Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

Salute, Sicurezza e Sistemi del Verde

Ciclo 35°

Settore Concorsuale: 05/E1 Chimica Generale

Settore Scientifico Disciplinare: BIO/10 Biochimica

TITOLO TESI

Evaluation of the real nutritional value of processed food to improve their effect on health and increase their sustainability

Presentata da:
Chiarello Elena

Coordinatore Dottorato:
Prof.ssa Patrizia Tassinari

Supervisore:
Prof.ssa Alessandra Bordoni

Co-supervisori:
Prof. Francesco Capozzi
Prof. Marcello Lanari

Esame finale anno 2023

Abstract

Nutrition plays a decisive role on human health, in terms of preventing noncommunicable diseases (NCDs) and improving quality of life. In this context, nutritionists, food technologists and food industries are cooperating in order to develop strategies to improve the nutritional value of foods. Although highly appreciated by consumers for their nutritional and sensory characteristics, cured meats and dairy products are criticized for their high salt content and synthetic additives. This has led to the development of strategies to reduce and replace these ingredients and additives. Since the food matrix and technological processes can affect the bioaccessibility of nutrients, it is necessary to study their release during gastrointestinal digestion to determine the real nutritional value of foods. In the first part of this PhD project, the impact on the nutritional quality of the reduction of sodium content and of the replacement of synthetic nitrates/nitrites with a combination of nitrate-reducing starter microbial cultures, ascorbate and natural antioxidants was evaluated in Parmigiano Reggiano Cheese and salami, respectively. For this purpose, an *in vitro* digestion model system combined with different analytical techniques such as spectrophotometric techniques, Nuclear Magnetic Resonance (NMR), Gas Chromatography (GC) and Liquid chromatography–high resolution mass spectrometry (LC–HRMS) was used. The main results showed that fatty acids and proteins release increased over time during digestion, with slight differences between the different prototypes. At the end of digestion, the innovative formulation/processing did not negatively affect fatty acids release and proteins hydrolysis, and led to the formation of a higher number of putative bioactive peptides.

Sugars are another controversial nutrient. In fact, glucose represents the main energy substrate in human metabolism, but the excessive intake of sugars is closely correlated with metabolic diseases. As for other nutrients, after gastrointestinal digestion, monosaccharides are potentially available for intestinal absorption. However, after the intestinal uptake their release in the blood stream depends on their metabolic fate within the enterocyte. In the second part of this PhD project, the absorption and metabolism of glucose, fructose and sucrose was evaluated using intestinal cell line. A faster absorption of fructose than glucose was observed, and a different modulation of the synthesis/transport of other metabolites by monosaccharides was shown.

Cultured intestinal cells were also used to verify the stability and intestinal uptake of vitamin A and vitamin D_3 delivered to cells through two vehicles. It was shown that the presence of lipids (mixed micelles) not only protected the vitamin from external factors such as light, heat and oxygen, but also improved bioavailability compared with solubilization of vitamins in ethanol.

Overall, the results obtained in this PhD project confirmed that considering only the chemical composition of foods is not sufficient to determine their nutritional value. The bioaccessibility and bioavailability of nutrients must be considered as well, knowing that it is modulated by the interactions between different food components and by the different processing. Furthermore, it was confirmed that the use of the *in vitro* digestion system and cultured intestinal cells is an effective tool for the nutritional evaluation of foods.

Index

CHAPTER-1

Introduction

According to the 1948 World Health Organization (WHO) definition, health is a state of complete physical, social and mental well-being, and not just the absence of disease or infirmity. Lifestyle, environmental and social factors represent the three sides of the pyramid from which study and research arise towards new and increasingly sustainable strategies that evolve with a contemporary and dynamic society. Modern society's hurried lifestyle is reflecting an increase in sickness and death among the population, as it is increasingly meant to simplify and smooth out key everyday routines, such as eating [1].

In recent years there has been great interest in human nutrition as a preventive and / or corrective tool of healthy state, because of the several potential benefits that reflect on human body. Nutrition research focuses not only on the physical-chemical and structural functions of the major nutrients, but also on how they are linked to psychological and physical well-being, promoting healthy eating habits to enhance public health.

Human nutrition research, food science and technology, and collaboration with medical specialists and environmental experts are all aimed at reversing the trend toward unhealthy habits and at restoring the value and the importance of a healthy diet.

Food contains nutrients such as carbohydrates, fats, proteins, vitamins, and minerals, which have a substantial impact on metabolism and influence the vital activities of the human body. Several studies have shown that proper nutrition can prevent numerous diseases, such as cardiovascular diseases (CVD), cancer, obesity and type 2 diabetes [2]. These diseases are the leading causes of death in industrialized countries, and a change in lifestyle and eating habits can significantly reduce the risk of chronic disease development [3]. As an example, a 2% increase of energy intake from trans-fatty acid is associated to a 23 % greater risk of coronary heart disease [4], while replacing 5% energy intake as saturated fat with polyunsaturated fat leads to a 19% reduction in coronary heart disease [5]. In a case-control study involving 52 countries, it has been shown that the diet is one of the most important risk factors for the occurrence of myocardial infarction and that eating fruits and vegetables significantly reduces the risk of developing an heart attack [6]. Also, Kulkarni et colleagues

have reported that a proper diet including higher intake of folate, antioxidant vitamins and whole grains can reduce deleterious vascular effects in the heart due to the action of phytochemicals' scavenging of the free radicals produced during atherogenesis [7].

Processed foods represent a wide group of foods that underwent physical-chemical technological processes such as heat treatments, fermentation and/or the addition of additive compounds to improve shelf-life and sensory characteristics, and to implement microbiological safety. [8]. Processed foods are often high in saturated fat, sugar or salt, and additives.

The use of salt (NaCl) to preserve food has been a common practice since ancient times. Nowadays, salt is not only used to improve shelf life; in meat, it enhances water-holding capacity and yield after cooking, and has also a tenderising effect on raw meat [9]. Furthermore, salt is added in diary-product to regulate the activity of starter culture microorganisms, to modify enzyme activity and to influence the water content of the cheese during maturation [9].

Despite its positive technological effects, excessive salt consumption is correlated to a blood pressure increase and consequently to an increased risk of cardiovascular diseases. In fact, cohort studies have shown that a 5 g per day increase in salt intake (2000 mg sodium) is linked to a 17% higher risk of a cardiovascular disease and a 23% higher risk of stroke. [10]. Excess dietary salt intake has also been associated with obesity, insulin resistance, non-alcoholic fatty liver disease (NAFLD) [11]. Furthermore, excessive salt intake has directly been associated with increasing risk of gastric cancer [12]. On the contrary, two meta-analyses have shown that modest salt reduction causes a blood pressure decrease in both hypertensive and normotensive individuals [13]; [14]. Based on these critical evidences, WHO has recommended a reduction in salt consumption, keeping sodium intake lower than 2 g per day. Food reformulation to lower salt content in products, a proper consumer education, front-of-pack labelling schemes and salt taxation, are all strategies which can facilitate compliance to this recommendation [15].

Cured meat and cheese are usually rich in salt, and since they are heavily consumed, their contribution to sodium intake is high. In Italy, some cured meat and cheese products adhere to the European Union's (EU) quality program for agricultural products and foodstuffs [16]

These food products are protected by production regulations that preserve and promote their quality and authenticity (Protected Denomination of Origin – PDO, or Protected Geographical Indication -PGI). Cured meat and cheese, particularly the PDO or PGI ones, are highly appreciated by consumers. Besides their excellent sensory traits, they have important nutritional characteristics. Indeed, they are good sources of essential nutrients as high-value proteins, vitamin B12, iron, calcium and other minerals.

Proteins are primarily a source of amino acids and nitrogen, which are used as raw materials in the synthesis of body proteins [17]. Particularly, animal-derived proteins contain the correct amount of all essential amino acids, which cannot be synthesized by the human body and therefore must be introduced through a suitable diet. Animal proteins contribute to disease prevention and good aging as well as in performing key activities in metabolic and physiological processes. Adequate protein consumption has been linked to lower overall mortality and morbidity in the elderly, owing to its protective effect against age/lifestyle-related diseases such as sarcopenia, cardiovascular disease, osteoporosis and obesity [18]. Furthermore, during food fermentation and ripening as well as during digestion, proteins undergo hydrolysis by several enzymes, splitting peptide bonds and possibly releasing bioactive peptide. These molecules are supposed to possess biological functions (anti-hypercholesterolemic, anti-hypertensive, anti-thrombotic, and anti-inflammatory), that could contribute to the reduction of disease risk [19].

Cured meat and cheese are therefore foods like two-faced Janus. On the one hand, they have a nutrient content that makes them extremely important for human nutrition, but they also have characteristics that impose a limitation in their consumption. Among these negative characteristics, not only salt but also nitrite/nitrate addition must be considered.

Nitrates and nitrites are naturally present in many foods and are also added in some foods to prevent the growth of bacteria Clostridium botulinum, to control the lipids rancidity by inhibiting their oxidation, and to improve specific sensory properties such as flavour and colour [20]. Several studies have shown that excessive nitrite intake is positively correlated to the increased risk of diseases such as cancer [21]. Indeed, nitrites are precursors to the formation of carcinogenic compounds as N-nitrosamines (NAs) and polycyclic aromatic hydrocarbons (PAH) that are formed during the production of processed meat. Epidemiologic and clinical studies have linked excessive nitrate and nitrite consumption with increased risk

of gastrointestinal cancers, thyroid dysfunction and thyroid cancer [22], chronic obstructive pulmonary disease in women [23], and delays in children's stature and weight development [24]. It is recommended to limit nitrites consumption to 3.7 mg per kilogram of body weight per day.

The well-known adverse effects of salt, sugars and nitrites/nitrates and the growing consumer interest in healthy food have prompted the food industry to develop strategies limiting the concentration of these components in processed foods.

Reduction or replacement of sodium with a low-sodium mixture, use of flavour enhancers such as monosodium glutamate or yeast extract, changing the physical form of salt, and improving salt diffusion via high pressure treatment or ultrasound technology are strategies currently used to reduce sodium content in processed foods [25].

Nowadays, one of the meat industry's challenges is to find a solution to decrease added and residual nitrite in cured meat in order to reduce nitrite intake. Although no ideal synthetic nitrate/nitrite substitutes have been identified yet due to their multifunctional effects [26]; [27], some potential alternatives such as the natural-source alternatives have been evaluated. Because of their strong compatibility with processed meat products, plant extracts are commonly used as natural nitrate sources since they do not impart any off-flavours [28]. In recent studies it has been shown that adding celery powder to cooked sausages efficiently prevents quality deterioration during storage [29], and that a meat emulsion made with pre-converted nitrite from red beets (10%) and ascorbic acid has comparable quality to another one made with sodium nitrite [30]. In addition, the application of nitrate-reducers starter culture could be an upward trend for reducing this additive. Coagulase-negative staphylococci were discovered to possess a nitric oxide synthase (NOS)-encoding gene, which enables NO generation and colour formation in meat products without nitrite/nitrate addition [31]. In fermented meat products, Staphylococcus vitulinus is regularly isolated and investigated as a potential starting culture [32]; [33]; [34].

Dietary sugars are the main sources of energy for the human body and they play an important role in the human diet as well as in the food industry. In fact, sugars contribute to food structure and functionality, affecting the textural properties. The different chemical and physical properties of sugars, such as solubility, crystallization, melting behaviour, moisture absorption ability, and powder flow characteristics could affect processing, storage and interaction with other molecules [35]. Sucrose, glucose and fructose are the main sugars found in food either naturally existing or added during processing. According to the definition of the European Food Safety Agency (EFSA) "free sugars" include both those naturally present in honey, syrups, fruit, vegetable or concentrated juices and also added sugars. [36].

Over the past fifty years, there has been a 33 % increased amount of sugars intake from processed food and beverages including fruit-flavoured drinks, frozen desserts, and sports drinks. Excessive sugar intake is positively correlated to obesity, type 2 diabetes, and cardiovascular diseases [37]. Therefore, the WHO has recommended a reduction of free sugars intake to less than the 5% of the total energy intake in both adults and children [38]. Some nutritional approaches have been implemented to limit sugar consumption, replacing it with artificial sweeteners, or reducing its content. While developing potential strategies to reduce sugar intake, feasibility, cost, effectiveness, and likely consumer acceptability must be considered. [39]. As an example, the sugar replacement with artificial sweeteners in chocolate has resulted in variations in both the sweetness and the texture or colour of the final product [40]. Moreover, the sugar reduction up to 50% in frozen-dessert formulations has caused an increase in ice content, a harder texture, and a lower melting rate [41].

Nutrition professionals, public and private institutions are encouraging consumers to reduce sugar and total energy intake through responsible advertising and increasing consumer education activities [39]. Meanwhile, scientific research in biochemistry and nutrition is focusing on the study of intestinal metabolism and transport of sugars. Sugar absorption causes metabolic changes in the enterocyte, which in turn may regulate the availability of monosaccharides and other metabolites in the blood stream. A better understanding of the biochemical events that occur within the intestinal cell after exposure to a sugar load could help a better interpretation of the effects of sugar consumption on health.

In recent decades, the effect of food on health and the effect of food processing has been extensively studied by nutritionists and food technologists in terms of the quality and quantity of nutrients, without evaluating the molecular organization. The so-called “matrix effect” is described as the whole of the chemical components of food and their molecular interactions, the chemical composition of food, and the way those components are structurally organized at micro- and macroscopic levels [42]. The food matrix effect can influence the nutrient bioaccessibility, i.e., the fraction of a nutrient that is released from the food matrix during gastrointestinal digestion, and potentially available for intestinal absorption [43]. Indeed, the food matrix affects the transformation of foods during digestion, modulating the release of nutrients, their accessibility to absorption by intestinal epithelial cells, and therefore the nutrient bioavailability. The antagonistic or synergistic interactions of the molecules that are present in food have an impact on digestibility and bioaccessibility. [44] In turn, bioaccessibility modulates bioavailability, i.e. the portion of a nutrient that is absorbed by the intestine and delivered to the bloodstream and to the peripheral tissues [45]. The bioavailability of nutrients is highly variable and is influenced by a variety of factors, including physiochemical properties (such as chemical binding form), the presence or absence of other food components that improve or inhibit absorption, the metabolization after absorption, and host-related factors (genetic factors, age, and lifestyle) [46].

Bioaccessibility and bioavailability are of paramount importance in human nutrition, since the health benefits of nutrients and bioactive components are related to the amount of the absorbed compounds. The chemical composition (i.e. the concentration of nutrients and bioactives), of foods does not completely reflect the possible effects in the human body, since bioaccessibility and bioavailability are not taken into account [47]. Therefore, the evaluation of nutrient bioaccessibility represents a pivotal point to estimate the real nutritional value of food.

Food processing can modify the physicochemical interactions of food and so the bioaccessibility of nutrients. The effect of technological processes on bioaccessibility of carotenoids and flavonoids from foodstuffs appears to be associated with the treatments applied. In a study, it has been shown that high-pressure homogenization treatments are more

suitable than traditional pasteurization treatments because the high-pressure homogenization enhances the bioaccessibility of carotenoids [48].

In vitro digestion assay is a valuable method for researching and understanding changes, interactions, and the bioaccessibility of nutrients, drugs, and non-nutritive substances [49]. For ethical reasons, *in vivo* investigations are limited. In addition, they are expensive and the sample collection can be problematic. *In vitro* investigations are simple to conduct and allow multiple samples to be run simultaneously. To encourage a wide range of researchers to use the *in vitro* method, it is designed to be feasible with ordinary laboratory equipment and without any specific training. In fact, it is faster, less expensive, and less labour-intensive, as well as free of ethical constraints [50]. *In vitro* digestion can be used to assess digestion end-points or the kinetics of very specific digestion phases, such as small intestinal hydrolysis, although it may oversimplify complex physiological digesting processes [51]. Even if *in vitro* methods do not fully reflect the complexity of *in vivo* digestion, they are considered useful screening tools for diet-related issues such as digestibility, bioavailability, release of bioactive compounds, and structural changes in food [52].

Several *in vitro* digestion methods have been developed to mimic physiological conditions and the sequence of events that occur during the digestion in the human gastrointestinal tract. In 2014, the COST Infogest network, an international consortium of over 200 scientists from 32 countries, attempted to harmonize the parameters required in a simulated food digestion process, developing an international consensus for the standardized *in vitro* digestion protocol suitable for mechanistic investigations and hypothesis generation because of their reproducibility, controllability, and ease of sampling at the site of interest [50]. This method emulates the oral, gastric and intestinal phases conditions that occur *in vivo* using simulated digestive fluids, digestive enzymes (oral α-amylase, gastric pepsin, and intestinal pancreatin) and bile salts, modulating pH and temperature, and reproducing peristaltic intestinal movements.

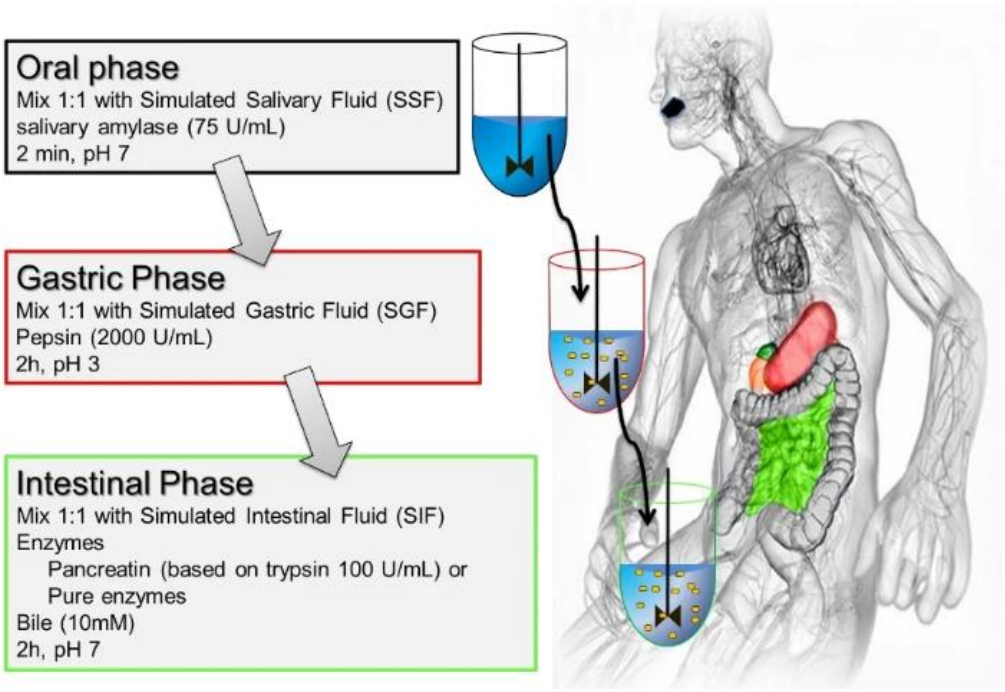


Fig.1 Simulated *in vitro* digestion [50]

Although the harmonized protocol was based on human data, the physiological applicability of the harmonized protocol to *in vivo* digestion was not experimentally clarified. Therefore, a recent study [53] has compared *in vitro* digestion with human jejunal samples performed on isolated casein and whey protein, finding significant similarities between the two systems at intestinal level. Also, it has been proven that *in vitro* protein digestion of skim milk powder using Infogest protocol was comparable to *in vivo* protein hydrolysis of skim milk powder in pigs' gastrointestinal tract [54]. Because of the proven validity, the Infogest static protocol is the most frequently used method to evaluate the bioaccessibility of nutrients.

