O, Septier C, Chabanet C, Lucchi G, et al. (2012). Salivary Protein Profiles and Sensitivity to the Bitter Taste of Caffeine. Chemical Senses 37, 87–95.
7. Galindo MM, Voigt N, Stein J, van Lengerich J, Raguse JD, et al. (2012). G protein-coupled receptors in human fat taste perception. Chemical Senses 37, 123-139.
8. Grabenhorst F, Rolls ET, Parris BA & d'Souza AA. (2010). How the Brain Represents the Reward Value of Fat in the Mouth. Cerebral Cortex 20, 1082 - 1091.
9. Hammond RA, Ornstein JT, Fellows LK, Dubé L, Levitan R et al. (2012). A model of food reward learning with dynamic reward exposure. Frontiers in Computational Neuroscience 6, 82.
10. Hamosh M. (1978). Rat lingual lipase: factors affecting enzyme activity and secretion. American Journal of Physiology - Regulatory, Integrative, and Comparative Physiology 299, 1050-1058.
11. Hamosh M. (1990). Lingual and gastric lipases. Nutrition 6, 421-8.
12. Henkin RI, Law JS & Nelson NR. (1988). The role of zinc on the trophic growth factors nerve growth factor and gustin. In Trace elements in man and animal. Vol 6. [Hurley LS, Keen CL, Lonnerdal B, Rucker RB, editors] New York, NY: Plenum Press. 385–8.
13. Henkin RI, Martin BM & Agarwal RP. (1999a). Efficacy of exogenous oral zinc in treatment of patients with carbonic anhydrase VI deficiency. The American Journal of Medical Sciences 318, 392–405.
14. Henkin RI, Martin BM & Agarwal RP. (1999b). Decreased parotid saliva gustin/carbonic anhydrase VI secretion: an enzyme disorder manifested by gustatory and olfactory dysfunction. The AmericanJournal of Medical Sciences. 318, 380-91.
15. Humphrey SP & Williamson RT. (2001). A review of saliva: Normal composition, flow, and function. The Journal of prosthetic dentistry 85, 162-9.
16. Kawai T & Fushiki T. (2003). Importance of lipolysis in oral cavity for oro-sensory detection of fat. American Journal of Physiology - Regulatory, Integrative, and Comparative Physiology 285, 447-454.
17. Khan NA & Besnard P. (2009). Oro-sensory perception of dietary lipids: New insights into the fat taste transduction. Biochimica et Biophysica Acta 1791, 149–155.

18. Kulkarni B, Mattes R. (2013). Evidence for presence of nonesterified fatty acids as potential gustatory signaling molecules in humans. Chemical Senses 38,119-27.

19. Kurooka S, Okamoto S & Hashimoto M. (1977). Novel and Simple Colorimetric Assay for Human Serum Lipase. The Journal of Biochemistry 81, 361-369.

20. Ledikwe JH, Ello-Martin J, Pelkman CL, Birch LL, Mannino ML et al. (2007). A reliable, valid questionnaire indicates that preference for dietary fat declines when following a reduced-fat diet. Appetite 49, 74–83.

21. Lenoir M, Serre F, Cantin L & Ahmed SH. (2007). Intense Sweetness Surpasses Cocaine Reward. PlosONE 8, e698.

22. Mandel AL & Breslin PA. (2012). High endogenous salivary amylase activity is associated with improved glycemic homeostasis following starch ingestion in adults. Journal of Nutrition 142, 853-8.

23. Mandel AL, Peyrot des Gachons C, Plank KL, Alarcon S & Breslin PA. (2010). Individual Differences in AMY1 Gene Copy Number, Salivary a-Amylase Levels, and the Perception of Oral Starch. PlosONE 5, e13352.

24. Matsuo R. (2000). Role of Saliva in the Maintenance of Taste Sensitivity. Critical Reviews in Oral Biology & Medicine 11, 216-229.

25. Nater UM, Rohleder N, Schlotz W, Ehlert U & Kirschbaum C. (2007). Determinants of the diurnal course of salivary alpha-amylase. Psychoneuroendocrinology 32, 392–401.

26. Navazesh M. & Kumar SK. (2008). Measuring salivary flow: challenges and opportunities. The Journal of American Dental Association 139 Suppl:35S-40S.

27. Neyraud E, Palicki O, Schwartz C, Nicklaus S & Feron G. (2012). Variability of human saliva composition: possible relationships with fat perception and liking. Archives of oral biology 57, 556-66.

28. Nguyen T & Lau DCW. (2012). The Obesity Epidemic and Its Impact on Hypertension. Canadian Journal of Cardiology 28, 326–333.

29. Noble RE. (2000). Salivary alpha-amylase and lysozyme levels: a non-invasive technique for measuring parotid vs. submandibular/sublingual gland activity. Journal of Oral Science 42, 83–6.

30. Padiglia A, Zonza A, Atzori E, Chillotti C, Calò C et al. (2010). Sensitivity to 6-n-propylthiouracil is associated with gustin (carbonic anhydrase VI) gene polymorphism, salivary zinc, and body mass index in humans. American Journal of Clinical Nutrition 92, 539-45.

31. Pangborn RM, Bos KEO & Stern JS. (1985). Dietary fat intake and taste response to fat in milk by under-, normal, and overweight women. Appetite 6, 25–40.

32. Passilly-Degrace P, Chevrot M, Bernard A, Ancel D, Martin C, et al. (2014). Is the taste of fat regulated? Biochimie, 96: 3-7.

33. Pepino MY, Love-Gregory L, Klein S & Abumrad NA. (2012). The fatty acid translocase gene, CD36, and lingual lipase influence oral sensitivity to fat in obese subjects. Journal of lipid research 53, 561-6.

34. Perry GH, Dominy NJ, Claw KG, Lee AS, Fiegler H et al. (2007). Diet and the evolution of human amylase gene copy number variation. Nature Genetics, Letters 39, 1256-1260.

35. Salles C, Chagnon MC, Feron G, Guichard E, Laboure H, et al. (2011). In mouth mechanisms leading to flavor release and perception. Critical Reviews in Food Science and Nutrition 51, 67-90.

36. Simons PJ, Kummer JA, Luiken JJFP & Boon L. (2011). Apical CD36 immunolocalization in human and porcine taste buds from circumvallate and foliate papillae. Acta Histochemica 113, 839– 843.

37. Smeets AJPG & Westerterp-Plantenga MS. (2006). Satiety and substrate mobilization after oral fat stimulation. British Journal of Nutrition 95, 795–801.

38. Stewart JE, Feinle-Bisset C, Golding M, Delahunty C, Clifton PM et al. (2010). Oral sensitivity to fatty acids, food consumption and BMI in human subjects. British Journal of Nutrition 104, 145–152.

39. Stratford JM & Contreras RJ. (2009). Saliva and other taste stimuli are important for gustatory processing of linoleic acid. American Journal of Physiology Regulatory, Integrative and Comparative Physiology 297, 1162-1170.

40. Stunkard AJ & Messick S. (1985). The three-factor eating questionnaire to measure dietary restraint, disinhibition, and hunger. Journal of Psychosomatic Research. 29, 71–83.

41. Tepper BJ & Nurse RJ. (1997). Fat perception is related to PROP taster status. Physiology & Behavior 61, 949-54.

42. Thoma MV, Kirschbaum C, Wolf JM & Rohleder N. (2012). Acute stress responses in salivary alpha-amylase predict increases of plasma norepinephrine. Biological Psychology 91, 342-348.

43. Toepel U, Knebel JF, Hudry J, le Coutre & Murray MM. (2010). Advantageous Object Recognition for High-Fat Food Images. In Fat Detection: Taste, Texture, and Post Ingestive Effects. Chapter 9, [Montmayeur, J.P., le Coutre, J. editors].

44. Voho A, Chen J, Kumar M, Rao M & Wetmur J. (2006). Lipase expression and activity in saliva in a healthy population. Epidemiology. 17, S336-S336.

45. Wise RA. (2004). Dopamine, learning and motivation. Nature Review Neuroscience 5, 483–94.

Chapter 3. Food liking enhances the plasma response of 2-arachidonoylglycerol and of pancreatic polypeptide upon modified sham feeding in humans

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Abstract

Background. Food palatability increases food intake and may lead to overeating. Mechanisms behind this observation are still largely unknown.

Objective. The aims of this study were i) to elucidate the plasma responses of endocannabinoids, N-acylethanolamines and gastrointestinal peptides to a palatable (sweet, SW), unpalatable (bitter, BT) and sensory acceptable (tasteless control, CT); and ii) to verify if some of these bioactives can serve as plasma biomarkers of food liking in humans.

Methods. Three puddings providing 60 kcal (of which 35% from proteins, 62% from carbohydrates and 3% from fats) but with different taste were developed. Twenty healthy subjects (11 women / 9 men, mean age 28 years and BMI 22.7 kg/m^2), selected because they liked the puddings in the order SW>CT>BT, participated in a randomized crossover study based on a modified sham feeding (MSF) protocol. Blood samples at baseline and every 5 min up to 20 min following the MSF were analyzed for gastrointestinal peptides, endocannabinoids, and N-acylethanolamines. Thirty minutes after MSF, energy intake at an *ad libitum* breakfast was measured.

Results. Following MSF, no response was observed with 7 out of the 9 gastrointestinal peptides measured. Ghrelin concentration at 20 min after SW and BT was 25% lower than after CT ($p=0.04$) and pancreatic polypeptide response after SW was 23% greater than after BT ($p=0.02$). The plasma response of 2-arachidonoylglycerol after SW was 37% and 15% higher than BT ($p<0.001$) and CT ($p=0.03$), respectively. Trends for greater responses of anandamide ($p=0.06$), linoleoylethanolamide ($p=0.07$), palmitoylethanolamide ($p=0.06$) and oleoylethanolamide ($p=0.09$) after SW than BT were found. No differences in subsequent energy intake were recorded.

Conclusion. Data demonstrated that food palatability influenced some plasma endocannabinoids and N-acylethanolamines concentration during the cephalic phase response and indicated that 2-arachidonoylglycerol and pancreatic polypeptide can be used as biomarkers of food liking in humans.

Introduction

Food palatability is a very complex property deriving from the interaction of intrinsic food characteristics such as taste, smell, texture, temperature, visual appearance, sound, trigeminal input and personal feature driven by previous experience and cultural subdomains (1-3).

Among tastes, sweet acts as a key that unlocks activation of brain 'liking' systems, whereas bitter elicits aversion and disgust (4). These preferences are innate in animals as well as in humans and might have had a role in human evolution to detect plant poisonous substances (5). Nevertheless, social factors may lead to conditioned learning preference for bitter foods, as happen for coffee or some bitter vegetables (6-7).

Food palatability is generally assessed in humans using psychometric methods that are based on tasting of food and self-recording of a score for food pleasantness on hedonic visual analogue scales (VAS) or fixed point scales (FPS) (8). Cephalic phase response of eating can be efficiently investigated by using a modified sham feeding (MSF) protocol. In MSF experiments the food is tasted, chewed, but finally expectorated (9). The magnitude of the cephalic phase response is relatively small compared to the post-ingestive processes (10). However, using MSF it is possible to assess the biochemical variations without the confounding effects of nutrient digestion for a period of 10 to 60 minutes (9, 11). Both sensory and cognitive stimuli such as sight, smell, taste or even the thought of food can elicit cephalic responses to food (12). At these pre-ingestive phase, the activation of endocrine and exocrine gastrointestinal (GI) secretions is mostly mediated by the efferent component of the vagus nerve while involving different brain centers (13).

Food palatability may influence food intake in both a positive and a negative manner. In general, the higher is palatability of the food the higher is the intake (14), and the cephalic phase response plays a major role in this phenomenon (15). In fact, high palatable foods induced the secretion of pancreatic polypeptide (PP); sweet palatable stimulus being more effective than salty stimulus (10). On the other hand, oral stimulation with a high fat meal mobilizes lipid metabolites in blood (16), but it does not elicit significant differences in plasma responses of PP, insulin, glucagon and glucose compared to the fat free food (17). Interestingly, the cephalic response to an unpalatable bitter tasting food was reported to cause a delay in gastric emptying (18), however no data are available about the associated hormonal response.

Mounting evidence indicated that the endocannabinoid anandamide (AEA) and several other acylethanolamines (NAEs), including oleoylethanolamide, palmitoylethanolamide (PEA), and

linoleoylethanolamide (LEA), may contribute with GI hormones to the regulation of food intake (19). Moreover, both the ECs, AEA and 2-arachidonoyl glycerol (2-AG), may modulate the palatability of foods by altering peripheral sweet taste responses (20) as they increased in peripheral tissues and brain of animals sham fed with a lipid or nutrient mixed meal (21). A major role of 2-AG and ghrelin in hedonic eating in humans was also recently demonstrated by Monteleone et al. (22). However, the role of ECs and NAEs in the cephalic phase response to palatable and unpalatable food is largely unknown.

In this context, the main objective of this study was to investigate the modifications of the concentration of plasma endocannabinoids (ECs), N-acylethanolamines (NAEs) and GI peptides due to the cephalic phase response to a palatable (sweet, SW), unpalatable (bitter, BT) and sensory acceptable (tasteless control, CT) food.

To this purpose, a MSF protocol was carried out using foods specifically designed to have different taste and the same macronutrient composition and selecting participants according to the hedonic value attributed to those foods. The plasma response of GI peptides, of ECs and NAEs to gustatory stimulation every 5 minutes over the following 20 minutes and the effects on appetite sensation as well as was on subsequent energy intake were measured.

Methods

Food products

Experimental foods

Three milk puddings differing in taste - control (CT) with no taste, sweet (SW) and bitter (BT) - were developed and used as test foods. Their macronutrient composition is summarized in **Table 1**. The three foods were isocaloric, providing 60 kcal, 3% of daily estimated energy needs. CT was formulated using skimmed milk (91 %), wheat flour (7 %) and gelatin (2%). Bitter taste of BT was obtained by addition to CT of *Gentiana lutea* extract (1%), whereas sweet taste of SW was provided by addition to CT of Stevia® (1.3%) and sorbitol (9.8%).

Table 1. Energy values and macronutrient composition of the experimental puddings.

	Portion	**Energy**	**Protein**	**Carbohydrate**	**Fat**
	g	**kcal**	**g (*% E*)**[1]	**g (*% E*)**[1]	**g (*% E*)**[1]
Control (CT)	100	61.8	5.5 (*35.6*)	9.5 (*61.5*)	0.2 (*2.9*)
Sweet (SW)	100	56.6	5.0 (*35.3*)	8.7 (*61.5*)	0.2 (*3.2*)
Bitter (BT)	100	62.2	5.5 (*35.4*)	9.6 (*61.7*)	0.2 (*2.9*)

[1]Percentage of energy

Test Meal

The test meal consisted of a variety of foods that were selected from those normally eaten for breakfast/snack by the volunteers taking into account their nutritional content (low/high) and taste (sweet/savory). The foods were selected among those getting by participants an average preference of 6 on a scale from 0 to 10 (data not shown). The test meal was offered on a tray where foods were always presented in the same order and subjects were allowed to eat *ad libitum* as much as they wanted. The test meal macronutrient composition is given in **Table 2**.

Table2. Energy values and macronutrient composition of the test meal by serving size.

	Portion g	Energy kcal	Protein g	Carbohydrate g	Fat g	Dietary fiber g
Jelly roll	28	102	1.6	17.5	2.6	1.0
Chocolate snack	42	195	2.4	18.3	15.3	1.2
Vanilla cream filled biscuits	55	278	3.2	35.9	13.2	1.4
Biscuits	60	295	4	38.5	13.5	2.0
Dietary fiber rich biscuits	28	120	3	16.5	3.5	4.0
Salty biscuits	18	90	1.4	11.5	4.0	0.36
Cheese finger snack	23.5	129	1.9	13.2	7.6	0.4
Corn flakes	27	102	1.9	22.7	0.24	0.8
Chocolate cereal	34	131.6	2.8	25.9	1.4	2.1
Semi-skim milk	125	59	4	6.3	2	0
Fruit Juice	125	55.3	0.3	13.4	0	0.4

Subjects

Recruitment was performed at the Department of Agricultural and Food Science of University of Naples. Subjects were recruited by public announcements in local public sites, online social networks, and among students and staff of the Department. Subjects were excluded from the study if they had a BMI≥25 kg/m^2, any chronic illnesses such as diabetes, hypertension, or heart arrhythmia, if they were smokers or they were taking any medication, with food allergy, dieting or those who were under a controlled dietary regimen over the previous three months. Twenty-eight subjects were screened on the basis of a palatability test were they had to express hedonic values for the three experimental foods (BT, SW and CT). Subjects reporting a low preference (but not disgust) for BT, average preference for CT and high preference for SW were included in the study. Twenty participants were recruited and completed the protocol. Eating behavior was assessed using a validated Italian translation of the three factors eating questionnaire TFEQ (23) and volunteers with a restraint score >9 were excluded from participation (24). The participants' characteristics are summarized in the **Table 3**. Subjects were unaware of the exact aim of the study and entered into the study by signing a written informed consent. All experimental procedures were approved by the Ethics Committee of the University of Naples.

Table 3. General characteristics of participants.

		Range
Subjects, n	20	
Sex, M/F	11/9	
Age, y [1]	28±1	24-36
BMI [1]**, kg/m²**	22.7±0.61	20.2-24.6
Fasting blood glucose [1]**, mg/dL**	83.4±2.3	78-86
Restraint [1,2]**, score**	6.7±0.6	5-9
Disinhibition [1,2]**, score**	6.3±1.3	3-12
Hunger [1,2]**, score**	3.1±0.7	1-6

[1] Values are means ±SEMs, n=20.
[2] Results from the Three Eating Factor Questionnaire.

Study design

Eligible subjects who accepted to enter into the study were instructed about the MSF protocol and they underwent a training test two days before the first experimental day.

The experimental tests were conducted on three separate days (one for each pudding) in a randomized crossover design and with a 1-week wash-out period from each other. The experimental tests took place early in the morning with fasting subjects. Participants were instructed to consume a standardized dinner on the evening before each test and to refrain from eating and drinking energy-containing foods and beverages from 22:00h on the day before each test day.

Once arrived to the nutritional laboratory of the Department of Agricultural and Food Science, subjects had a 10 min rest and then they were instructed to rate their hunger, thirst, fullness and desire to eat on a 100 mm VAS, anchored on the left as ‘‘not at all’’ and on the right as ‘‘extremely’’. The questionnaire comprised 4 main questions (How satiated do you feel? How full do you feel? How thirsty are you now? How great is your desire to eat?), and subjects were asked to answer by indicating on the scale the point corresponding to their sensations. Then, to be sure that all the subjects were in a fasted state blood glucose concentration by finger pricking was measured and experimental testing was postponed if blood glucose was higher than 100 mg/dL (10). Subsequently, a peripheral catheter was placed into an arm vein for venous blood collection at specific time points. After a 15 min acclimation period, subjects were submitted to the first blood drawing (baseline, t0) and 5 min later they started the MSF protocol. During MSF subjects were presented with one of experimental foods and were instructed to palpate the food until the point at which they would normally swallow and then to expectorate the food into a plastic cup.

They were continually reminded not to swallow any food and they repeated the procedure for 3 minutes, in order to elicit a cephalic phase response (10). A recovery measure of the dried weight of food in the spit material was performed to check that subjects did not swallow (25).

Blood samples were collected at 5, 10, 15 and 20 min later MSF started. Ten minutes later the last blood drawing (30 min after the start of MSF), each subject rated thirst and appetite sensations on VAS questionnaire and a tray containing weighted and nutritionally known portions of selected foods was offered for breakfast. Subjects sat separately and they were not allowed to see or talk to each other while having their breakfast. Energy intake (EI) at the breakfast was measured by weighting the foods left by subjects in their trays. A scheme of the experimental study design is shown in **Figure 1**.

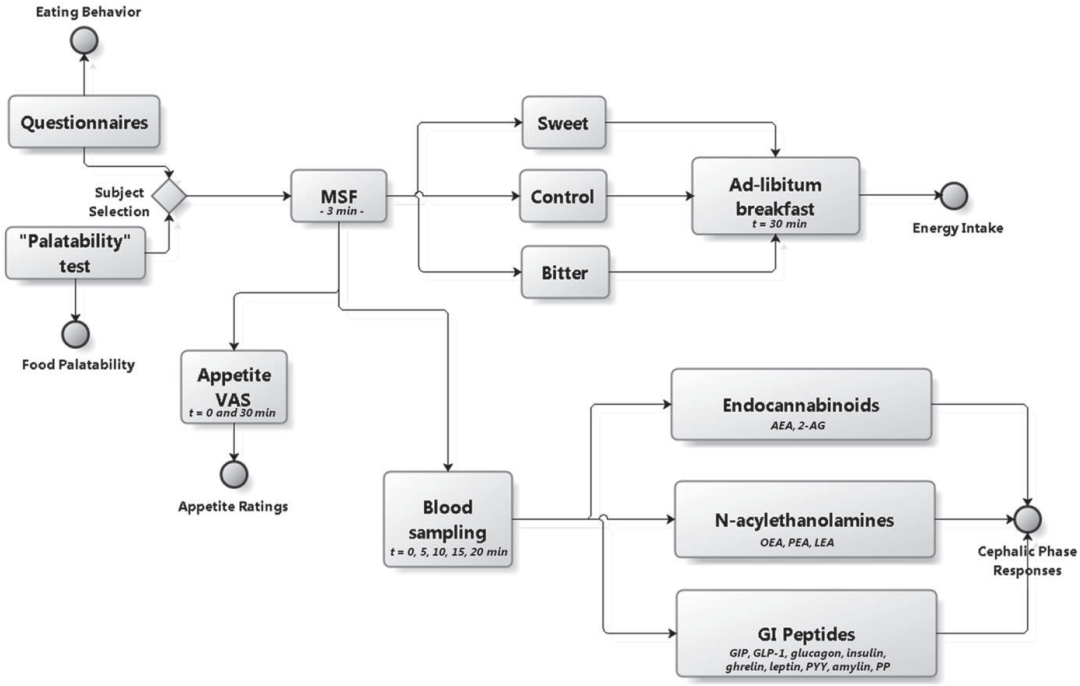

Figure 1. **Study design.** Outcomes and times are reported in circles and in boxes, respectively.

Biochemical analysis

Blood glucose

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

Blood collection

For each time point, 3 mL of blood were collected into two different vacutainer® tubes with EDTA. Only the blood for the GI peptide analysis (one tube) was immediately mixed with a protease inhibitor mix consisting of dipeptidylpeptidase IV (DPPIV) inhibitor (Millipore’s DPPIV inhibitor; St Charles, MO, USA), protease inhibitor cocktail (Sigma, St. Louis, MO, USA) and 4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF, Pefabloc® SC, Roche-diagnostics). After sample preparation, both tubes were centrifuged at 4000 g for 10 min and plasma samples were aliquoted and frozen at -80°C, within 30 min from collection.

Plasma gastrointestinal peptides

Plasma GI peptides were determined in duplicate in 25 µL of plasma by using Milliplex® human hormone magnetic bead panel (Merck Millipore, Millipore S.p.A., Via XI Febbraio, 99, 20090 Vimodrone - MI) and by using Luminex Technology (Bio-Plex; Bio-Rad, Nazareth, Belgium). The procedure described in the booklet was strictly followed from the collection of samples up to the data analysis. The Milliplex® immunoassays kits allowed the simultaneous quantification of the following biomarkers: ghrelin, leptin, glucose-dependent insulinotropic peptide (GIP), glucagon-like peptide -1 (GLP-1), glucagon, insulin, polypeptide pancreatic (PP), total amylin and total peptide YY (PYY). The sensitivity levels of the assay (in pg/mL) correspond to the following: ghrelin 2.0; leptin, 27.0; GIP, 0.6; GLP-1, 7.0; glucagon, 6.0; insulin, 58.0; PP, 2.0; total amylin, 14.0; PYY, 8.0. The intra-assay variation (%CV) was 2% for ghrelin, total amylin and PYY; 3% for GIP, glucagon, insulin and leptin; 4% for PP and 7% for GLP-1. The inter-assay variation (%CV) was 4% for leptin; 5% for GIP; 6% for insulin; 7% for PP and glucagon; 8% for ghrelin; 10% for GLP-1; 11% for PYY and 33% for amylin.

Plasma endocannabinoids and N-acylethanolamines

Plasma concentrations of the ECs (AEA and 2-AG), and the NAEs (OEA, LEA, and PEA), were simultaneously quantified by liquid chromatography-mass spectrometry as previously described by Mennella et al. (26). Briefly 500 µL of sample was mixed with 3 volumes of acetone and centrifuged for protein precipitation. The supernatant was extracted with 1.5 mL of methanol/chloroform (1:2) containing 5 pmol of d8-anandamide as internal standard. The organic phase was then dried under nitrogen, and re-suspended in 100 µL of acetonitrile:water (1:1) before chromatrographic separation. This was performed using an HPLC apparatus equipped with two micropumps Perkin-Elmer series 200 (Norwalk, CT, USA). A Synergi Max RP 80 column, 50x2.1 mm (Phenomenex, USA) was used and the flow rate was set to 0.2 mL/min. Injection volume was 10 µL. Mobile phases and the gradient program were the same as in (18). MS/MS analyses were performed on an API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Canada). All the analyses were performed with a TurboIonSpray source set as previously described (26) 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 were already published (26).