This *in vitro* digestion method can be also used to detect the effects of technological processing on the nutritional value of foods.

Similarly, intestinal cell models are often employed to investigate bioavailability in spite of expensive animal models with restricted screening possibilities, since they are simple, efficient, and highly reproducible. [55]. Among intestinal cell models, Caco-2 cell line is widely used to reproduce the intestinal epithelial barrier. The Caco-2 cell line can be used to identify mechanisms for the transport of dietary components across the intestinal epithelium, to determine how dietary matrices affect molecules transport across the intestinal epithelial

layer, to investigate the molecular features and relevance of efflux systems in the intestinal epithelium, as well as to investigate and determine relationships between molecules during intestinal epithelial transport [56]. The Caco-2 cell line is originally derived from a colon carcinoma, developed through a research made by Jorgen Fogh at Sloan-Kettering Institute for Cancer Research [57]. Caco-2 cells possess the morphological and functional characteristics of small intestine, expressing brush borders, tight junctions, intestinal efflux and uptake transporters [57]. To better simulate the intestinal environment, Caco-2 cells are cultured on permeable filter inserts, called trans well, made of polycarbonate, polyester or polyethylene terephthalate that improves their morphological and functional differentiation [58]. After 21 days of differentiation, Caco-2 cells form a continuous barrier between the upper and the lower compartments, simulating the internal milieu (apical chamber) and the intestinal lumen (basolateral chamber) (Fig.2).

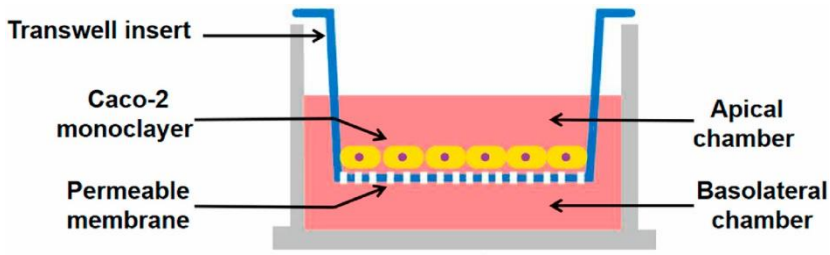


Fig 2. Caco-2 cells on a tissue culture insert. [55]

Many studies have been performed in recent years using the differentiated Caco-2 cell model to evaluate the bioavailability of macronutrients [59]; [59], micronutrients [60]; [61], and phytochemicals [62].

Although Caco-2 cells spontaneously differentiate and express morphological and functional characteristics of mature enterocytes, they have significant drawbacks when compared to normal intestinal epithelium. In fact, Caco-2 cells are enterocytes while intestinal epithelium consist of different cell types, which can influence the absorption of molecules. In addition, Caco-2 cells do not produce mucus, which is present *in vivo* intestinal epithelium [63]. Notwithstanding, this cell model represents a valid tool to identify the appropriate strategies to improve or reduce nutrient/bioactive bioavailability. Indeed, the vehicles and molecules-

interactions play a key role in conveying nutrients across the intestinal epithelium. It was demonstrated that polyphenols interfere with intestinal sugar transporters, by inhibiting glucose and fructose absorption, reducing postprandial glycemia and lowering body mass index [64]. The interaction between fatty acids and lipophilic vitamins has been proven to be a good approach to improve their bioavailability. In fact, the different fatty acids can enhance the vitamin D absorption and, in particular, it has been shown that oleic acids significantly improved cholecalciferol basolateral efflux across Caco-2 cells. [65]. On the contrary, phytosterols can interfere with the bioavailability of vitamins, specifically decreasing vitamin D uptake by a competition for micellar incorporation and for apical uptake. [66].
Overall, understanding nutrient interplay is essential to predict nutrient/bioactive absorption rate and therefore bioavailability and possible physiological effects.

Although it has long been known that the amount of the different food components absorbed in the intestine and delivered to the bloodstream does not correspond to their concentration in the food, most of past research on foods and their effects on health has been based on the food chemical composition and the concentration of single nutrients, without considering the effect of gastrointestinal digestion and neglecting the release of nutrients by the food matrix. This discrepancy has led to misleading results that do not take into account what physiologically occurs in the body.
Indeed, although the physiological effect/function is linked to the food component we cannot fail to consider how the component is delivered. The food matrix can influence the kinetics of release and absorption of molecules during the digestive process, and the available fraction of an ingested nutrient depends on different factors such as its chemical status, its release from the food matrix, the interactions with other food molecules, the presence of suppressors or cofactors, and the formation of stable compounds that are slowly metabolized [67].
Therefore, the use of *in vitro* digestion and intestinal cell models, that allow the assessment of nutrient/bioactive component absorption, represents a strong innovation in the field of food science aimed at consumer health and well-being.

References

1. Farhud, D.D. Impact of Lifestyle on Health. *Iran J Public Health* 2015, *44*, 1442-1444.

2. Powell-Wiley, T.M.; Poirier, P.; Burke, L.E.; Després, J.-P.; Gordon-Larsen, P.; Lavie, C.J.; Lear, S.A.; Ndumele, C.E.; Neeland, I.J.; Sanders, P., et al. Obesity and Cardiovascular Disease: A Scientific Statement From the American Heart Association. *Circulation* 2021, *143*, e984-e1010, doi:doi:10.1161/CIR.0000000000000973.

3. Freedman, N.D.; Park, Y.; Subar, A.F.; Hollenbeck, A.R.; Leitzmann, M.F.; Schatzkin, A.; Abnet, C.C. Fruit and vegetable intake and head and neck cancer risk in a large United States prospective cohort study. *Int J Cancer* 2008, *122*, 2330-2336, doi:10.1002/ijc.23319.

4. Forouhi, N.G.; Krauss, R.M.; Taubes, G.; Willett, W. Dietary fat and cardiometabolic health: evidence, controversies, and consensus for guidance. *BMJ* 2018, *361*, k2139, doi:10.1136/bmj.k2139.

5. Zong, G.; Li, Y.; Wanders, A.J.; Alssema, M.; Zock, P.L.; Willett, W.C.; Hu, F.B.; Sun, Q. Intake of individual saturated fatty acids and risk of coronary heart disease in US men and women: two prospective longitudinal cohort studies. *BMJ* 2016, *355*, i5796, doi:10.1136/bmj.i5796.

6. Neuhouser, M.L. The importance of healthy dietary patterns in chronic disease prevention. *Nutrition Research* 2019, *70*, 3-6, doi:https://doi.org/10.1016/j.nutres.2018.06.002.

7. Asgary, S.; Rastqar, A.; Keshvari, M. Functional Food and Cardiovascular Disease Prevention and Treatment: A Review. *Journal of the American College of Nutrition* 2018, *37*, 429-455, doi:10.1080/07315724.2017.1410867.

8. Sadler, C.R.; Grassby, T.; Hart, K.; Raats, M.; Sokolović, M.; Timotijevic, L. Processed food classification: Conceptualisation and challenges. *Trends in Food Science & Technology* 2021, *112*, 149-162, doi:https://doi.org/10.1016/j.tifs.2021.02.059.

9. Hutton, T. Sodium Technological functions of salt in the manufacturing of food and drink products. *British Food Journal* 2002, *104*, 126-152, doi:10.1108/00070700210423635.

10. Strazzullo, P.; D'Elia, L.; Kandala, N.-B.; Cappuccio, F.P. Salt intake, stroke, and cardiovascular disease: meta-analysis of prospective studies. *BMJ* 2009, *339*, b4567, doi:10.1136/bmj.b4567.

11. Lanaspa, M.A.; Kuwabara, M.; Andres-Hernando, A.; Li, N.; Cicerchi, C.; Jensen, T.; Orlicky, D.J.; Roncal-Jimenez, C.A.; Ishimoto, T.; Nakagawa, T., et al. High salt intake causes leptin resistance and obesity in mice by stimulating endogenous fructose production and metabolism. *Proceedings of the National Academy of Sciences* 2018, *115*, 3138-3143, doi:doi:10.1073/pnas.1713837115.

12. D'Elia, L.; Rossi, G.; Ippolito, R.; Cappuccio, F.P.; Strazzullo, P. Habitual salt intake and risk of gastric cancer: A meta-analysis of prospective studies. *Clinical Nutrition* 2012, *31*, 489-498, doi:10.1016/j.clnu.2012.01.003.

13. He, F.J.; Tan, M.; Ma, Y.; MacGregor, G.A. Salt Reduction to Prevent Hypertension and Cardiovascular Disease: JACC State-of-the-Art Review. *Journal of the American College of Cardiology* 2020, *75*, 632-647, doi:https://doi.org/10.1016/j.jacc.2019.11.055.

14. Aburto, N.J.; Ziolkovska, A.; Hooper, L.; Elliott, P.; Cappuccio, F.P.; Meerpohl, J.J. Effect of lower sodium intake on health: systematic review and meta-analyses. *BMJ : British Medical Journal* 2013, *346*, f1326, doi:10.1136/bmj.f1326.

15. Santos, J.A.; Tekle, D.; Rosewarne, E.; Flexner, N.; Cobb, L.; Al-Jawaldeh, A.; Kim, W.J.; Breda, J.; Whiting, S.; Campbell, N., et al. A Systematic Review of Salt Reduction Initiatives Around the World: A Midterm Evaluation of Progress Towards the 2025 Global Non-Communicable Diseases Salt Reduction Target. *Advances in Nutrition* 2021, *12*, 1768-1780, doi:10.1093/advances/nmab008.

16. Council, E.P.a.o.t. Regulation (EU) No 1151/2012 of the European Parliament and of the Council of 21 November 2012 on quality schemes for agricultural products and foodstuffs. OJ L, 2012; Vol. , pp 1–29.

17. Wu, G.; Bazer, F.W.; Johnson, G.A.; Herring, C.; Seo, H.; Dai, Z.; Wang, J.; Wu, Z.; Wang, X. Functional amino acids in the development of the pig placenta. *Molecular Reproduction and Development* 2017, *84*, 870-882, doi:https://doi.org/10.1002/mrd.22809.

18. Wu, G. Dietary protein intake and human health. *Food & Function* 2016, *7*, 1251-1265, doi:10.1039/C5FO01530H.

19. Tapal, A.; Vegarud, G.E.; Sreedhara, A.; Kaul Tiku, P. Nutraceutical protein isolate from pigeon pea (Cajanus cajan) milling waste by-product: functional aspects and digestibility. *Food & Function* 2019, *10*, 2710-2719, doi:10.1039/C8FO01933A.

20. Dawson, P.L.; Acton, J.C. 22 - Impact of proteins on food color. In *Proteins in Food Processing (Second Edition)*, Yada, R.Y., Ed. Woodhead Publishing: 2018; https://doi.org/10.1016/B978-0-08-100722-8.00023-1pp. 599-638.

21. Said Abasse, K.; Essien, E.E.; Abbas, M.; Yu, X.; Xie, W.; Sun, J.; Akter, L.; Cote, A. Association between Dietary Nitrate, Nitrite Intake, and Site-Specific Cancer Risk: A Systematic Review and Meta-Analysis. *Nutrients* 2022, *14*, 666.

22. Ward, M.H.; Kilfoy, B.A.; Weyer, P.J.; Anderson, K.E.; Folsom, A.R.; Cerhan, J.R. Nitrate Intake and the Risk of Thyroid Cancer and Thyroid Disease. *Epidemiology* 2010, *21*.

23. Jiang, R.; Camargo, C.A., Jr; Varraso, R.; Paik, D.C.; Willett, W.C.; Barr, R.G. Consumption of cured meats and prospective risk of chronic obstructive pulmonary disease in women. *The American Journal of Clinical Nutrition* 2008, *87*, 1002-1008, doi:10.1093/ajcn/87.4.1002.

24. Cîrţînă, D. Considerations regarding the toxicity of nitrosamines in food. *Annals of'Constantin Brancusi'University of Targu-Jiu. Engineering Series* 2020.

25. Pimpin, L.; Kranz, S.; Liu, E.; Shulkin, M.; Karageorgou, D.; Miller, V.; Fawzi, W.; Duggan, C.; Webb, P.; Mozaffarian, D. Effects of animal protein supplementation of mothers, preterm infants, and term infants on growth outcomes in childhood: a systematic review and meta-analysis of randomized trials. *The American Journal of Clinical Nutrition* 2019, *110*, 410-429, doi:10.1093/ajcn/nqy348.

26. Munekata, P.E.S.; Pateiro, M.; Domínguez, R.; Pollonio, M.A.R.; Sepúlveda, N.; Andres, S.C.; Reyes, J.; Santos, E.M.; Lorenzo, J.M. Beta vulgaris as a Natural Nitrate Source for Meat Products: A Review. *Foods* 2021, *10*, 2094.

27. Fraqueza, M.J.; Laranjo, M.; Elias, M.; Patarata, L. Microbiological hazards associated with salt and nitrite reduction in cured meat products: control strategies based on antimicrobial effect of natural ingredients and protective microbiota. *Current Opinion in Food Science* 2021, *38*, 32-39, doi:https://doi.org/10.1016/j.cofs.2020.10.027.

28. Alahakoon, A.U.; Jayasena, D.D.; Ramachandra, S.; Jo, C. Alternatives to nitrite in processed meat: Up to date. *Trends in Food Science & Technology* 2015, *45*, 37-49, doi:https://doi.org/10.1016/j.tifs.2015.05.008.

29. Jin, S.-K.; Choi, J.S.; Yang, H.-S.; Park, T.-S.; Yim, D.-G. Natural curing agents as nitrite alternatives and their effects on the physicochemical, microbiological properties and sensory evaluation of sausages during storage. *Meat Science* 2018, *146*, 34-40, doi:https://doi.org/10.1016/j.meatsci.2018.07.032.

30. Choi, Y.S.; Kim, T.K.; Jeon, K.H.; Park, J.D.; Kim, H.W.; Hwang, K.E.; Kim, Y.B. Effects of Pre-Converted Nitrite from Red Beet and Ascorbic Acid on Quality Characteristics in Meat Emulsions. *Korean J Food Sci Anim Resour* 2017, *37*, 288-296, doi:10.5851/kosfa.2017.37.2.288.

31. Huang, P.; Xu, B.; Shao, X.; Chen, C.; Wang, W.; Li, P. Theoretical basis of nitrosomyoglobin formation in a dry sausage model by coagulase-negative staphylococci: Behavior and expression of nitric oxide synthase. *Meat Science* 2020, *161*, 108022, doi:https://doi.org/10.1016/j.meatsci.2019.108022.

32. Huang, P.; Shao, X.; Zhu, M.; Xu, B.; Chen, C.; Li, P. Sucrose enhances colour formation in dry sausages by up-regulating gene expression of nitric oxide synthase in Staphylococcus vitulinus. *International Journal of Food Microbiology* 2020, *315*, 108419, doi:https://doi.org/10.1016/j.ijfoodmicro.2019.108419.

33. López, C.M.; Sentandreu, M.A.; Vignolo, G.M.; Fadda, S.G. Low molecular weight peptides derived from sarcoplasmic proteins produced by an autochthonous starter culture in a beaker sausage model. *EuPA Open Proteomics* 2015, *7*, 54-63, doi:https://doi.org/10.1016/j.euprot.2015.05.001.

34. Casquete, R.; Benito, M.J.; Martín, A.; Ruiz-Moyano, S.; Córdoba, J.J.; Córdoba, M.G. Role of an autochthonous starter culture and the protease EPg222 on the sensory and safety properties of a traditional Iberian dry-fermented sausage "salchichón". *Food Microbiology* 2011, *28*, 1432-1440, doi:https://doi.org/10.1016/j.fm.2011.07.004.

35. Wang, Y.; Truong, T.; Li, H.; Bhandari, B. Co-melting behaviour of sucrose, glucose & fructose. *Food Chemistry* 2019, *275*, 292-298, doi:https://doi.org/10.1016/j.foodchem.2018.09.109.

36. EFSA Panel on Dietetic Products, N.; Allergies. Dietary reference values for vitamin D. *EFSA Journal* 2016, *14*, e04547, doi:https://doi.org/10.2903/j.efsa.2016.4547.

37. Stanhope, K.L. Sugar consumption, metabolic disease and obesity: The state of the controversy. *Crit Rev Clin Lab Sci* 2016, *53*, 52-67, doi:10.3109/10408363.2015.1084990.

38. Breda, J.; Jewell, J.; Keller, A. The Importance of the World Health Organization Sugar Guidelines for Dental Health and Obesity Prevention. *Caries Research* 2019, *53*, 149-152, doi:10.1159/000491556.

39. Di Monaco, R.; Miele, N.A.; Cabisidan, E.K.; Cavella, S. Strategies to reduce sugars in food. *Current Opinion in Food Science* 2018, *19*, 92-97, doi:https://doi.org/10.1016/j.cofs.2018.03.008.

40. Azevedo, B.M.; Morais-Ferreira, J.M.; Luccas, V.; Bolini, H.M.A. Bittersweet chocolates containing prebiotic and sweetened with stevia (Stevia rebaudiana Bertoni) with different Rebaudioside A contents: multiple time–intensity analysis and physicochemical characteristics. *International Journal of Food Science & Technology* 2017, *52*, 1731-1738, doi:https://doi.org/10.1111/ijfs.13470.

41. Abbasi, S.; Saeedabadian, A. Influences of lactose hydrolysis of milk and sugar reduction on some physical properties of ice cream. *Journal of Food Science and Technology* 2015, *52*, 367-374, doi:10.1007/s13197-013-1011-1.

42. Capuano, E.; Oliviero, T.; van Boekel, M.A.J.S. Modeling food matrix effects on chemical reactivity: Challenges and perspectives. *Critical Reviews in Food Science and Nutrition* 2018, *58*, 2814-2828, doi:10.1080/10408398.2017.1342595.

43. Etcheverry, P.; Grusak, M.; Fleige, L. Application of in vitro bioaccessibility and bioavailability methods for calcium, carotenoids, folate, iron, magnesium, polyphenols, zinc, and vitamins B6, B12, D, and E. *Frontiers in Physiology* 2012, *3*, doi:10.3389/fphys.2012.00317.

44. Cömert, E.D.; Gökmen, V. Effect of food combinations and their co-digestion on total antioxidant capacity under simulated gastrointestinal conditions. *Curr Res Food Sci* 2022, *5*, 414-422, doi:10.1016/j.crfs.2022.02.008.

45. Rodrigues, D.B.; Marques, M.C.; Hacke, A.; Loubet Filho, P.S.; Cazarin, C.B.B.; Mariutti, L.R.B. Trust your gut: Bioavailability and bioaccessibility of dietary compounds. *Current Research in Food Science* 2022, *5*, 228-233, doi:https://doi.org/10.1016/j.crfs.2022.01.002.