Statistical analysis

Results are expressed as the mean ± SEM. Analysis of variance was done to exclude any difference at baseline between treatments for both appetite scores and biochemical parameters. Since no differences were found the results were analyzed and expressed as the absolute changes from the baseline to reduce possible effects of inter-subject fasting variability. The total area under the curves (AUC) of desire to eat, fullness and satiety ratings as well as of GI hormone, EC and NAE plasma concentrations from the baseline until 20 min after MSF were also estimated using the linear trapezoidal rule. The subjective appetite sensations recorded after the MSF with the three puddings and the response of hormones and ECs were compared and tested for the effect of treatment and of time as factors by the analysis of variance (ANOVA) for repeated measures. For all the tests, following a significant main effect in ANOVA, individual means were compared using Tukey's test. Results were considered statistically significant for $p<0.05$. Bivariate correlation analysis between variables was carried out using the Pearson product-moment correlation coefficient. Statistical analyses were performed using Statistical Package for Social Sciences (version 16.0; SPSS, Inc., Chicago, IL, USA).

Results

Puddings recovery

The recovery of puddings was calculated on the total weight (immediately after the MSF) and on the dried weight (after freeze-drying) of the chewed puddings. A recovery of 102 ± 6.71 % on the total weight and 98.3 ± 5.80 % on the dried weight, respectively, was found.

Appetite ratings

Subjective appetite ratings were recorded before (baseline) and after the MSF, immediately before the *ad libitum* breakfast. No difference in appetite scores at baseline among treatments and between time x treatments was found (**Figure 2**). Interestingly, the desire to eat after the MSF with CT negatively correlated with individual disinhibition (F2) ($r=-0.41$; $p=0.001$) and hunger factor (F3) ($r=-0.47$; $p=0.02$); moreover F2 negatively correlated with fullness ($r=-0.43$; $p=0.01$) and satiety ($r=-0.34$; $p=0.02$).

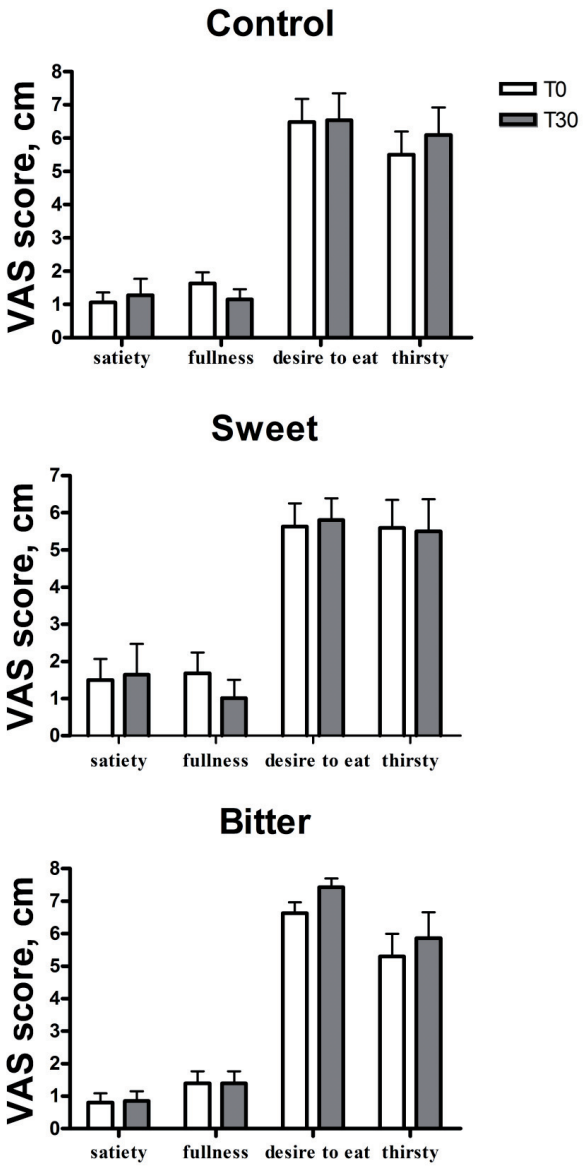

Figure 2: Appetite ratings recorded at baseline (t = 0 min) and before *ad-libitum* test meal (t =30 min) following MSF with Control, Sweet and Bitter pudding. No significant differences were found within and between the treatments. Data are means ± SEMs, n=20. VAS, Visual Analogue Scale.

Energy intake at test meal

No differences in energy intake (592±49 kcal, 608±63 kcal and 604±48 kcal after CT, SW and BT, respectively; $p>0.05$) or macronutrient composition of meals following the different puddings were found.

GI peptides

No significant differences in specific GI peptides at baseline among treatments were found. No significant changes of plasma GIP, GLP-1, glucagon, insulin, leptin, total amylin and PYY concentrations over the 20 min following the MSF for any experimental condition was found (**Figure 3**).

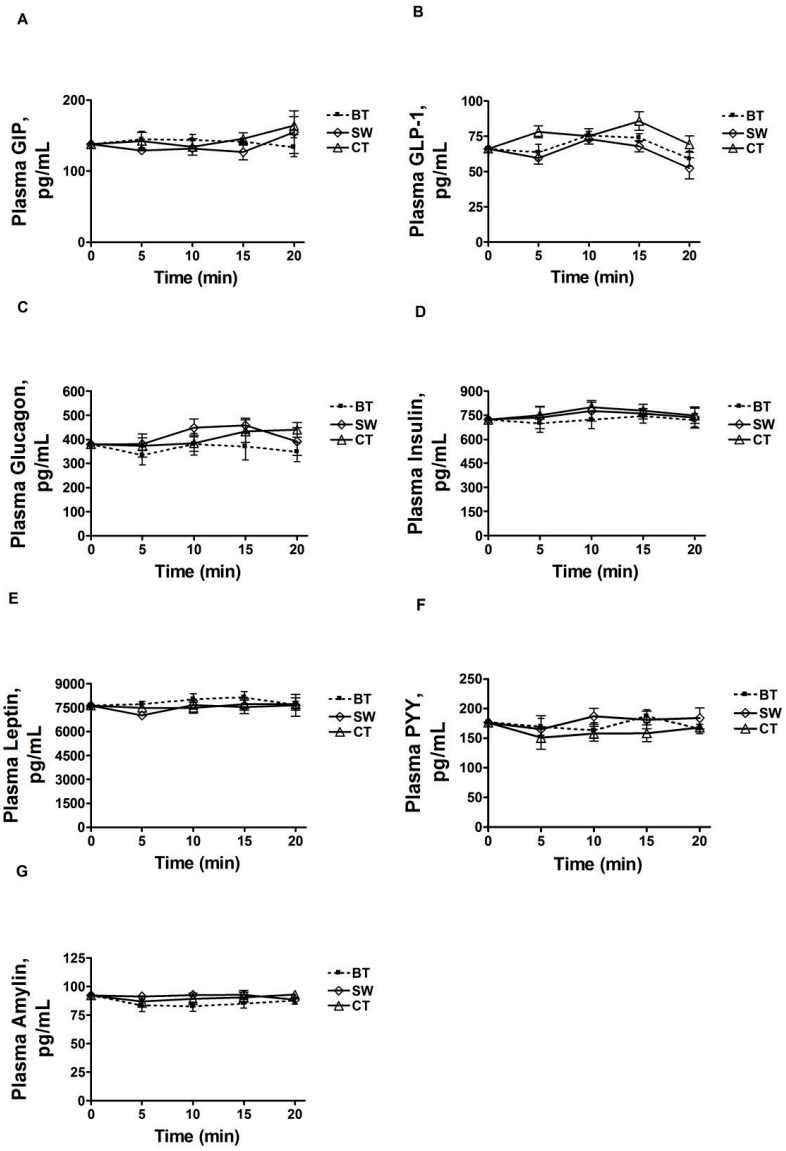

Figure 3: Concentration-time curves of plasma GI peptides following sensory stimulation with palatable (sweet, SW), unpalatable (bitter, BT) and sensory acceptable (tasteless control, CT) pudding. Data are means ± SEMs, n=20. No significant differences were found over time within and between treatments for these 7 out of 9 GI peptides monitored.

The time-concentration curves of plasma ghrelin and PP are shown in **Figure 4**. Ghrelin concentration was significantly lower 20 min after BT (p=0.04) and SW (p=0.04) than after CT

whereas PP concentration was significantly lower 15 min and 20 min after BT (p=0.02 and p=0.03, respectively) than after SW. As a consequence, $AUC_{10\text{-}20}$ of PP following BT was significantly lower than after SW (p=0.02) and tended to be lower than after CT (p=0.06). Moreover, a tendency for a reduced response of PP over all 20 min following BT vs SW was found ($AUC_{0\text{-}20}$ being $1.65 \cdot 10^3 \pm 138$ pg/mL·min vs $2.15 \cdot 10^3 \pm 144$ pg/mL·min, respectively; p=0.07).

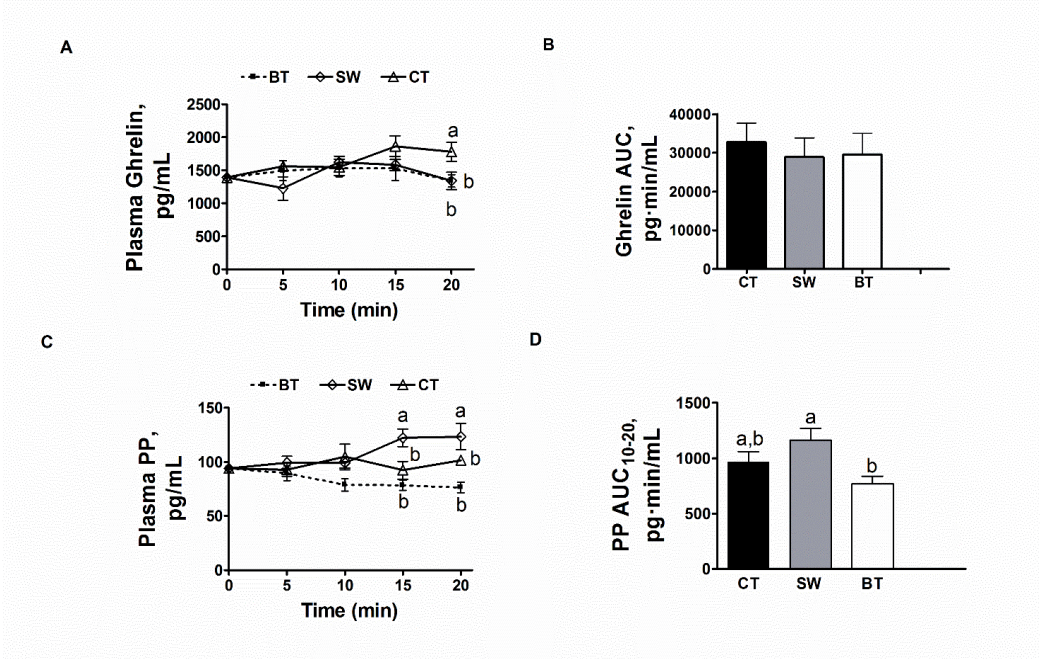

Figure 4. Plasma response of ghrelin and pancreatic polypeptide (PP) found in subjects following sensory stimulation with palatable (sweet, SW), unpalatable (bitter, BT) and sensory acceptable (tasteless control, CT) pudding. Data are means ± SEMs, n=20. Labelled means at a time without a common letter differ, $P < 0.05$.

Endocannabinoids and N-acylethanolamines

Plasma concentration-time curves and AUC of each EC and NAE are shown in **Figure 5**. No significant differences in concentrations of the specific molecules at baseline and over time compared to baseline were found between the treatments. However, the direction of plasma responses was always after SW>CT>BT and significant differences in 2-AG, AEA, and PEA concentrations at some time points following SW and BT were also observed. These differences were for 2-AG at 10 min (p=0.001), at 15 min (p=0.004) and at 20 min (p=0.001), for AEA at 10 min (p=0.015), and for PEA at 20 min (p=0.042). However, only the AUC of 2-AG after SW was

15% higher than after CT ($p=0.03$) and 37% higher than after BT ($p<0.10 \cdot 10^{-2}$). Moreover, a trend for greater responses of AEA ($p=0.06$), LEA ($p=0.07$) and PEA ($p=0.06$) over 20 min following SW than BT were observed; for OEA the same trend was found when $AUC_{10\text{-}20}$ was considered ($p=0.09$).

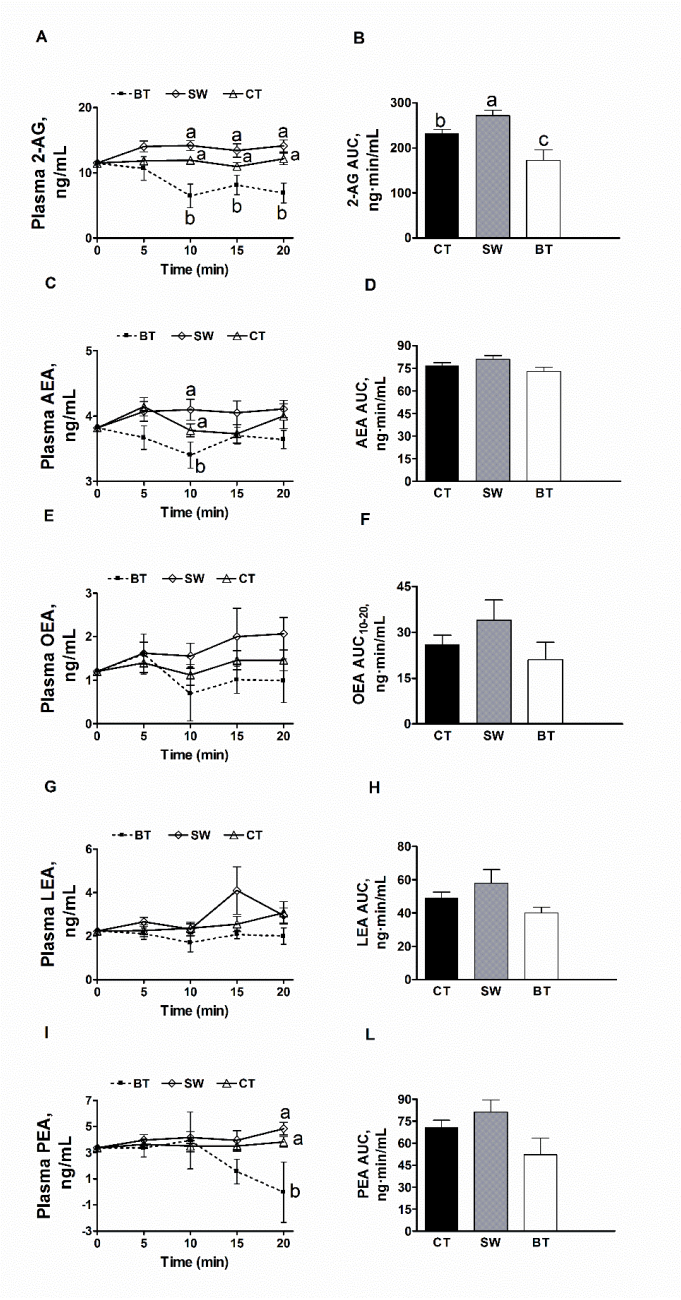

Figure 2. Plasma response of endocannabinoids (ECs) and N-acylethanolamines (NAEs) found in subjects following sensory stimulation with palatable (sweet, SW), unpalatable (bitter, BT) and sensory acceptable (tasteless control, CT) pudding. Data are means ± SEMs, n=20. Labelled means at a time without a common letter differ, $P < 0.05$.

Associations between variables

Pearson's correlation analysis between variables showed several significant associations summarized in **Table 4**. Data showed that energy intake at *ad libitum* meal test were positively correlated with the desire to eat only after MSF with CT and SW, whereas they were inversely associated with fullness and satiety feelings only after MSF with CT. Moreover the desire to eat after SW stimulation was also positively associated with ghrelin concentration at 20 min and plasma ghrelin response (AUC) negatively correlated with the individual disinhibition factor (F2 score). Interestingly, individual disinhibition factor positively correlated with plasma response of AEA, OEA, LEA and PEA following SW whereas it negatively correlated with plasma response of OEA and LEA following BT. This biochemical response was in turn positively associated with the response of PP and reduced individual desire to eat only after BT. Indeed, only after the MSF with CT the concentration of PP at 20 min positively correlated with satiety score and PP response (AUC) following CT positively correlated with that of OEA.

Table 4. Associations between some variables monitored during the MSF experiments with CT, BT and SW, n=20.

	CT		BT		SW	
	r	***P***	***r***	***P***	***r***	***P***
Energy intake (kcal)[1]						
Desire to eat (cm) [2]	0.46	0.008	0.15	0.67	0.72	0.01
Fullness (cm) [2]	-0.55	0.01	0.36	0.29	0.05	0.9
Satiety (cm) [2]	-0.51	0.006	0.59	0.07	0.25	0.5
Plasma ghrelin (pg/mL) [3]						
Desire to eat (cm) [2]	-0.61	0.11	-0.19	0.58	0.71	0.032
Fullness (cm) [2]	0.42	0.22	0.18	0.6	0.26	0.49
Satiety (cm) [2]	0.54	0.11	0.21	0.56	0.36	0.34
Plasma PP (pg/mL) [3]						
Desire to eat (cm) [2]	0.26	0.83	-0.36	0.55	0.02	0.96
Fullness (cm) [2]	0.85	0.09	-0.36	0.54	0.19	0.74
Satiety (cm) [2]	0.85	0.04	0.15	0.81	0.16	0.78
Plasma PP (pg/mL•min) [4]						
AEA (pg/mL•min) [4]	0.32	0.29	0.33	0.2	0.2	0.93
2-AG (pg/mL•min) [4]	0.21	0.74	0.26	0.39	0.32	0.18
OEA (pg/mL•min) [4]	0.86	0.03	0.84	0.04	0.26	0.56
LEA (pg/mL•min) [4]	0.24	0.69	0.85	0.04	0.36	0.37
PEA (pg/mL•min) [4]	0.31	0.16	0.29	0.39	0.49	0.18
Individual disinhibition (factor)[5]						
AEA (pg/mL•min) [4]	-0.01	0.96	-0.26	0.29	0.65	0.001
2-AG (pg/mL•min) [4]	0.12	0.60	-0.36	0.11	0.16	0.47
OEA (pg/mL•min) [4]	0.16	0.47	-0.39	0.03	0.58	0.004
LEA (pg/mL•min) [4]	0.02	0.92	-0.41	0.04	0.49	0.02
PEA (pg/mL•min) [4]	0.38	0.08	-0.31	0.16	0.59	0.04

[1] Energy intake were measured at *ad libitum* test meal offered to subjects 20 min after MSF procedure
[2] Appetite feelings measured by Visual Analogue Scale before *ad libitum* test meal
[3] Plasma concentration of gastro-intestinal peptides 20 min after MSF were measured by multiplexed assay
[4] Plasma concentration of PP, ECs and NAEs every 5 min over 20 min following MSF were measured by LC/MS/MS method and $AUC_{(0\text{-}20)}$ was calculated for each parameter
[5] Individual disinhibition indicates an eating behavior trait and was measured by TFEQ

Discussion

The present study is the first to describe the cephalic response induced by three foods specifically developed to be palatable and sweet (SW), unpalatable and bitter (BT), sensory acceptable and tasteless (CT) in healthy subjects by measuring 9 GI peptides, 2 ECs and 3 NAEs in plasma and by evaluating the subsequent appetite and energy intake.

The semisolid pudding was used as test food because it could easily induce a cephalic phase response upon a MSF protocol (10). Its low fat and sugar content reduced their confounding effects on ECs and insulin release (25, 27-29). A pudding could also be produced in three formulations differing only for the taste and sharing the same macronutrient composition, texture

and color. In fact, the low temperatures used for preparation prevented the heat-induced formation of compounds that could influence sensory properties of the final products (30).

The most important result of this study was the demonstration of a significant effect of food palatability (liking) on the plasma response of 2-AG. Moreover, the oral stimulation with the palatable food (SW) elicited a tendency to increased plasma responses of all ECs and NAEs compared to unpalatable (BT) food.

The arousal of ECs tone upon the consumption of rewarding fatty food was already demonstrated in animals (21) and when it was induced by sweet and fatty taste it could even enhance the taste sensitivity and drive the food preference (31-33). This might result in a self-reinforcing mechanism that could have been a fundamental role in human evolution (34).

The finding that the magnitude of the 2-AG response increases with the liking of the food is in line with a previous study by Monteleone et al. (22). They suggested that wanting of high palatable food increased plasma 2-AG in non-fasting subjects and found that food liking heightened the 2-AG tone over the 120 min following consumption of high palatable compared to a non-palatable food. Thus, the link established in the present study between circulating concentration of 2-AG in response to the hedonic value of the chewed food in fasting subjects demonstrates a main role of this compound in the cephalic phase of eating. This suggests a potential involvement of 2-AG in the habit of eating in absence of metabolic needs. Further studies should demonstrate the latter hypothesis in hedonic eaters.

As the foods used in this study had the same macronutrient composition the results also suggest that the taste and liking value of a food influence the 2-AG spillover from the brain, the peripheral organs, or vascular endothelium independently from food macronutrient composition and energy value. Further human studies may verify this hypothesis. The reduced tone of 2-AG and AEA following bitter stimulus is in line with Janssen et al. (35) who demonstrated that intra-gastric administration of bitter compounds increased satiety and reduced food intake in rats. The data of our study suggest that a decrease in 2-AG plasma levels might have contributed to the satiating effect elicited by bitter compounds in those animals (35). After ingestion, bitter compounds may bind to the taste receptors distributed throughout the GI tract (36) and may trigger a satiating effect thanks to the reduction of the ECs tone.

No response of 7 out of 9 GI peptides monitored was recorded following the MSF with the puddings. This result confirmed our expectations and strengthened the decision of using puddings

with a low fat and carbohydrate content to evaluate the plasma ECs response without the potential interference of insulin, which is known to lower ECs concentration after eating (37). However, SW elicited a 23% greater response of PP than BT, thus confirming that individual liking of a food modulates the response of PP: the higher is the food palatability, the higher is the PP response (10).

Despite a general low response of ghrelin was recorded, it positively correlated with the 2-AG response after SW. Similarly, positive correlations were found between PP and OEA after both CT and BT. All these correlations suggest the existence of a link between GI hormones, ECs and NAEs responses in humans.

Taken together, data demonstrated that food palatability is associated with modifications in plasma ECs, NAEs and PP concentrations during cephalic phase of eating. Although these modification were expected to induce orexigenic effect (38), we found that they did not influence subsequent appetite sensations and eating behavior in the short term. This might be due to the short length (3 min) of the MSF as in a previous study Wijlens et al. (39) demonstrated that the duration of the orosensory stimulation modulate the subsequent appetite and energy intakes: the longer the stimulation, the lower the hunger and energy intakes (39).

The correlations between individual disinhibition factor and biochemical response to MSF are worth of notice. “Disinhibition” (which is the propensity to overeating) is recognized as closely related to food sensitivity and to the onset of eating (40). Hence, it would be positively associated with increased food intake as well as with response to palatability (41). Data showed that over MSF with SW individual disinhibition positively correlated with LEA and OEA responses and negatively correlated with ghrelin (that in turn correlated with desire to eat); whereas following MSF with BT a negative correlation between disinhibition and OEA and LEA (which negatively correlated with the desire to eat) was found.

In other words, present data indicate that individual propensity to overeating is associated with NAEs response in the early phase of eating and LEA and OEA may trigger disinhibited eating (that is eating in absence of metabolic needs) in normal weight subjects. A specific study with overweight and obese subjects should be performed to verify this hypothesis.

This study had also some limitations. The first one is related to the low fat and carbohydrate content of puddings which might have caused the lack of a response of most of the of GI peptides measured and the general low responses of all biochemical parameters thus possibly causing an underestimation of the physiological response occurring when a pudding with regular amount of

fats and sugar is tasted. Secondly, no specific correction was made for multiple comparisons, this possibly leading to some false-positive findings.

In conclusion, data obtained in this study provided some fundamental clues on the physiological role of ECs, NAEs and GI peptides during a cephalic phase response to palatable and unpalatable food. It was demonstrated that:

- food palatability influenced plasma responses of ECs and NAEs over 20 min following oral stimulation, without swallowing;
- 2-AG and PP were the main physiological biomarkers of ECs and GI hormone response associated to palatable foods, respectively;
- food palatability did not influence appetite, energy intake or food choice following a 3 minutes oral stimulation in healthy and fasting subjects.

As greater biochemical responses are expected during post-ingestive phase than pre-ingestive, these data support the idea that bitter compounds may modulate GI hormones, ECs and NAEs upon ingestion in humans and may elicit satiety and reduce energy intake, as already shown in animals (35).