46. Schonfeldt, H.; Pretorius, B.; Hall, N. Bioavailability of Nutrients. *Encyclopedia of Food and Health* 2016, 10.1016/B978-0-12-384947-2.00068-4, doi:10.1016/B978-0-12-384947-2.00068-4.

47. Santos, D.I.; Saraiva, J.M.A.; Vicente, A.A.; Moldão-Martins, M. 2 - Methods for determining bioavailability and bioaccessibility of bioactive compounds and nutrients. In *Innovative Thermal and Non-Thermal Processing, Bioaccessibility and Bioavailability of Nutrients and Bioactive Compounds*, Barba, F.J., Saraiva, J.M.A., Cravotto, G., Lorenzo, J.M., Eds. Woodhead Publishing: 2019; https://doi.org/10.1016/B978-0-12-814174-8.00002-0pp. 23-54.

48. Stinco, C.M.; Sentandreu, E.; Mapelli-Brahm, P.; Navarro, J.L.; Vicario, I.M.; Meléndez-Martínez, A.J. Influence of high pressure homogenization and pasteurization on the in vitro bioaccessibility of carotenoids and flavonoids in orange juice. *Food Chemistry* 2020, *331*, 127259, doi:https://doi.org/10.1016/j.foodchem.2020.127259.

49. Lucas-González, R.; Viuda-Martos, M.; Pérez-Alvarez, J.A.; Fernández-López, J. In vitro digestion models suitable for foods: Opportunities for new fields of application and challenges. *Food Research International* 2018, *107*, 423-436, doi:https://doi.org/10.1016/j.foodres.2018.02.055.

50. Minekus, M.; Alminger, M.; Alvito, P.; Ballance, S.; Bohn, T.; Bourlieu, C.; Carrière, F.; Boutrou, R.; Corredig, M.; Dupont, D., et al. A standardised static in vitro digestion method suitable for food – an international consensus. *Food & Function* 2014, *5*, 1113-1124, doi:10.1039/C3FO60702J.

51. Egger, L.; Ménard, O.; Portmann, R. Quantitative Characterization of Digestion Processes. In *Interdisciplinary Approaches to Food Digestion*, Gouseti, O., Bornhorst, G.M., Bakalis, S., Mackie, A., Eds. Springer International Publishing: Cham, 2019; 10.1007/978-3-030-03901-1_8pp. 159-184.

52. Espert, M.; Borreani, J.; Hernando, I.; Quiles, A.; Sanz, T.; Salvador, A. Structural changes of filling creams after in vitro digestion. Application of hydrocolloid based emulsions as fat source. *LWT* 2019, *112*, 108223, doi:https://doi.org/10.1016/j.lwt.2019.05.121.

53. Sanchón, J.; Fernández-Tomé, S.; Miralles, B.; Hernández-Ledesma, B.; Tomé, D.; Gaudichon, C.; Recio, I. Protein degradation and peptide release from milk proteins in human jejunum. Comparison with in vitro gastrointestinal simulation. *Food Chem* 2018, *239*, 486-494, doi:10.1016/j.foodchem.2017.06.134.

54. Egger, L.; Schlegel, P.; Baumann, C.; Stoffers, H.; Guggisberg, D.; Brügger, C.; Dürr, D.; Stoll, P.; Vergères, G.; Portmann, R. Physiological comparability of the harmonized INFOGEST in vitro digestion method to in vivo pig digestion. *Food Research International* 2017, *102*, 567-574, doi:https://doi.org/10.1016/j.foodres.2017.09.047.

55. Ding, X.; Hu, X.; Chen, Y.; Xie, J.; Ying, M.; Wang, Y.; Yu, Q. Differentiated Caco-2 cell models in food-intestine interaction study: Current applications and future trends. *Trends in Food Science & Technology* 2021, *107*, 455-465, doi:https://doi.org/10.1016/j.tifs.2020.11.015.

56. Ponce de León-Rodríguez, M.d.C.; Guyot, J.-P.; Laurent-Babot, C. Intestinal in vitro cell culture models and their potential to study the effect of food components on intestinal inflammation. *Critical Reviews in Food Science and Nutrition* 2019, *59*, 3648-3666, doi:10.1080/10408398.2018.1506734.

57. Lea, T. Caco-2 Cell Line. In *The Impact of Food Bioactives on Health: in vitro and ex vivo models*, Verhoeckx, K., Cotter, P., López-Expósito, I., Kleiveland, C., Lea, T., Mackie, A., Requena, T., Swiatecka, D., Wichers, H., Eds. Springer International Publishing: Cham, 2015; 10.1007/978-3-319-16104-4_10pp. 103-111.

58. Bednarek, R. In Vitro Methods for Measuring the Permeability of Cell Monolayers. *Methods and Protocols* 2022, *5*, 17.

59. Goulart, A.J.; Bassan, J.C.; Barbosa, O.A.; Marques, D.P.; Silveira, C.B.; Santos, A.F.; Garrido, S.S.; Resende, F.A.; Contiero, J.; Monti, R. Transport of amino acids from milk whey by Caco-2 cell monolayer after hydrolytic action of gastrointestinal enzymes. *Food Research International* 2014, *63*, 62-70, doi:https://doi.org/10.1016/j.foodres.2014.01.037.

60. Sandberg, A.-S. The Use of Caco-2 Cells to Estimate Fe Absorption in Humans - a Critical Appraisal. *International Journal for Vitamin and Nutrition Research* 2010, *80*, 307-313, doi:10.1024/0300-9831/a000038.

61. Faria, M.A.; Araújo, A.; Pinto, E.; Oliveira, C.; Oliva-Teles, M.T.; Almeida, A.; Delerue-Matos, C.; Ferreira, I.M.P.L.V.O. Bioaccessibility and intestinal uptake of minerals from different types of home-cooked and ready-to-eat beans. *Journal of Functional Foods* 2018, *50*, 201-209, doi:https://doi.org/10.1016/j.jff.2018.10.001.

62. Yi, W.; Akoh, C.C.; Fischer, J.; Krewer, G. Absorption of Anthocyanins from Blueberry Extracts by Caco-2 Human Intestinal Cell Monolayers. *Journal of Agricultural and Food Chemistry* 2006, *54*, 5651-5658, doi:10.1021/jf0531959.

63. Lu, Y.; Qi, J.; Wu, W. Chapter 20 - Lipid nanoparticles: In vitro and in vivo approaches in drug delivery and targeting. In *Drug Targeting and Stimuli Sensitive Drug Delivery Systems*, Grumezescu, A.M., Ed. William Andrew Publishing: 2018; https://doi.org/10.1016/B978-0-12-813689-8.00020-3pp. 749-783.

64. Loureiro, G.; Martel, F. The effect of dietary polyphenols on intestinal absorption of glucose and fructose: Relation with obesity and type 2 diabetes. *Food Reviews International* 2019, *35*, 390-406, doi:10.1080/87559129.2019.1573432.

65. Goncalves, A.; Gleize, B.; Roi, S.; Nowicki, M.; Dhaussy, A.; Huertas, A.; Amiot, M.-J.; Reboul, E. Fatty acids affect micellar properties and modulate vitamin D uptake and basolateral efflux in Caco-2 cells. *The Journal of Nutritional Biochemistry* 2013, *24*, 1751-1757, doi:https://doi.org/10.1016/j.jnutbio.2013.03.004.

66. Goncalves, A.; Gleize, B.; Bott, R.; Nowicki, M.; Amiot, M.-J.; Lairon, D.; Borel, P.; Reboul, E. Phytosterols can impair vitamin D intestinal absorption in vitro and in mice. *Molecular Nutrition & Food Research* 2011, *55*, S303-S311, doi:https://doi.org/10.1002/mnfr.201100055.

67. Parada, J.; Aguilera, J.M. Food Microstructure Affects the Bioavailability of Several Nutrients. *Journal of Food Science* 2007, *72*, R21-R32, doi:https://doi.org/10.1111/j.1750-3841.2007.00274.x.

CHAPTER-2

Aim of the study

The ever-increasing progress of modern societies in tandem with industrial expansion, urban upgrading, and economic development have contributed to changes in dietary and lifestyle patterns, with a significant impact on the health and nutrition of the population. Despite the positive effects related to better living conditions, the importance of a healthy and balanced diet has been downgraded by the rush to buy food products increasingly available on the market without assessing the effect of food on health [1].

Over the past decade, scientific research in human nutrition has highlighted that a healthy diet and lifestyle contribute to the prevention and control of non-communicable diseases, and that several compounds in foods increase the likelihood of these diseases.

According to the report published by the World Health Organization (WHO) in 2017, chronic non-communicable diseases remain the leading cause of death worldwide, half of which could be prevented by adopting lifestyle changes and a healthy and balanced diet [2]. The pressing emergency of reversing the direction has urged nutrition experts to apply nutritional strategies to safeguard human health. In addition, consumers have grown aware of the importance of pursuing healthy eating, excluding foods that contain potentially health-threatening ingredients from the diet.

In response to this emergency, food industries, in collaboration with food technologists and nutritionists, have intervened by modifying food production technologies, making them more sustainable, and replacing synthetic ingredients with natural ones or reducing salt, fat and sugar content.

However, modification of technological processes and food formulations should not alter the taste and flavour of foods to avoid consumers distrust and detachment. Also, it shall be noted that the full or partial replacement of potentially harmful compounds does not necessarily improve the nutritional value of foods. In fact, replacement of saturated fats with unsaturated ones is considered an improvement of nutritional value, but if they are replaced with sugars the final result is very different. As well, processing has a role since it can impact on the bioaccessibility and bioavailability of food components. Therefore, the chemical composition

of a food only partially reflects its nutritional value, which must be evaluated after examining also what happens following the digestion of the food. In fact, to evaluate the positive effects of foods on health it is essential to understand how nutrients and food components interact with each other, how they are converted during gastrointestinal digestion, and how the production process interferes with their release from the food matrix and their intestinal absorption.

One of the aims of this PhD project was to evaluate the impact of technological processes and innovative formulations such as reduction of sodium content and replacement of synthetic nitrate/nitrite on the nutritional quality of foods of animal origin.

Although after digestion the nutrients released from the food matrix are potentially available for intestinal absorption, their uptake by the intestinal cells is strongly influenced by the interaction with other molecules. As well, nutrient bioavailability is influenced by the metabolic events occurring within enterocytes. Sugars are controversial nutrients; although glucose represents the main energy substrate in human metabolism, excess intake of sugars increases the risk of diseases, particularly diabetes. Another aim of this PhD thesis was to evaluate the absorption and diffusion of glucose, fructose and sucrose using Caco-2 cells as an *in vitro* intestinal model. The same model was used to study the absorption of fat-soluble vitamins incorporated into different vehicles.

The results obtained will contribute to a deeper understanding of the impact of new food formulations and innovative technological processes on the real nutritional value of food, and to the development of innovative, healthier foods.

References

1. Ancuța Rus, V. The Role of Healthy Diet and Lifestyle in Preventing Chronic Diseases. *Journal of Interdisciplinary Medicine* 2019, *4*, 57-58, doi:doi:10.2478/jim-2019-0009.
2. World Health, O. *Noncommunicable diseases country profiles 2018*; World Health Organization: Geneva, 2018.

CHAPTER-3

Impact of a Shorter Brine Soaking Time on nutrient bioaccessibility and peptides formation in 30 months ripened Parmigiano Reggiano Cheese

Already published in "*Molecules"* 2022, *27*(3), 664; https://doi.org/10.3390/molecules27030664

Introduction

High salt intake is a key contributing factor for the prevalence of non-communicable diseases (NCDs) around the world. High-salt diets are linked to elevated blood pressure, a major risk factor for heart diseases and stroke, which in turn are among the leading causes of death worldwide [1,2]. Despite the current WHO recommendations for sodium (Na) consumption by adults (< 2 g per day), in Europe the overall sodium intake varies between 2.7 and 7.1 g per day (7 to 18 g salt per day) in most countries [3], and processed foods might provide about 20% of the total Na intake [4]. Since salt reduction has been identified as 1 of the 5 priority interventions in response to the global NCD crisis [5], there is a growing interest in processing procedures lowering salt content, particularly when applied to highly appreciated and nutritional valued foods. Parmigiano-Reggiano cheese (PRC) is a long ripened, hard cheese made up of a combination of partially skimmed and whole raw milk, with the addition of a natural whey starter, principally consisting of thermophilic starting lactic acid bacteria [6]. The PRC is included in the list of foods with a Protected Designation of Origin (PDO), according to which the aging must last at least 12 months, starting from the forming of the cheese. The most valuable cheeses are those that ripen from 24 to 36 months, during which hydrolytic phenomena occur and the organoleptic quality improves. EU regulation 1151/2012 ensures the way of production, quality, and area of origin of PDO products. In particular, PRC must be produced and transformed only in the Northern Italian provinces of Parma, Reggio Emilia, Modena and in some parts of Bologna and Mantova. PRC is highly appreciated worldwide not only for its taste, but also for its nutritional characteristics [7]. PRC contains high-value nutritional proteins that are also functionally and biologically attractive, and their hydrolysates formed during ripening are supposed to be sources of bioactive peptides [8,9]. Recently, we investigated on the impact of reducing the brining time in salt solution on PRC sensory properties and peptide content [10], but information is still lacking about possible

modification of the bioaccessibility of protein and fatty acids, and on the release of biologically active peptides during digestion. Processing can deeply modify both nutrient content and bioaccessibility, i.e., the quantity of a compound that is released from the food matrix in the gastrointestinal tract, becoming available for intestinal absorption [11]. Modification in bioaccessibility due to changes in the supramolecular organization and in the network of interactions between molecules, or to nutrient localization within compartments may impact on the nutritional value of food [12]. Indeed, in a previous work we reported the impact of different ripening time (15 and 30 months) on the kinetics of protein hydrolysis and the formation of peptides and small organic compounds during PRC in vitro digestion [13].

Aim of the study

The aim of the present work was to evaluate whether the reduction of brine soaking time modifies fatty acid and protein bioaccessibility of a low-salt PRC produced and ripened for 30 months according to the PDO specification. To this aim, conventional (C-PRC) and hyposodic (Hypo-PRC) underwent in vitro static gastrointestinal digestion according to the INFOGEST protocol [14]. Sampling was performed at end of the gastric phase, in the middle and at the end of the duodenal phase, and fatty acid and protein bioaccessibility was assessed. Digested samples were also analysed by nuclear magnetic resonance (NMR) and liquid chromatography coupled with high resolution mass spectrometry (LC-HRMS), and the release of bioactive peptide sequences was verified using a bioinformatic approach.

Materials and methods

Parmigiano Reggiano Cheese (PRC)

PRC was produced and ripened according to the Protection Designation of Origin specification, which includes restrictions to its geographical area of production, cows feeding and cheese manufacturing. PRC was produced in a local dairy by using conventional (18 days, 3 samples from 3 different wheels) and reduced (12 days, 3 samples from 3 different wheels) brine soaking times in a saturated sodium chloride solution (about 36% w/w). Average salt content was 2.32 ± 0.06% dry matter (DM) and 1.90 ± 0.20% DM in C-PRC and short brine

soaking time Hypo-PRC, respectively ($p = 0.0253$). A detailed description of PRC production and proximate analysis of C-PRC and Hypo-PRC is reported in a previous work [10].

In vitro Digestion

In vitro digestion was performed in triplicate according to the INFOGEST protocol [14]. To simulate chewing, PRC was chopped before starting oral digestion. In vitro digestion lasted for 242 min (2 min of oral digestion, 120 min of gastric digestion, and 120 min of intestinal digestion) at 37°C. During the process, several consecutive enzymatic reactions took place by addition of simulated saliva, simulated gastric juice (containing 2,000 U/ ml pepsin) at pH 3, and simulated pancreatic juice (containing 10 mM bile and 100 U/ml pancreatin) at pH 7. Samples were taken at the end of the gastric phase (G120), after 60 minutes (D60) and at the end of the duodenal phase (D120). Digested samples were centrifuged at 50,000 × g for 15 min. To remove any turbidity, supernatants were filtered with 0.2 μm membranes and stored at −80 °C until further analysis.

Fatty acids bioaccessibility

Total lipids were extracted according to Bligh & Dyer [15], with slight modifications. Briefly, 6 ml of methanol, 3 ml of chloroform and 2.4 ml of distilled water were sequentially added to 0.1 g of food or 0.8 ml of digested sample followed by through mix with stirring magnet at the maximum intensity for 3 minutes. Successively, 3 ml of chloroform and 3 ml of distilled water, each one followed by through mix with stirring magnet for 1 minute, were added. The solution was kept overnight at 4°C, then the upper layer was removed by suction, and the lower chloroform layer was transferred to a test tube. Pentadecanoic acid (1 mg) was added as internal standard, and the chloroform layer was dried under nitrogen infusion. After methylation [16], the quantitative and qualitative content of fatty acids (as methyl esters - FAMEs) was determined by fast-GC (GC-2030AF; Shimadzu, Kyoto, Japan) using a capillary column (30 mt, 0.2 μm film thickness) with a programmed temperature gradient (50–250 °C, 10 °C/min). The gas chromatographic peaks were identified based on their retention time ratios relative to methyl stearate and predetermined by use of authentic samples [17]. Gas chromatographic traces and quantitative evaluations were obtained using a Lab Solution software (Shimadzu, Kyoto, Japan) and normalized for the dilution factor due to the addition of digestive fluids. FAMEs from chemicals added during in vitro digestion system

were subtracted, and bioaccessibility was calculated as FAME concentration in digested sample/FAME concentration in PRC before digestion × 100 [18].

Protein bioaccessibility

Protein concentration was determined spectrophotometrically by o-phthaldialdehyde (OPA) [19] and Coomassie assay [20], and by measuring the absorbance at 280 nm [21] using L-isoleucine, bovine serum albumin and non-fat dry milk as standard, respectively. Values were normalized for the dilution factor due to the addition of digestive fluids, and protein content from enzymes added during in vitro digestion was subtracted. Bioaccessibility was calculated as protein mass in digested sample/protein mass in PRC before digestion × 100 [18].

HR-NMR Spectroscopy

HR-NMR analysis was performed in digested sample as previously reported [22], with slight modification. Samples were thawed, centrifuged first at 2300 x g for 5 min at 4 °C to eliminate the coarser particles and then at 50,000 g for 5 min at 4 °C to eliminate the fine particulates. Afterward, each sample was vortexed for 30 s, then 750 µl of supernatant was taken and added to 120 µl of 100 mM phosphate buffer with 10 mM trimethylsilylpropanoic acid (TSP), molecular weight (MW) 172.27 g/mol (Cambridge Isotope Lab Inc., Tewksbury, MA, USA) and brought to pH 7.0 HR-NMR spectra were recorded at 298 K on a Bruker US+ Avance III spectrometer operating a proton frequency of 600.13 MHz, equipped with a BBI-z probe and a SampleCase™ sampler for automation. The spectra were collected with a 90° pulse of 13.1 µs with 10 W of power, a relaxation delay of 5 s and an acquisition time of 2.3 s; for each sample 256 scans were obtained, resulting in 32 K data points over a 7194,245 Hz encompassing a spectral width of 12 ppm. The residual signal of monodeuterated water (HOD) was suppressed using the NOESYGPPR1D sequence (a standard pulse sequence provided in the Bruker library), which incorporated the first increment of the NOESY pulse sequence plus a spoil gradient. A Fourier transform was exploited to extract the frequency-domain spectrum of 64 K data points from the raw time-domain FID. TopSpin version 3.5.6 was used to automatically execute phase and base line fixes (Bruker BioSpin, Karlsruhe, Germany). The chemical shifts were internally referenced to the DSS at 0.000 ppm. Data are expressed as arbitrary units [12].

Bioactive peptide release and identification

Digested samples were centrifuged at 14,000 rpm for 40 min at 4 °C with a Centrifuge 5810 R (Eppendorf s.r.l, Milan, Italy) to remove any particulate matter formed during freezing/thawing. The supernatants were filtered through 0.45 µm PTFE filters and directly injected in the UPLC-MS system. Samples were separated by a reverse-phase Acquity UPLC Peptide BEH 300 C18 column (1.7 µm, 2.1 × 150 mm, Waters, Milford, MA, USA) in an UPLC system coupled with ESI and MS (UPLC Acquity I-class, with a Vion IMS QTof Mass Spectrometer, Waters, Milford, MA, USA). Gradient elution was set as follows with eluent A (H_2O with 0.2% CH_3CN and 0.1% HCOOH) and eluent B (CH_3CN with 0.1% HCOOH)): 0 to 7 min, isocratic 100% A; 7 to 50 min, linear gradient from 100% A to 50% A; 50-52.6 min 50% A, 52.6-53 min from 50% A to 0% A, 53-58.2 min 0% A, 58.2-59 min from 0% A to 100% A, 59-72 min 100% A and reconditioning. Flow rate was set at 0.20 ml min-1, injection volume 1 µl, column temperature 35 °C, sampler temperature 18 °C, and the total run time was 72 min. Detection was performed using a Vion IMS QTof Mass Spectrometer (Waters, Milford, MA, USA) with the following parameters. Experiment type: peptide map (IMS), experiment type: MSe, source type: ESI, polarity: positive, analyser mode: sensitivity, mode: standard transmission, capillary: 3.00 kV, sample cone voltage: 40 V, source offset voltage: 80 V, source temperature: 120°C, desolvation temperature: 450 °C, cone gas flow: 50 L/h, desolvation gas flow: 800 L/h. MSe mode: high definition MSe, acquisition time: 0-58.2 min, scan range: 100-2000 m/z, scan time: 0.4 s, low collision energy: 6 V, high collision energy ramp: 20 to 45 V, automatic lock correction (leucine enkephaline). The software used for data processing was UNIFI (Waters Corporation, Milford MA, USA). The expected component list comprised the following protein Uniprot accession numbers: P02666 (β-casein), P02662 (αS1-casein), P02663 (αS2-casein), P02668 (κ-casein), P02754 (β-lactoglobulin), P00711 (α-lactalbumin). Variable amino acid modifications were included as: deamidation (N, Q) pyroglutamic acid N-term (E, Q), oxidation (single or double, M or W), phosphorylation (S, T, Y). Peptide semiquantitative data were obtained normalizing integral areas. Released bioactive peptides were identified using a bioinformatic approach. The whole set of peptide sequences under analysis was searched into two benchmark database of peptide bioactivity, namely BIOPEP (http://www.uwm.edu.pl/biochemia/index.php/en/biopep) [23]

and AHTPDB (http://crdd.osdd.net/raghava/ahtpdb/) [24], which were queried automatically and systematically employing a script pipeline developed «in-house».

Statistical analysis

Statistical differences were determined by the one-way analysis of variance (ANOVA) followed by Tukey's post hoc test using Prism software ver. 7.0 (GraphPad, San Diego, CA, USA). Different letters indicate significant differences (at least $p < 0.05$).

Results

Fatty acids bioaccessibility

In agreement with Malacarne et al. [25], the main fatty acids found in undigested PRC were palmitic acid > oleic acid > myristic acid > stearic acid, which accounted for approximately the 85% of total fatty acid with no differences between C- and Hypo-PRC (supplementary table 1). The release of fatty acids from the food matrix increased over time, and no differences were detected between C-PRC and Hypo-PRC at the end of digestion. Notwithstanding, the time course of fatty acid release was not the same in the two samples, bioaccessibility of myristic, stearic, oleic, total MUFA and total fatty acids being higher in Hypo-PRC at D60 (table 1).

	G120		D60		D120	
	C-PRC	**Hypo-PRC**	**C-PRC**	**Hypo-PRC**	**C-PRC**	**Hypo-PRC**
C8:0	1.53±0.58b	1.87±0.12b	11.70±3.73ab	1.08±1.53b	16.32±6.09a	16.69±1.82a
C10:0	1.24±0.34c	2.05±0.16c	22.18±3.55ab	16.76±6.50b	31.36±4.48a	32.10±3.63a
C12:0	1.55±0.17c	2.51±0.11c	27.32±4.78b	35.60±3.62ab	42.51±4.30a	38.21±1.20a
C14:0	1.48±0.23c	3.00±0.05c	26.30±4.92b	45.17±1.24a	43.99±9.63ab	34.19±5.46ab
C16:0	1.52±0.38b	2.89±0.02b	22.32±4.81ab	43.83±5.05a	39.80±11.00a	28.24±4.79a
C16:1 n-7	0.00±0.00c	1.32±1.86c	33.92±5.47b	49.02±3.33ab	53.47±9.05a	44.45±4.37ab
C18:0	1.84±0.37c	2.88±0.08c	20.68±5.26bc	43.23±5.89a	37.24±10.12a	25.30±5.6ab
C18:1 n-9	0.96±0.74c	2.03±0.15c	27.99±6.26b	45.29±6.01a	47.79±5.10a	47.86±3.24a
C18:2 n-6	0.53±0.92c	1.07±1.52c	34.01±5.20b	47.58±7.64ab	56.43±9.48a	48.29±4.37ab
ΣSFA	1.47±0.30b	2.69±0.05b	21.72±4.69a	39.12±2.00a	37.58±8.64a	27.95±4.27a
ΣMUFA	0.88±0.68c	1.98±0.00c	28.40±6.24b	45.60±5.76a	48.22±5.38a	47.60±2.69a
ΣPUFA	0.53±0.92c	1.07±1.52c	34.01±5.20b	47.58±7.64ab	56.43±9.48a	48.29±4.37ab
Total	1.28±0.40c	2.45±0.07c	23.95±5.13b	41.13±3.15a	40.97±7.59a	33.90±2.48ab

Table 1. Fatty acid bioaccessibility in conventional Parmigiano-Reggiano cheese (C-PRC) and hyposodic Parmigiano-Reggiano cheese (Hypo-PRC) at different digestion time. Data are means ± SD of three independent in vitro digestions, each one in duplicate. Fatty acid bioaccessibility is expressed as % and was calculated as fatty acid methyl esters concentration in digested/ fatty acid methyl esters concentration in PRC before digestion x 100. Statistical analysis was by the one-way ANOVA (C8:0: $p = 0.0017$; C16:0: $p = 0.0002$; C10:0, C12:0, C14:0, C16:1 n-7, C18:0, C18:1 n-9, C18:2 n-6, ΣSFA, ΣMUFA, ΣPUFA and total fatty acids: $p < 0.0001$) with Tukey's post-hoc test (different letters indicate significant differences). G120: end of gastric phase; D60: 60 minutes of duodenal phase; D120: end of duodenal phase.

Protein bioaccessibility

Protein bioaccessibility was evaluated by 3 different spectrophotometric methods (OPA, Coomassie, absorbance at 280 nm), all evidencing a time-dependent release of protein/peptides/amino acids from the matrix. Regardless the analytical methodology, no differences were detected between C- and Hypo-PRC at any digestion time, and maximal bioaccessibility was already achieved at D60 (Figure 1).

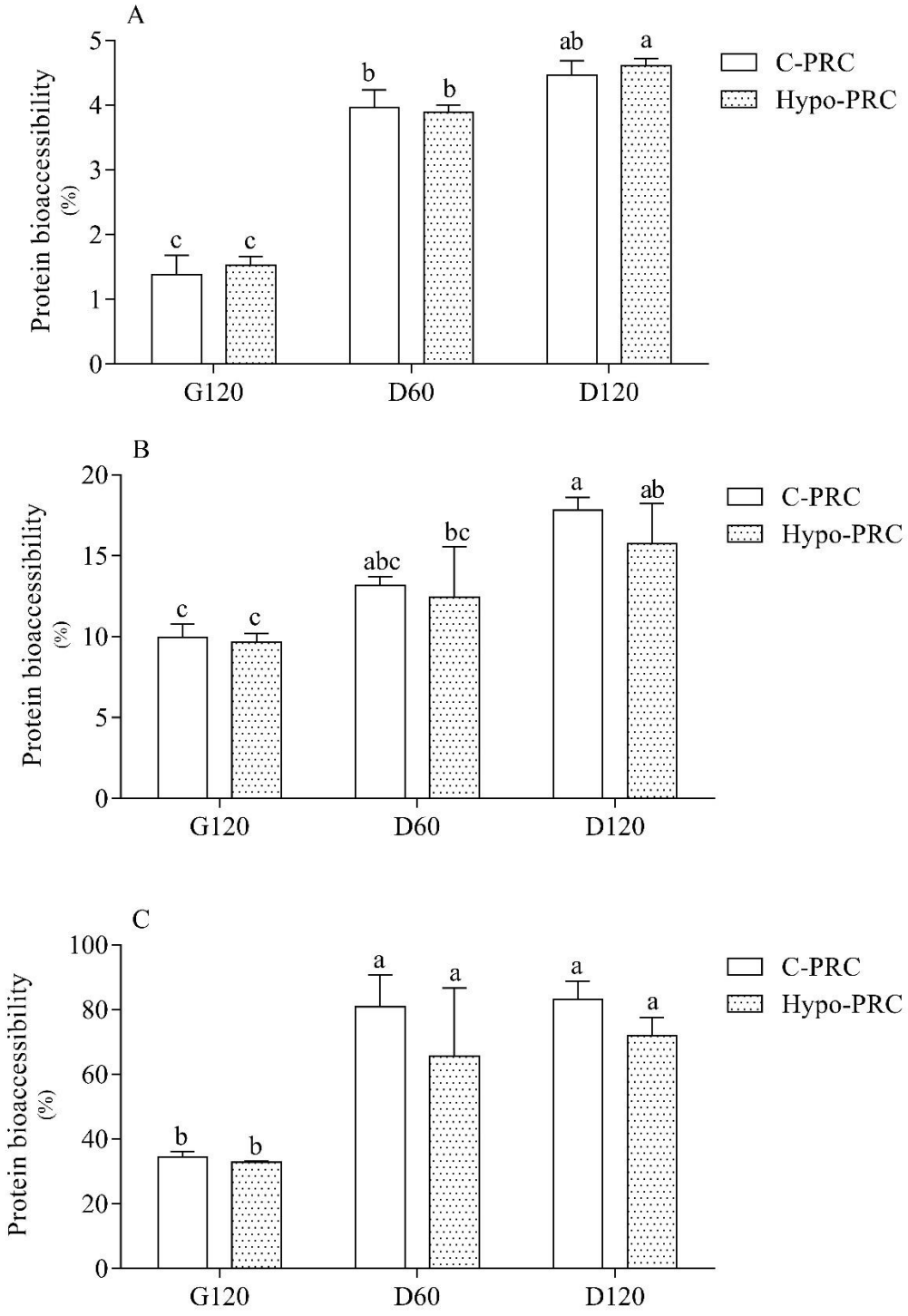


Figure 1. Protein bioaccessibility assessed by OPA (A), Coomassie assay (B) and absorbance at 280 nm (C) in conventional Parmigiano-Reggiano cheese (C-PRC) and hyposodic Parmigiano-Reggiano cheese (Hypo-PRC) at different digestion time. Data are means ± SD of 3 independent in vitro digestions, each analysed in triplicate. Protein bioaccessibility is expressed as % and it was calculated as the ratio x 100 between the proteins mass in the digestion fluid and the total proteins mass in the original undigested PRC. Statistical analysis was by the one-way ANOVA (A and C: $p<0.0001$; B: $p < 0.005$) with Tukey's post-hoc test (different letters indicate significant differences). G120: end of gastric phase; D60: 60 minutes of duodenal phase; D120: end of duodenal phase.

HR-NMR spectroscopy

In figure 2, the NMR spectrum of the digestion fluids after the three digestion phases is shown as traces with different colours, subdivided in three different spectral regions, collecting signals from hydrogen atoms located on aromatic, alpha -carbon and aliphatic side chains of amino acids, respectively.

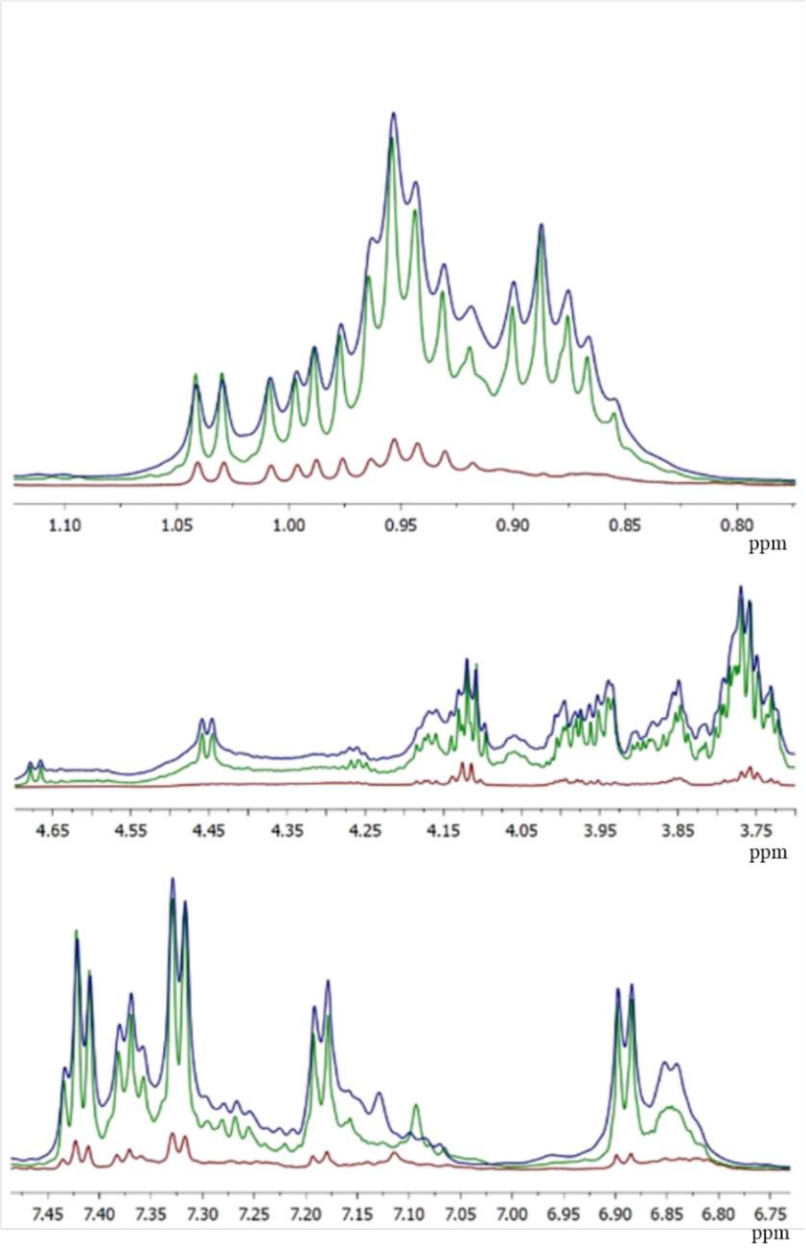


Figure 2. Proton HR-NMR spectrum of PRC digestion fluids at G120 (brown trace), D60 (green) and D120 (blue) phases. Top panel shows the upfield region, where mainly aliphatic hydrogen atoms of branched side chains of amino acids resonate. Middle panel shows the midfield spectral region, where hydrogen atoms bound to amino acids alpha-carbon resonate. Finally, bottom panel shows the downfield spectral region, where hydrogen atoms belonging to aromatic amino acids resonate.

More in detail, the progression of in vitro digestion of the two PRC evaluated by the appearance of NMR signal in branched, aromatic, and α-amino acid proton spectral region is reported in figure 3. The α-amino acid proton region includes the signals of all amino acids, both in the free state and bound to peptides or in soluble proteins. In each spectral region, the maximum release was already achieved at D60 with no significant difference between C- and Hypo-PRC except for branched amino acids, release of which was higher in Hypo- than C-PRC at the end of the gastric digestion.