Acknowledgments

Authors' contributions to manuscript: PV designed the study; IM and FZ conducted the research; IM, FZ and RF analyzed data or performed statistical analysis; IM, PV and VF wrote the paper; PV had primary responsibility for final content. All authors have read and approved the final manuscript.

References

1. Yeomans MR, Blundell JE, Leshem M. Palatability: response to nutritional need or need-free stimulation of appetite? *Br J Nutr* 2004; 92 Suppl 1:S3-14.
2. Hyde R J, Witherly SA. Dynamic contrast: a sensory contribution to palatability. *Appetite* 1993; 21:1-16.
3. Nakano K, Kyutoku Y, Sawa M, Matsumura S, Dan I, Fushik T. Analyzing comprehensive palatability of cheese products by multivariate regression to its subdomains. *Food Science & Nutrition* 2013; 1: 369-376. doi: 10.1002/fsn3.48.
4. Berridge KC. 'Liking' and 'wanting' food rewards: Brain substrates and roles in eating disorders. *Physiol Behav* 2009; 97:537-50. doi: 10.1016/j.physbeh.2009.02.044.
5. Drewnowski A, Gomez-Carneros C. Bitter taste, phytonutrients, and the consumer: a review. *Am J Clin Nutr* 2004; 72:1424-35.
6. Prescott MJ, Buchanan-Smith HM, Smith AC. Social interaction with non-averse group-mates modifies a learned food aversion in single- and mixed-species groups of tamarins (Saguinus fuscicollis and S. labiatus). *Am J Primatol*; 2005; 65:313-26.
7. Galef BG Jr. Social Interaction Modifies Learned Aversions, Sodium Appetite, and Both Palatability and Handling-Time Induced Dietary Preference in Rats. *J Comp Psychol* 1986; 100:432-9.
8. Guy-Grand B, Lehnert V, Doassans M, Bellisle F. Type of test-meal affects palatability and eating style in humans. *Appetite*. 1994; 22:125-34.
9. Teff KL, Mattes RD, Engelman K. Cephalic phase insulin release in normal weight males: verification and reliability. *Am J Physiol* 1991; 261:E430–6.
10. Teff. K. Cephalic phase pancreatic polypeptide responses to liquid and solid stimuli in humans. *Physiol Behav* 2010; 99:317-23. doi: 10.1016/j.physbeh.2009.11.009.
11. Katschinski M. Nutritional implications of cephalic phase gastrointestinal responses. *Appetite* 2000; 34:189-96.
12. Buss C, Kraemer-Aguiar LG, Maranhão PA, Marinho C, de Souza MD, Wiernsperger, N, Bouskela E. Novel findings in the cephalic phase of digestion: A role for microcirculation? *Physiology & Behavior* 2012; 105:1082–1087.
13. Zafra MA, Molina F, Puerto A. The neural/cephalic phase reflexes in the physiology of nutrition. *Neurosci. Biobehav Rev* 2006; 30:1032–1044.
14. Sørensen LB, Møller P, Flint A, Martens M, Raben A. Effect of sensory perception of foods on appetite and food intake: a review of studies on humans. *Int J Obes Relat Metab Disord*. 2003; 27:1152-66.
15. Smeets PA, Erkner A, de Graaf C. Cephalic phase responses and appetite. Nutrition Reviews Vol. 68(11):643–655
16. Smeets AJ, Westerterp-Plantenga MS. Satiety and substrate mobilization after oral fat stimulation. *Br J Nutr*. 2006;95:795–801.
17. Crystal SR1, Teff KL. Tasting fat: cephalic phase hormonal responses and food intake in restrained and unrestrained eaters. *Physiol* Behav. 2006;89(2):213-20).
18. Wicks D, Wright J, Rayment P, Spiller R. Impact of bitter taste on gastric motility. Eur J Gastroenterol Hepatol. 2005;17(9):961-5.

19. Hansen HS1, Diep TA. N-acylethanolamines, anandamide and food intake. Biochem Pharmacol. 2009;78(6):553-60.

20. Niki M1, Jyotaki M, Yoshida R, Ninomiya Y. Reciprocal modulation of sweet taste by leptin and endocannabinoids. Results Probl Cell Differ. 2010;52:101-14.

21. Di Patrizio NV, Astarita G, Schwartz G, Li X, Piomelli D. Endocannabinoid signal in the gut controls dietary fat intake. *Proc Natl Acad Sci* 2011; 108:12904-8.

22. Monteleone P, Piscitelli F, Scognamiglio P, Monteleone AM, Canestrelli B, Di Marzo V, Maj M. Hedonic eating is associated with increased peripheral levels of ghrelin and the endocannabinoid 2-arachidonoyl-glycerol in healthy humans: a pilot study. *J Clin Endocrinol Metab* 2012; 97:E917-24.

23. Stunkard AJ, Messick S. The three-factor eating questionnaire to measure dietary restraint, disinhibition and hunger. *J Psychosom Res*. 1985; 29:71-83.

24. Crystal SR, Teff KL. Tasting fat: Cephalic phase hormonal responses and food intake in restrained and unrestrained eaters. *Physiol Behav* 2006; 89:213-20.

25. Smeets AJ, Lejeune MP, Westerterp-Plantenga MS. Effects of oral fat perception by modified sham feeding on energy expenditure, hormones and appetite profile in the postprandial state. *Br J Nutr*. 2009; 101:1360-8.

26. Mennella I, Savarese M, Ferracane R, Sacchi R, Vitaglione P. Oleic acid content of a meal promotes oleoylethanolamide response and reduces subsequent energy intake in humans. *Food Funct*. 2014; 10. DOI: 10.1039/C4FO00697F

27. Di Patrizio NV, Astarita G, Schwartz G, Li X, Piomelli D. Endocannabinoid signal in the gut controls dietary fat intake. *Proc Natl Acad Sci* 2011; 108:12904-8. doi: 10.1073/pnas.1104675108.

28. Abdallah L, Chabert M, Louis-Sylvestre J. Cephalic phase responses to sweet taste. *Am J Clin Nutr* 1997; 65:737-43.

29. Teff KL, Devine J, Engelman K. Sweet taste: effect on cephalic phase insulin release in men. *Physiol Behav* 1995; 57:1089-95.

30. Wang Y, Ho CT. Flavour chemistry of methylglyoxal and glyoxal. *Chem Soc Rev* 2012; 41:4140-9. doi: 10.1039/c2cs35025d.

31. Yoshida R, Ohkuri T, Jyotaki M, Yasuo T, Horio N, Yasumatsu K, Sanematsu K, Shigemura N, Yamamoto T, Margolskee RF et al. Endocannabinoids selectively enhance sweet taste. *Proc Natl Acad Sci* 2010; 107:935-9. doi: 10.1073/pnas.0912048107.

32. Jyotaki M, Shigemura N, Ninomiya Y. Modulation of sweet taste sensitivity by orexigenic and anorexigenic factors. *Endocr J* 2010;57:467-75.

33. Di Patrizio NV, Joslin A, Jung KM, Piomelli D. Endocannabinoid signaling in the gut mediates preference for dietary unsaturated fats. *FASEB J*. 2013; 27:2513-20. doi: 10.1096/fj.13-227587.

34. Fattore L, Melis M, Fadda P, Pistis M, Fratta W. The endocannabinoid system and nondrug rewarding behaviours. *Exp Neurol*. 2010; 224:23-36. doi: 10.1016/j.expneurol.2010.03.020.

35. Janssen S, Laermans J, Verhulst PJ, Thijs T, Tack J, Depoortere I. Bitter taste receptors and α-gustducin regulate the secretion of ghrelin with functional effects on food intake and gastric emptying. *Proc Natl Acad Sci*. 2011; 108:2094-9. doi: 10.1073/pnas.1011508108.

36. Trivedi BP. Neuroscience: hardwired for taste. *Nature.* 2012; 486:S7-9. doi: 10.1038/486S7a.

37. Di Marzo V, Verrijken A, Hakkarainen A, Petrosino S, Mertens I, Lundbom N, Piscitelli F, Westerbacka J, Soro-Paavonen A, Matias I et al. Role of insulin as a negative regulator of plasma endocannabinoid levels in obese and nonobese subjects. *Eur J Endocrinol* 2009;161:715-22. doi: 10.1530/EJE-09-0643.

38. Cristino L, Becker T, Di Marzo V. Endocannabinoids and energy homeostasis: an update. *Biofactors*. 2014; 40:389-97. doi: 10.1002/biof.1168.

39. Wijlens AG, Erkner A, Alexander E, Mars M, Smeets PA, de Graaf C. Effects of oral and gastric stimulation on appetite and energy intake. *Obesity* 2012; 20:2226-32.

40. French SA, Epstein LH, Jeffery RW, Blundell JE, Wardle J. Eating behavior dimensions. Associations with energy intake and body weight. A review. *Appetite* 2012; 59:541-9.

41. Yeomans MR, Chambers L. Satiety-relevant sensory qualities enhance the satiating effects of mixed carbohydrate-protein preloads. *Am J Clin Nutr* 2011; 94:1410-7. doi: 10.3945/ajcn.111.011650.

Chapter 4. Oleic acid content of a meal promotes oleoylethanolamide response and reduces subsequent energy intakes in humans

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Abstract

Animal data suggest that dietary fat composition may influence endocannabinoids (ECs) response and dietary behavior. This study tested the hypothesis that fatty acid composition of a meal can influence short-term response of ECs and subsequent energy intakes in humans. Fifteen volunteers in three occasions were randomly offered a meal containing 30g of bread and 30 mL of one of three selected oils: sunflower oil (SO), high oleic sunflower oil (HOSO) and virgin olive oil (VOO). Plasma ECs concentrations and appetite ratings over 2h and energy intakes over 24h following the experimental meal were measured. Results showed that after HOSO and VOO circulating oleoylethanolamide (OEA) was significantly higher than SO; concomitantly, a significant reduction of energy intake was found. For the first time oleic acid content of a meal was demonstrated to increase post-prandial response of circulating OEA and to reduce energy intakes at subsequent meal in humans.

Introduction

Endocannabinoids (ECs) are a class of lipid mediators acting as endogenous ligands of the G protein-coupled cannabinoid receptors. In the early nineties, the two primary ECs were discovered: the arachidonoylethanolamide (AEA) and the 2-arachidonoylglycerol (2-AG)[1,2]. AEA together with palmitoylethanolamide (PEA), oleoylethanolamide (OEA), linoleoylethanolamide (LEA) belong to the chemical group of *N*-acylethanolamines (NAEs)[3-5]. All these compounds take part to a wide range of biological processes: pain, anxiety and depression, nausea, addiction and withdrawal[6], innate immunity[7]. Moreover, they are involved in feeding regulation by influencing metabolic and reward system[8]. In particular, AEA and 2-AG showed orexigenic properties in rodents as they dose-dependently increased food intake by central and peripheral administration[9,10] and were shown to be modulated by fasting and feeding states in brain[11]. In humans, a role of 2-AG in hedonic eating was demonstrated by Monteleone et al.[12] who found a significant increase of 2-AG concentration in plasma 2 h after consumption of a high palatable meal but not after consumption of non-palatable meal.

On the contrary oral or intraperitoneal administration of OEA, as well as its duodenal increase, determined a decrease of food intake in mice and rats[13-20] (for a review of the literature see Piomelli[21]); the mechanism underlying such effect being recently demonstrated to involve the histaminergic system.[22]

The chemical composition of the ingested food plays a primary role in the OEA formation: infusion into the duodenum of glucose or proteins did not show any effect, whereas among several fats, only oleic acid elicited OEA production in animals[23].

Interestingly, in humans Joosten and co-workers[24] found that fasting and non-fasting plasma concentrations of AEA, OEA, PEA and stearoylethanolamide (SEA) were positively associated with both serum total free fatty acids and their specific fatty acid precursors namely arachidonic, oleic, palmitic and stearic acid, respectively.

However, in humans the evidence of diet influence on ECs system is still scarce and limited on macronutrient ratios[25]. Moreover, to the best of our knowledge, the post-prandial ECs response was never associated to appetite cues and following energy intakes in humans.

The objective of this study was to test the hypothesis that fatty acid composition of a meal, and mainly its oleic acid content, can influence short-term response of ECs and subsequent energy intakes in humans. To this purpose three equicaloric meals with the same macronutrient

composition but containing oils providing different amounts of oleic acid were offered to healthy and fasted volunteers. Blood drawings were performed over the following two hours and energy intakes at subsequent meal and over the following 24h were measured by self-recorded food diaries.

Materials and methods

Materials

AEA, LEA, OEA, PEA, 2-AG and d8-AEA were purchased by Cayman (Cayman Chemical, Ann Arbor, MI). Ethanol (EtOH), methanol (MeOH), chloroform, acetone, water, were from Merck (Darmstadt, Germany). Plastic vacutainer® serum tubes (16x100mm, 10ml) were purchased from Becton & Dickinson (1 Becton Drive, Franklin Lakes, NJ, USA). Polypropylene 1.5 ml tubes were from Eppendorf (Hamburg, Germany), 12 × 75 mm glass tubes from Corning (Corning S.r.l., Via Mercantini 5,Turin, Italy). Verex™ Vial, 9 mm, screw top, µVial i3 (Qsert) and PTFE/Silicone Cap were purchased from Phenomenex (Torrance, CA, USA). Sunflower seed oil, high oleic sunflower oil and virgin olive oil were provided by the Oleifici Mataluni (Montesarchio, Benevento, Italy).

Subjects

Healthy subjects were selected among students and staff of Department of Agriculture of "Federico II" University of Naples. Thirty five subjects were screened. Subjects taking any kind of drug, or presenting endocrine, hepatic, renal, tumoral, autoimmune, cardiovascular, hematological, neurological or psychiatric diseases, sleep disorders, or allergies requiring treatment, as well as those who experimented variation of their body weight over the previous three months or who were on a restrictive diet, were excluded. The 51-items Three Factor Eating Questionnaire (TFEQ) was used to exclude restraint subjects (score in the restraint subscale F1>8)[26]. Fifteen subjects were eligible and they were enrolled to participate after signing an informed written consent. They were 7 Male and 8 Female, between 22 and 40 years old with a BMI between 18.1 and 25.0 kg/m². All experimental procedures were approved by the Ethics Committee of the University of Naples.

Meals

Three oils differing for their fatty acid compositions were used in this study (**Table 1**). They were sunflower seed oil (SO), high oleic sunflower oil (HOSO) and virgin olive oil (VOO).

Table 1: Fatty acid composition and differences among sunflower seed oil (SO), high oleic sunflower oil (HOSO) and virgin olive oil (VOO) used in this study.

Fatty acid	composition (%)			difference (%)	
	SO	HOSO	VOO	HOSO - SO	VOO - SO
Myristic acid	0.07	0.05	0.01	-0.02	-0.06
Palmitic acid	6.42	4.42	12.08	-2.00	5.66
Palmitoleic acid	0.14	0.16	0.69	0.02	0.55
Heptadecanoic acid	0.04	0.03	0.04	-0.01	0.00
Heptadecenoic acid	0.02	0.04	0.06	0.02	0.04
Stearic acid	3.29	2.67	2.34	-0.62	-0.95
Oleic acid trans	0.04	0.04	0.00	0.00	-0.04
Oleic acid	33.20	79.04	75.26	45.84	42.06
Linoleic acid trans	0.45	0.09	0.00	-0.36	-0.45
Linoleic acid	55.02	11.99	8.25	-43.03	-46.77
Arachidic acid	0.24	0.24	0.28	0.00	0.04
Linolenic acid	0.07	0.04	0.67	-0.03	0.60
Eicosenoic acid	0.17	0.24	0.25	0.07	0.08
Behenic acid	0.64	0.73	0.04	0.09	-0.60
Lignoceric acid	0.21	0.23	0.01	0.02	-0.20

Thirty milliliters of each oil together with 30 g white bread were offered to fasting subjects in 3 different occasions. Each meal provided an energy intake of 357 kcal, of which 75.9% came from lipids, 2.9% from proteins and 21.2% from carbohydrates. A higher content of lipids than a nutritionally balanced meal was used in order to exclude the potential confounding factors from other meal components on both short-term physiological response of ECs and appetite cues.

Study protocol

The study was conducted at the Department of Agriculture of the University of Naples. It was a randomized intervention trial with a cross-over design. Volunteers were invited to reach the nutrition laboratory at 8:00 a.m. in a fasting condition from 10 hours on three occasions with a 1-week wash-out period from each other. On the evening before each test volunteers were instructed to consume a standardized dinner and to refrain from eating and drinking alcoholic or energy-drinks from 22:00h. Once arrived to the laboratory participants had a 10 min rest and they were instructed to rate their hunger, fullness, satiety, thirst and desire to eat on 100 mm visual analogue scales (VAS)[27] anchored on the left as ‘‘not at all’’ and on the right as ‘‘extremely’’. The questionnaire comprised 3 main questions (How great is your desire to eat?, How full do you feel?, How satiated do you feel?), and subjects were asked to answer indicating on the scale the point corresponding to their sensations. After the first blood drawing (baseline) each subject was asked to seat in a specific position isolated from the others, and was presented a tray containing the experimental meal

including the type of oil he/she was randomized to consume in that occasion. Subjects were asked to consume the meal within 15 minutes and the compliance was evaluated by controlling that the glass and plate containing the foods were empty at the end of breakfast. At the following 30, 60, 120 minutes subjects rated their appetite sensations on VAS and underwent to blood drawings. After the last blood drawing, before participants left the laboratory, they were instructed to fill a 24h-food diary by recording the exact time, the types and amount (weight) of foods and beverages consumed from the moment they left the laboratory until the day after. On the next day volunteers had to return their 24h-food diary to the expert nutritionist of the research group and were submitted to a 24h diet recall interview in order to assess the compliance and to validate the 24h-food diary.

Biochemical analysis

Blood was collected in vacutainer® serum tubes and centrifuged at 2400 x g per 10 min at 4 °C. Serum was aliquoted (by 500 µL) and kept frozen at -80 °C until analysis [28]. Concentration of AEA, LEA, OEA, PEA, 2-AG were determined by isotopic dilution liquid chromatography-mass spectrometry as described previously by Cote and co-workers [29]. Five hundred microliters of each sample were added in polypropylene 1.5 mL tubes and protein precipitation was obtained by adding 3 volumes of acetone and centrifuging at 14000 x g per 10 min at 4°C. The supernatants were collected, transferred into 12 × 75 mm glass tubes and subjected to lipid extraction adding 1.5 mL of methanol/chloroform (1:2) containing 5 pmol of d8-anandamide as internal standard. The organic phase was then dried under nitrogen, the resulting residue re-suspended in 100 µL of acetonitrile:water (1:1) and centrifuged (4°C; 2400 g; 10 min).

Chromatographic separation was performed using an HPLC apparatus equipped with two micropumps Perkin-Elmer series 200 (Norwalk, CT, USA). A Synergi Max RP 80 column, 50x2.1 mm (Phenomenex, USA) was used and the flow rate was set to 0.2 mL/min. Injection volume was 10 µL. Mobile phase A consisted of H2O, 0.2% formic acid, while mobile phase B was CH3CN. The gradient program was as follows: 50-79 % B (10 min), 79-95 % B (1 min), constant at 95% B (2 min), finally returning to the initial conditions in 2 min. MS/MS analyses were performed on an API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Canada). All the analyses were performed with a TurboIonSpray source with the following settings: drying gas (air) was heated to 300 °C, capillary voltage (IS) 5000 V. The declustering potential (DP) and the collision energy (CE) were optimized for each compound by directly infusion of standard solutions (10 µg/mL) into the mass spectrometry at a flow rate of 6 µl/min, using a Model 11 syringe pump (Harvard, Apparatus, Holliston, MA, USA). The acquisition was carried out in MRM (Multiple

Reaction Monitoring) in positive ion mode for each compound. Data acquisition and processing were performed using Analyst software v. 1.4. Acquisition parameters are summarized in **Table 2**.

Table 2: Acquisition parameters used for the LC/MS/MS analysis.

	Precursor Ion $[M+H]^+$	**Product Ion $[M+H]^+$**	**DP**	**CE**
AEA	348.0	62	40	35
OEA	326.0	62	40	35
LEA	324.0	62	60	30
PEA	300.0	62	60	30
2-AG	396.5	379.5	35	11
		287.3		14
		268.9		18
AEA-d8	356.5	63.2	50	31
		209.3		18

Data analysis and statistics

The sample size needed to detect an effect of meal composition on primary outcome (post-prandial response of ECs) and secondary outcome (the effect of meal composition on subsequent energy intakes) was estimated on the basis of previous studies. A sample size of 13 participants was calculated to be adequate to find changes in ECs response significantly different using variation in accordance with Monteleone et al.[12] and Gatta-Cherifi et al. [30] , with an 80% power and an $\alpha = 0.05$. A sample size of 12 subjects was adequate to detect a 19% difference in energy intake with a power of 80% and $\alpha = 0.05$ using variation in accordance with findings of our previous studies [31,32].

Statistical analyses were performed using the statistical package SPSS for Windows (version13).

The results of both appetite scores and of biochemical analyses were analyzed and expressed as the absolute changes from the baseline to reduce possible effects of inter-subject fasting variability. The subjective appetite sensations recorded after the consumption of the three types of oils and the ECs curves were compared and tested for the effect of treatment and of time as factors using ANOVA for repeated measures. The total area under the curves (AUC) for hunger, fullness and satiety

ratings (from baseline over 2 h from breakfast consumption) as well as for ECs blood concentrations were also estimated using the linear trapezoidal rule. Differences in the AUC values were analyzed by one-way ANOVA and by Newman-Kleus multiple comparison test as post hoc. Differences were considered significant at $p<0.05$.

Results

Biochemical analysis

No significant difference of plasma concentrations of ECs at baseline among experimental meals was found. **Figure 1** (**panel A**) shows the variations of plasma concentration of AEA, 2-AG, LEA and PEA over 2 hours following the three meals. A tendency for reduced concentrations of AEA, 2-AG and PEA irrespective to the type of breakfast consumed was found. However, OEA concentrations following HOSO and VOO were 23.7% and 20.5% significantly higher than that following SO consumption, $AUC_{0\text{-}120}$ being 858±54 pmol•min/mL, and 823±28 pmol•min/mL vs 654±70 pmol•min/mL, respectively (**Figure 1**, **panel B**). LEA concentrations did not change over time upon the three meals.

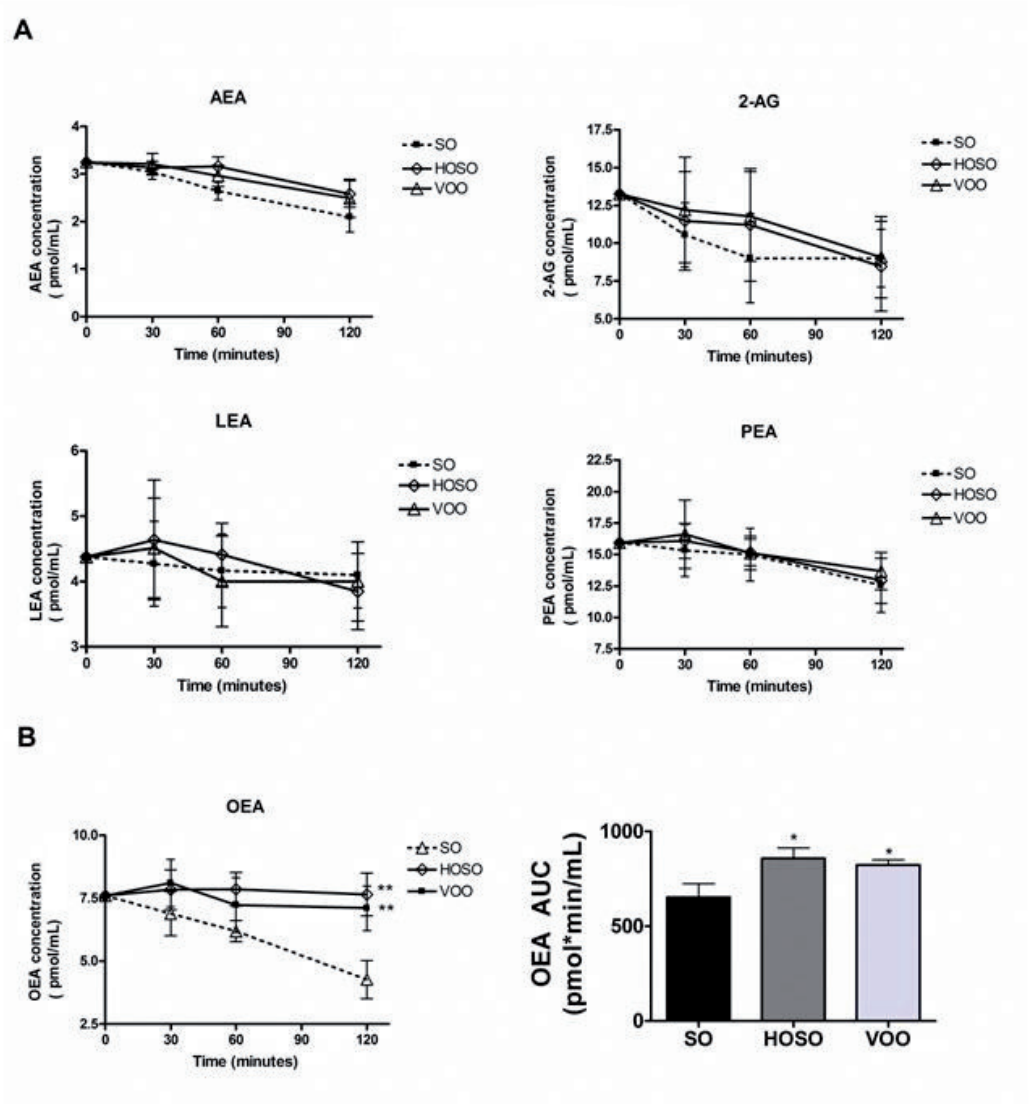

Figure 1: Post-prandial response of endocannabinoids - A) Concentration-time curves of AEA, 2-AG, LEA and PEA over 120 min following experimental meals; no significant difference of concentrations at baseline and following time points among experimental meals was found; B) Concentration-time curve and AUC of OEA over 120 min following experimental meals; no significant difference of baseline concentrations among experimental meals was found. Values are expressed as means ± SEM. * $p<0.05$ vs SO; ** $p<0.001$ vs SO).