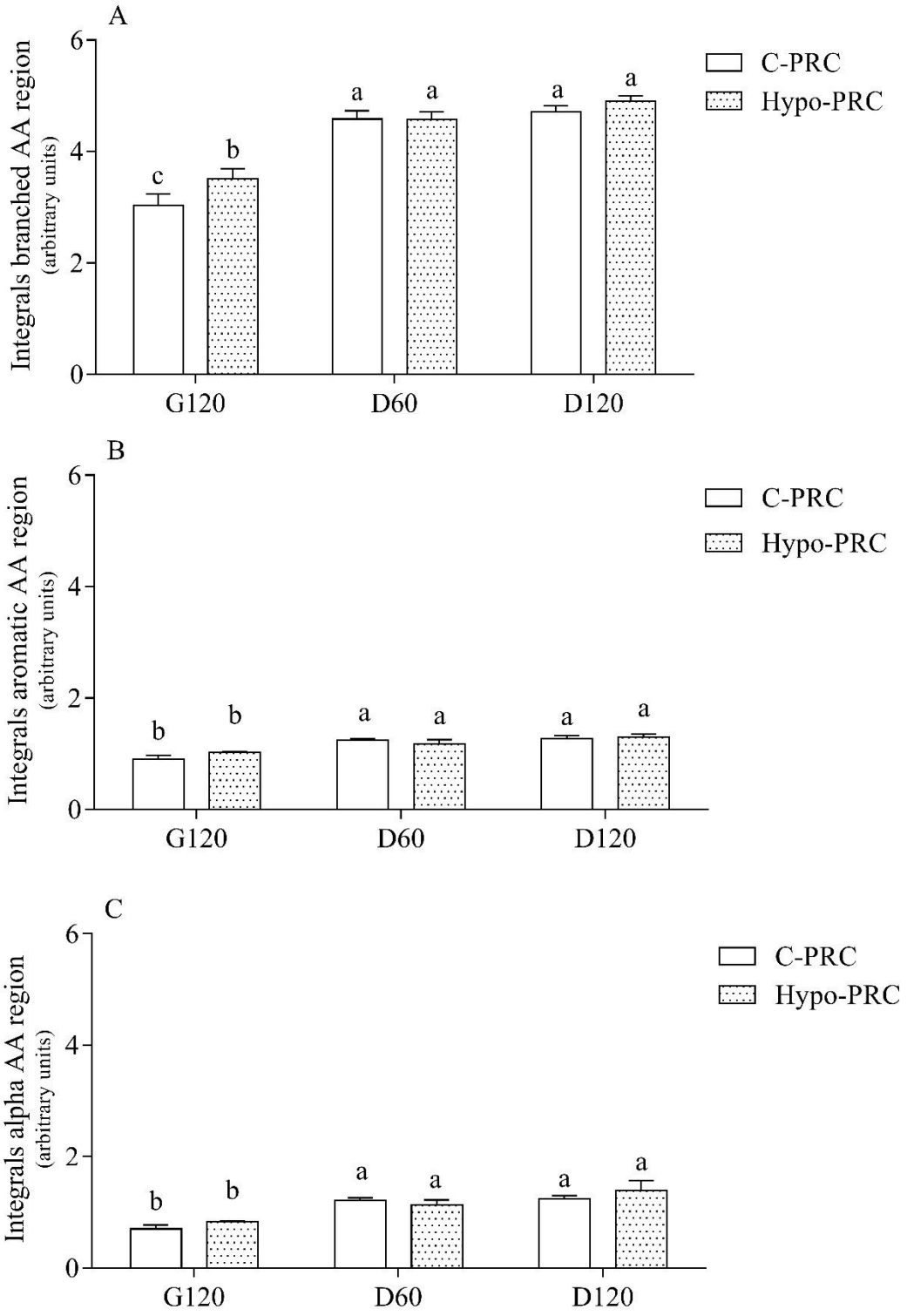


Figure 3. Integral area of branched (A), aromatic (B), and α-proton (C) amino acid region in conventional Parmigiano-Reggiano cheese (C-PRC) and hyposodic Parmigiano-Reggiano cheese (Hypo-PRC) at different digestion times. Data are means ± SD of 3 independent in vitro digestions, each one in triplicate. Integrals are expressed as arbitrary units. Statistical analysis was by the one-way ANOVA (all integrals $p < 0.0001$) with Tukey's post-hoc test (different letters indicate significant differences). G120: end of gastric phase; D60: 60 minutes of duodenal phase; D120: end of duodenal phase.

Peptides formation

The total number of peptides generated at different digestion time from parent proteins was evaluated by LC-HRMS (Table 2). On average, less than 80 peptides were found in not-digested PRC, and in vitro digestion increased their number to > 85 at G120 and > 100 at D60 and D120. In both C- and Hypo-PRC, most of the peptides were from β-casein (60%) and αS1-casein (20%), whereas less than 10% of the identified sequences came from αS2-casein, and κ-casein. The number of peptides from β-casein increased mainly during the duodenal phase, while those from κ -casein were mainly released during the gastric phase. Regarding whey proteins, a small number of peptides from β-lactoglobulin was released during the duodenal digestion. These results are in agreement with recent digestomic studies on milk proteins, which have shown that β-lactoglubulin is resistant to the action of pepsin during digestion [26; 27]. Overall, no significant differences were detected between C- and Hypo-PRC.

Protein source	Not digested[a]		G120		D60		D120		ANOVA
	C-PRC	Hypo-PRC	C-PRC	Hypo-PRC	C-PRC	Hypo-PRC	C-PRC	Hypo-PRC	
β-casein	41.0±0.0c	41.0±0.0c	46.33±2.52c	50.0±2.0bc	66.0±5.2a	60.0±3.46ab	65.33±9.45a	66.0±2.0a	P<0.0001
αS1-casein	25.0±0.0a	25.0±0.0a	19.67±0.58a	21.67±1.15a	25.0±3.61a	23.3±2.61a	21.67±3.79a	24.33±3.06a	P=0.0992
αS2-casein	9.0±0.0a	9.0±0.0a	7.0±1.0ab	7.33±1.15ab	6.67±0.58b	8.33±0.58ab	7.33±1.53ab	8.0±0.0ab	P=0.0167
κ-casein	3.0±0.0c	3.0±0.0c	9.33±0.58a	9.67±1.15a	3.33±0.58c	6.0±1.0b	3.33±1.15c	3.67±0.58c	P<0.0001
β-LG (A and B isoforms)	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b	1.0±0.0a	0.67±0.58ab	1.0±0.0a	0.67±0.58ab	P=0.0004
Total	78.0±0.0c	78.0±0.0c	82.33±2.31bc	88.67±4.16abc	102.0±8.72a	98.0±6.56ab	98.67±12.50ab	102.67±4.93a	P=0.0002

Table 2. Number of different peptides released in conventional Parmigiano-Reggiano cheese (C-PRC) and hyposodic Parmigiano-Reggiano cheese (Hypo-PRC) at different digestion time. a [10]. Data are means ± SD of three different PRC and independent in vitro digestions. Statistical analysis was performed by the one-way ANOVA with Tukey's post-hoc test (different letters indicate significant differences). G120: end of gastric phase; D60: 60 minutes of duodenal phase; D120: end of duodenal phase, LG: lactoglobulin

Bioinformatic analysis

Bioactive sequences detected in not digested PRC and at different time points of in vitro digestion, their semiquantitative content and putative biological activity are reported in table 3. Seven peptides with a documented bioactive sequence were found in not digested PRC, with no difference in their semiquantitative content between C- and Hypo-PRC. At the end of gastric phase, bioactive peptides originally found in not digested samples were not anymore detectable or their concentration was significantly reduced, while new sequences were detected in both C- and Hypo-PRC, although in different amount. All bioactive peptides detected at the end of the gastric phase were further hydrolysed during the duodenal phase, and 15 peptides were detected in both C- and Hypo-PRC at the end of in vitro digestion. Among them, 4 (PQNIPPL, VYPFPGPI, LHLPLPL, PGPIPN) were more abundant in Hypo-PRC than C-PRC.

Peptide sequence	Protein source	Not digested		G120		D60		D120		Reported activity (uM IC_{50})
		C-PRC	Hypo-PRC	C-PRC	Hypo-PRC	C-PRC	Hypo-PRC	C-PRC	Hypo-PRC	
LHLPLP	β-CN (133-138)	62±3.8a	64.7±7.8a	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b	ACE inhibition (5.5)
RPKH	αS1-CN (1-4)	0.9±0.1a	0.9±0.1a	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b	ACE inhibition (> 1863)
YKVPQL	αS1-CN (104-109)	0.6±0.1a	0.6±0.1a	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b	ACE inhibition (> 22)
NLHLPLPLL	β-CN (132-140)	25.2±1.9a	25.5±2.7a	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b	ACE inhibition (15)
RELEEL	β-CN (1-6)	1.5±0.4ab	2.0±0.5a	0.9±0.1b	0.0±0.0c	0.0±0.0c	0.0±0.0c	0.0±0.0c	0.0±0.0c	ACE inhibition (1000)
LLYQEPVLGPVRGPFPIIV	β-CN (191-209)	2.6±0.3a	2.7±0.3a	0.3±0.0b	0.3±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b	ACE inhibition and immunomodulating (nr)
RPKHPIKHQGLPQEVLNENL LRF	αS1-CN (1-23)	4.7±1.3a	5.22±1.2a	0.3±0.1b	0.3±0.1b	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b	Antibacterial (nr)
AYFYPEL	αS1-CN (143-149)	0.0±0.0c	0.0±0.0c	0.26 ± 0.0a	0.22±0.04b	0.0±0.0c	0.0±0.0c	0.0±0.0c	0.0±0.0c	ACE inhibition (6.6)
DAYPSGAW	αS1-CN (157-164)	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.05±0.01a	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b	ACE inhibition (98)
LKKISQRYQKFALPQYLKT	αS2-CN (164-182)	0.0±0.0c	0.0±0.0c	1.5 ± 0.0a	1.4±0.06b	0.0±0.0c	0.0±0.0c	0.0±0.0c	0.0±0.0c	Haemolytic (nr)
PLW	αS1-CN (196-199)	0.0±0.0b	0.0±0.0b	0.1±0.01a	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b	ACE inhibition (36)
VENLHLPLPLL	β-CN (129-139)	0.0±0.0c	0.0±0.0c	0.4±0.01a	0.35±0.03b	0.0±0.0c	0.0±0.0c	0.0±0.0c	0.0±0.0c	Anticancer (nr)
VYQHQKAMKPWIQPKTKVIP YVRYL	αS2-CN (183-207)	0.0±0.0b	0.0±0.0b	2.3±0.4a	2.4±0.1a	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b	Antibacterial (nr)
YQKFPQY	αS2-CN (89-95)	0.0±0.0a	0.0±0.0a	0.8±0.08a	0.7±0.0b	0.0±0.0c	0.0±0.0c	0.0±0.0c	0.0±0.0c	ACE inhibition (20.1)
TPEVDDEALEK	β-Lg (125-135)	0.0±0.0c	0.0±0.0c	0.0±0.0c	0.0±0.0c	0.4±0.05a	0.2±0.0b	0.0±0.0c	0.0±0.0c	DPP-IV inhibition (319.5)
VPYPQ	β-CN (177-181)	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.1±0.01a	0.1±0.01a	0.0±0.0b	0.0±0.0b	Antioxidant (nr)
FYPEL	αS1-CN (145-149)	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.7±0.0a	0.0±0.0b	0.0±0.0b	Antioxidant (nr)
HLPLP	β-CN (134-138)	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.5±0.2b	1.7±0.8a	0.4±0.08b	0.4±0.1b	ACE inhibition (41)
EMPFPK	β-CN (108-113)	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b	2.5±0.4a	2.8±0.4a	2.7±0.1a	2.4±0.1a	ACE inhibition (423)
HLPLPL	β-CN (134-139)	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.5±0.2b	1.7±0.8a	0.4±0.08b	0.4±0.1b	Antiamnestic (10)
KEDVPSE	αS1-CN (83-89)	0.0±0.0c	0.0±0.0c	0.0±0.0c	0.0±0.0c	0.06± 0.0b	0.1±0.04a	0.1±0.0c	0.1±0.0c	ACE inhibition (41)
LPLPL	β-CN (135-139)	0.0±0.0c	0.0±0.0c	0.0±0.0c	0.0±0.0c	0.8±0.3b	1.8±0.3a	1.1±0.2b	1.1±0.3b	DPP-IV inhibition (325)
LPYP	k-CN (56-59)	0.0±0.0c	0.0±0.0c	0.0±0.0c	0.0±0.0c	1.1±0.3ab	1.5±0.1a	1.1±0.2ab	0.9±0.2b	ACE inhibition (5)
NIPPLTQTPV	β-CN (73-82)	0.0±0.0c	0.0±0.0c	0.0±0.0c	0.0±0.0c	0.9±0.2ab	0.5±0.09bc	1.3±0.6a	0.9±0.2ab	ACE inhibition (173)
PQNIPPL	β-CN (71-77)	0.0±0.0d	0.0±0.0d	0.0±0.0d	0.0±0.0d	0.02±0.0c	0.05±0.01b	0.025±0.0c	0.1±0.01a	DPP-IV inhibition (1500)
VVPPFLQPE	β-CN (83-91)	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b	1.5±0.1a	1.6±0.6a	1.5±0.5a	2.0±0.2a	Antioxidant (nr)
VYPFPGPI	β-CN (59-66)	0.0±0.0d	0.0±0.0d	0.0±0.0d	0.0±0.0d	2.9±0.2c	4.0±0.2b	4.2±0.6b	5.2±0.4a	Antiamnestic (110)
YPEL	αS1-CN (146-149)	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b	1.4±0.2a	1.5±0.2a	1.2±0.1a	1.3±0.1a	Antioxidant (nr)
YPFPGPI	β-CN (60-66)	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.8±0.06a	1.1±0.4a	0.9±0.1a	0.8±0.007a	ACE inhibition (500)
YPVEPF	β-CN (114-119)	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b	7.6± 1.0a	9.0±1.8a	7.7±0.8a	8.7±0.8a	Opioid (nr)
LHLPLPL	β-CN (133-139)	0.0±0.0c	0.0±0.0c	0.0±0.0c	0.0±0.0c	0.0±0.0c	0.0±0.0c	0.2±0.09b	0.3±0.03a	ACE inhibition (432.7)
PGPIPN	β-CN (63-68)	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.09±0.0a	Immunomodulating (nr)

Table 3. Relative bioactive peptides abundance in conventional Parmigiano-Reggiano cheese (C-PRC) and hyposodic Parmigiano-Reggiano cheese (Hypo-PRC) at different digestion time. Data are means ± SD of three samples. Semiquantitative content was performed normalizing single peptide area against the sum of all peptides areas. Statistical analysis was performed by the one-way ANOVA (all peptides $p<0.0001$) with Tukey's post-hoc test (different letters indicate significant differences). ACE: angiotensin converting enzyme; DPP-IV: dipeptidyl peptidase 4; G120: end of gastric phase; D60: 60 minutes of duodenal phase; D120: end of duodenal phase.

Discussion

Sodium chloride (NaCl) is one of the most widely used additives in the food processing and the adverse effects of high sodium (Na) consumptions on blood pressure and NCD have been well documented [28,29]. The overall concern about Na dietary intake has boosted the development of new approaches to decrease the amount of salt added to food products [30,31]. In this study, we investigated the possible impact of an about 20% salt reduction (2.32 ± 0.06% dry matter (DM) and 1.90 ± 0.20% DM in C-PRC and Hypo-PRC, respectively) on the nutritional value of 30 months ripened PRC. Indeed, the nutritional value of food does not exclusively depend on its chemical composition as the bioaccessibility of nutrients is also a critical aspect to consider. The release/production of bioactive compounds, including bioactive peptides upon digestion of protein-rich food, is crucial as well. Since both bioaccessibility and release/generation of bioactive compounds can be affected by food processing [12,32,33], we evaluated fatty acids and protein bioaccessibility, and peptides formation in C-PCR and experimental Hypo-PCR.

The different salt concentration modulated the kinetics of fatty acid release from the food matrix during in vitro digestion. This effect could reflect a higher hydrolysis degree of triacylglycerol during ripening. Salaberría et al. [34] already evidenced an accelerate lipolysis process in low-salt PRC. The faster lipolysis could be due to the decrease in the NaCl:moisture ratio, which could increase the amount of water available for triglycerides hydrolysis. Anyway, at the end of in vitro digestion fatty acid bioaccessibility was similar in C- and po-PRC.

Like fatty acids, protein bioaccessibility increased over time during digestion. Since our aim was to verify the time course and extent of protein hydrolysis during digestion, the use of the three spectrophotometric methods was mandatory since they selectively quantify proteins with different molecular weight. The evident disparity between the three absolute value of bioaccessibility reflected the powerlessness of the Coomassie assay to detect small peptides and amino acids [35], the latter instead detected by OPA assay [36]. Comparing results from the three methods, we assume that most proteins were hydrolysed to fragments between 3000 Dalton and peptides bigger than five amino acids in length, which are detected by the absorbance in UV spectra only. The highest release of protein fragments below 3000 Dalton,

a dimension compatible with peptides formation, was already reached in the mid of intestinal digestion (D60). The kinetic of protein hydrolysis and peptide/amino acid release was similar in C- and Hypo-PRC protein, which evidenced the same protein bioaccessibility at the end of the in vitro digestion.

Since the coexistence of soluble protein fragments larger than 3000 Dalton was still compatible with the above data, NMR spectroscopy was applied to verify their presence in the digested fractions. High resolution NMR spectroscopy provides information about the whole set of molecules present in solution if they have hydrogen atoms included in their structure, practically all organic molecules, including amino acids and their oligo-/polymers. A simple inspection of the resulting spectra provides quantitative and qualitative information such as i) the solute concentrations, that are linearly correlated to the signal area independently from the molecule the hydrogen atom belongs to, ii) the type of functional group hosting the identified atoms, for instance aromatic or aliphatic moieties, and iii) the size or flexibility of the molecules which the atom is bound to. The latter property is revealed by the signal width: the broader the signal the larger and more rigid the molecule is, as expected by their shorter relaxation times, in turn reflected in the signal width [37]. The clear increment in the area of NMR signals demonstrated that digestion was hydrolysing soluble fragments from insoluble proteins. It is worth to note that even in the last phase of digestion there were both narrow and broad signals, evidencing the co-presence of small peptides, with MW <3kDa and larger protein fragments with MW >3kDa. In not digested samples, the same bioactive peptides were present at similar concentration in both C- and Hypo-PRC. Among them, the sequences NLHLPLPLL (from β-CN, residues 132-140), LHLPLP (from β-CN, residues 133-138), YKVPQL (from αS1-CN, residues 104-109) were already identified as ACE-inhibitory peptides in 12-months-ripened PRC [9], while the ACE-inhibitory and immunomodulating peptide LLYQEPVLGPVRGPFPIIV (β-CN 191-209) was detected in 24-months ripened PRC extracts [38]. All peptides detected in not digested PRC were almost completely degraded during gastric digestion, and new bioactive sequences were released. In digested samples, most identified sequences derived from the hydrolysis of high molecular weight peptides already identified in undigested cheese [10] and from the cleavage of intact α- and β-casein and whey proteins. At the end of gastric digestion, a total of 10 bioactive peptides were originated from β-casein, αS1-casein, and αS2-casein, confirming the high

affinity of pepsin for these proteins [39,40]. Again, all peptides originated during gastric digestion were further hydrolysed during the duodenal phase, which generated most of the bioactive peptides. Among these sequences, many fragments contained a PXP motif or a proline residue near to the carboxylic terminus. These structural motifs increase the resistance to the hydrolysis by digestive enzymes, which do not readily hydrolyse proline-containing peptides [41]. Most of the bioactive sequences detected at the end of the in vitro digestion were ACE-inhibitory. Among them, HLPLP (β-CN 134-138) and LHLPLPL (β-CN 133-139) originated from the hydrolysis of NLHLPLPLL (β-CN 132-140) and VENLHLPLPLL (β-CN 130-140) identified in not digested samples. Among bioactive peptides formed during duodenal digestion, and thus theoretically absorbed through the intestinal barrier, the sequences HLPLP, KEDVPSE, and LHLPLPL could elicit ACE-inhibitory activity.

Although the same bioactive peptides were detected in C- and Hypo-PRC, semiquantitative analysis revealed that most of them were present at higher concentration in Hypo-PRC at the end of digestion. Previous in vitro study performed on myoglobin evidenced that NaCl reduced protein digestibility promoting the exposure of hydrophobic amino acids, making the binding of phenylalanine with its surrounding environment within the protein core stronger, and eventually resulting in a protein organization less prone to undergo an enzyme-dependent hydrolysis [42]. Notwithstanding, it is not easy to explain how salt concentration selectively modulated the formation of biologically active peptides during gastrointestinal digestion, and further studies are needed to clarify this important aspect. To the best of our knowledge, the present study is the first one investigating the kinetics of bioactive fragment formation during the different phase of digestion. Indeed, although the evolution of bioactive peptide formation with ripening was already addressed [38], and several studies reported bioactive peptides released from PRC after in vitro digestion [38,43,44], exhaustive information regarding the fate of these peptides was still lacking. Our results represent a further step to uncover the hidden functionality of foods that is linked to bioactive peptide formation.