Energy intakes at subsequent lunch and over 24h

All participants returned a well done 24-food diary and were submitted to 24-h diet recall interview. Data indicated that no difference in timing of subsequent lunch was present among participants following the three experimental meals. All subjects had their lunch always 3h after the experimental meal. However, subjects had a significant 261 kcal and 250 kcal energy reduced lunch after HOSO and VOO compared to SO, respectively (**Figure 2**).

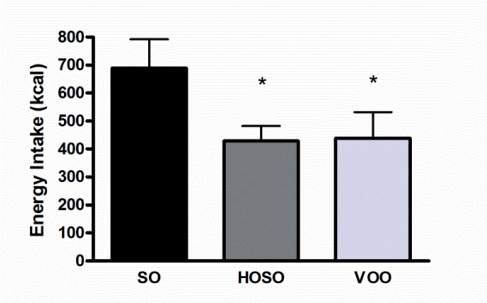

Figure 2: Energy intakes - Energy intakes (kcal) consumed during the lunch subsequent each experimental meal expressed as means ± standard deviation. * $p<0.05$ for VOO and HOSO vs SO. No significant difference of energy intakes over the 24h was found (1787 ±602 kcal 1803±542 kcal and 1646 ±430 kcal following HOSO, VOO and SO, respectively).

Appetite ratings

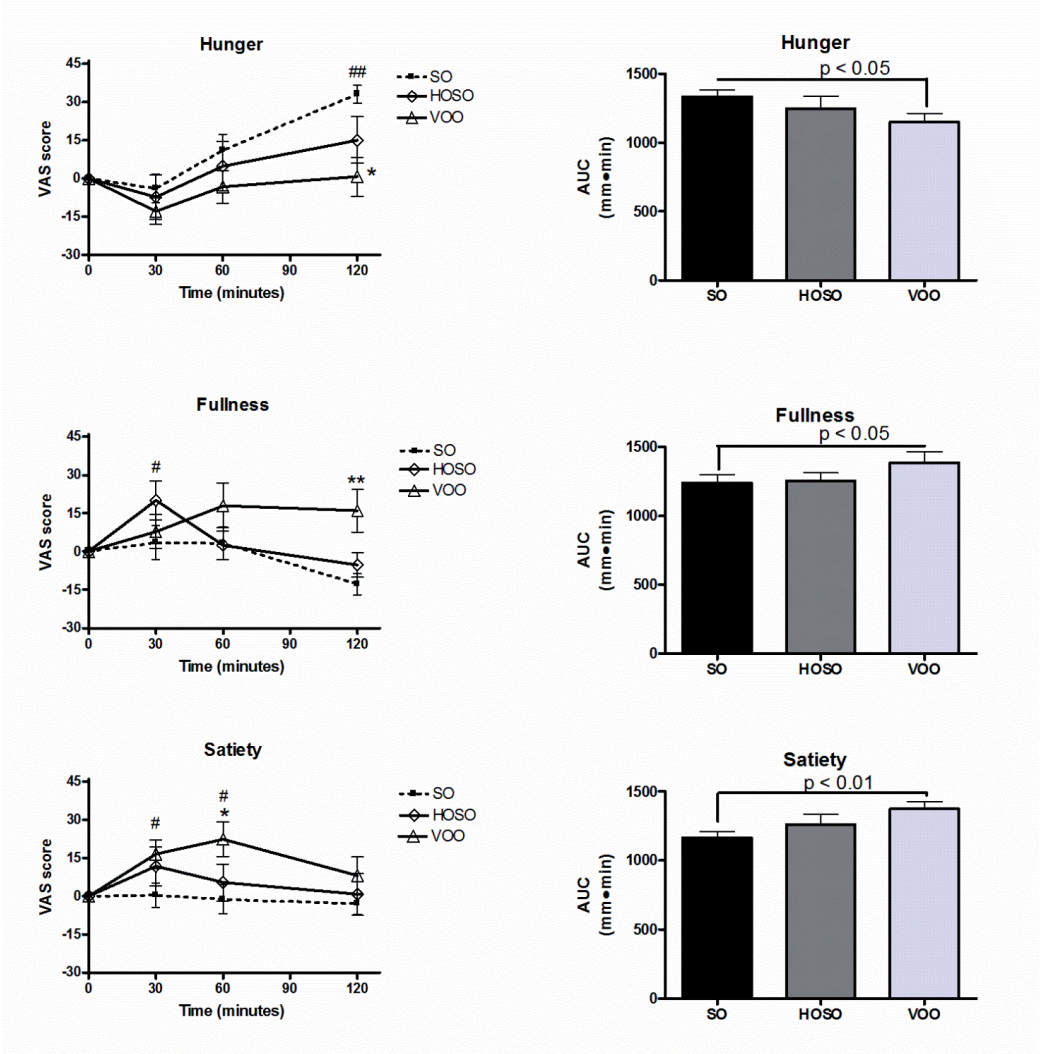

Figure 3: Appetite - Appetite rating-time curves and AUC of appetite sensations over 120 min following experimental meals. Values are expressed as means ± SEM. No significant difference of appetite sensations at baseline among experimental meals was found. At 120 min: *p<0.01 for hunger following VOO vs SO; **p<0.001 for fullness following VOO vs SO; # p<0.01 from baseline; ## p<0.001 from the baseline. AUCs of hunger, fullness and satiety after VOO are significantly different from SO.

No significant difference of sensations of hunger, fullness and satiety at baseline among experimental meals was found. **Figure 3** shows appetite ratings and AUC over the 2h following the

consumption of breakfasts containing VOO, HOSO or SO. A trend of hunger reduction at 30 min and return to baseline value over the following 60 min after the three meals were recorded. Only after 120 min from SO consumption subjects perceived a hunger sensation higher than baseline and that perceived after HOSO and VOO consumption. Interestingly, increased fullness and satiety compared to baseline were found between 30 min and 60 min after meals containing HOSO and VOO, but not after SO. These perceptions were prolonged at 120 min only following VOO consumption. Looking at the appetite sensations over the 2h after the breakfasts ($AUC_{0\text{-}120}$), significant reductions of hunger and increase of fullness and satiety were found after VOO compared to SO consumption.

Discussion

The main finding of this study is that the content of oleic acid ingested at a meal influences post-prandial ECs response, appetite sensations and energy intake at subsequent meal in humans.

Few human studies investigated the response of ECs to meals with specific chemical composition. In this study a trend to reduced postprandial concentrations of all ECs except that of OEA after HOSO and VOO and of LEA after all experimental conditions were found.

The reduced post-prandial concentrations of ECs were in accordance with findings of previous studies[24,33,30]. A physiological reason to this response might be linked to the peripheral action of post-prandial insulin and to the direct influence of meal lipids on ECs biosynthesis/hydrolysis route in the upper intestine. In fact, Di Marzo and co-workers[27] suggested that insulin reduces ECs levels in a way inversely related to insulin resistance and it is known that dietary monounsaturated or polyunsaturated fatty acids can increase post-prandial insulin sensitivity in healthy subjects[34]. Thus, it is likely that the consumption of a meal rich in unsaturated fatty acids might have generally reduced ECs response through insulin.

On the other hand, the consumption of meals providing higher amount of oleic acid (such as that including HOSO and VOO vs SO) might sustain post-prandial concentration of OEA independently from insulin action. In fact, oleic acid may act as precursor of OEA formation in the intestine as previously demonstrated in animals[35-37] and/or trigger some physiological mechanisms modulating its selective spillover from the intestinal membrane phospholipids. This hypothesis is consisting with a previous study demonstrating that the consumption of virgin olive oil and high-oleic sunflower oil determined, over the following 2 hours, a significant increase of circulating oleic acid-

rich phospholipids[38], which are known to be the precursors of intestinal biosynthesis of ECs at level of mucosa, epithelial cells and serosa[39]. In addition, a strict connection between circulating ECs and free fatty acids was recently suggested in humans by Joosten and co-workers[24].

Other factors than fatty acid composition of oils might have influenced post-prandial response of LEA whose concentration did not change vs baseline after the three meals.

It could not be excluded in the present study that different cephalic responses triggered by oral taste and/or different preference for the oils might contribute to influence the circulating pattern of ECs, through their well-known interaction with the gut metabolism. That dietary fat (but not other nutrients) can modify gut metabolism of ECs through oral sensing and selectively mobilize ECs in the upper gut, also influencing dietary behavior, was demonstrated in rats[40]. On the other hand a link between circulating 2-AG and food preference was found by Monteleone and co-workers[12] who showed increased plasma 2-AG in humans after consumption of their preferred food but not after the non-preferred one[12].

Further human studies should clarify the role of meal lipid composition on formation of different ECs induced by cephalic response.

Strikingly, both the meals eliciting the highest post-prandial OEA response (VOO and HOSO vs SO) were associated with the highest reductions of energy intakes at subsequent meal. These findings were in disagreement with the animal study conducted by Gaetani and co-workers[15], where OEA administration in free-feeding rats reduced only meal frequency without altering meal size, whereas they were perfectly in line with Provensi and co-workers [22].

Social and cognitive cue could majorly influence the timing of eating in humans compared to animals thus rendering insignificant the effect of OEA response on time of eating while evidencing OEA effect on food intake at subsequent meal in our free-living participants. Several researchers aimed at ranking the effect of lipid composition on satiety[41-43]. Alfenas and co-workers[44] proposed that the satiety effect of fatty acids was linked to their oxidation rate: the higher is the number of double bonds, the faster is the rate of oxidation, the higher is satiety. However other studies did not confirm this suggestion[45,46]. Several differences among the studies, including the amount and source of fats provided to the volunteers might cause such discrepancies rendering the debate still open.

Only appetite ratings after consumption of the meal containing VOO were coherent with the reduced energy intake compared to the meal with SO. This might be a matter of dietary habits and cognitive factors on appetite sensations. In fact, data from food frequencies questionnaire (not

shown) indicated that all study participants were used to consume virgin olive oil as conditioning fat, while the consumption of seed oils was sporadic. Familiarity with a food and expected satiation are interrelated. More familiar foods are expected to be more filling[47] and measures of expected satiety are highly correlated with actual satiety[48]. From a mechanistic point of view it could not be excluded that non-fat components present in VOO (but not in SO or HOSO) such as several volatile compounds (attributing to VOO the characteristic aroma) and phenolic compounds might contribute to the effect of VOO on energy intake and appetite regulation as recently suggested in the elegant study by Frank and co-workers[49] or reviewed by Panickar[50], respectively.

Conclusion

In conclusion, in this study for the first time it was demonstrated that oleic acid content of a meal can increase post-prandial response of circulating OEA and it may reduce energy intakes at subsequent meal in humans. The present data offer a concept to design new food ingredients for energy intake control using edible oils rich in oleic acid. Further studies should evaluate whether these findings can be reproduced also in overweight/obese subjects and in the context of meals nutritionally balanced for macronutrients ratio.

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References

1. W. A. Devane, L. Hanus, A. Breuer, R. G. Pertwee, L. A. Stevenson, G. Griffin, D. Gibson, A. Mandelbaum, A. Etinger and R. Mechoulam. *Science*, 1992, **258**: 1946–1949.
2. T. Sugiura, S. Kondo, A. Sukagawa, S. Nakane, A. Shinoda, K. Itoh, A. Yamashita and K. Waku, *Biochem Biophys Res Commun*, 1995, **215**:89–97.
3. S. Kondo, T. Sugiura, T. Kodaka, N. Kudo, K. Waku and A. Tokumura. *Arch. Biochem. Biophys*, 1998, **354**: 303–310.
4. H. H. Hansen, C. Ikonomidou, P. Bittigau, S. H. Hansen and H. S. Hansen. *J. Neurochem*, 2001, **76**: 39–46.
5. H. H. Schmid, P. C. Schmid and V. Natarajan. *Prog. Lipid Res*, 1990, **29**: 1–43.
6. J. F. Blankman and F. C. Benjamin. *Pharmacol. Rev*., 2013, **65**:849–871.
7. A. C. Kendall and A. Nicolaou. *Progress in Lipid Research*, 2013, **52**: 141–164.
8. V. Di Marzo, A. Ligresti and L. Cristino. *International Journal of Obesity*. 2009, **33**: S18–S24.
9. C. M. Williams and T. C. Kirkham. *Psychopharmacology (Berl)*, 1999, **143**: 315–317.
10. T. C. Kirkham, C. M. Williams, F. Fezza and V. Di Marzo. *Br. J. Pharmacol*, 2002, **136**: 550–557.
11. T. F. Gamage and A. H. Lichtman. *Pediatr. Blood Cancer*., 2012*,* **58**: 144–148.
12. P. Monteleone, F. Piscitelli, P. Scognamiglio, A.M. Monteleone, B. Canestrelli, V. Di Marzo, and M. Mai. *J. Clin. Endocrinol. Metab*. 2012, **97**:E917-24.
13. F. Rodríguez de Fonseca, M. Navarro, R. Gómez, L. Escuredo, F. Nava, J. Fu, E. Murillo-Rodríguez, A. Giuffrida, J. LoVerme, S. Gaetani, S. Kathuria, C. Gall and D. Piomelli. *Nature*, 2001, **414**: 209-212.
14. J. Fu, S. Gaetani, F. Oveisi, J. Lo Verme, A. Serrano, F. Rodríguez De Fonseca, A. Rosengarth, H. Luecke, B. Di Giacomo, G. Tarzia and D. Piomelli. *Nature*. 2004, **425**: 90-93.
15. S. Gaetani, F. Oveisi and D. Piomelli. *Neuropsychopharmacology*, 2003, **28**: 1311–1316.
16. F. Oveisi, S. Gaetani, K. T. Eng and D. Piomelli. *Pharmacol Res.* 2004, **49**:461-6.
17. M. J. Nielsen, G. Petersen, A. Astrup and H.S. Hansen. *J. Lipid Res*. 2004, **45**: 1027–1029.
18. K. Proulx, D. Cota, T. R. Castañeda, M. H. Tschöp, D. A. D'Alessio, P. Tso, S. C. Woods and R. J. Seeley. *Am. J. Physiol. Regul. Integr. Comp. Physiol*., 2005, **289**: R729-737.
19. G. Astarita, B. Di Giacomo, S. Gaetani, F. Oveisi, T. R. Compton, S. Rivara, G. Tarzia, M. Mor, and D. Piomelli. *J. Pharmacol Exp. Ther*., 2006, **318**: 563-70.
20. J. Fu, J. Kim, F. Oveisi, G. Astarita, and D. Piomelli. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, 2008, **295**: R45-50.
21. D. Piomelli. *Trends in Endocrinology and Metabolism*, 2013, **24**: 332-341.
22. G. Provensi, R. Coccurello, H. Umehara, L. Munari, G. Giacovazzo, N. Galeotti, D. Nosi, S. Gaetani, A. Romano, A. Moles, R. Blandina, M. B. Passani*. Proc Natl Acad Sci U S A*. www.pnas.org/cgi/doi/10.1073/pnas.1322016111
23. G. J. Schwartz, J. Fu, G. Astarita, X. Li, S. Gaetani, P. Campolongo, V. Cuomo and D. Piomelli. *Cell Metab*., 2008, **8**: 281-8.

24. M. Joosten, M. G. Balvers, K. C. Verhoeckx, H. F. Hendriks and R. F. Witkamp. *Nutr Metab*., 2010, **14**;7:49.

25. S. S. Naughton, M. L. Mathai, D. H. Hryciw and A. J. McAinch. *Int. J. Endocrinol*., 2013: 361895.

26. A. J. Stunkard and S. Messick. *Journal of Psychosomatic Research*, 1985, **29**: 71-83.

27. S. M. Green, H. J. Delargy, D. Joanes and J. E. Blundell. *Appetite*, 1997, **29**: 291–304.

28. V. Di Marzo, A. Verrijken, A. Hakkarainen, S. Petrosino, I. Mertens, N. Lundbom, F. Piscitelli, J. Westerbacka, A. Soro-Paavonen, I. Matias, L. Van Gaal and M. R. Taskinen. *European Journal of Endocrinology*, 2009, **161**: 715–722.

29. M. Côté, I. Matias, I. Lemieux, S. Petrosino, N. Alméras, J. P. Després and V. Di Marzo. *International Journal of Obesity*, 2007, **31**: 692–699.

30. B. Gatta-Cherifi, I. Matias, M. Vallée, A. Tabarin, G. Marsicano, P. V. Piazza and D. Cota. *Int J Obes (Lond)*, 2012, **36**:880-5.

31. P. Vitaglione, R. Barone Lumaga, A. Stanzione, L. Scalfi and V. Fogliano. *Appetite*, 2009, **53:** 338–344.

32. R. Barone Lumaga, D. Azzali, V. Fogliano, L. Scalfi and P. Vitaglione. *Food Funct*, 2012, **3**: 67–75

33. I. Matias, M. P. Gonthier, P. Orlando, V. Martiadis, L. De Petrocellis, C. Cervino, S. Petrosino, L. Hoareau, F. Festy, R. Pasquali, R. Roche, M. Maj, U. Pagotto, P. Monteleone and V. Di Marzo. *J Clin Endocrinol Metab*., 2006, **91**: 3171-80.

34. E. Gatti, D. Noe, F. Pazzucconi, G. Gianfranceschi, M. Porrini, G. Testolin and C. R. Sirtori. *Eur.J. Clin. Nutr*. 1992, **46**: 161–166.

35. G. Petersen, C. Sørensen, P. C. Schmid, A. Artmann, M. Tang-Christensen, S. H. Hansen, P. J. Larsen, H. H. Schmid, and H. S. Hansen. *Biochim Biophys. Acta,* 2006, **1761**: 143-150.

36. J. Fu, G. Astarita, S. Gaetani, J. Kim, B. F. Cravatt, K. Mackie and D. Piomelli. *J. Biol. Chem*., 2007, **282**:1518-1528.

37. A. Artmann, G. Petersen, L. I. Hellgren, J. Boberg, C. Skonberg, C. Nellemann, S. H. Hansen and H. S. Hansen. *Biochimica et Biophysica Acta*, 2008, **1781**: 200–212.

38. R. Abia, Y. M. Pacheco, E. Montero, V. Ruiz-Gutierrez and F. J. Muriana. *Life Sci*, 2003, **72**:1643-54.

39. F. Borrelli, and A. A. Izzo. *Best Pract Res Clin Endocrinol Metab*., 2009, **23**:33-49.

40. N. V. Di Patrizio, G. Astarita, G. Schwartz, X. Li, and D. Piomelli. *Proc Natl Acad Sci U S A*. 2011, **108**:12904-8.

41. M. I. Friedman. *Am J Clin Nutr*, 1998**, 67**:513S-518S.

42. C. L. Lawton, H. J. Delargy, J. Brockman, F. C. Smith and J. E. Blundell. *Br J Nutr.*, 2000, **83**:473-82.

43. P. Casas-Agustench, P. López-Uriarte, M. Bulló, E. Ros, A. Gómez-Flores and J. Salas-Salvadó. *Clin Nutr*. 2009, **28**:39-45.

44. R. C. Alfenas and R. D. Mattes. *Obes Res*., 2003, **11**:183-7.

45. C. G. MacIntosh, S. H. Holt and J. C. Brand-Miller. *J Nutr*., 2003, **133**:2577-80.

46. A. Flint, B. Helt, A. Raben, S. Toubro and A. Astrup. *Obes Res*., 2003, **11**:1449-55.

47. N. Brogden and E. Almiron-Roig. Appetite, 2010, **55**:551-5.

48. J. M. Brunstrom, N. G. Shakeshaft and N. E. Scott-Samuel. *Appetite*, 2008, **51**:604-14.

49. S. Frank, K. Linder, L. Fritsche, M. A. Hege, S. Kullmann, A. Krzeminski, A. Fritsche, P. Schieberle, V. Somoza, J. Hinrichs, R. Veit, H. Preissl. *Am J Clin Nutr*, 2013, **98**:1360-6.

K. S. Panickar. *Mol Nutr Food Res.*, 2013, **57**: 34-47.

Chapter 5. Microencapsulated bitter compounds (from Gentiana Lutea) reduce daily energy intakes in humans

Ilario Mennella, Vincenzo Fogliano, Rosalia Ferracane, Marco Arlorio, Franco Pattarino, Paola Vitaglione. To be submitted at The Journal of Nutrition.

Abstract

Mounting evidence showed that bitter tasting compounds may modulate appetite and eating behavior through bitter receptors in the gastrointestinal tract. Human studies were performed by nasogastric administration of bitter tasting compounds.

The aim of this study was to evaluate the influence on human appetite and energy intakes of microencapsulated bitter compounds consumed in a food.

A new microencapsulated food ingredient (EBI) with a core of bitter *Gentiana Lutea* root extract and a coating of ethylcellulose-stearate (to mask the bitterness in the mouth but allowing the delivery of bitter seicoridoids in the gastro-intestinal tract), was developed and included in a palatable vanilla pudding (EBIP). A cross-over randomized study was performed: 20 healthy subjects consumed at breakfast EBIP (providing 100 mg of seicoridoids) or the control pudding (CP), without bitter compounds, in two different occasions. Blood samples, glycaemia and appetite ratings were collected at baseline, and 30 min, 1, 2, and 3 h after breakfast. Gastro-intestinal peptides, endocannabinoids (ECs) and N-acylethanolamines (NAEs) were measured in plasma samples. Energy intakes were measured at an *ad libitum* lunch offered 3h after breakfast and over the remaining part of the day (post-lunch) through food diaries.

No difference of plasma gastro-intestinal hormone, glucose, ECs and NAEs responses as well as appetite ratings between EBIP and CP over 3h post-breakfasts was found.. EBIP determined a 30% kcal reduction of energy intakes ($p<0.05$) over post-lunch period compared to CP .

In conclusion, it was demonstrated that microencapsulated bitter seicoridoids from gentian root are effective to reduce daily energy intakes in humans.

Introduction

The knowledge that cells with taste receptors are not only in the mouth but they are widely distributed in the human digestive tract (trachea, stomach, intestine, bile duct) posed the basis of several researches aimed at understanding the physiological roles of these taste circuits in humans (1). Mounting evidence indicated that taste cells in the gastrointestinal tract play a major role in nutrient sensing and in triggering a specific gut-brain response (2). In fact, taste receptors agonist elicit in enteroendocrine cells *in vitro* and in animals *in vivo* the release of peptides (including CCK, GIP, PYY and GLP1) that are involved in the regulation of gastrointestinal secretion and motility, glucose homeostasis, food intake and satiety (3). In particular, several studies on cell lines and animals suggested that bitter-tasting compounds may modulate energy intake by delaying gastric emptying through inhibition of gastric contractility and triggering the secretion of CCK, GLP-1 and ghrelin (4-8). In addition a link between the bitter taste receptors and the endocannabinoids system (ECs), a group of molecules also involved in the regulation of energy homeostasis and reward (9), has been suggested. In fact, Tomassini et al. (2013) showed that the sensitivity to the bitter compound PROP was associated with higher plasma levels of the endocannabinoids (ECs) 2-arachidonoylglycerol (2-AG) and arachidonoylethanolamide (AEA). They speculated that the genetic of bitter taste receptors may influence the eating behavior through the modulation of the ECs system (10,11).

Moreover, a long-term study conducted in high fat diet fed rats demonstrated that isohumulones, the bitter compounds derived from hops in beer, improved insulin sensitivity and lipid metabolism through activation of both proliferator-activated receptor (PPAR) -α and –γ (12). An effect of isohumulones in controlling glycemia in mild diabetic subjects was also ascertained by the same authors and confirmed by Obara and co-workers (2009). Contrasting results were found in the two studies on the ability of hop derived bitter compounds to control body weight upon a 12 week intervention (12,13).

However, in *in vivo* studies bitter compounds were always used as gastric gavage or drugs in form of capsules, in order to skip the natural aversion for this taste and possible anticipated physiological responses induced by oral tasting. Therefore the use of bitter compounds as a food ingredients with specific benefic effects is limited by the repulsion for this taste. The aversion for bitter is not an intrinsic value of this tastant, but it comes from the ability of bitter compounds to stimulate brain center of disgust (14). In an elegant experiment Mueller et al. (2005) clearly demonstrated that the

behaviour attraction or aversion for bitter compounds is mediated by the specific activation of taste receptors cells, rather than a property of the tastant molecules (15).