Conclusion

Based on herein reported results, we can conclude that reduction of salt content in ripened PRC did not significantly affect fatty acid and protein bioaccessibility and led to the formation of higher number of bioactive peptides after gastrointestinal digestion. Of note, ACE-inhibitors peptides were more abundant in Hypo- than C-PRC. The presence of these peptides has been reported in various fermented milk products, the antihypertensive effect of which has been proved by various in vitro and in vivo (animal and human trials) experiments [45]. Considering that in a previous study we evidenced that the sensory profiles were remarkably similar in C- and Hypo-PRC, without any off-flavour development [10], our results confirmed that reduction of salt content in ripened PRC represents a promising strategy to reduce Na intake, potentially protecting consumers' health. In this light, the evaluation of the actual ACE-inhibitory activity of digested C- and Hypo-PRC deserves further studies in biological systems.

References

1. Cai, W.; Lang, M.; Jiang, X.; Yu, Q.; Zhou, C.; Zou, W.; Zhang, X.; Lei, J. Correlation among high salt intake, blood pressure variability, and target organ damage in patients with essential hypertension: Study protocol clinical trial (SPIRIT compliant). *Medicine* 2020, *99*, e19548, doi:10.1097/MD.0000000000019548.

2. Pan, H.; Hibino, M.; Kobeissi, E.; Aune, D. Blood pressure, hypertension and the risk of sudden cardiac death: a systematic review and meta-analysis of cohort studies. *Eur J Epidemiol* 2020, *35*, 443-454, doi:10.1007/s10654-019-00593-4.

3. Thomas-Danguin, T.; Guichard, E.; Salles, C. Cross-modal interaction as a strategy to enhance salty taste and to maintain liking of low-salt food: a review. *Food & Function* 2019, *10*, doi:10.1039/C8FO02006J.

4. Inguglia, E.S.; Zhang, Z.; Tiwari, B.K.; Kerry, J.P.; Burgess, C.M. Salt reduction strategies in processed meat products – A review. *Trends in Food Science & Technology* 2017, *59*, 70-78, doi:https://doi.org/10.1016/j.tifs.2016.10.016.

5. Ali, S.H.; Luo, R.; Li, Y.; Liu, X.; Tang, C.; Zhang, P. Application of Mobile Health Technologies Aimed at Salt Reduction: Systematic Review. *JMIR Mhealth Uhealth* 2019, *7*, e13250, doi:10.2196/13250.

6. Solieri, L.; Bianchi, A.; Giudici, P. Inventory of non starter lactic acid bacteria from ripened Parmigiano Reggiano cheese as assessed by a culture dependent multiphasic approach. *Syst Appl Microbiol* 2012, *35*, 270-277, doi:10.1016/j.syapm.2012.04.002.

7. Bottesini, C.; Paolella, S.; Lambertini, F.; Galaverna, G.; Tedeschi, T.; Dossena, A.; Marchelli, R.; Sforza, S. Antioxidant capacity of water soluble extracts from Parmigiano-Reggiano cheese. *International Journal of Food Sciences and Nutrition* 2013, *64*, 953-958, doi:10.3109/09637486.2013.821696.

8. Mazorra-Manzano, M.A.; Robles-Porchas, G.R.; González-Velázquez, D.A.; Torres-Llanez, M.J.; Martínez-Porchas, M.; García-Sifuentes, C.O.; González-Córdova, A.F.;

Vallejo-Córdoba, B. Cheese Whey Fermentation by Its Native Microbiota: Proteolysis and Bioactive Peptides Release with ACE-Inhibitory Activity. *Fermentation* 2020, *6*, 19.

9. Solieri, L.; Baldaccini, A.; Martini, S.; Bianchi, A.; Pizzamiglio, V.; Tagliazucchi, D. Peptide Profiling and Biological Activities of 12-Month Ripened Parmigiano Reggiano Cheese. *Biology* 2020, *9*, 170.

10. Loffi, C.; Bortolazzo, E.; Garavaldi, A.; Musi, V.; Reverberi, P.; Galaverna, G.; Sforza, S.; Tedeschi, T. Reduction in the Brining Time in Parmigiano Reggiano Cheese Production Minimally Affects Proteolysis, with No Effect on Sensory Properties. *Foods* 2021, *10*, 770.

11. Santos, D.I.; Saraiva, J.M.A.; Vicente, A.A.; Moldão-Martins, M. 2 - Methods for determining bioavailability and bioaccessibility of bioactive compounds and nutrients. In *Innovative Thermal and Non-Thermal Processing, Bioaccessibility and Bioavailability of Nutrients and Bioactive Compounds*, Barba, F.J., Saraiva, J.M.A., Cravotto, G., Lorenzo, J.M., Eds. Woodhead Publishing: 2019; https://doi.org/10.1016/B978-0-12-814174-8.00002-0pp. 23-54.

12. Bordoni, A.; Laghi, L.; Babini, E.; Di Nunzio, M.; Picone, G.; Ciampa, A.; Valli, V.; Danesi, F.; Capozzi, F. The foodomics approach for the evaluation of protein bioaccessibility in processed meat upon in vitro digestion. *Electrophoresis* 2014, *35*, 1607-1614, doi:10.1002/elps.201300579.

13. Bordoni, A.; Picone, G.; Babini, E.; Vignali, M.; Danesi, F.; Valli, V.; Di Nunzio, M.; Laghi, L.; Capozzi, F. NMR comparison of in vitro digestion of Parmigiano Reggiano cheese aged 15 and 30 months. *Magnetic Resonance in Chemistry* 2011, *49*, S61-S70, doi:https://doi.org/10.1002/mrc.2847.

14. Minekus, M.; Marie, A.; Alvito, P.; Ballance, S.; Bohn, T.; Bourlieu, C.; Carrière, F.; Boutrou, R.; Corredig, M.; Dupont, D., et al. A standardised static in-vitro digestion method suitable for food – an international consensus. *Food & Function* 2014, 10.1039/C3FO60702J, doi:10.1039/C3FO60702J.

15. Bligh, E.G.; Dyer, W.J. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 1959, *37*, 911-917, doi:10.1139/o59-099.

16. Ghini, V.; Di Nunzio, M.; Tenori, L.; Valli, V.; Danesi, F.; Capozzi, F.; Luchinat, C.; Bordoni, A. Evidence of a DHA Signature in the Lipidome and Metabolome of Human Hepatocytes. *International Journal of Molecular Sciences* 2017, *18*, 359.

17. Bub, A.; Malpuech-Brugère, C.; Orfila, C.; Amat, J.; Arianna, A.; Blot, A.; Di Nunzio, M.; Holmes, M.; Kertész, Z.; Marshall, L., et al. A Dietary Intervention of Bioactive Enriched Foods Aimed at Adults at Risk of Metabolic Syndrome: Protocol and Results from PATHWAY-27 Pilot Study. *Nutrients* 2019, *11*, 1814.

18. Di Nunzio, M.; Betoret, E.; Taccari, A.; Dalla Rosa, M.; Bordoni, A. Impact of processing on the nutritional and functional value of mandarin juice. *Journal of the Science of Food and Agriculture* 2020, *100*, 4558-4564, doi:https://doi.org/10.1002/jsfa.10514.

19. Spellman, D.; McEvoy, E.; O'Cuinn, G.; FitzGerald, R.J. Proteinase and exopeptidase hydrolysis of whey protein: Comparison of the TNBS, OPA and pH stat methods for quantification of degree of hydrolysis. *International Dairy Journal* 2003, *13*, 447-453, doi:https://doi.org/10.1016/S0958-6946(03)00053-0.

20. Valli, V.; Danesi, F.; Gianotti, A.; Di Nunzio, M.; Taneyo Saa, D.L.; Bordoni, A. Antioxidative and anti-inflammatory effect of in vitro digested cookies baked using different types of flours and fermentation methods. *Food Research International* 2016, *88*, 256-262, doi:https://doi.org/10.1016/j.foodres.2015.12.010.

21. Di Nunzio, M.; Toselli, M.; Verardo, V.; Caboni, M.F.; Bordoni, A. Counteraction of oxidative damage by pomegranate juice: influence of the cultivar. *J Sci Food Agric* 2013, *93*, 3565-3573, doi:10.1002/jsfa.6234.

22. Di Nunzio, M.; Picone, G.; Pasini, F.; Chiarello, E.; Caboni, M.F.; Capozzi, F.; Gianotti, A.; Bordoni, A. Olive oil by-product as functional ingredient in bakery

products. Influence of processing and evaluation of biological effects. *Food Research International* 2020, *131*, 108940, doi:https://doi.org/10.1016/j.foodres.2019.108940.

23. Minkiewicz, P.; Iwaniak, A.; Darewicz, M. BIOPEP-UWM Database of Bioactive Peptides: Current Opportunities. *International Journal of Molecular Sciences* 2019, *20*, 5978.

24. Kumar, R.; Chaudhary, K.; Sharma, M.; Nagpal, G.; Chauhan, J.S.; Singh, S.; Gautam, A.; Raghava, G.P.S. AHTPDB: a comprehensive platform for analysis and presentation of antihypertensive peptides. *Nucleic Acids Research* 2014, *43*, D956-D962, doi:10.1093/nar/gku1141.

25. Malacarne, M.; Summer, A.; Franceschi, P.; Formaggioni, P.; Pecorari, M.; Panari, G.; Mariani, P. Free fatty acid profile of Parmigiano–Reggiano cheese throughout ripening: Comparison between the inner and outer regions of the wheel. *International Dairy Journal* 2009, *19*, 637-641, doi:https://doi.org/10.1016/j.idairyj.2009.04.004.

26. Picariello, G.; Miralles B.; Mamone G.; Sanchez-Rivera, L.; Isidra Recio, I.; Addeo, F.; Ferranti, P. Role of intestinal brush border peptidases in the simulated digestion of milk proteins. *Molecular Nutrition Food Research* 2015, *59*, 948-956, doi.org/10.1002/mnfr.201400856.

27. Picariello, G.; Iacomino, G.; Mamone, G.; Ferranti, P.; Fierro, O.; Gianfrani C.; Di Luccia A.; Addeo, F. Transport across Caco-2 monolayers of peptides arising from in vitro digestion of bovine milk proteins. *Food Chemistry 2013*, 139, 203-2012.

28. Bennett, J.E.; Stevens, G.A.; Mathers, C.D.; Bonita, R.; Rehm, J.; Kruk, M.E.; Riley, L.M.; Dain, K.; Kengne, A.P.; Chalkidou, K., et al. NCD Countdown 2030: worldwide trends in non-communicable disease mortality and progress towards Sustainable Development Goal target 3.4. *The Lancet* 2018, *392*, 1072-1088, doi:https://doi.org/10.1016/S0140-6736(18)31992-5.

29. Filippini, T.; Malavolti, M.; Whelton, P.K.; Naska, A.; Orsini, N.; Vinceti, M. Blood Pressure Effects of Sodium Reduction: Dose-Response Meta-Analysis of

Experimental Studies. *Circulation* 2021, *143*, 1542-1567, doi:10.1161/circulationaha.120.050371.

30. Bhat, Z.F.; Morton, J.D.; Mason, S.L.; Bekhit, A.E.A. The application of pulsed electric field as a sodium reducing strategy for meat products. *Food Chem* 2020, *306*, 125622, doi:10.1016/j.foodchem.2019.125622.

31. Zhu, Y.; Yan, Y.; Yu, Z.; Wu, T.; Bennett, L.E. Effects of high pressure processing on microbial, textural and sensory properties of low-salt emulsified beef sausage. *Food Control* 2022, *133*, 108596, doi:https://doi.org/10.1016/j.foodcont.2021.108596.

32. Carbonell-Capella, J.M.; Buniowska, M.; Barba, F.J.; Esteve, M.J.; Frígola, A. Analytical Methods for Determining Bioavailability and Bioaccessibility of Bioactive Compounds from Fruits and Vegetables: A Review. *Comprehensive Reviews in Food Science and Food Safety* 2014, *13*, 155-171, doi:https://doi.org/10.1111/1541-4337.12049.

33. Ferranti, P.; Nitride, C.; Nicolai, M.A.; Mamone, G.; Picariello, G.; Bordoni, A.; Valli, V.; Di Nunzio, M.; Babini, E.; Marcolini, E., et al. In vitro digestion of Bresaola proteins and release of potential bioactive peptides. *Food Research International* 2014, *63*, 157-169, doi:https://doi.org/10.1016/j.foodres.2014.02.008.

34. Salaberría, F.; Marzocchi, S.; Bortolazzo, E.; Carrín, M.E.; Caboni, M.F. Study of the Effect of NaCl on Lipolysis in Parmigiano Reggiano Cheese. *ACS Food Science & Technology* 2021, *1*, 54-59, doi:10.1021/acsfoodscitech.0c00025.

35. Urbinati, E.; Di Nunzio, M.; Picone, G.; Chiarello, E.; Bordoni, A.; Capozzi, F. The Effect of Balsamic Vinegar Dressing on Protein and Carbohydrate Digestibility is Dependent on the Food Matrix. *Foods* 2021, *10*, 411.

36. Egger, L.; Schlegel, P.; Baumann, C.; Stoffers, H.; Guggisberg, D.; Brügger, C.; Dürr, D.; Stoll, P.; Vergères, G.; Portmann, R. Physiological comparability of the harmonized INFOGEST in vitro digestion method to in vivo pig digestion. *Food*

Research International 2017, *102*, 567-574, doi:https://doi.org/10.1016/j.foodres.2017.09.047.

37. Deng, R.; Seimys, A.; Mars, M.; Janssen, A.E.M.; Smeets, P.A.M. Monitoring pH and whey protein digestion by TD-NMR and MRI in a novel semi-dynamic in vitro gastric simulator (MR-GAS). *Food Hydrocolloids* 2022, *125*, 107393, doi:https://doi.org/10.1016/j.foodhyd.2021.107393.

38. Martini, S.; Conte, A.; Tagliazucchi, D. Effect of ripening and in vitro digestion on the evolution and fate of bioactive peptides in Parmigiano-Reggiano cheese. *International Dairy Journal* 2020, *105*, 104668, doi:https://doi.org/10.1016/j.idairyj.2020.104668.

39. Qi, W.; Su, R.X.; He, Z.M.; Zhang, Y.B.; Jin, F.M. Pepsin-Induced Changes in the Size and Molecular Weight Distribution of Bovine Casein During Enzymatic Hydrolysis. *Journal of Dairy Science* 2007, *90*, 5004-5011, doi:10.3168/jds.2007-0230.

40. Sams, L.; Amara Ep Douzi, S.; Puppo, R.; Lebrun, R.; Paume, J.; Giallo, J.; Carrière, F. Characterization of pepsin from rabbit gastric extract, its action on β-casein and the effects of lipids on proteolysis. *Food & Function* 2018, *9*, doi:10.1039/C8FO01450G.

41. Tagliazucchi, D.; Helal, A.; Verzelloni, E.; Bellesia, A.; Conte, A. Composition and properties of peptides that survive standardised in vitro gastro-pancreatic digestion of bovine milk. *International Dairy Journal* 2016, *61*, 196-204, doi:10.1016/j.idairyj.2016.06.002.

42. Liu, H.; Li, Q.; Zhao, D.; Zhang, M.; Jiang, S.; Li, C. Changes in the structure and digestibility of myoglobin treated with sodium chloride. *Food Chemistry* 2021, *363*, 130284, doi:https://doi.org/10.1016/j.foodchem.2021.130284.

43. Basiricò, L.; Catalani, E.; Morera, P.; Cattaneo, S.; Stuknytė, M.; Bernabucci, U.; De Noni, I.; Nardone, A. Release of angiotensin converting enzyme-inhibitor peptides during in vitro gastrointestinal digestion of Parmigiano Reggiano PDO cheese and

their absorption through an in vitro model of intestinal epithelium. *J Dairy Sci* 2015, *98*, 7595-7601, doi:10.3168/jds.2015-9801.

44. Bottari, B.; Quartieri, A.; Prandi, B.; Raimondi, S.; Leonardi, A.; Rossi, M.; Ulrici, A.; Gatti, M.; Sforza, S.; Nocetti, M., et al. Characterization of the peptide fraction from digested Parmigiano Reggiano cheese and its effect on growth of lactobacilli and bifidobacteria. *Int J Food Microbiol* 2017, *255*, 32-41, doi:10.1016/j.ijfoodmicro.2017.05.015.

45. Rai, A.K.; Sanjukta, S.; Jeyaram, K. Production of angiotensin I converting enzyme inhibitory (ACE-I) peptides during milk fermentation and their role in reducing hypertension. *Critical Reviews in Food Science and Nutrition* 2017, *57*, 2789-2800, doi:10.1080/10408398.2015.1068736.

Supplementary

	C-PRC	Hypo-PRC
C8:0	216.37±16.58	220.90±43.74
C10:0	552.78±28.06	563.87±81.72
C12:0	684.14±29.52	671.87±70.75
C14:0	2191.56±151.62	2120.22±115.49
C16:0	5993.19±350.51	5832.99±330.59
C16:1 n-7	350.49±14.09	349.37±49.00
C18:0	1807.86±497.96	1789.42±295.13
C18:1 n-9	4286.09±634.15	4288.07±104.86
C18:2 n-6	505.73±124.86	505.97±64.26
ΣSFA	11445.91±968.33	11199.26±245.87
ΣMUFA	4636.58±624.78	4637.44±98.28
ΣPUFA	505.73±124.86	505.97±64.26
Total	16588.22±1709.90	16342.67±369.40

Supplementary table 1. Fatty acid methyl esters content in conventional Parmigiano-Reggiano cheese (C-PRC) and hyposodic Parmigiano-Reggiano cheese (Hypo-PRC). Data are means ± SD of three independent analysis and are expressed as mg of fatty acid methyl esters/100g PRC. Statistical analysis was by evaluated by unpaired t-test (* at least $p<0.05$).

CHAPTER-4

Cleaning the label of cured meat. Effect of the replacement of nitrates/nitrites on nutrients bioaccessibility, peptides formation, and cellular toxicity of in vitro digested salami

Already published in " *Int. J. Mol. Sci".* 2022, *23*(20), 12555; https://doi.org/10.3390/ijms232012555

Introduction

Meat is a high-protein, mineral-rich food that also contains several vitamins, particularly type B ones [1]. Processed meat is described as meat that has been cured, salted, or smoked (e.g., ham or bacon) to increase its shelf-life and/or color, and taste [2]. Between 66% and 99% of Europeans consume processed meat, with an average amount consumed per day ranging between 10 and 80 g [3]. Most processed meats contain nitrites (NO_2-) and nitrates (NO_3-) [4], which are authorized as food additives in the European Union under Commission Regulation (EU) No 1129/2011. The addition of nitrates/nitrites has positive traits, including color fixation, flavor enhancement, antioxidant activity, and antimicrobial (Clostridium botulinum) preservation [5]. However, their use in meat products is restricted in many countries. In fact, nitrites and nitrates react with amines produced by the decomposition of proteins [6-8], resulting in the production of nitrosamines, carcinogen and inflammatory agents.