On the other hand, social interactions may influence the food palatability leading to recognize certain bitter foods, such as coffee or beer, as high palatable (16). Food palatability has a crucial role in eating behavior. The relation between food palatability and the appetite sensations or the energy intakes (EI) is not clear, but several studies demonstrated that hunger and food intakes increase as palatability increases (17).

Among novel technologies encapsulation has been demonstrated as a reliable strategy to design a palatable food enriched with functional compounds with unpleasant taste. In fact, the formation of a specific external coating around the desired compound avoid the perception in the mouth and allow the delivery of the encapsulated compound directly in the gastrointestinal tract (18).

In this frame, this study tested the hypothesis that a new food ingredient consisting of bitter compounds encapsulated with a coating material able to mask oral taste and to release the core material in the intestine, might influence appetite in humans. To this purpose a concentrated extract of Gentiana lutea root with a known amount of bitter compounds, was encapsulated in a stearic acid coating and was included in a new vanilla pudding. The appetite rating, gastrointestinal hormones, ECs and NAEs response over 3h from consumption of the new pudding and the control pudding (without encapsulated ingredient) were recorded in healthy subjects in two different occasions.

Materials and methods

Encapsulated bitter ingredient (EBI)

An aqueous extract of gentian lutea root was provided by (Polcaro Fitopreparazioni SRL). In order to mask bitterness in the mouth and to allow the release of bitter compounds in the intestine, the gentian root extract was submitted to spray drying technique and coated with ethylcellulose and stearate coating following the procedure described by (19). An encapsulated bitter ingredient (EBI) containing 8% of bitter compounds including (Loganic acid 11,5%; Swertiamarin 9,5%; Gentiopicroside 53,9%; Sweroside 25,1%;) was obtained and used in pudding preparation.

Puddings

Two iso-caloric and macronutrient equal vanilla puddings were formulated. They were an EBI-enriched pudding (EBIP) and a control pudding (CP). EBIP contained 1.25 g of EBI per a 100 g portion, providing 100 mg of bitter compounds. CP contained the same amount of EBI coating except the bitter core. The two puddings were also very similar for sensory properties as assessed by a preliminary test conducted with volunteers (data not shown). In order to provide about 20% of the total daily EI three biscuits were served together with one of the two pudding for individual breakfast. The macronutrient composition, energy values and bitter compound content of the two puddings and breakfasts were reported in Table 1.

Table 1. Nutritional composition and bitter compounds content of the two puddings (encapsulated bitter ingredient -EBI- pudding and Control pudding), biscuits and of the breakfasts offered to volunteers.

	EBI pudding	Control pudding	Biscuits	EBI breakfast	Control Breakfast
Carbohydrates (g)	9.5	9.5	35.6	45.1	45.1
Protein (g)	5.5	5.5	3.2	8.7	8.7
Fat (g)	0.2	0.2	10.4	10.6	10.6
Energy (kcal)	62	62	252	314	314
Bitter compounds (mg)	100	---	---	100	---

Subjects

Recruitment was performed at the Department of Agricultural and Food science of University of Naples among students and staff of the Department. Subjects were excluded from the study if they had a BMI≥25 kg/m^2, any chronic illnesses such as diabetes, hypertension, if were smokers or taking any prescription medication, or those who were under a controlled dietary regimen or loosed weight over the previous three months, and pregnant or lactating women. Fifteen participants were recruited (8F/7M, aged between 22 to 33 years; mean age 26 ± 3.45 years) and participating to two experimental sessions.

Study design

The study had a 2 side cross-over randomized design with a 1-week wash-out period. On the evening before the experimental day volunteers were instructed to consume a standardized dinner until 22:00h. On the experimental day fasted subjects arrived at 08:15 h to the nutritional laboratory of the Department of Agricultural and Food Science. After 10 minutes they rated their appetite sensations (hunger, thirst, fullness and desire to eat) on a 100 mm visual analogue scale (VAS), anchored on the left as ‘‘not at all’’ and on the right as ‘‘extremely’’. Subsequently, a venous catheter was placed into an arm vein and a baseline blood sample was collected and glycaemia was measured by finger-pricking. After 30, 60, 120 and 180 minutes that subjects consumed their breakfast (in 15 min), appetite sensations, glycaemia and blood samples were collected. At 3h post-breakfast an *ad libitum* lunch was offered to the volunteers who were free to take all the time they wanted to consume a self-selected meal. During the lunch each subject sate in a single place cabin separated by the others and energy intake (EI) were measured as the differences between the amount of food contained in each plate taken and the foods left in the plate by the subject. Before leaving the laboratory, subjects were asked to fill out a food diary over the following 24 hours to register all foods and beverages consumed together with the post-ingestive sensations.

All experimental procedures were approved by the Ethics Committee of the University of Naples and subjects joined the protocol only after signing an informed agreement document.

Test meal

The food offered in the test meal included a variety of typical Italian dishes. They were chosen on the basis of a food preference questionnaire filled out during the enrollment procedure: those not rated by participants as very high or very low in a hedonic scale from 0 to 10 were selected (data not shown). The dishes were prepared following always the same recipes in order to accurately know the energy value and the macronutrient composition. Food was portioned in coded plates and subject were free to compose their tray and eat *ad libitum*.

Biochemical analysis

Blood collection

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

Gastrointestinal peptides

Luminex Technology (Bio-Plex; Bio-Rad, Nazareth, Belgium) was used to determine GI peptides. A magnetic bead panel kit provided by Milliplex® (Merck Millipore, Millipore S.p.A.) allowed the simultaneous determination of the following nine hormones: ghrelin, glucagon, glucagon-like peptide -1 (GLP-1), glucose-dependent insulinotropic peptide (GIP), insulin, leptin, polypeptide pancreatic (PP), total amylin and total peptide YY (PYY). The sensitivity levels of the assay (in pg/mL) were: ghrelin 2.0; leptin, 27.0; GIP 0.6; GLP-1 7.0; glucagon 6.0; insulin 58.0; PP 2.0; total amylin 14.0; PYY 8.0.

The intra-assay variation (%CV) was 2% for ghrelin, total amylin and PYY; 3% for GIP, glucagon, insulin and leptin; 4% for the PP; 7% for the GLP-1. The inter-assay variation (%CV) was 4% for leptin; 5% for GIP; 6% for insulin; 7% for PP and glucagon; 8% for ghrelin; 10% for GLP-1; 11% for PYY and 33% for amylin.

Endocannabinoids and N-acylethanolamines

Two ECs, 2-arachidonoylglycerol (2-AG) and arachidonoylethanolamide (AEA), three NAEs, linoleoylethanolamide (LEA), oleoylethanolamide (OEA) and palmitoylethanolamide (PEA), were determined by LC/MS/MS. Samples were extracted following the method set up by Lam et al (2010). Briefly 1ml of sample were added into Oasis HLB 1 cc, 30 mg cartridges (Waters) preconditioned with 1 mL methanol and 1 mL H2O. The cartridges were washed with 1 mL 40% aqueous methanol and eluted in 1 mL acetonitrile. The eluants were then dried under nitrogen, and re-suspended in 100 µL of acetonitrile:water (1:1) before chromatrographic separation (20).

This was performed using an HPLC apparatus equipped with two micropumps Perkin-Elmer series 200 (Norwalk, CT, USA). A Synergi Max RP 80 column, 50x2.1 mm (Phenomenex, USA) was used and the flow rate was set to 0.2 mL/min. Injection volume was 20 µL. MS/MS analyses were performed on an API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Canada). All the analyses were performed with a TurboIonSpray source set as previously described (*21*). 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 were already published (*21*)**.**

Blood glucose

Blood glucose concnetration was measured by finger pricking and using a bedside glucometer (OneTouch Sure Step; Life Scan Inc., Milpitas, CA, USA). Accuracy of the glucometer was evaluated by the manufacturer using least-squares linear regression analysis and found to be 98% ‘‘clinically accurate’’ when compared with reference (YSI2700) results.

Statistical analysis

Results are expressed as means ± SEM. If no difference was found between treatments at baseline appetite sensations and biochemical analysis were analyzed and expressed as the absolute changes from the t0 to reduce possible effects of inter-subject fasting variability. The subjective appetite sensations and the response of hormones and ECs were tested for the effect of treatment and of time as factors by the analysis of variance (ANOVA) for repeated measures. The linear trapezoidal rule was used to calculate the total area under the curves (AUC) for the appetite sensations and biochemical markers. Results were considered statistically significant for $p < 0.05$. Statistical analyses were performed using Statistical Package for Social Sciences (version 16.0; SPSS, Inc., Chicago, IL, USA).

Results

Appetite

Figure 1 showed the profiles of appetite sensations over 180 min after breakfasts. As expected all the sensations positively (for fullness and satiety) or negatively (for hunger and desire to eat)

peaked at 30 min and returned to baseline values at 180 min after breakfasts. No significant differences within and between treatments were found.

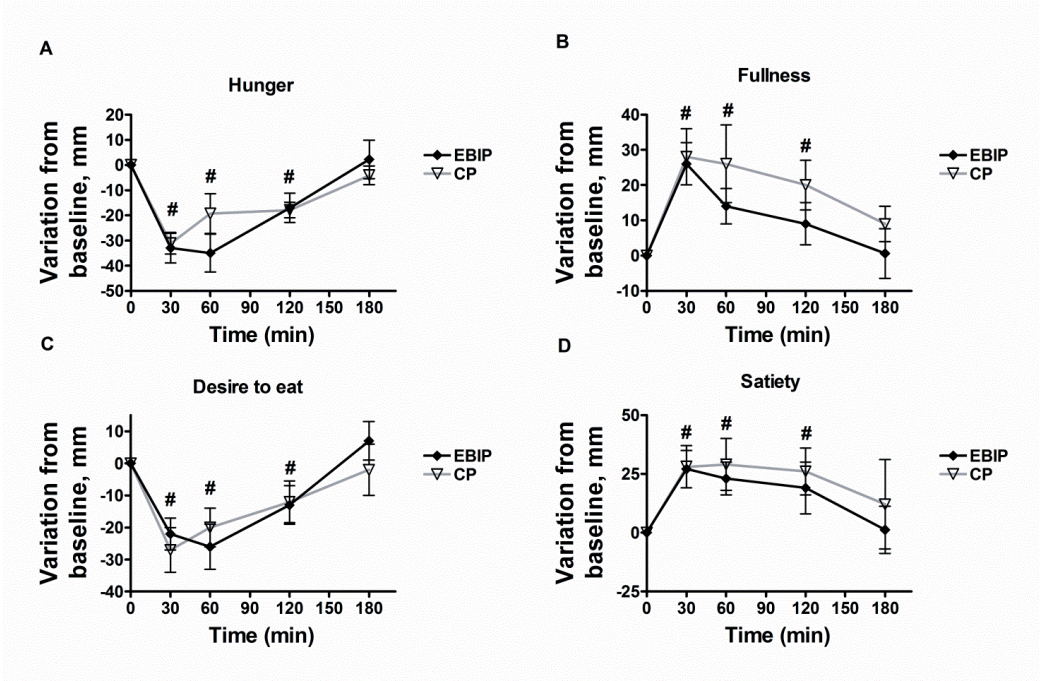


Figure 1. Time course over the following 3hours of the appetite sensations. Results are expressed as mean variation from baseline ±SEM. No differences were found between the two experimental conditions. #p<0.05 vs baseline.

Energy intake

Figure 2 showed the energy intakes (EI) at the *ad libitum* lunch and over the remaining part of the experimental day. A non significative 10% lower EI at lunch (823 ±87 kcal vs 911 ±55 kcal, p>0.05) but a significant 30% lower EI during the post-lunch time period (596 ±63kcal vs 848 ± 99kcal, p=0.04) when subjects had EBIP at breakfast compared to CP, were recorded. This caused a 22% lower 24h EI during the experimental day with EBIP than CP (1419 ±113kcal vs 1759 ±121kcal; p<0.05). No differences in the macronutrient composition of lunch and post-lunch meals between the two experimental days were found.

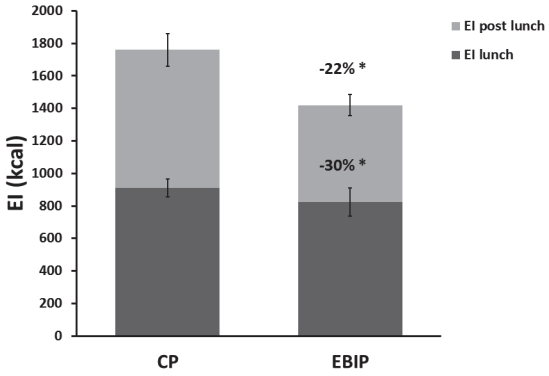


Figure 2. Energy intakes (EI) at *ad libitum* lunch, over the remaining part of the experimental day (post-lunch), and total daily EI (sum of lunch and post-lunch EI) after breakfast with EBIP or CP. Data are reported as means ± SEM. *p<0.05 vs EI at lunch and daily EI after CP.

Blood glucose

Blood glucose response over 180 min after breakfasts were reported in **Figure 3**. A significant increase at 30 min following both breakfasts compared to baseline was found. At 60 min blood glucose concentration sloped down and returned to the baseline values at 180 min. No significant differences between treatments were found.

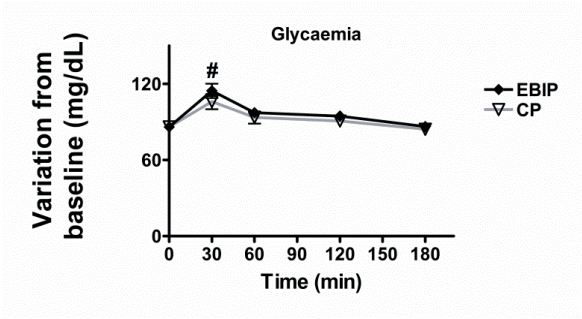


Figure 3. Blood glucose variations over the 3hours post breakfasts. Results are expressed as mean variation from baseline ±SEM. #p<0.05 vs baseline.

Gastrointestinal peptides

Amylin, ghrelin, glucagon, GIP, insulin, leptin, PP, PYY and GLP variations from baseline over the three hours following the breakfast were reported in **Figure 4**. At 30 min after both breakfasts ghrelin concentration showed a significant reduction ($p<0.05$) while amylin, GIP, insulin and PP increased ($p<0.05$) compared to baseline. At the same time point GLP-1significantly peaked only after EBIP consumption ($p<0.05$). All the hormones, with the exception of the PP and GIP, returned to the baseline levels within 180 min after the breakfasts. Glucagon, leptin and PYY did not changed after any breakfast. No significant differences between the two experimental breakfasts for all the hormones investigated were found. Interestingly, a trend for a higher response of GLP-1 after EBIP than after CP was found (AUC_{0-180} after EBIP was 14660 pg•min/ml and after CP was 10553 pg•min/ml, p=0,09).

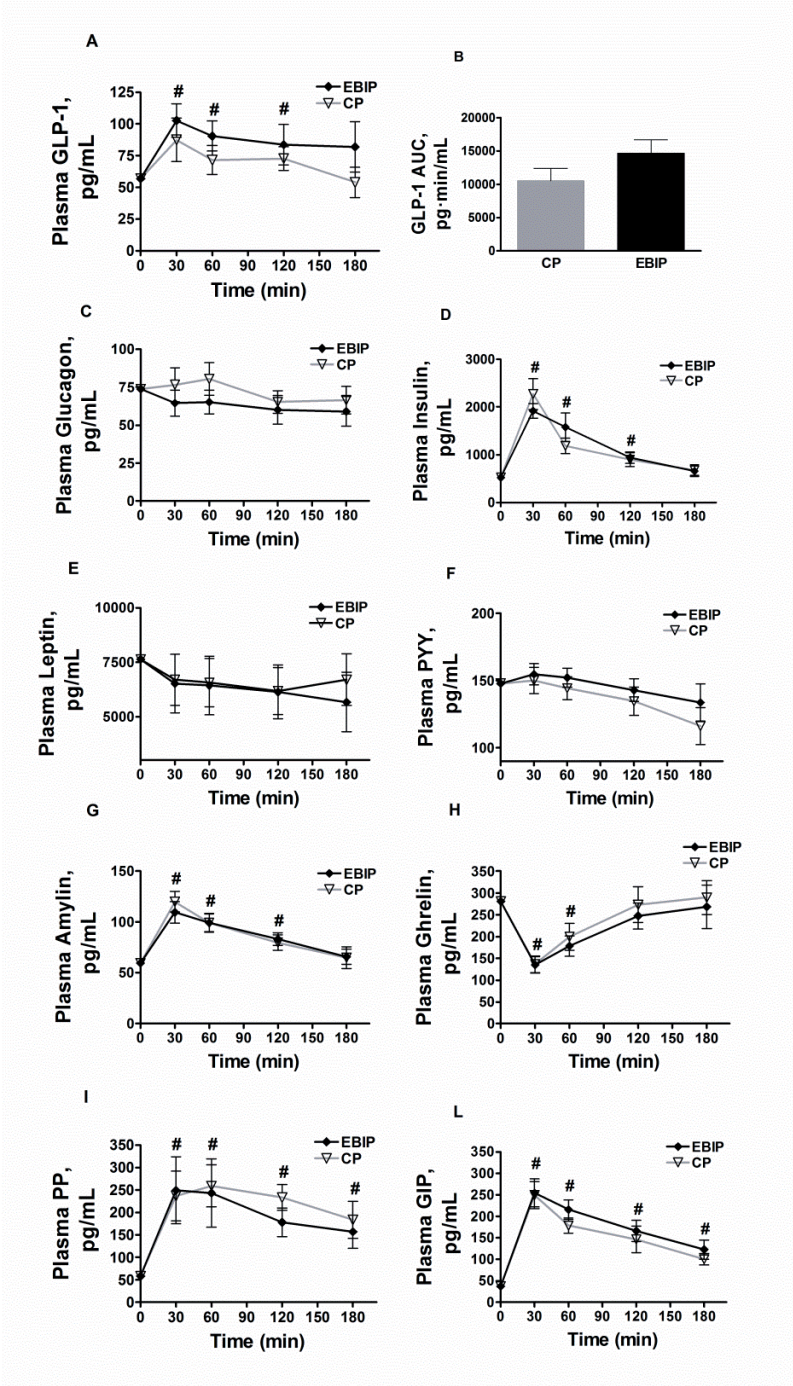


Figure 4. Hormones variations over the 3hours post breakfasts. Results are expressed as mean variations from baseline ±SEM. #p<0.05 vs baseline.

Endocannabinoids and N-acylethanolamines

A significant decrease from baseline of plasma levels for AEA, LEA, OEA and PEA was assessed after both breakfasts up to 3hours post ingestion ($p<0.05$). 2-AG showed a significant reduction from baseline only at 180 min after both breakfasts. No differences between the two puddings were recorded over time for any EC and NAE monitored (**Figure 5**).

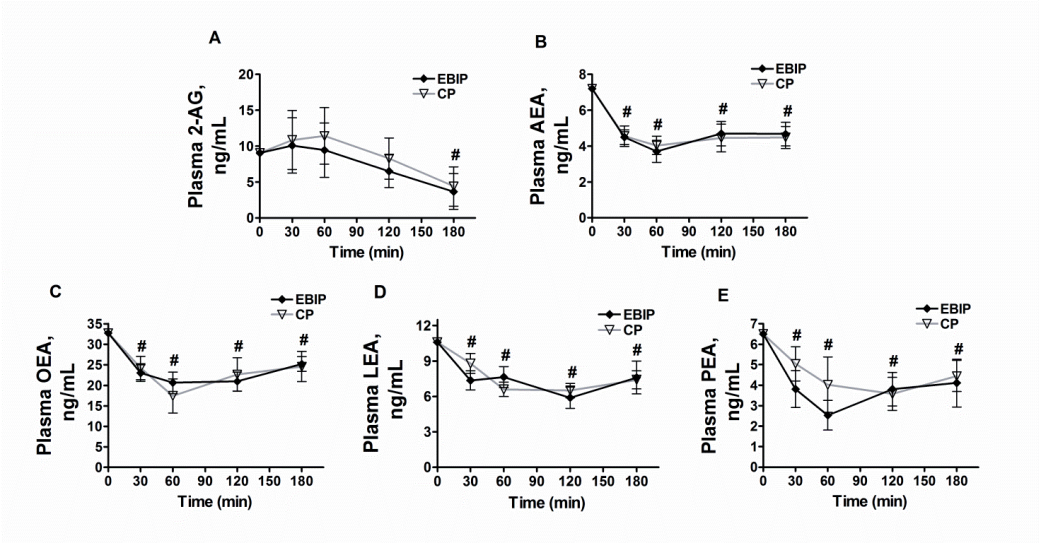


Figure 5. ECs and NAEs variations over the 3hours post breakfasts. Results are expressed as mean variations from baseline ±SEM. #$p<0.05$ vs baseline.

Discussions

Some *in vitro* and *in vivo* studies showed that the activation of bitter receptors in the gastrointestinal tract may result in the secretion of hormones modulating appetite and eating behavior (4-8). This study was designed to assess the efficacy of microencapsulated bitter compounds to modulate appetite and energy intakes in humans. A new food ingredient consisting of a microencapsulated *Gentiana lutea* root extract was developed and included in a palatable vanilla pudding. *Gentiana lutea* bitterness is due to the secoiridoid monoterpenes and their acylglucoside derivatives such as amarogentin, that is known as one of the most bitter among natural compounds (22). Microencapsulation of the extract was used to mask the bitterness of *Gentiana lutea* seicoridoids in the mouth and to guarantee their release in the intestine upon consumption of the pudding containing EBI. Pudding was chosen as test food because during its preparation ingredients never reached a temperature higher than 60°C and only slow agitation was necessary, that preserved the stability of EBI in the final product.

Data indicated that EBIP (providing 100 mg of microencapsulated bitter compounds) did not influence energy intakes at 3h after their consumption but lead to a 22% reduced energy intakes later on within 24h compared to CP. These results were partially in agreement with Janssen and co-workes (7) reporting that gastric administration of bitter compounds in mice determined an immediate release of ghrelin and increase of food intake but an anorexigenic effect starting from the next 4 h after treatment (resulting in a 49% reduction of 24h food intake in mice treated with bitter compound compared to placebo). In that study the food intake reduction was also associated with an inhibition of gastric emptying that was not mediated by cholecystokinin and GLP-1 but involved a direct inhibitory effect of bitter compounds on gastric contractility (7). The capacity of intragastric administration of bitter solutions to delay gastric emptying was recently confirmed in pigs (8) and fasting humans (23), whereas it did not underpin the reduction of appetite over 4 h post-consumption of a meal ingested 30 min after bitter administration (24). The strong point of this study was the use of microencapsulated gentian extract (EBI). It allowed us to evaluate the effect of bitter tasting compounds on appetite and eating behavior in humans in a more physiological and less stressful manner than previous studies using nasogastric administration (23,24). On the other hand, the protocol here adopted did not allow to ascertain the physiological mechanism underlying the post-lunch effect of EBI. It was excluded that adverse gastrointestinal events or nausea occurred in the subjects and influenced their food behavior following EBIP but it could not be excluded that multiple mechanisms including delayed gastric emptying (mediated or not by CCK and GLP-1) or direct effect of anorexigenic hormones were triggered by bitter compounds once they were

delivered in the intestine from the microencapsulated ingredient. That anorexic GLP-1 might be majorly involved in the satiating effect of EBI was suggested from the finding that it was the only hormone slightly increasing (28%) over the three hours after breakfast with EBIP vs CP thus sustaining the 10% reduced (not significant) energy intake after EBIP consumption. This hypothesis was supported by the fact that stimulation of GLP-1 secretion by bitter compounds was already found in the mouse GI enteroendocrine cell line STC-1 stimulated by the bitter agonists phenylthiocarbamide and denatonium benzoate (6). Moreover, in humans it was suggested that a disruption of the TAS2R receptors may affect GLP-1 secretion and as a consequence altered glucose and insulin homeostasis (5). However, the absence of a significant difference between EBIP and CP consumption on individual appetite sensations, glycaemia, ECs, NAEs and gastro-intestinal peptide response (except the slight effect on GLP-1) over 3 h after breakfast (until lunch) as well as on energy intakes at lunch was coherent with a high stability of EBI along the first part of gastro-intestinal tract. Moreover, the effect of EBI on post-lunch food intakes confirmed an intestinal delivery of the bitter core (secoiridoids) from the microcapsules with a ethylcellulose-stearate coating (25) and might be consistent with a stronger stimulation of GLP-1 and other anorexic hormones secretion from the enteroendocrine cells.The activation of bitter receptors in the gastrointestinal tract stimulate ghrelin secretion resulting in a short term orexigenic effect (7), but its release is predominant in the stomach (26). This may explain the efficacy of the ancient habit to drink bitter herbs infusions to aid digestion or the popular use of bitter wines as aperitifs, leading to a short term increase of appetite. Data altogether suggested that when bitter compounds are masked to bitter receptors in the stomach (due to coating material or because they are included in a food or a meal) the ghrelin production is reduced, therefore a long-term anorexigenic effect is prevalent on the short-term orexigenic effect.