Consumers' interest in food ingredients and production methods is on the rise, and the "clean label" trend has forced the food industry to exclude some synthetic additives and replace them with more "natural" ones [9]. As nitrates/nitrites remain among the food additives most feared by consumers [10], the use of natural compounds as alternatives for their full/partial replacement represents a challenge for the meat industry. Although ideal substitutes of synthetic nitrates/nitrites have not yet been found [11,12], some candidates have been recently evidenced. Their use, alone or in combination, could make it possible to produce healthier processed meat products with good sensory characteristics.

In an effort to produce healthier meat products, it must be considered that processing (including formulation and fermentation) deeply modifies both the content and

bioaccessibility of nutrients [13,14]. Bioaccessibility, i.e., the fraction of the total amount of a substance that is released from the food matrix during digestion and potentially becomes available for absorption [15], can be modified by changes in the supramolecular architecture and in the network of interactions between molecules, as well as the location of nutrients within compartments, with an impact on the nutritional value of food [16]. Indeed, in a recent work we reported the impact of different sodium chloride content and ripening time on the kinetics of protein hydrolysis and on the formation of peptides and small organic compounds during in vitro digestion of Parmigiano Reggiano cheese [17].

Aim of the study

The aim of the present study was to investigate whether a modification in salami processing that allows the replacement of synthetic nitrates/nitrites affects the bioaccessibility of fatty acid, the hydrolysis of proteins, and the release of bioactive peptides after in vitro digestion of the final products. Two innovative formulations not containing nitrites were prepared: the first (SA) was supplemented with nitrate-reducing microbial starter cultures (MSC) and sodium ascorbate (0.3%); the second (SMA) was added with MSC, sodium ascorbate (0.3%) and plant extracts from grapeseed, green tea and olive. The two innovative formulations were compared with the "positive control" (C-NO_2) added with sodium nitrite, potassium nitrate and MSC, and the "negative control" (C-0) containing neither MSC nor additives (nitrite, polyphenols and ascorbate). The sensory characteristics (color, texture and rancid flavor) of the innovative formulations were comparable to those of the positive control, without negative sensory properties induced by the presence of the plant extracts.

Conventional and experimental salami formulations were subjected to in vitro static gastrointestinal digestion according to the INFOGEST protocol, and the kinetics of fatty acids and protein release from the food matrix were followed by sampling at the end of the gastric phase, in the middle and at the end of the intestinal phase. Digested samples were also analyzed by high-resolution nuclear magnetic resonance (HR-NMR) and liquid chromatography coupled with high-resolution mass spectrometry (LC–HRMS), and the formation of bioactive peptide sequences was demonstrated using a bioinformatic methodology. To further address health concern on processed meat and nitrite use, the digested samples were analyzed for their impact on cell proliferation and genotoxicity in a

human colon cancer cell line (HT-29), currently used to study the relationship between food and cellular physiology/metabolism in vitro [18].

Materials and Methods

Chemicals

Unless specified, chemicals and solvents were of the highest analytical grade and purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich (St. Louis, MO, USA).

Salami formulation, preparation, and fermentation

Salami were manufactured at the Stazione Sperimentale per l'Industria delle Conserve Alimentari (SSICA, Italy). Four different salami formulations were tested. For all the formulations, the salami mixture consisted of lean muscle tissue (75%) and minced bacon (25%). The meat was weighed, cut into small pieces, ground in a meat mincer (∅= 6 mm plate), and then mixed with salt (2.5%), dextrose (0.2%), ascorbate (0.05%) and natural flavors.

The positive control formulation (C-NO_2) was added with sodium nitrite, potassium nitrate and nitrate-reducing microbial starter cultures (MSC). MSC (Chr. Hansen, S.p.A., Via Quintino Sella 3/A, Parma, Italy) contained lactic acid bacteria and nitrate-reducing coagulase negative staphylococcaceae, and was inoculated as common manufacturing practices to properly drive the fermentation phase and to promote the development of aroma during the ripening phase.

Two innovative formulations not containing nitrites were prepared: the first (SA) was added with MSC and sodium ascorbate (0.3%); the second (SMA) was added with MSC, sodium ascorbate (0.3%) and plant extracts from grapeseed, green tea and olive (Indena S.p.A., Via Ortles 12, Milan, Italy), characterized according to their total polyphenols and nitrate content to provide 0.4 g/kg of bioactive polyphenols and less than 1 ppm of total nitrate to the meat mixture. Finally, the "negative control" (C-0) was prepared with neither MSC nor additives (nitrite, polyphenols and ascorbate).

The salami formulations were prepared in a mixer and stuffed into natural casings separately (∅ = 55 mm) to obtain salami with an average weight of 470 ± 25 g. The recipe for the preparation of the salami batter is reported in Table 4. The positive controls (C-NO_2) were subjected to a traditional hot drying method, while the nitrite-free formulations were subjected to a first cold drying until when the water activity and pH were low enough to avoid microbiological risk, then were conventionally ripened. Ripening was conducted in a temperature and atmosphere-controlled climate camera at 13-15°C, with a relative humidity of 75-83 %, and ended when weight loss of a 38-40% was achieved.

Each salami formulation was prepared in triplicate in three different days. A detailed description of salami processing has been recently reported [19].

The consumer test performed at SSICA did not reveal any differences in terms of color, texture and rancid flavor between control and experimental salami, without negative sensory properties induced by the plant extracts (data not shown).

Ingredient (g)	**CNO_2**	**C0**	**SA**	**SMA**
Lean muscle tissue	750	750	750	750
Fat muscle tissue	250	250	250	250
Salt	25	25	25	25
Sugar	2	2	2	0
Spice mix[1]	0.3	0.3	0.3	0.3
Sodium ascorbate	0.5	0	0.5	0.5
Sodium nitrite	0.05	0	0	0
Potassium nitrate	0.08	0	0	0
Polyphenol mix[2]	0	0	0	0.86
Coagulase-negative Staphylococcaceae	0.25	0	0.25	0.25
Lactic acid Bacteria	0.125	0	0.125	0.125

[1] Garlic: black pepper (1:10 w/w)

[2] Grapeseed extract: olive extract: green tea extract (1:1:3 w/w) for a total gallic acid equivalent content of 60g /100g

In vitro digestion

According to the standardized INFOGEST protocol [20], the digestion process was performed on 45 g of salami for 242 min (2 min of oral, 120 min of gastric and 120 min of intestinal digestion) at 37 °C. To simulate chewing, salami formulations were chopped before starting oral digestion. During the process, several consecutive enzymatic reactions took place by addition of simulated saliva (15.1 mM KCl, 3.7 mM KH_2PO_4, 13.6 mM $NaHCO_3$, 0.15 mM $MgCl_2(H2O)_6$, 0.06 mM $(NH_4)_2CO_3$, 0.75 mM $CaCl_2$ pH 7), simulated gastric juice (6.9 mM KCl, 0.9 mM KH_2PO_4, 25 mM $NaHCO_3$, 47.2 mM NaCl, 0.12 mM $MgCl_2(H2O)_6$, 0.5 mM $(NH_4)_2CO_3$, 75 µM $CaCl_2$ containing 2,000 U/ml pepsin) at pH 3, and simulated pancreatic juice (6.8 mM KCl, 0.8 mM KH_2PO_4, 85 mM $NaHCO_3$, 38.4 mM NaCl, 0.33 mM $MgCl_2(H2O)_6$, 0.3 mM CaCl2 containing 10 mM bile and 100 U/ml pancreatin) at pH 7. Samples were taken at the end of the gastric phase (G120), after 60 minutes (D60) and at the end of the duodenal phase (D120). In G120 samples, the pH was increased to 7 with 35% NaOH to stop pepsin hydrolytic action and reported to 3 with 37% HCl. Samples at D60 and D120 were acidified to pH 3 with 37% HCl to stop pancreatic hydrolysis and reported to 7 with 35% NaOH. Digested samples were centrifuged at 50,000 × g for 15 min and the surfaced upper oil phase discarded. To remove any turbidity, the supernatant consisting in the aqueous micellar phases were filtered with 0.2 µm cellulose acetate membranes and stored at −80 °C until further analysis. In each salami formulation, digestion was performed in triplicate and triplicates were then combined.

Fatty acids bioaccessibility

Total lipids were extracted according to Folch et al. [21]. After methyl-esterification [22], the quantitative and qualitative content of fatty acid methyl esters (FAMEs) was determined by fast-GC (GC-2030AF; Shimadzu, Kyoto, Japan) using a capillary column (30 mt, 0.2 µm film thickness) with a programmed temperature gradient (50–250 °C, 10°C/min). The gas chromatographic peaks were identified using authentic samples based on their retention time. FAMEs from chemicals added during in vitro digestion were subtracted and quantitative evaluations were normalized for the dilution factor due to the addition of digestive fluids. Bioaccessibility was assessed as FAME content in digested sample/FAME content in the corresponding sample be-fore digestion × 100 [23].

Protein hydrolysis

In undigested salami, protein content was determined by Kjeldahl method [24]. In digested samples, protein/peptide concentration was determined spectrophotometrically by three different methods, namely measuring the absorbance at 280 nm [17], the Coomassie assay [25], and the o-phthaldialdehyde (OPA) assay [26], using non-fat dry milk, bovine serum albumin, and L-isoleucine as standard, respectively. Protein content from enzymes added during in vitro digestion was subtracted and values were normalized for the dilution factor due to the addition of digestive fluids.

HR ^{1}H NMR spectroscopy

Digested samples were centrifuged at 2,300g for 5 min at 4 °C to eliminate any particulate matter formed during freezing/thawing and then 750 µl of supernatant was taken and added to 120 µl of 100 mM phosphate buffer with 10 mM trimethylsi-lylpropanoic acid (TSP). HR-NMR analysis was recorded at 298 K on a Bruker US+ Avance III spectrometer operating a proton frequency of 600.13 MHz as previously reported [17].

Bioactive peptides determination and identification

Digested samples were centrifuged at 14,000 rpm for 40 min at 4°C to remove any particulate matter formed during freezing/thawing. The supernatants were filtered through 0.22 µm PTFE filters and directly injected into the LC-HRMS system, as previously reported [17]. The software used for data processing was UNIFI (Waters Corporation, Milford MA, USA). The following protein Uniprot accession numbers were employed in processing: P68137 (actin), Q9TV61 (myosin-1), Q9TV62 (myosin-4), Q9TV63 (myosin-2), P79293 (myosin-7), F2Z5B6 (tropomyosin alpha-1 chain), P08835 (albumin), P02189 (myoglobin), F1SHL9 (pyruvate kinase). A minimum threshold of three amino acids was set in the processing parameters. Variable amino acid modifications were included as deamidation (N, Q) pyroglutamic acid N-term (E, Q), oxidation (single or double, M or W), phosphorylation (S, T, Y). Peptide semiquantitative data were obtained as normalized areas. Released bioactive sequences were identified using a bioinformatic approach.The whole set of peptide sequences under analysis was searched in two bench-mark databases of peptide bioactivity, namely BIOPEP (http://www.uwm.edu.pl/biochemia/index.php/en/biopep) [27] and AHTPDB

(http://crdd.osdd.net/raghava/ahtpdb/) [28], which were queried automatically and systematically employing a scripted pipeline developed «in-house».

HT-29 cell culture and supplementation

The human colon adenocarcinoma cell line HT-29 was kindly obtained from the Northern Ireland Centre for Food and Health. Cells were grown in DMEM added with 100 U/ml penicillin, 100 μg/ml streptomycin, 2mM L-glutamine and 10% fetal bovine serum, in a humidified CO2 (5%) incubator at 37 °C. For antiproliferative experiments, cells were seeded at 5x103 cells/well into 96-well plates and supplemented with scalar concentration (1:200 - 1:100) of D120 digested samples in free serum DMEM for 24h. For genotoxic experiments, cells were supplemented with a 1:150 concentration of D120 digested samples or 2 mM ethylmethanesulfonate (EMS) in free serum DMEM for 24h. To avoid interference, in each experiment some cells (B) received the same dilution of the solution obtained from a 'blank' digestion, comprising an in vitro digestion per-formed without the addition of any food.

Cell proliferation

Anti-proliferative effect was evaluated by using CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega Corporation, Madison WI, USA), following the manufacturer's instructions. After treatment, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) was added to each well and absorbance of new soluble formazan product in DMEM was measured after 4 hours at 485 nm by Tecan SpectraFluor Plus plate reader (Tecan, Männedorf, Switzerland). Cell viability was expressed as percent respect unsupplemented cells, assigned as 100%.

Genotoxicity assay

Genotoxic effect was evaluated by alkaline single-cell gel electrophoresis (Comet Assay) [29], with minor modifications. After supplementation, cells were washed twice with PBS and trypsinized. Cells were resuspended in 90 μl of low melting 0.7% agarose and transferred onto degreased microscope slides previously dipped in 1% normal melting agarose for the first layer and covered with a third layer of low melting 0.7% agarose. Cell lysis was carried out overnight at 4 °C by a lysis buffer (2.5 M NaCl, 100 mM EDTA, 8 mM Tris–HCl, 1% Triton X-100 and 10% dimethyl sulfoxide, pH 10). The electrophoretic migration (0.78 V/cm,

300 mA for 20 min) was performed in an alkaline buffer (1 mM EDTA, 300 mM NaOH, 0 °C, pH > 13). DNA was stained with 75 µl ethidium bromide (10 µg/ml) before examination at 100x magnification under a Leica DMLS fluorescence microscope (excitation filter BP 515–560 nm, barrier filter LP 580 nm), using an automatic image analysis system (Comet Assay IV—Perceptive Instruments Ltd., Bury St Edmunds, UK). The total percentage of fluorescence in the tail (TI, tail intensity) provided representative data on genotoxic effects (Figure S3). For each sample, coded and evaluated blind, 100 cells were analyzed, and the median value of TI was calculated.

Statistical analysis

Statistical differences were evaluated by the one-way analysis of variance (ANOVA) followed by Tukey's post hoc test using Prism software ver. 7.0 (GraphPad, San Diego, CA, USA). Different letters indicate significant differences (at least $p < 0.05$).

Results

Fatty acid composition and bioaccessibility

As reported by Herranz et al. [30], the major fatty acids in undigested salami were oleic > palmitic > stearic > linoleic acid, which accounted for approximately 94% of the total fatty acid. No differences were detected between the different formulations with the exception of minor fatty acids (myristic, α-linolenic, and gondoic acid) (Table S1).

The bioaccessibility of fatty acid increased during digestion, particularly during the intestinal phase (Table 1). Although the maximum total bioaccessibility was already achieved after 60 min of duodenal digestion in C0 and SMA, at the end of digestion (D120) it was similar in all formulations. Comparing the different salami formulations at each digestion time, at D60 the bioaccessibility of stearic acid was lower in CNO_2 than SMA (Table S2).

	CNO_2			C0			SA			SMA		
	G120	**D60**	**D120**	**G120**	**D60**	**D120**	**G120**	**D60**	**D120**	**G120**	**D60**	**D120**
14:0	0.5±0.7c	30.6±4.2b	37.3±0.2a	0.3±0.2b	38.0±6.5a	43.6±6.4a	0.4± 0.1b	37.7±1.8a	42.1±3.1a	0.7±0.4b	38.6±2.4a	40.0±0.6a
16:0	0.8±0.4c	29.2±3.0b	37.4±2.7a	0.4±0.2b	39.2±8.3a	45.6±7.9a	1.0± 0.1c	32.3±1.1b	43.9±4.2a	0.9±0.5b	40.2±2.2a	37.5±0.0a
16:1 n-7	0.4±0.5b	27.0±4.7a	31.7±0.3a	0.0±0.0b	32.6±5.7a	37.1±5.7a	0.0±0.0b	29.8±3.1a	37.9±4.8a	0.1±0.1b	35.0±2.8a	38.2±2.5a
18:0	0.9±0.6b	28.2±1.2a	38.4±8.5a	0.6±0.1b	40.2±8.9a	47.3± 0.8a	1.2± 0.1c	30.8±1.0b	43.5±4.6a	1.0±0.3c	30.4±2.2b	40.3±1.8a
18:1 n-9	0.6±0.4b	31.0±4.7a	37.5±0.6a	0.2±0.1b	37.2±7.9a	43.1±6.6a	0.8± 0.0c	33.0±2.7b	42.3±4.0a	0.6±0.5c	39.0±2.4b	41.8±2.8a
18:2 n-6	0.6±0.5c	36.6±3.8b	45.2±3.0a	0.3±0.1b	39.9±8.7a	47.2±6.3a	0.4± 0.1c	42.4±5.4b	54.4±6.2a	0.6±0.4b	44.0±3.5a	48.1±2.1a
18:3 n-3	0.0±0.0b	40.3±14.0a	45.2±4.5a	0.6±1.1b	38.7±19.7a	51.2±6.1a	0.0± 0.0b	41.2±8.1a	51.2±4.1a	0.0±0.0b	44.3±6.7a	49.8±1.1a
20:1 n-9	0.0±0.0b	22.1±7.5a	29.3±0.8a	0.0±0.0b	30.1±7.3a	38.1±5.1a	0.0± 0.0b	25.0±2.5a	35.6±5.8a	0.0±0.0b	30.4±0.8a	33.4±3.6a
20:4 n-6	0.0±0.0b	46.3±3.2a	70.3±17.3a	0.0±0.0b	53.0± 16.7a	70.0±14.4a	0.0± 0.0b	49.5±12.3a	70.3±14.6a	0.0±0.0c	45.0±2.3b	50.8±3.2a
Total	0.6±0.4c	30.7±3.8b	38.3±2.5a	0.33±0.1b	38.3±8.2a	44.7±7.4a	0.8± 0.0c	33.3±1.3b	44.1± 3.6a	0.7±0.4b	39.9±2.4a	39.8±1.3a

Table 1. Time-course of fatty acid bioaccessibility in the different salami formulations. Data are means ± SD of in vitro digestion of three independent samples analyzed in duplicate. Fatty acid bioaccessibility is expressed as %, and it was calculated as concentration of fatty acid methyl ester in digested salami/concentration in salami before digestion x 100. Statistical analysis was by one-way ANOVA (always $p < 0.05$) with Tukey's post-hoc test comparing each salami formulation at the three digestion time points (different letters indicate statistical significance). G120: end of gastric phase; D60: mid duodenal phase; D120: end of duodenal phase; C-NO_2: salami with sodium nitrite, potassium nitrate and with nitrate-reducing microbial starter cultures; C-0: salami containing neither nitrate-reducing microbial starter cultures nor additives (nitrite, polyphenols and ascorbate); SA: salami with nitrate-reducing microbial starter cultures and sodium ascorbate; SMA: salami with nitrate-reducing microbial starter cultures, sodium ascorbate and plant extracts.