In conclusion, in the present study it was demonstrated that microencapsulation of bitter compounds, finalized to bitter taste masking effect and a slow and targeted delivery in the intestine, is an effective tool to produce new food ingredients to control energy intakes. These data together with previous studies suggested that the kinetic of bitter compounds delivery along the gastro-intestinal tract is fundamental in modulation of appetite behavior finalized to energy intake reduction in humans. However, further studies aiming at clarifying the biochemical response underpinning the long term effect on daily food intake here shown with EBI are warranted to model the kinetic of the bitter compounds delivery in the gastro-intestinal tract. Modelling delivery of bitter compounds in the respect of physiological effects may be important to foresee the effect of the ingredient on body weight control in overweight subjects upon a continued consumption.

References

1. Trivedi B. P. Hardwired for taste. *Nature.* 2012; 486:S7-S9.
2. Rønnestad I, Akiba Y, Kaji I, Kaunitz JD. Duodenal luminal nutrient sensing. *Curr Opin Pharmacol*. 2014; 19C:67-75. doi: 10.1016/j.coph.2014.07.010.
3. Sterninia C, Anselmia L, Rozengurta E. Enteroendocrine cells: a site of 'taste' in gastrointestinal chemosensing. *Curr Opin Endocrinol Diabetes Obes*. 2008; 15: 73–78. doi:10.1097/MED.0b013e3282f43a73.
4. Chen MC, Wu SV, Reeve JR Jr, Rozengurt E. Bitter stimuli induce Ca2+ signaling and CCK release in enteroendocrine STC-1 cells: role of L-type voltage-sensitive Ca2+ channels. *Am J Physiol Cell Physiol.* 2006; 291:C726-39.
5. Dotson, C. D., Zhang, L., Xu, H., Shin, Y.-K., Vigues, S., Ott, S. H., Elson, A. E. T., Choi, H.-J., Shaw, H., Egan, J. M., Mitchell, B. D., Li, X., Steinle, N. I., and Munger, S. D. Bitter taste receptors influence glucose homeostasis. *PLoS ONE*. 2008; 3,e3974.
6. Jeon, T. I., Zhu, B., Larson, J. L., and Osborne, T. F. SREBP-2 regulates gut peptide secretion through intestinal bitter taste receptor signaling in mice. *J. Clin. Invest*. 2008; 118, 3693–3700.
7. Janssen S, Laermans J, Verhulst PJ, Thijs T, Tack J, Depoortere I. Bitter taste receptors and α-gustducin regulate the secretion of ghrelin with functional effects on food intake and gastric emptying. *Proc Natl Acad Sci*. 2011; 108:2094-9. doi: 10.1073/pnas.1011508108
8. Mani, Venkatesh; Hollis, James; and Gabler, Nicholas K. Bitter Compounds Decrease Gastric Emptying and Influence Intestinal Nutrient Transport. *Animal Industry Report*. AS 658, ASL R2725.
9. Cristino L., Becker T, Di Marzo V. Endocannabinoids and energy homeostasis: An update. *Biofactors.* 2014; 40:389-97. doi: 10.1002/biof.1168.
10. Tomassini Barbarossa I, Carta G, Murru E, Melis M, Zonza A, Vacca C, Muroni P, Di Marzo V, Banni S. Taste sensitivity to 6-n-propylthiouracil is associated with endocannabinoid plasma levels in normal-weight individuals. *Nutrition.* 2013; 29:531-6. doi: 10.1016/j.nut.2012.09.018.
11. Tepper BJ, Banni S, Melis M, Crnjar R, Tomassini Barbarossa I. Genetic sensitivity to the bitter taste of 6-n-propylthiouracil (PROP) and its association with physiological mechanisms controlling body mass index (BMI). *Nutrients.* 2014; 6:3363-81. doi: 10.3390/nu6093363.
12. Yajima H, Ikeshima E, Shiraki M, Kanaya T, Fujiwara D, Odai H, Tsuboyama-Kasaoka N, Ezaki O, Oikawa S, Kondo K. *J Biol Chem*. 2004; 279:33456-62.
13. Obara K, Mizutani M, Hitomi Y, Yajima H, Kondo K. Isohumulones, the bitter component of beer, improve hyperglycemia and decrease body fat in Japanese subjects with prediabetes. *Clin Nutr.* 2009; 28:278-84. doi:10.1016/j.clnu.2009.03.012.
14. Berridge KC. 'Liking' and 'wanting' food rewards: Brain substrates and roles in eating disorders. *Physiology & Behavior*. 2009; 97:537–550. doi:10.1016/j.physbeh.2009.02.044
15. Mueller KL, Hoon MA, Erlenbach I, Chandrashekar J, Zuker CS, Ryba NJ. The receptors and coding logic for bitter taste. *Nature.* 2005; 434:225-9.
16. Prescott MJ1, Buchanan-Smith HM, Smith AC. Social interaction with non-averse group-mates modifies a learned food aversion in single- and mixed-species groups of tamarins (Saguinus fuscicollis and S. labiatus). *American Journal of Primatology.* 2005; 65:313–326.

17. Sørensen LB1, Møller P, Flint A, Martens M, Raben A. Effect of sensory perception of foods on appetite and food intake: a review of studies on humans. *Int J Obes Relat Metab Disord*. 2003; 27:1152-66.

18. Champagne CP, Fustier P. Microencapsulation for the improved delivery of bioactive compounds into foods. *Curr Opin Biotechnol.* 2007; 18:184-90.

19. Shirai Y, Sogo K, Yamamoto K, Kojima K, Fujioka H, Makita H, Nakamura Y. A novel fine granule system for masking bitter taste. *Biol Pharm Bull*. 1993; 16:172-7.

20. Lam PM, Marczylo TH, Konje JC. Simultaneous measurement of three N-acylethanolamides in human bio-matrices using ultra performance liquid chromatography-tandem mass spectrometry. *Anal Bioanal Chem*. 2010; 398:2089-97. doi: 10.1007/s00216-010-4103-z.

21. Mennella I, Savarese M, Ferracane R, Sacchi R, Vitaglione P. Oleic acid content of a meal promotes oleoylethanolamide response and reduces subsequent energy intake in humans. *Food Funct*. 2015; 6:204-10. doi: 10.1039/c4fo00697f.

22. WHO monographs on selected medicinal plants. *World Health Organization*. 2001; ISBN 978 92 4 154702 4.

23. Deloose E, Corsetti M, Van Oudenhove L, Depoortere I, Tack J.F. In Man Intragastric Administration of the Bitter Compound Denatonium Benzoate Decreases Hunger and the Occurrence of Gastric Phase III in the Fasting State. *AGA Abstracts*. 2013; Su 2070, S-548.

24. Verschueren S, Janssen P, Andrews C.N., Verbeke K, Depoortere I, F. Tack J. The Effect of the Bitter Taste Receptor Agonist Denatonium Benzoate on Gastric Emptying, Satiety and Return of Hunger After a Meal in Healthy Volunteers. *AGA Abstracts.* 2013; Su 2071, S-548.

25. McClements DJ, Li Y. Structured emulsion-based delivery systems: Controlling the digestion and release of lipophilic food components. *Advances in Colloid and Interface Science.* 2010*;* 159:213–228.

26. Sakata I, Sakai T. Ghrelin Cells in the Gastrointestinal Tract. *International Journal of Peptides*. 2010; 2010, 1-7.

Chapter 6. General Discussions

In humans the appetite control is determined by complex interactions between sensory and metabolic aspects. There are internal signals which are comprised of sensory, cognitive, post-ingestive and post-absorptive factors that form the satiety cascade (Blundell et al, 2010). Moreover there are many external cues, including food visibility, social norms, marketing suggestions, portion sizes, and others, which also influence meal initiation and termination. It should be expected that internal signals may perfectly balance timing and the amount of food intake following the alternations of hunger and satiety sensations. However, in today's environment the continuous exposition to external cues undermines the food intake control by the internal signals and may easily lead to overconsumption (Bilman, 2014). Therefore, the scenario of the factors that take part in the appetite control is huge and extremely complex.

The experiments carried out in the present thesis focused on pre-ingestive and post-absorptive physiological factors which can affect the satiety cascade. A summary of the studies and the major results are showed in table 6.1. Two studies related to the pre-ingestive phase investigated the role of the saliva and the endocannabinoids (ECs) system in the food preference and liking. Two other studies were designed to explore the potential satiating effect on the short term energy intake of specific food ingredients. A wide panel of biomarkers were investigated in order to describe the physiological factors behind the individual dietary behaviour.

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

Chapter	*Objectives*	*Methods and Subjects*	*Outcomes*	*Major findings*	*Conclusions*
2	to investigate the relationship between salivary lipase and α-amylase activities as well as zinc concentration with food preference and choice in subjects with different nutritional status (normal weight NW and overweight OW).	*Study:* observational study *Measures:* • Dietary behaviour and preferences • Saliva composition *Subjects:* • Healthy, Age, y: 29(19-54) • Number: 42(22M/20F) • BMI kg/m^2: 23 NW 22.2±1.7; 19 OW 28.5±2.1	• salivary α-amylase and lipase activity • salivary zinc concentration • fat preference questionnaire • food frequency questionnaire.	• higher α-amylase and lipase activity and lower zinc concentration in overweight than in normal weight subjects. • positive correlation between lipase activity and individual preference for high-fat foods and fat content of the diets.	the salivary enzymatic activity may influence the food preference and food consumption in humans.
3	to assess the endocannabinoids (ECs), N-acylethanolamines (NAEs) and gastrointestinal (GI) peptides response to a palatable (sweet, SW), unpalatable (bitter, BT) and sensory acceptable (tasteless control, CT) food stimuli.	*Study:* randomized crossover sham feeding-designed study *Measures:* • Blood biomarkers • Energy intakes (EI) *Subjects:* • Healthy, Age, y: 28(24-36) • Number: 20(11M/9F) • BMI, kg/m^2: 22.7±0.61	• ECs • NAEs, • GI pepitides, • appetite ratings • EI	• higher 2-arachidonoylglycerol (2-AG) and polypeptide pancreatic (PP) response after SW pudding vs BT or CT pudding ($p<0.05$). • food palatability did not influence appetite, EI or food choice.	2-AG and PP may be biomarkers of food liking in humans.
4	to test if a meal high in sunflower oil (SO) or high oleic sunflower oil (HOSO) or in virgin olive oil (VOO) can influence the short-term response of ECs and subsequent EI in humans.	*Study*: randomized crossover *Measures:* • Blood biomarkers • EI *Subjects:* • Healthy, Age, y: 29(22-40) • Number: 15(7M/8F) • BMI, kg/m^2 : 23.2±0.73	• ECs, • NAEs, • EI	• oleoylethanolamide (OEA) higher levels after the HOSO and VOO consumption vs SO. • A significant reduction of EI was also found.	a meal with high content of oleic acid may suistain the OEA plasma levels and reduce the following short term EI.
5	to evaluate if the ingestion of encapsulated bitter ingredients (EBI) contained in a food may influence subsequent EI.	*Study*: randomized crossover human study *Measures:* • Blood biomarkers • EI *Subjects:* • Healthy, Age, y: 26 (22-33) • Number: 15 (7M/8F) • BMI, kg/m^2: 21.1±1.6	• ECs, • NAEs, • GI peptides, • appetite ratings • EI	• No differences in Ecs, NAEs, GI peptides and appetite sensations. • A significant reduction in the EI was found over post-lunch period after the consumption of the pudding enriched in EBI vs control pudding.	microencapsulated bitter compounds can be a valid food ingredient to reduce daily EI in humans.

Development of satiety enhancing food: a matter of taste

The Western society is an obesogenic environment where people are continuously exposed to high energy-dense foods. The free availability and the heavy, continuous promotion of high-fat and/or -sugar foods determine the individual inability to control energy intakes (Berthoud, 2011). In other words, the environmental overstimulation of non-homeostatic processes behind food intake far exceed the homeostatic ones, thus leading to a dramatic increase of obesity. In this context, an effective strategy to control energy intake and reduce obesity should act through individual unconsciousness. For overweight and obese people it is extremely difficult to adhere to a diet rich in healthy and low-calorie foods such as fruits and vegetables for a long period. There is a need to develop healthy foods that can trigger satiety and cause an unconscious reduction of energy intake guarantying the food reward in the long period. Effective satiety enhancing foods require they are at least as tasty and gratifying as the unhealthier items they are intended to replace (Halford et al, 2012). A major challenge is to combine the desired functionality with consumers acceptance, as well as manufacturing and business feasibility (Mela, 2013). Consumers acceptance for healthier foods is conditioned by their perception that unhealthy foods are inherently tastier and vice versa. This consumer belief may represent another obstacle to the acceptance of healthy food since people think they are less palatable than their unhealthy alternative (Tremblay et al, 2013). Many healthy foods sold in groceries and specialized stores have a very high protein or fibre content which negatively affects food structure, flavor, and texture properties. Therefore, resulting in food products with very low overall palatability. This can partly explain the negative consumer perception for products promoted as healthy with a satiating effect (Blatt et al, 2011; Fromentin et al, 2012; Van Kleef et al, 2012). Moreover, in designing a food for appetite control it is also worth taking into consideration the enhancement of palatability is associated to an increase of hunger sensations, food ingestion speed, and global duration of a meal (Yeomans, 1996) and therefore may lead to greater food intake and higher risks of over-consumption (Bellisle et al, 1984; de Castro et al, 2000; Yeomans et al, 2004). Cephalic phase response, continued cephalic stimulation, and sensory specific satiety signals are involved in this process (Smeets et al, 2010). Therefore a big challenge for the food scientists will be to find the right balance between the hedonic value and the functionality of satiety enhancing foods, with the final aim to increase the palatability (to promote acceptance and consumption) without weakening satiety (without promoting over-consumption) (Trembaly et al, 2013).

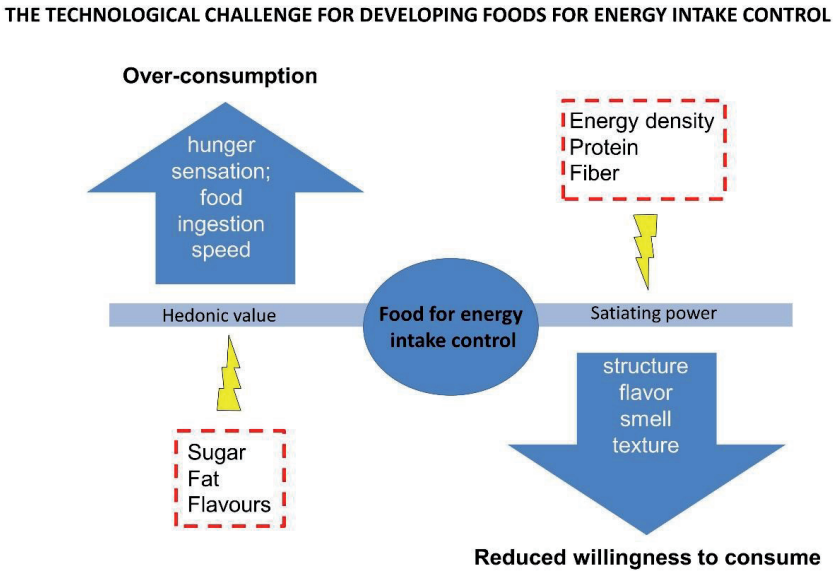


Figure 1. Human dietary behaviour and choices are governed by hedonic, homeostatic and environmental factors (Berthoud, 2011). Foods can influence these factors and control energy intakes (Tremblay et al, 2013). Mounting evidence indicates that satiating foods unconsciously leading to a reduction of energy intake are an effective strategy; the long term consumption of these foods is still an issue (Van Kleef et al, 2012). Foods with an high hedonic value may lead to an overconsumption, (Bellisle et al, 1984; de Castro et al, 2000; Yeomans et al, 2004) whereas modification of the food aiming at increasing satiating power may lead to a disruption of the palatability and a reduced willingness to consume (Tremblay et al, 2013; Van Kleef et al, 2012). In this context, the real challenge of food technologists and scientists is to find the right balance between the hedonic value and the satiating power of the food. This objective can be achieved by finely modulating the food factors influencing the two arms of the balance and the interconnected physiological pathways. The most promising food factors to modulate hedonic value are the content of sugar and fats as well as the food flavour. Whereas the energy density of the food and its protein and dietary fiber content mainly influence the satiating power of the food.

The other pillar to develop an effective satiety enhancing food is the elucidation of the physiological processes behind the hedonic value of a food. Mounting evidence showed that saliva composition and ECs system may have a role in eating behaviour. However there is still a lack of knowledge about their role in food preference and liking. A primary objective of this thesis was to study the saliva composition as an individual factor that can influence sensory perception and the ECs system as the physiological system able to influence food liking and choice.

The role of salivary enzymes in taste perception and food preferences

A series of physiological factors may influence the subjective perception and therefore the overall liking of foods. The simple sight and smell of foods is enough to stimulate saliva secretion by major salivary glands: the parotid, sublingual, and submandibular glands. In addition to these, there is a large and still undefined number (600-1000) of salivary minor glands which share some common characteristics: they have a quite simple structure consisting of a small clusters of secretory cells with a short excretory duct; with the exception of the von Ebner's glands, which secrete serous saliva, they are mucous; they have little or no sympathetic innervations (Hand et al, 1999; Riva et al. 2000). By contrast with the major glands which fully secrete their fluid only upon stimulation, the minor glands function more or less continuously during the day and at night. Moreover the salivary flow is depended on the density and number of small glands: a high variability among humans have been reported (Won et al, 2001; Gaubenstock et al, 1990; Hamada et al, 1974). Saliva has a fundamental role in taste perception influencing the way molecules are released from a food matrix and its diffusion in the mouth. Some animal studies also demonstrated that the macronutrient composition of the diet influence the saliva composition. A summary of the saliva interactions with food and its effect on taste perception are resumed in the table 6.2.

Table 6.2 Saliva properties influencing taste perception and the components involved in this job

Saliva property	Component	Possible effect on taste perception	References
cover the top of the taste pore	whole saliva	maintain the external milieu of the taste receptor cells.	Matsuo 2000
dissolve and diffuse tastant molecules	whole saliva	help the taste recognition	Matsuo 2000
hypotonicity	whole saliva	enhance salty taste	Humphrey et al. 2001
emulsifing properties	lysozyme	facilitate the fat texture perception	Siletti et al. 2007
proteolytic activity	cystatin SN	enhance bitter perception	Dsamou et al. 2012
amylolitic activity	salivary amylase	increase the oral perception of starch viscosity	Mandel et al. 2010
lypolitic activity	salivary lipase	contribute to oral fat detection	Kulkarni et al. 2014
lypolitic activity and protein composition	salivary lipase, lipocalin	increase the fat-liking	Neyraud et al. 2011

The study of saliva composition and its physiological role in food preferences had a double objective:

- to shade lights on the mechanisms occurring during the cephalic phase of eating that can influence food digestion, satiation and reward processes following food ingestion;
- to seek biological biomarkers of liking that, as present in saliva, may be an advantageous alternative to plasma biomarkers, due to the non-invasiveness, stress-free and repeated samples possibility associated to saliva sampling compared to plasma one (Aydin, 2007).

Several molecules found in saliva have been already proposed as biomarkers of some biological conditions. Salivary cortisol has been suggested as a marker of body's stress response to acute exercise, whereas testosterone/cortisol ratio is an indicator of anabolic/catabolic adaptations of training (Papacosta et al, 2011). A role for alpha-amylase as biomarker of sympathetic nervous system (SNS) activity to the acute stress induction was also proposed since its activity predicts the plasma levels of a well-established SNS indicators norepinephrine (Aydin, 2007).

With regard to taste perception and preference, the salivary enzymatic activity is of great interest. In fact, alpha-amylase is the most abundant protein in human saliva (Hirtz et al, 2005). It was demonstrated that this enzyme plays a significant role in the oral perception of starch viscosity (Mandel et al, 2010), creaminess (De Wijk et al, 2004) and the release of flavour compounds (Ferry et al, 2004). In the last years, the suggestion of a specific taste for fat in addition to the traditional five taste qualities, sweet, umami, salty, sour, and bitter (Chandrashekar et al, 2006) stimulate also the interest for the salivary lipolytic activity. Whether a triglyceride degrading enzyme exists in human saliva is still disputed, although a recent research conducted by Voigt et al. detected lipolytic activities in minor salivary and identified gene coding for lingual lipase (Voigt et al, 2014).

In the study described in chapter 2, two main questions were answered regarding the role of lingual lipase. Firstly, we confirmed that a lipolytic activity can be detected in human saliva (in accordance with Voigt et al, 2014). Secondly, we found that individual nutritional status is associated with this activity: the higher the BMI the higher the lingual lipase activity.

A gap of knowledge still exists on the biological factors influencing salivary enzymatic activity. Genetic, nutritional and behavioural factors may be involved. Interestingly, a research carried out on two populations with different diet pattern found out that in the population which historically have a diet rich in carbohydrates, the AMY1 gene copies (the gene that code for salivary amylase) are higher compared to the protein-rich food eaters (Perry et al, 2007). Moreover, there is evidence

that a diet high in starch may upregulate salivary amylase expression (Squires, 1953). In agreement with these studies, we found that both salivary amylase activity and lingual lipase activity are correlated with carbohydrates and total dietary fats intake, respectively. A previous study reported that amylase concentration in saliva and salivary enzymatic activity are significantly correlated, but these results were not so strong as expected (Mandel et al, 2010). It is possible that salivary enzymatic activity may be also affected by other factors such as genetic polymorphism, post translation modification or interactions with other proteins, such as mucins (Iontcheva et al, 1997). An intriguing hypothesis is that also oral microflora may consistently take part to the total salivary enzymatic activity.

Mandel et al. (2012) found that in normal weight people high levels of salivary alpha amylase activity may help in maintaining the homeostasis after food intake reducing the postprandial blood glucose response. By contrast, in a previous article the same authors hypothesized that salivary alpha amylase activity directly correlated with postprandial glycaemia levels, since the action of the enzyme increase the free sugars release from the food matrix (Mandel et al, 2010). An explanation for this unexpected result can be that a higher breakdown of starch by alpha amylase in the oral cavity activates a higher cephalic phase insulin response, which helps to tolerate glucose better once the food is ingested. (Mandel et al, 2012).

All these researches were mainly conducted on alpha amylase, whereas there is still a gap of knowledge on lingual lipase. Therefore, we decided to investigate if similar mechanisms also exist about factors that can influence lingual lipase activity. Further studies are necessary to establish causal mechanisms among the enzyme activity, nutritional status, and dietary habits. Similar to the salivary amylase, it can be hypothesized that also genetic factors influence the amount and the activity of the enzyme. Moreover, it can be suggested that in addition to its role on fat perception (Voigt et al, 2014), lingual lipase has also a role in food liking and may have a role in anticipating post-prandial response to food (figure 2). Understanding the basic mechanisms underlying the rationale of salivary biomarkers will help us to learn how the changes that occur in foods during oral processing impact our hedonic evaluation, preference, and ingestion of such foods.

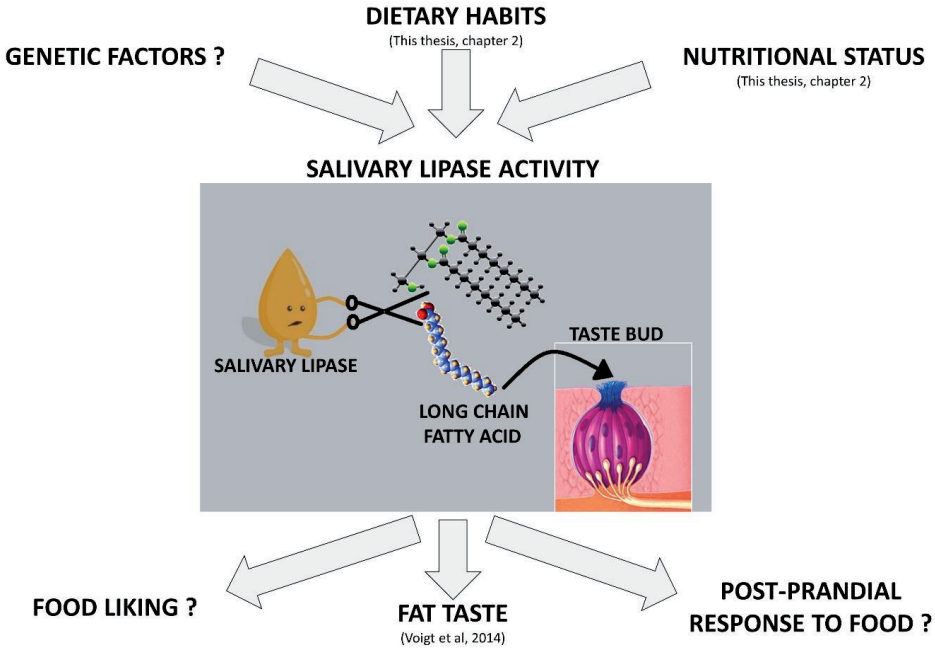


Figure 2. Factors influencing the salivary lipase activity its potential effects on physiological functions. Genetic, dietary and nutritional factors may affect the salivary lipase activity. The enzyme begins the digestion of triglycerides, releasing free fatty acids that can bind their taste receptors. As a consequence fat is sensed in the mouth and this can be related to food liking and postprandial lipids metabolism through a cephalic phase mechanism.

Endocannabinoids system upon the cephalic phase of eating: a biomarker of food liking

Non-homeostatic mechanisms of eating and food reward are fundamental in the short, medium, and long term. In the short term they influence food choice, since people eat what they like and avoid food they dislike. In the medium term, they may modulate the variety of the diet through the sensory specific satiety that may elicits the termination of eating a specific food. In the long term, they influence the learning associations and create the memory of foods influencing the individual food pattern and the food choice in a specific context (Smeets et al, 2010).

Food reward consist of three main components which normally occur together but have separate neurological pathways: liking (the hedonic impact of a food), wanting (motivation to obtain foods) and learning (associative conditioning and cognitive processes) (Berthoud et al, 2011).

The hedonic value of a food is mainly established during the cephalic phase of eating and can be divided in a first cephalic response, acting before meal ingestion, and in a continued cephalic stimulation acting upon food ingestion until meal termination (Smeets et al, 2010). Literature data supported the idea that cephalic phase response (CPR) may prepare the body to maintain homeostasis during the digestion (Teff, 2000; Nederkoorn et al, 2000). High palatable food stimulate a higher CPR thus allowing a high amount of food to be consumed. Cephalic stimuli consist of a series of secretory and non-secretory responses. However, few hormones also involved in the appetite control, such as insulin, pancreatic polypeptide, ghrelin, are stimulated during the CPR. This background suggested that other factors also related to satiety and appetite control should be stimulated during the cephalic phase in response to food liking, wanting, and learning associations. ECs and related species, such as N-acylethanolamines (NAEs) may act as a link between the homeostatic and the non-homeostatic (hedonic) system (Di Marzo, 2009).

In the study described in the chapter 3, we aimed at determining the role of ECs during the cephalic phase of eating a high palatable, acceptable and unpalatable food. In general, the ECs system sustains several rewarding behaviours, such as cooperative playing and sexual activity, stimulating dopamine neurons in ventral tegmental area and substantia nigra (Wise, 2009). The neurotransmitter dopamine targets brain areas involved in reinforcement, learning, emotional memory, habit formation: they are all hallmarks of reward-related behaviours (Fattore et al, 2010). Eating is another rewarding behaviour, whose power may depend from the macronutrient composition of the consumed food (Berthoud et al, 2012). Several limbic and cortical brain regions, stimulated by several neurotransmitters, including ECs, are involved in the rewarding value of the food. In the present thesis we demonstrated that, even before food ingestion, taste perception itself is able to

induce a plasma ECs response. In particular, Chapter3 shows that food perceived as high palatable stimulated an increase in 2-AG plasma levels whereas food perceived as unpalatable produced a reduction of 2-AG plasma levels. The peculiarity of our experimental design was that the macronutrient composition of the tested foods were identical and study participants were selected on the basis of the hedonic values for the specific test foods, thus the hedonic values could be assumed as the main drivers of the ECs response. This lead to the conclusion that food palatability can influence the ECs plasma concentration and 2-AG play a pivotal role in food liking. A previous preliminary study already suggested wanting and ingestion of high palatable foods stimulate an higher 2-AG plasma response compared to a control food (Monteleone et al, 2012). All in all, this data suggests that the 2-AG is involved in the two main components that regulate the rewarding value of foods: liking and wanting. Further studies should investigate the role of 2-AG in learning, the third component of food reward.

From a physiological point of view the ECs system has a general anabolic effect, promoting food intake and increasing pleasure and motivation to eat (Cristino et al, 2014). Therefore, the cephalic phase ECs response may be part of the mechanisms which allow a greater food intake when the hedonic value of the food is high. Similarly to the cephalic phase response of insulin, 2-AG response can have a role in the preparatory mechanisms behind eating which helps body to maintain homeostasis. These findings can have interesting implications for the designing of foods aiming at the reduction of calorie intake. The measure of specific ECs can offer the possibility to quantify responses that up to now were approached by qualitative attribute such as liking and palatability. It is clear that the combination of the biochemical and sensory approach is a powerful way to compare the satiating and rewarding capacity also of different category of foods (i.e. dessert vs bakery).

Macronutrients in appetite regulation: modulating the endocannabinoids system from reward to satiety

A hierarchy has been observed for the satiating efficacies of the macronutrients being protein the most and fats less satiating macronutrient. Macronutrient formulation influence the form, texture, and microstructure of foods thus influencing taste and satiety (Lundin et al, 2008). A number of studies reported that proteins interaction among each other or with other food constituents as well as their isoelectric points may influence protein digestions and, as a consequences, the post-prandial appetite cues (Ross et al, 2003; Yildirim et al, 1998; Wooster et al, 2007; Bowen et al, 2006; Boirie et al, 1997; Hall et al, 2003).

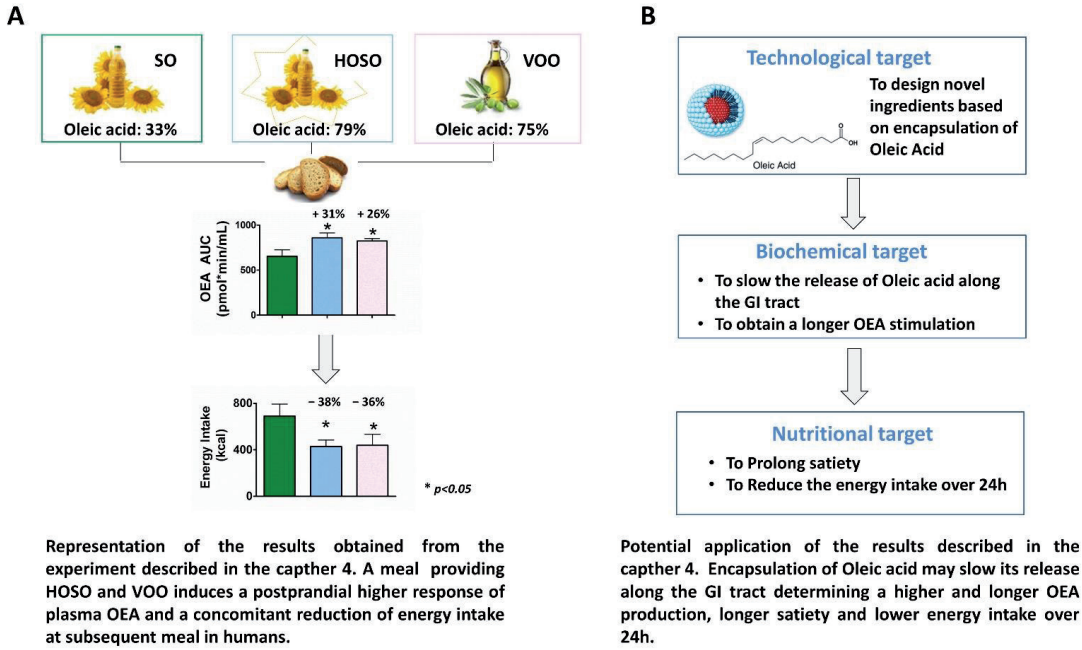
The primary physiological effect of dietary fibre (such as cellulose, pectin, galactomannans, glucans) influencing appetite is the slowing of stomach emptying associated to a greater fullness due to a prolonged permanence of the food in the stomach (Lundin et al. 2008). The solubility of the dietary fibres and the ability to increase the viscosity of a meal are the main physical characteristics of these food components associated to the stomach emptying rate.

Despite they are recognized as the macronutrient with the lower satiety quotient fatty acids have an important role in satiety and subsequent energy regulation. All fats are digested in form of emulsion: in fact the lipases act on the surface of insoluble fat droplets (Fave et al, 2004; Mu et al, 2004). The emulsion surface area is the key physicochemical factor affecting fat digestion (Lundin et al, 2008), generally the higher the surface the faster the digestion. Another property of fatty acids extensively investigated in relation to satiety is the degree of saturation (Casas-Agustench et al, 2009). Higher is the number of double bonds faster is the rate of oxidation (Casas-Agustench et al, 2009). In other words, saturated fatty acids are metabolised through the mitochondrial beta oxidation slower than monounsaturated fatty acids (MUFA) which in turn are metabolised slower than polyunsaturated fatty acids (PUFA) (Casas-Agustench et al, 2009). It was suggested that the fats which are not stored, but directly oxidised have a higher satiety quotient, thus the satiating power should be PUFA>MUFA>SFA. However, most of the studies conducted to test this hypothesis did not find any significant variations among the different fats: either, the length and the number of double bonds, did not influence the following energy intakes (Casas-Agustench et al, 2009; Flint et al, 2003; Lawton et al, 2000, Alfenas et al, 2003; MacIntosh et al, 2003; Burton-Freeman et al, 2004; Burton-Freeman et al, 2005). Unfortunately, none of the reported studies evaluated the biochemical plasma response to fats ingestion thus making impossible a mechanistic explanation (de Graaf et al, 2004).

On the other hand the use of fats to stimulate satiety proved to be successful when fatty acids precursors of the oleoylethanolamide (OEA), an anorexigenic compound belonging to ECs related species, were used. In fact, animal studies showed that high intake of oleic acid can induce the production of OEA in the gastrointestinal tract resulting in a satiating effect. Oleic fatty acid was the unique compound among glucose, proteins and other fatty acids able to support the OEA plasma levels (Schwartz et al, 2008) demonstrating that the presence of a precursor in the gastrointestinal tract may stimulate the ECs anabolism (Piomelli, 2013). To the best of our knowledge, no studies were present in literature on the influence of diet on the ECs plasma levels and the scarce researches were focused more on the macronutrient ratios rather than specific fatty acids intakes (Naughton et al, 2013).

In this context, we performed the study described in the chapter 4. It aimed to investigate whether a high intake of oleic acid may modulate the response of OEA and influence the subsequent energy intake in humans. The results previously reported open a series of interesting possibilities in designing novel functional ingredient for appetite control. In fact, we showed that OEA plasma levels may be increased by a high intake of its precursor oleic acid and this resulted in a reduction of 24h energy intake. Virgin olive oil and high-oleic acid sunflower oil were used in that study as a source of oleic acid. These oils are rich in triolein which is digested releasing oleic acids along the gastrointestinal tract. Our results are in agreement with a previous animal study investigating the intraduodenal effect of non-esterified oleic acid (Schwartz et al, 2006) and showing that the presence of free oleic acid in the gut stimulates the OEA production. It was suggested that three receptors, CD36, GPR120, and GPR40, are implicated in fatty acids sensors in the intestine (Sclafani et al, 2012). So, it can be hypothesized that the activation of specific fats taste receptors in the gastrointestinal tract can be a way to develop novel ingredients for appetite control.

The use of well-designed ingredients may increase the effect of food developed for appetite control: the release of the ingredient in a more focused area of the gastrointestinal tract as well as the direct use of oleic acid instead of triolein may increase the OEA endogenous production (figure 3). From a technological point of view, the targeted release of an ingredient in a specific site along the gastro-intestinal tract may be achieved using the encapsulation technology (Kuang et al, 2010).



Representation of the results obtained from the experiment described in the capther 4. A meal providing HOSO and VOO induces a postprandial higher response of plasma OEA and a concomitant reduction of energy intake at subsequent meal in humans.

Potential application of the results described in the capther 4. Encapsulation of Oleic acid may slow its release along the GI tract determining a higher and longer OEA production, longer satiety and lower energy intake over 24h.

Figure 3. Results and potential application from the experiment described in the chapter 4. SO, sunflower oil; HOSO, high oleic sunflower oil; VOO virgin olive oil; OEA, Oleoylethanolamine; GI, gastrointestinal; AUC, area under the curve.

Moreover, further experiments should verify if also other ECs may be influenced by the intake of their precursors. Studies conducted in rats demonstrated that a low dose of the cannabinoid receptor agonist THC, may enhance the palatability of sucrose solution and reduce rejection of a highly unpalatable quinine solution (Jarrett et al, 2005; Jarrett et al, 2007). Moreover, another research in rats demonstrated that 2-AG and AEA administration selectively enhance sweet taste (Yoshida et al, 2010). If these mechanisms will be confirmed in humans, it will be possible to enhance the palatability of a food as well as its sweet taste (and consequent overall hedonic value of the food) by sustaining the 2-AG and AEA plasma tone during eating. This would lead to a faster satisfaction upon eating due to the sensory specific satiety mechanism and an overall reduction of energy intake upon a meal or of eating occasions during the day. Future studies should test whether the use of fatty acids that are ECs precursors in a specific amount and formulation may be used to design palatable foods for appetite control.

Taste receptors in the gastrointestinal tract: a chemoreceptor system to sense nutrients

The detection of nutrients in the gastrointestinal tract plays a crucial role in regulating appetite and satiety, stomach and intestine motility and initiate neural and hormonal pathways necessary for normal digestive function (Reinehr et al, 2015). Cells that can detect the nutrients in the gastrointestinal tract are located on the surface of the luminal content, close to afferent nerve terminals (Steinert et al, 2011). Enterocytes, brush cells and entero-endocrine cells (EEC) are all involved in chemosensory mechanisms. However, with regard to appetite control EEC have the main role. In fact, more than 20 types of EEC secrete a variety of hormones, including CCK, GIP, GLP, PYY, representing the largest endocrine organ of the human body (Janessen et al, 2012). Interestingly, recent studies abolished the concept of the one cell-one hormone and corroborated the idea that a lineage of EEC may secrete a group of related peptides (Habib et al, 2012; Egerod et al, 2012). When the receptors for fats are activated in the gastrointestinal tract they induce a cascade of satiating peptides including CCK, GLP-1 and PYY. In addition to nutrients, receptors also exist for their metabolites. For example, OEA was found to be a moderate agonist for the receptor GPR119 (Overton et al, 2006). This is expressed on the surface of intestinal endocrine K and L-cells and its activation results in the production of the satiating peptides GIP, GLP and PYY in vitro and in vivo in rodents (Chu et al 2008; Lauffer et al. 2009; Rüttimann et al, 2009; Cheung et al, 2009). Moreover, the administration of GPR119 agonist reduces food intake and body weight in obese rats, but this effect is lost in GPR119 $^{-/-}$ rats (D'Alessio et al, 2007).

All in all, these data suggest that the results we found following the intake of oils rich in oleic acid might be due to a wide activation of the fat taste receptors in the gut which in turn lead to a greater production of OEA. In addition to the taste receptors activation, which can already stimulate the release of satiating peptides, the metabolite OEA may stimulate GIP, GLP and PYY through the activation of GPR119 resulting in a positive reinforcing feeback on satiety.

Micronutrients in appetite regulation: take the better from the bitter

Comparing evolutionary evidences, it has been inferred that taste probably evolved more than 500 million years ago when a primitive fish, developed a new kind of cell. Taste buds have been repeatedly tweaked over time to suit various animals' dietary needs, a shift in diet removed the need to sense certain chemicals in food or vice versa (Callaway, 2012). Among tastes, studies conducted on the elephant shark's genome, a very old species that lacks bitter taste receptors, suggests that these genes evolved more recently (Grus et al, 2009). It is widely accepted that bitter taste has evolved as a central warning signal against the ingestion of potentially toxic substance when life

moved on dry land and animals began to eat vegetables. In fact, almost all plants and vegetables produce bitter substances which widely differ in chemical structures and molecular weights. As a consequence, the receptors that recognize them are diverse being 25 in humans, each recognizing unique combinations of chemicals. In the mouth, bitter substances evoke disgust and avoidance. This instinctive rejection of intense bitter taste may be immutable because it has been crucial for human survival, and it seems that it still affects our food choice (Hofmann, 2009). Once, bitter compounds are ingested they can cause nausea and depending on the amount and the subject sensitivity may also induce vomit episodes. TAS2Rs bitter receptors were found in smooth-muscle strips in humans airways where their activation cause a relaxation of the muscle tissue (Deshpande et al, 2010). In vitro, animals and humans studies support the existence of a similar mechanism in the gut where the activation of the antral and duodenal smooth-muscle strips by bitter taste-receptor agonist may have an inhibitory effect on gastric contractility, probably mediated by the release of anorexigenic peptides (Janssen et al, 2010; Mani et al, 2012; Deloose et al, 2013; Verschueren et al, 2013) The decreased rate at which food passes through the stomach should lower the amount of food ingested reducing the ingestion of potential toxic compounds.

To the best of our knowledge, the research described in the chapter 5 is the first where a bitter ingredient was used in a food to investigate its effect on hunger and satiety in humans. In fact, to avoid the repulsion generated by the perception in the mouth previous studies were conducted using pills or by intragastric infusion (Yajima et al, 2004; Obara et al, 2009; Deloose et al, 2013; Verschueren et al, 2013). In our case, a novel food ingredient including bitter compounds and designed for appetite control was developed and included in a novel palatable food for its validation. We demonstrated, that the application of an appropriate microencapsulation technology on a suitable ingredient (a gentiana lutea bitter extract) allowed to develop a food able to reduce daily energy intakes. The microencapsulation of the bioactive extract was used to mask the bitter taste in the mouth and deliver the functional ingredient directly in the gastrointestinal tract (figure 4). We chose puddings as a food to validate the new ingredient because their formulation avoids thermal and mechanical stresses on the microcapsules. In fact, the microencapsulated ingredient was stable up to 80°C and under a moderate mechanical stress. In other words, it's worth underlying the importance of a proper ingredient design to control the effects of foods on satiety functions.

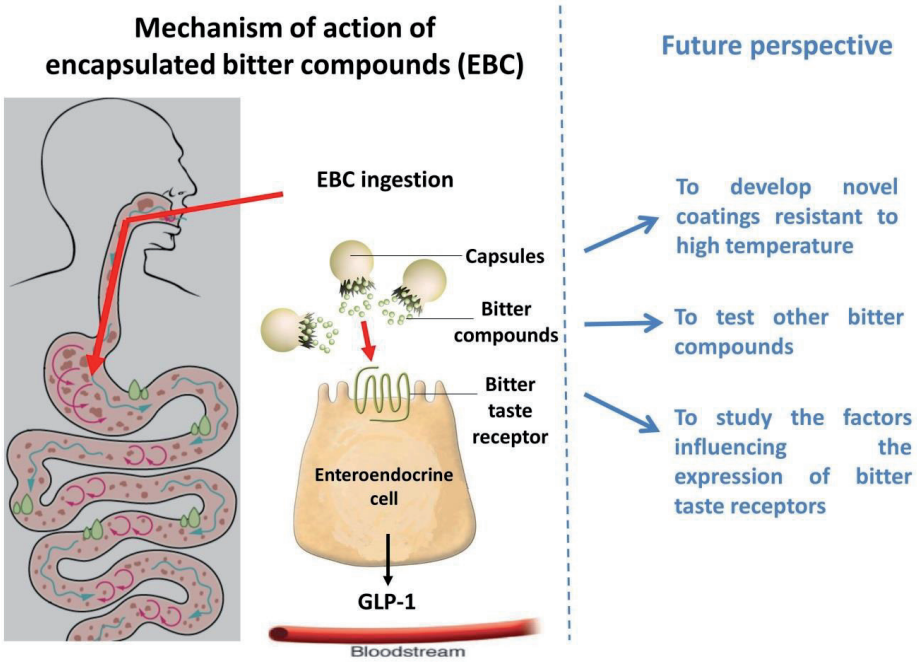


Figure 4. Physiological mechanism underlying the satiating effect of encapsulated bitter compounds tested in the chapter 5 and potential future applications of our findings. The capsules once ingested arrived intact in the gastrointestinal tract where they were slowly digested. The particles breakage released the bitter compounds that could bind the bitter taste receptors on the enteroendocrine cells. This binding elicited the release of anorexigenic peptides such as GLP-1 in the bloodstream. Further studies may focus on enhancing the satiating effect by testing novel coating and core materials and investigating the influence of genetics of bitter receptors on enteroendocrine response to bitter stimuli.

Further studies may aim to develop other types of ingredients with the same bioactive compounds and different coating materials in order to improve the thermal and mechanical resistance and allow their use in a wide type of foods. On the other hand, other bitter compounds having chemical structures and molecular weights different from Gentiana's bitter compounds should be evaluated in order to evaluate if the possible link with different receptors in GI tract may elicit, together with a different level of bitterness, also a different satiety response. In fact, as previously stated the chemical structures and molecular weights of bitter compounds determine the possibility to bind specific receptors resulting in a different bitter value. It was reported that some compounds may bind up to eight receptors whereas others such as the phenylthiocarbamide, binds to only one receptor (Meyerhof et al, 2010). In humans, variation in the bitter-taste receptor genes and expression of bitter receptors mRNA in taste cells have been reported to influence the bitter taste perception. In addition, the extent of mRNA expression correlated with the recent caffeine intake

thus suggesting(suggests) that the intake of bitter compounds may induce variation of the bitter receptors' gene expression (Lipchock et al, 2013). It can be hypothesized that compounds exerting higher bitter value in the mouth may also generate a greater anorexigenic effect. In addition, the genetic variability and the influence of the diet on the genetic expression may also result in a different subjective response to bitter compounds intake. Further sensory and nutrigenomics studies will test these hypotheses in order to achieve the maximum effect from a nutrient sensing approach in developing food ingredients for appetite control.

Conclusions

From the work described and discussed in the present thesis it can be concluded that:

fat food consumption is conditioned by individual taste perception and by salivary lipase activity. Further research is needed to clarify the genetic and dietary factors behind the activity of this enzyme and of the minor salivary glands influencing saliva composition.

Eating behaviour may be influenced by the ECs response that was found linked with food liking (even before food ingestion) and may in turn reinforce learning associations between unhealthy eating and food reward (pleasure).

Post-prandial ECs response may be modulated by lipid composition of the food and can influence energy intake.

Sensing of bitter tasting compounds along the GI tract may reduce energy intake in humans.

The translation of this acquired knowledge in developing new food/ingredients for energy intake control results in the conclusions that:

the hedonic value of the food has to be taken into high account by food developers

new ingredients must be designed to reach specific targets in the body, including the chemical taste receptors on the enteroendocrine cells.

The stability of bioactive compounds to the technological processes and to the GI digestive system, together with the necessity to mask some unwanted taste and flavour of bioactive compounds, must be guaranteed. Microencapsulation techniques can cope with these issues. However, the high inter-individual variability in chemical receptor genes expression has to be considered in a nutrigenomics prospective in order to analyse the relation among gene expression, taste perception and satiety

response. Future studies should combine physiology, nutrient sensing, and nutrigenomics in order to develop personalized foods for appetite control.

References

1. Alfenas RGC and Mattes RD. *Effect of Fat Sources on Satiety*. Obesity Research. 2003; Vol. 11 No. 2 February, 183
2. Aydin S. *A comparison of ghrelin, glucose, alpha-amylase and protein levels in saliva from diabetics*. J Biochem Mol Biol. 2007; Jan 31;40(1):29-35.
3. Bellisle F, Lucas F, Amrani R, Le Magnen J. *Deprivation, palatability and the micro-structure of meals in human subjects.* Appetite. 1984; 5,85–94.
4. Berthoud HR, Lenard NR, Shin AC. *Food reward, hyperphagia, and obesity*. Am J Physiol Regul Integr Comp Physiol. 2011; Jun;300(6):R1266-77. doi: 10.1152/ajpregu.00028.2011.
5. Berthoud HR, Münzberg H, Richards BK, Morrison CD. *Neural and metabolic regulation of macronutrient intake and selection*. Proc Nutr Soc. 2012; Aug;71(3):390-400. doi: 10.1017/S0029665112000559.
6. Berthoud HR. *Metabolic and hedonic drives in the neural control of appetite: who is the boss?*. Curr Opin Neurobiol. 2011; Dec;21(6):888-96. doi: 10.1016/j.conb.2011.09.004.
7. Bilman E. *Claiming satiety: consumer perception, interpretation & subsequent food intake.* Wageningen School of Social Sciences (WASS), PhD thesis, Wageningen University, Wageningen, NL. 2014; 172pages. ISBN: 978-94-6173-866-0.
8. Blatt AD, Roe LS, Rolls BJ. *Increasing the protein content of meals and its effect on daily energy intake*. J Am Diet Assoc. 2011; 111, 290–4.
9. Blundell J, de Graaf C, Hulshof T, Jebb S, Livingstone B, Lluch A, Mela D, Salah S, Schuring E, van der Knaap H, et al. *Appetite control: methodological aspects of the evaluation of foods.* Obesity Reviews. 2014; 11(3), 251-270.
10. Boirie Y, Dangin M, Gachon P, Vasson MP, Maubois JL, Beaufrere B. *Slow and fast dietary proteins differently modulate postprandial protein accretion*. Proc Natl Acad Sci USA. 1997;94: 14930–5.
11. Bowen J, Noakes M, Clifton PM. *Appetite regulatory hormone responses to various dietary proteins differ by body mass index status despite similar reductions in ad libitum energy intake.* J Clin Endocrinol Metab. 2006; 91: 2913–19.
12. Burton-Freeman B, Davis PA, O Schneeman B. *Interaction of fat availability and sex on postprandial satiety and cholecystokinin after mixed-food meals.* Am J Clin Nutr. 2004; 80:1207–14.
13. Burton-Freeman B. *Sex and Cognitive Dietary Restraint Influence Cholecystokinin Release and Satiety in Response to Preloads Varying in Fatty Acid Composition and Content.* J Nutr. 2005; Jun;135(6):1407-14.
14. Callaway E. *The lost appetites*. S16, Nature. 2012; vol 486,
15. Casas-Agustench P, López-Uriarte P, Bulló M, Ros E, Gómez-Flores A, Salas-Salvadó J. *Acute effects of three high-fat meals with different fat saturations on energy expenditure, substrate oxidation and satiety.* Clin Nutr. 2009; Feb;28(1):39-45. doi: 10.1016/j.clnu.2008.10.008
16. Chandrashekar, J.M. A. Hoon, N. J. Ryba, and C. S. Zuker. *The receptors and cells for mammalian taste*. Nature. 2006; 444:288– 294.

17. Cheung GW, Kokorovic A, Lam CK, Chari M, Lam TK. *Intestinal cholecystokinin controls glucose production through a neuronal network.* Cell Metab. 2009; Aug;10(2):99-109. doi: 10.1016/j.cmet.2009.07.005.

18. Chu ZL, Carroll C, Alfonso J, Gutierrez V, He H, Lucman A, Pedraza M, Mondala H, Gao H, Bagnol D, et al. *A role for intestinal endocrine cell-expressed g protein-coupled receptor 119 in glycemic control by enhancing glucagon-like Peptide-1 and glucose-dependent insulinotropic Peptide release*. Endocrinology. 2008; May;149(5):2038-47. doi: 10.1210/en.2007-0966.

19. Cristino L, Becker T, Di Marzo V. *Endocannabinoids and energy homeostasis: an update.* Biofactors. 2014 Jul-Aug;40(4):389-97. doi: 10.1002/biof.1168.

20. D'Alessio D, Lu W, Sun W, Zheng S, Yang Q, Seeley R, Woods SC, Tso P. Fasting and postprandial concentrations of GLP-1 in intestinal lymph and portal plasma: evidence for selective release of GLP-1 in the lymph system. Am J Physiol Regul Integr Comp Physiol. 2007; Dec;293(6):R2163-9.

21. De Castro JM, Bellisle F, Dalix AM. *Palatability and intake relationships in free-living humans: measurement and characterization in the French*. Physiol Behav. 2000; 68, 271–7.

22. de Graaf C, Blom WA, Smeets PA, Stafleu A, Hendriks HF. *Biomarkers of satiation and satiety.* Am J Clin Nutr. 2004; Jun;79(6):946-61.

23. de Wijk RA, Prinz JF, Engelen L, Weenen H. *The role of alpha-amylase in the perception of oral texture and flavour in custards*. Physiol Behav. 2004; Oct 30;83(1):81-91.

24. Deloose E, Corsetti M, Van Oudenhove L, Depoortere I, F. Tack J. *In Man Intragastric Administration of the Bitter Compound Denatonium Benzoate Decreases Hunger and the Occurrence of Gastric Phase III in the Fasting State*. AGA Abstracts. 2013; Su 2070, S-548

25. Deshpande DA, Wang WC, McIlmoyle EL, Robinett KS, Schillinger RM, An SS, Sham JS, Liggett SB. *Bitter taste receptors on airway smooth muscle bronchodilate by localized calcium signaling and reverse obstruction.* Nat Med. 2010; Nov;16(11):1299-304. doi:10.1038/nm.2237.

26. Di Marzo V, Ligresti A, Cristino L. *The endocannabinoid system as a link between homoeostatic and hedonic pathways involved in energy balance regulation.* Int J Obes (Lond). 2009; Jun;33 Suppl 2:S18-24. doi: 10.1038/ijo.2009.67.

27. Egerod KL, Engelstoft MS, Grunddal KV, Nøhr MK, Secher A, Sakata I, Pedersen J, Windeløv JA, Füchtbauer EM, Olsen J, et al. *A major lineage of enteroendocrine cells coexpress CCK, secretin, GIP, GLP-1, PYY, and neurotensin but not somatostatin.* Endocrinology. 2012; Dec;153(12):5782-95. doi: 10.1210/en.2012-1595.

28. Fattore L, Melis M, Fadda P, Pistis M, Fratta W. *The endocannabinoid system and nondrug rewarding behaviours*. Exp Neurol. 2010 Jul;224(1):23-36. doi:10.1016/j.expneurol.2010.03.020.

29. Fave G, Coste TC, Armand M. *Physicochemical properties of lipids: new strategies to manage fatty acid bioavailability.* Cell Mol Biol. 2004; 50: 815–31.

30. Ferry AL, Hort J, Mitchell JR. *Effect of amylase activity on starch paste viscosity and its implications for flavor perception.* J Texture Stud. 2004; 35: 511–524.

31. Flint A, Helt B, Raben A, Toubro S, Astrup A. *Effects of different dietary fat types on postprandial appetite and energy expenditure.* Obes Res. 2003; Dec;11(12):1449-55.

32. Fromentin G, Darcel N, Chaumontet C, Marsset-Baglieri A, Nadkarni N, Tomé D. *Peripheral and central mechanisms involved in the control of food intake by dietary amino acids and proteins.* Nutr Res Rev. 2012; 25, 29–39.

33. Gaubenstock LM, Leontyev VK. *Quantitative and topographic characteristics of minor salivary glands.* Stomatologia. 1990; 69: 28–31. 36.

34. Grus WE, Zhang J. *Origin of the genetic components of the vomeronasal system in the common ancestor of all extant vertebrates*. Mol Biol Evol. 2009; Feb;26(2):407-19. doi:10.1093/molbev/msn262.

35. Habib AM, Richards P, Cairns LS, Rogers GJ, Bannon CA, Parker HE, Morley TC, Yeo GS, Reimann F, Gribble FM. *Overlap of endocrine hormone expression in the mouse intestine revealed by transcriptional profiling and flow cytometry.* Endocrinology. 2012; Jul;153(7):3054-65. doi: 10.1210/en.2011-2170.

36. Halford JC, Harrold JA. *Satiety-enhancing products for appetite control: science and regulation of functional foods for weight management*. Proc Nutr Soc. 2012; May,71(2):350-62. doi: 10.1017/S0029665112000134.

37. Hall WL, Millward DJ, Long SJ, Morgan LM. *Casein and whey exert different effects on plasma aminoacid profiles, gastrointestinal hormone secretion and appetite*. Br J Nutr. 2003; 89: 239– 48.

38. Hamada T, Kawasoe Y, Sekino K, Nagasawa T, Tsuru H. *Palatal gland distribution*. J Dent Res. 1974; 53: 944.

39. Hand AR, Pathmanathan D, Field RB. *Morphological features of the minor salivary glands.* Arch Oral Biol. 1999; 44: S3–S10. 4.

40. Hirtz C, Chevalier F, Centeno D, Rofidal V, Egea JC, Rossignol M, Sommerer N, Deville de Périère D. *MS characterization of multiple forms of alpha-amylase in human saliva. Proteomics*. 2005; Nov;5(17):4597-607.

41. Hofmann T. *Identification of the key bitter compounds in our daily diet is a prerequisite for the understanding of the hTAS2R gene polymorphisms affecting food choice.* Ann N Y Acad Sci. 2009; Jul;1170:116-25. doi: 10.1111/j.1749-6632.2009.03914.x.

42. Iontcheva I, Oppenheim FG, Troxler RF. *Human salivary mucin MG1 selectively forms heterotypic complexes with amylase, proline-rich proteins, statherin, and histatins.* J Dent Res. 1997; 76(3): 734–43.

43. Janssen S, Depoortere I. *Nutrient sensing in the gut: new roads to therapeutics*? Trends Endocrinol Metab. 2013; Feb;24(2):92-100. doi: 10.1016/j.tem.2012.11.006

44. Janssen S, Laermans J, Verhulst PJ, Thijs T, Tack J, Depoortere I. Bitter taste receptors and α-gustducin regulate the secretion of ghrelin with functional effects on food intake and gastric emptying. Proc Natl Acad Sci U S A. 2011 Feb 1;108(5):2094-9. doi: 10.1073/pnas.1011508108.

45. Jarrett MM, Limebeer CL, Parker LA. *Effect of D9-tetrahydrocannabinol on sucrose palatability as measured by the taste reactivity test.* Physiol Behav. 2005; 86, 475–479.

46. Jarrett MM, Scantlebury J, Parker LA. *Effect of D9-tetrahydrocannabinol on quinine palatability and AM251 on sucrose and quinine palatability using the taste reactivity test.* Physiol Behav. 2007; 90, 425–430.

47. Kuang SS, Oliveira JC, Crean AM. *Microencapsulation as a tool for incorporating bioactive ingredients into food.* Crit Rev Food Sci Nutr. 2010; Nov;50(10):951-68. doi:10.1080/10408390903044222.

48. Lauffer LM, Iakoubov R, Brubaker PL. *GPR119 is essential for oleoylethanolamide-induced glucagon-like peptide-1 secretion from the intestinal enteroendocrine L-cell.* Diabetes. 2009; May;58(5):1058-66. doi: 10.2337/db08-1237.

49. Lawton CL, Delargy HJ, Brockman J, Smith FC, Blundell JE. *The degree of saturation of fatty acids influences post-ingestive satiety*. Br J Nutr. 2000; May;83(5):473-82.

50. Lipchock SV, Mennella JA, Spielman AI, Reed DR. Human bitter perception correlates with bitter receptor messenger RNA expression in taste cells. Am J Clin Nutr. 2013 Oct;98(4):1136-43. doi: 10.3945/ajcn.113.066688.

51. Lundin L, Golding M, Wooster TJ. *Understanding food structure and function in developing food for appetite control*. Nutrition & Dietetics. 2008; 65 (Suppl. 3): S79–S85 DOI: 10.1111/j.1747-0080.2008.00266.x

52. MacIntosh CG, Holt SH, Brand-Miller JC. *The degree of fat saturation does not alter glycemic, insulinemic or satiety responses to a starchy staple in healthy men*. J Nutr. 2003; Aug;133(8):2577-80.

53. Mandel AL, Breslin PA. *High endogenous salivary amylase activity is associated with improved glycemic homeostasis following starch ingestion in adults*. J Nutr. 2012 May;142(5):853-8. doi: 10.3945/jn.111.156984.

54. Mandel AL, Peyrot des Gachons C, Plank KL, Alarcon S, Breslin PA. Individual differences in AMY1 gene copy number, salivary α-amylase levels, and the perception of oral starch. PLoS One. 2010; Oct 13;5(10):e13352. doi: 10.1371/journal.pone.0013352.

55. Mani V, Hollis J, Gabler NK. *Bitter Compounds Decrease Gastric Emptying and Influence Intestinal Nutrient Transport*. Animal Industry Report. 2012; AS 658, ASL R2725.

56. Mela DJ. *Bringing satiety to the market through commercial foods: technology, product development and claims*. Woodhead Publishing Limited, 2013.

57. Meyerhof W, Batram C, Kuhn C, Brockhoff A, Chudoba E, Bufe B, Appendino G, Behrens M.. *The molecular receptive ranges of human TAS2R bitter taste receptors*. Chem. Senses. 2010; 35, 157–170.

58. Monteleone P, Scognamiglio P, Monteleone AM, Perillo D, Canestrelli B, Maj M. *Gastroenteric hormone responses to hedonic eating in healthy humans.* Psychoneuroendocrinology. 2013 Aug;38(8):1435-41. doi: 10.1016/j.psyneuen.2012.12.009.

59. Mu HL, Hoy CE. *The digestion of dietary triacylglycerols*. Prog Lipid Res. 2004; 43: 105–33.

60. Naughton S.S., Mathai M.L., Hryciw D.H. and McAinch A.J., 2013. *Fatty Acid modulation of the endocannabinoid system and the effect on food intake and metabolism.* Int. J. Endocrinol. 2013; 361895.

61. Nederkoorn C, Smulders FT, Jansen A. *Cephalic phase responses, craving and food intake in normal subjects*. Appetite. 2000;35:45–55.

62. Obara K, Mizutani M, Hitomi Y, Yajima H, Kondo K. Isohumulones, the bitter component of beer, improve hyperglycemia and decrease body fat in Japanese subjects with prediabetes. Clin Nutr. 2009; 28:278-84. doi:10.1016/j.clnu.2009.03.012.

63. Overton HA, Babbs AJ, Doel SM, Fyfe MC, Gardner LS, Griffin G, Jackson HC, Procter MJ, Rasamison CM, Tang-Christensen M, et al. *Deorphanization of a G protein-coupled receptor for oleoylethanolamide and its use in the discovery of small-molecule hypophagic agents.* Cell Metab. 2006; Mar;3(3):167-75.

64. Papacosta E, Nassis GP. *Saliva as a tool for monitoring steroid, peptide and immune markers in sport and exercise science*. J Sci Med Sport. 2011; Sep;14(5):424-34. doi:10.1016/j.jsams.2011.03.004.

65. Perry GH, Dominy NJ, Claw KG, Lee AS, Fiegler H, Redon R, Werner J, Villanea FA, Mountain JL, Misra R, et al. *Diet and the evolution of human amylase gene copy number variation*. Nat Genet. 2007; 39(10): 1256–60.

66. Piomelli D. *A fatty gut feeling*. Trends in Endocrinology and Metabolism. 2013; 24: 332-341.

67. Reinehr T, Roth CL. *The gut sensor as regulator of body weight.* Endocrine. 2015; May;49(1):35-50. doi: 10.1007/s12020-014-0518-1

68. Riva A, Puxeddu R, Uras L, Loy F, Serreli S, Testa Riva F. *A high resolution sem study of human minor salivary glands*. Eur J Morphol. 2000; 38: 219–226.

69. Roos N, Lorenzen PC, Sick H, Schrezenmeir J, Schlimme E. *Cross-linking by transglutaminase changes neither the in vitro proteolysis nor the in vivo digestibility of caseinate.* K Milchw Forsch. 2003; 55: 261–76.

70. Rüttimann EB, Arnold M, Hillebrand JJ, Geary N, Langhans W. *Intrameal hepatic portal and intraperitoneal infusions of glucagon-like peptide-1 reduce spontaneous meal size in the rat via different mechanisms*. Endocrinology. 2009; Mar;150(3):1174-81. doi: 10.1210/en.2008-1221.

71. Schwartz GJ, Fu J, Astarita G, Li X, Gaetani S, Campolongo P, Cuomo V, Piomelli D. *The lipid messenger OEA links dietary fat intake to satiety.* Cell Metab. 2008; 8: 281-8.

72. Sclafani A, Ackroff K. *Role of gut nutrient sensing in stimulating appetite and conditioning food preferences*. Am J Physiol Regul Integr Comp Physiol. 2012; 302(10): R1119–R1133.ù

73. Smeets PA, Erkner A, de Graaf C. *Cephalic phase responses and appetite*. Nutr Rev. 2010; Nov;68(11):643-55. doi: 10.1111/j.1753-4887.2010.00334.x

74. Squires BT. *Human salivary amylase secretion in relation to diet*. J Physiol. 1953; 119: 153–156.

75. Steinert RE, Beglinger C. *Nutrient sensing in the gut: interactions between chemosensory cells, visceral afferents and the secretion of satiation peptides*. Physiol Behav. 2011; Nov 30;105(1):62-70. doi: 10.1016/j.physbeh.2011.02.039.

76. Teff K. Nutritional implications of the cephalic-phase reflexes: endocrine responses. Appetite. 2000;34:206–213.

77. Tremblay A., Arguin H. *Functional foods, satiation and satiety*. Woodhead Publishing Limited, 2013.

78. Van Kleef E, Van Trijp JC, Van Den Borne JJ, Zondervan C. *Successful development of satiety enhancing food products: towards a multidisciplinary agenda of research challenges*. Crit Rev Food Sci Nutr. 2012; 52,611–28.

79. Verschueren S, Janssen P, N. Andrews C, Verbeke K, Depoortere I, F. Tack J. *The Effect of the Bitter Taste Receptor Agonist Denatonium Benzoate on Gastric Emptying, Satiety and Return of Hunger After a Meal in Healthy Volunteers*. AGA Abstracts. 2013; Su 2071, S-548.

80. Voigt N, Stein J, Galindo MM, Dunkel A, Raguse JD et al. *The role of lipolysis in human orosensory fat perception.* J Lipid Res. 2014 May;55(5):870-82. doi: 10.1194/jlr.M046029.

81. Wise RA. *Roles for nigrostriatal--not just mesocorticolimbic--dopamine in reward and addiction.* Trends Neurosci. 2009 Oct;32(10):517-24. doi: 10.1016/j.tins.2009.06.004.

82. Won S-H, Kho H-S, Kim Y-K, Chung S-C, Lee S-W. *Analysis of residual saliva and minor salivary gland secretions.* Arch Oral Biol. 2001; 46: 619–624.

83. Wooster TJ, Augustin MA. *Rheology of whey protein-dextran conjugate films at the air/water interface*. Food Hydrocolloids. 2007; 21: 1072–80.

84. Yajima H, Ikeshima E, Shiraki M, Kanaya T, Fujiwara D, Odai H, Tsuboyama-Kasaoka N, Ezaki O, Oikawa S, Kondo K. *Isohumulones, Bitter Acids Derived from Hops, Activate BothPeroxisome Proliferator-activated Receptor α and γ and Reduce Insulin Resistance.* J Biol Chem. 2004; 279:33456-62.

85. Yeomans MR, Blundell JE, Leshem M. *Palatability: response to nutritional need or need-free stimulation of appetite?* Br J Nutr. 2004; 92(Suppl 1),S3–14.

86. Yeomans MR. *Palatability and the micro-structure of feeding in humans: the appetizer effect*. Appetite. 1996; 27,119–33.

87. Yildirim M, Hettiarachchy NS. *Properties of films produced by cross-linking whey proteins and 11S globulin using transglutaminase.* J Food Sci. 1998; 63: 248–52.

88. Yoshida R, Ohkuri T, Jyotaki M, Yasuo T, Horio N, Yasumatsu K, Sanematsu K, Shigemura N, Yamamoto T, Margolskee RF, et al. *Endocannabinoids selectively enhance sweet taste*. Proc Natl Acad Sci USA. 2010; 107, 935–939.

Summary

Human dietary behaviour is driven by homeostatic, hedonic and environmental factors. Foods can influence these factors throughout extrinsic (marketing suggestions, portion sizes, form) and intrinsic characteristics (taste, flavour, smell, texture). In turn biochemical response and psychological traits influenced food taste, flavour, smell and texture perception determining the hedonic value of a meal. This interplay between the food and the subjective psychophysiological response determine the control of energy intake, therefore must be considered in developing food for appetite control.

In the present thesis four human studies are described. Of these two were conducted to investigate the role of the saliva and the endocannabinoids system in the food preference and liking during the cephalic phase of digestion. We found out that salivary enzymes activity are influenced by nutritional status, food preference and food habits. Moreover, food palatability influenced some plasma endocannabinoid and N-acylethanolamine concentrations during the cephalic phase response and indicated that 2-arachidonoylglycerol and pancreatic polypeptide can be used as biomarkers of food liking in humans. These findings can have interesting implications in designing foods for appetite control:

- salivary enzymatic activity must be considered because it influence taste and texture perception and consequently food choice;
- the measure of 2-arachidonoylglycerol can offer the possibility to merge the sensory and biochemical approach to compare the satiating and rewarding capacity of foods.

The other two studies investigated the potential satiating effect on the short term energy intake of specific food ingredients. As previous in animal studies shown, we demonstrated (chapter 4) that also in humans the circulating oleoylethanolamide levels can be modulated by the fatty acid composition of a meal and this can influence the short-term energy intake. Therefore, we highlighted the anorexigenic effect of the oleoylethanolamide that can be a target of specific food ingredients. In the study described in the chapter 5, we aimed in assessing the appetite control capability of bitter compounds. The ingredient was microencapsulated with the double aim to avoid the (not palatable) taste perception in the mouth and to deliver the compounds directly in the gastrointestinal tract and target the enteroendocrine bitter taste receptors. We showed that microencapsulated bitter compounds are effective to reduce daily energy intakes in humans. This study demonstrated that sense the taste receptors directly in the gastrointestinal tract may be a valid way to trigger satiety and control appetite.

The general conclusions of the present thesis are that a fine design of ingredients for appetite control is necessary to develop novel foods for appetite control that has to take in account from one side the hedonic value from the other side the functionality.

List of Publications

- Ilario Mennella, Vincenzo Fogliano, Rosalia Ferracane, Marco Arlorio, Franco Pattarino, Paola Vitaglione. *Microencapsulated bitter compounds (from Gentiana Lutea) reduce daily energy intakes in humans*. To be submitted at The Journal of Nutrition.

- Mennella I, Ferracane R, Zucco F, Fogliano V, Vitaglione P. *Food Liking Enhances the Plasma Response of 2-Arachidonoylglycerol and of Pancreatic Polypeptide upon Modified Sham Feeding in Humans*. J Nutr. 2015 Sep;145(9):2169-75. doi: 10.3945/jn.114.207704

- Mennella I, Savarese M, Ferracane R, Sacchi R, Vitaglione P. *Oleic acid content of a meal promotes oleoylethanolamide response and reduces subsequent energy intake in humans.* Food Funct. 2015 Jan;6(1):204-10. doi: 10.1039/c4fo00697f

- Vitaglione P, Mennella I, Ferracane R, Rivellese AA, Giacco R, Ercolini D, Gibbons SM, La Storia A, Gilbert JA, Jonnalagadda S, Thielecke F, Gallo MA, Scalfi L, Fogliano V. *Whole-grain wheat consumption reduces inflammation in a randomized controlled trial on overweight and obese subjects with unhealthy dietary and lifestyle behaviors: role of polyphenols bound to cereal dietary fiber*. Am J Clin Nutr. 2015 Feb;101(2):251-61. doi: 10.3945/ajcn.114.088120

- Ilario Mennella, Vincenzo Fogliano, Paola Vitaglione. *Salivary lipase and α-amylase activities are higher in overweight than in normal weight subjects: Influences on dietary behaviour*. Food Research International 66 (2014) 463–468. doi:10.1016/j.foodres.2014.10.008

- Bayod S, Mennella I, Sanchez-Roige S, Lalanza JF, Escorihuela RM, Camins A, Pallàs M, Canudas AM. *Wnt pathway regulation by long-term moderate exercise in rat hippocampus.* Brain Res. 2014 Jan 16;1543:38-48. doi: 10.1016/j.brainres.2013.10.048

- Vitaglione P, Barone Lumaga R, Ferracane R, Radetsky I, Mennella I, Schettino R, Koder S, Shimoni E, Fogliano V. *Curcumin bioavailability from enriched bread: the effect of microencapsulated ingredients*. J Agric Food Chem. 2012 Apr 4;60(13):3357-66. doi: 10.1021/jf204517k

Overview of completed training activities

Discipline specific activities	• 4th International Conference of Food Digestion INFOGEST; March 2015 ; Naples (poster presentation) • XIX Workshop on the Developments in the Italian PhD Research on Food Science, Technology & Biotechnology; September 2014; Bari (oral presentation) • Eurosense 2014 - the 6th European conference on Sensory and Consumer Research; September 2014; Copenhagen (poster presentation) • NSCPHD1155 Introduction to MATLAB for Multivariate Data Analysis; September 2014; University of Copenhagen • Course in "Human nutrition" school of nutrition of Salerno; May 2014; Salerno • 7th Workshop "Immunonutrition"; may 2014; Carovigno (Br, Italy) (poster presentation) • Sensory Perception and Food Preference 6th International Advanced Course; December 2013; - Graduate School VLAG, Wageningen University • 2nd International Conference on Cocoa Coffee and Tea; October 2013; Naples (poster presentation) • XVIII Workshop on the Developments in the Italian PhD Research on Food Science, Technology & Biotechnology, September 2013; Conegliano (poster presentation) • ENGIHR Conference The Intestinal Microbiota and Gut Health: Contribution of the Diet, Bacterial Metabolites, Host Interactions and Impact on Health and Disease; September 2013; Valencia
General courses	• Course in “Multivariate analysis for food data", May 2014; Graduate School VLAG, Wageningen University • PhD week; April 2014; Graduate School VLAG, Wageningen University • PhD course: "Applied Statistic"; December 2013; University of Naples • PhD course: "Data presentation and article preparation"; November 2013; University of Naples • PhD course: "Design and management of research projects"; October 2013; University of Naples • PhD course: " Precision and experimental errors "; September 2013; University of Naples
Optional courses and activities	• Preparing VLAG PhD project proposal • Design of Healthy foods, MSc course; October- December 2014; University of Naples • Functional Ingredients for Weight Management, MSc course October- December 2014; University of Naples • Molecular Gastronomy, MSc Course; October- December 2014; University of Naples

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