Protein hydrolysis

Protein content in undigested salami was similar: CNO_2 = 32.4 ± 0.9%; C0 = 33.9 ± 1.5%; SA = 31.8 ± 0.7%; SMA = 32.6 ± 0.5% (one-way ANOVA p = n.s.; Tukey's post-hoc test: n.s.). Protein hydrolysis during digestion was evaluated by three different spectrophotometric methods (OPA, Coomassie, and absorbance at 280 nm), all showing a time-dependent release of amino acids/peptides/proteins from the food matrix (Figure 1). Although the three assays have a different ability to detect protein fragments with different molecular mass [31,32], at D120 the same amino acid/peptide/protein concentration was detected in all samples by all methods. Nonetheless, some differences in the time course of protein hydrolysis were found between the formulations. Indeed, SA and SMA already achieved the highest protein hydrolysis at mid duodenal digestion (D60) as assessed by absorbance at 280 nm and Coomassie assay (for SMA only). Comparing the different salami formulations at each digestion point, no differences were found when measuring absorbance at 280 nm. On the contrary, a different efficiency of protein hydrolysis was highlighted by the OPA assay at G120 and by the Coomassie assay at D60 (Figure S1).

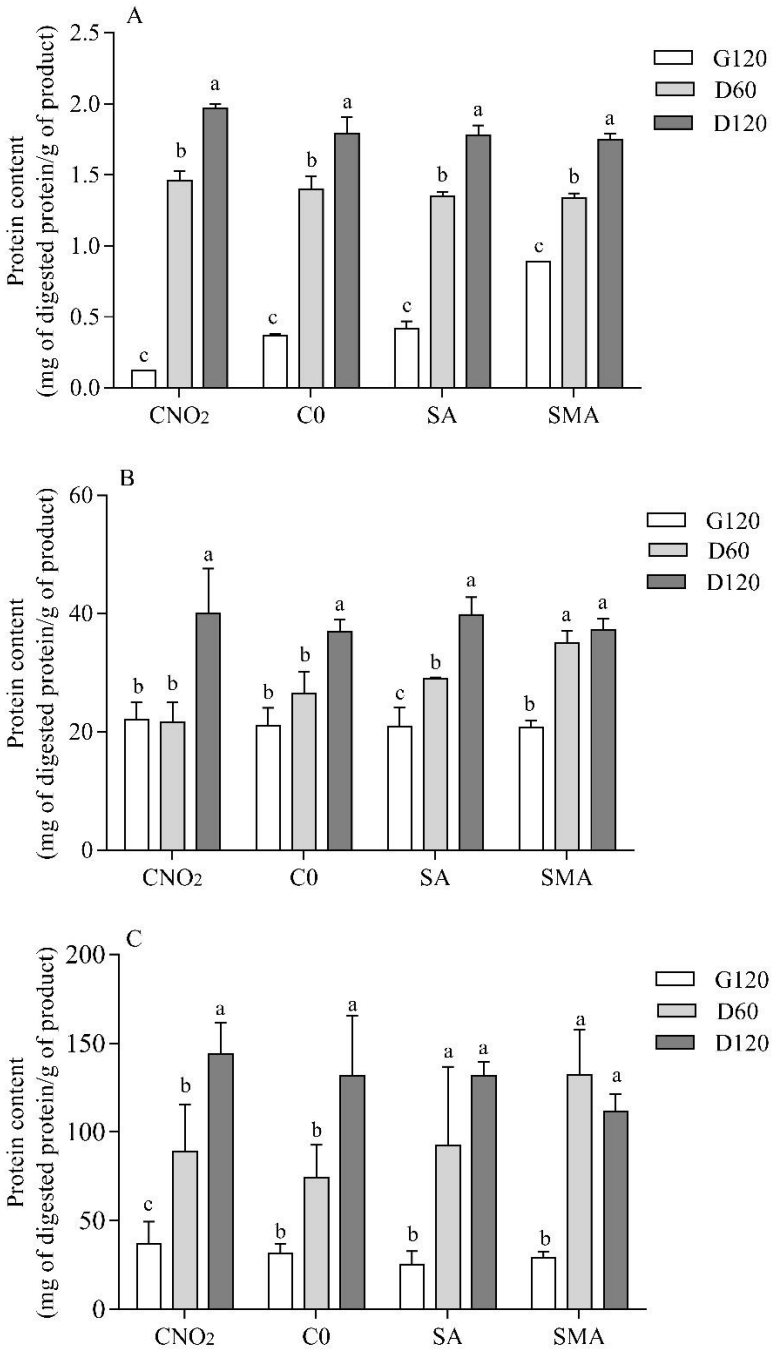


Figure 1. Time-course of protein hydrolysis in the different salami formulations. Protein content was assessed by OPA (A), Coomassie assay (B), and absorbance at 280 nm (C), and it is expressed as mg of protein digested/g of product. Data are means ± SD of in vitro digestion of three independent samples analyzed in triplicate. Protein content is expressed as mg of protein digested/g of product. Statistical analysis was by one-way ANOVA (always $p < 0.05$) with Tukey's post-hoc test comparing each salami formulation at the three digestion time points (different letters indicate statistical significance). G120: end of gastric phase; D60: 60 min of duodenal phase; D120: end of duodenal phase; C-NO_2: salami with sodium nitrite, potassium nitrate and with nitrate-reducing microbial starter cultures; C-0: salami containing neither nitrate-reducing microbial starter cultures nor additives (nitrite, polyphenols and ascorbate); SA: salami with nitrate-reducing microbial starter cultures and sodium ascorbate; SMA: salami with nitrate-reducing microbial starter cultures, sodium ascorbate and plant extracts.

HR ^{1}H-NMR spectroscopy

The progression of the release of the aliphatic (A), total (B), and aromatic (C) amino acid regions at different digestion times in the four formulations of salami is shown in Figure 2, as expressed by the integral areas recorded in specific diagnostic region of the NMR spectra. The α-proton amino acid region (region B) comprises the signals of hydrogen nuclei that are present individually in all amino acids, both in the free and bound state to peptides or in soluble proteins [32]. However, only hydrogen nuclei belonging to soluble molecules generate detectable signals, thus providing direct evidence of the solubilization of fragments derived from the fibrillar insoluble proteins that are released into the digestion fluid. In each spectral region, the release of amino acids or soluble peptides from the food matrix increased over time during the digestion, with the highest release achieved with statistical significance at D120 with the exception of SMA salami, for which there was not statistical difference between D60 and D120, due to higher variance of this salami at the longest digestion time. If the aromatic region of the NMR spectra is considered for comparison, there is not statistical difference even between the gastric phase (G120) and at the middle of the duodenal phase (D60). When comparing different salami formulation at the same digestion time, integrals of the α-proton amino acid region appeared statistically lower in SMA than in C0 at the middle of duodenal digestion (Figure S2).

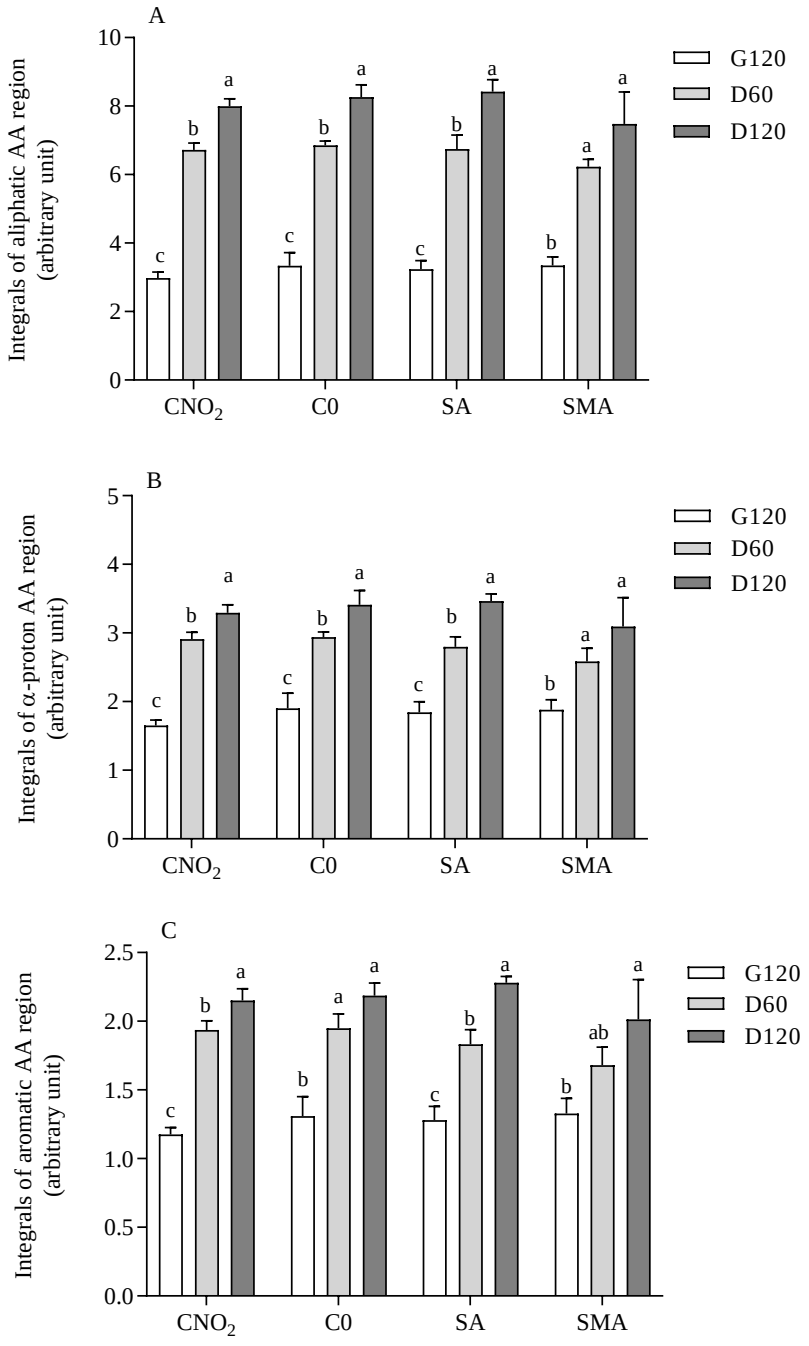


Figure 2. Integral area of aliphatic, α-proton, and aromatic amino acid region at different digestion times in different salami. Data are means ± SD of in vitro digestion of three independent samples analyzed in duplicate. Integrals of aliphatic (A), α-proton (B), and aromatic (C) amino acid region are expressed as signal areas. Statistical analysis was by one-way ANOVA (always $p < 0.05$) with Tukey's post-hoc test comparing the three digestion times in each salami formulation (different letters indicate significant differences). G120: end of gastric phase; D60: 60 minutes of duodenal phase; D120: end of duodenal phase; AA: amino acids; C-NO_2: salami with sodium nitrite, potassium nitrate and with nitrate-reducing microbial starter cultures; C-0: salami containing neither nitrate-reducing microbial starter cultures nor additives (nitrite, polyphenols and ascorbate); SA: salami with nitrate-reducing microbial starter cultures and sodium ascorbate; SMA: salami with nitrate-reducing microbial starter cultures, sodium ascorbate and plant extracts.

Peptides formation

The total number of peptides in the undigested and digested salami formulations is shown in Table 2. In all not digested formulations, most of the identified peptides were generated from myofibrillar proteins (78%), particularly from actin (24%) and myosin VII (28%), while only 12% and 10% of the sequences came from sarcoplasmic and not-identified proteins, respectively. The number of total and myofibrillar proteins-derived peptides (MF peptides) was not affected by in vitro digestion, except in C0. In contrast, the number of peptides from sarcoplasmic proteins (SP peptides) decreased in CNO_2 at all times of digestion and in C0 at G120. In all samples, peptides from not-identified proteins (NI peptides), phosphorylase B kinase, fructose B phosphate aldolase, troponin T, myosin VI, myosin VII heavy chain, and myosin I light chain were fully hydrolyzed during in vitro digestion, while peptides from tropomyosin α-1 chain and myosin IV were produced. The mean peptide length significantly decreased during duodenal digestion.

Table 2. Number of peptides in not digested and digested salami at different digestion time. Data are means ± SD of not digested or *in vitro* digestion of three independent samples analyzed in duplicate. Statistical analysis was performed by one-way ANOVA with Tukey's post-hoc test comparing each salami before digestion and at the three digestion times (different letters indicate significant differences). G120: end of gastric phase; D60: 60 minutes of duodenal phase; D120: end of duodenal phase. MF: myofibrillar; SP: sarcoplasmic; HC: heavy chain; LC: light chain; PK: pyruvate kinase; FBA: fructose bisphosphate aldolase; PBK: phosphorylase B kinase; NI: not identified; ND: not digested, AA: aminoacids; n.s.: not significant; C-NO_2: salami with sodium nitrite, potassium nitrate and with nitrate-reducing microbial starter cultures; C-0: salami containing neither nitrate-reducing microbial starter cultures nor additives (nitrite, polyphenols and ascorbate); SA: salami with nitrate-reducing microbial starter cultures and sodium ascorbate; SMA: salami with nitrate-reducing microbial starter cultures, sodium ascorbate and plant extracts.

Protein source (UNIPROT)	Type	CNO_2				ANOVA	C0				ANOVA
		ND	G120	D60	D120	p value	ND	G120	D60	D120	p value
Actin (P68137)	MF	12.0±0.0a	25.5±6.4a	10.0±4.2a	11.5±0.7a	< 0.05	12.0±0.0b	22.5±0.7a	9.5±2.1b	14.0±1.4b	< 0.05
Myosin I (Q9TV61)	MF	4.0±0.0a	0.5±0.7a	1.5±2.1a	2.0±1.4a	n.s.	4.0±0.0a	2.5±0.7ab	0.5±0.7b	2.5±0.7b	< 0.05
Myosin II (Q9TV63)	MF	1.0±0.0a	0.0±0.0a	0.5±0.7a	0.5±0.7a	n.s.	1.0±0.0a	0.0±0.0a	0.5±0.7a	1.0±0.0a	n.s.
Myosin IV (Q9TV62)	MF	0.0±0.0a	3.0±1.4a	7.0±4.2a	6.5±2.1a	n.s.	0.0±0.0b	3.5±0.7b	4.5±2.1b	11.0±1.4a	< 0.05
Myosin VI (Q29122)	MF	3.0±0.0a	0.0±0.0b	0.0±0.0b	0.0±0.0b	< 0.05	3.0±0.0a	0.0±0.0b	0.0±0.0b	0.0±0.0b	< 0.05
Myosin VII (P79293)	MF	14.0±0.0a	1.0±0.0a	11.0±4.2a	13.5±6.4a	n.s.	14.0±0.0a	3.0±1.4c	8.5±2.1b	19.0±0.0a	< 0.05
Myosin I LC (A1XQT6)	MF	3.0±0.0a	0.0±0.0b	0.0±0.0b	0.0±0.0b	< 0.05	3.0±0.0a	0.0±0.0b	0.0±0.0b	0.0±0.0b	< 0.05
Myosin VII HC (K7GMH0)	MF	1.0±0.0a	0.0±0.0b	0.0±0.0b	0.0±0.0b	< 0.05	1.0±0.0a	0.0±0.0b	0.0±0.0b	0.0±0.0b	< 0.05
Tropomyosin α-1 chain (F2Z5B6)	MF	0.0±0.0c	0.0±0.0c	5.0±0.0a	3.5±0.7b	< 0.05	0.0±0.0a	0.0±0.0a	3.0±1.4a	4.5±0.7a	< 0.05
Troponin T (Q75NG6)	MF	1.0±0.0a	0.0±0.0b	0.0±0.0b	0.0±0.0b	< 0.05	1.0±0.0a	0.0±0.0b	0.0±0.0b	0.0±0.0b	< 0.05
PK (F1SHL9)	SP	3.0±0.0a	1.0±0.0c	3.0±0.0a	2.0±0.0b	< 0.05	3.0±0.0a	1.0±0.0a	4.0±2.8a	3.5±0.7a	n.s.
Albumin (P008835)	SP	1.0±0.0a	0.5±0.7a	0.5±0.7a	1.0±0.0a	n.s.	1.0±0.0a	0.0±0.0a	0.5±0.7a	0.5±0.7a	n.s.
FBA (F1RJ25)	SP	1.0±0.0a	0.0±0.0b	0.0±0.0b	0.0±0.0b	< 0.05	1.0±0.0a	0.0±0.0b	0.0±0.0b	0.0±0.0b	< 0.05
PBK (F1RP07)	SP	1.0±0.0a	0.0±0.0b	0.0±0.0b	0.0±0.0b	< 0.05	1.0±0.0a	0.0±0.0b	0.0±0.0b	0.0±0.0b	< 0.05
NI		5.0±0.0a	0.0±0.0b	0.0±0.0b	0.0±0.0b	< 0.05	5.0±0.0a	0.0±0.0b	0.0±0.0b	0.0±0.0b	< 0.05
MF peptides		39.0±0.0a	30.0±5.7a	35.0±15.6a	37.5±6.4a	n.s.	39.0±0.0a	31.5±3.5a	26.5±9.2b	52.0±4.2a	n.s.
SP peptides		6.0±0.0a	1.5±0.7b	3.5±0.7b	3.0±0.0b	< 0.05	6.0±0.0a	1.0±0.0b	4.5±2.1ab	4.0±0.0ab	< 0.05
Total peptides		50.0±0.0a	31.5±4.9a	38.5±16.3a	40.5±6.4a	n.s.	50.0±0.0ab	32.5±3.5bc	31.0±7.1c	56.0±4.2a	< 0.05
Average peptides lengths (in AA)		11.0±0.0a	12.0±0.0a	6.0±0.0b	6.0±0.0b	< 0.05	11.0±0.0a	12.5±0.7a	6.0±0.0b	6.0±0.0b	< 0.05

Protein source (UNIPROT)	Type	SA				ANOVA	SMA				ANOVA
		ND	G120	D60	D120	p value	ND	G120	D60	D120	p value
Actin (P68137)	MF	12.0±0.0b	23.5±3.5a	10.0±1.4b	11.0±1.4b	< 0.05	12.0±0.0b	23.0±1.4a	12.5±2.1b	14.0±0.0b	< 0.05
Myosin I (Q9TV61)	MF	4.0±0.0a	0.5±0.7a	1.0±1.4a	1.0±1.4a	n.s.	4.0±0.0a	2.0±0.0a	1.0±1.4a	1.5±2.1a	n.s.
Myosin II (Q9TV63)	MF	1.0±0.0a	0.5±0.7a	0.5±0.7a	1.0±0.0a	n.s.	1.0±0.0a	0.0±0.0b	1.0±0.0a	1.0±0.0a	< 0.05
Myosin IV (Q9TV62)	MF	0.0±0.0c	2.5±0.7bc	7.0±0.0a	5.0±1.4ab	< 0.05	0.0±0.0a	2.5±0.7a	6.0±1.4a	8.5±4.9a	n.s.
Myosin VI (Q29122)	MF	3.0±0.0a	0.0±0.0b	0.0±0.0b	0.0±0.0b	< 0.05	3.0±0.0a	0.0±0.0b	0.0±0.0b	0.0±0.0b	< 0.05
Myosin VII (P79293)	MF	14.0±0.0ab	0.5±0.7b	11.5±6.4ab	15.5±3.5ab	< 0.05	14.0±0.0a	1.5±0.7b	12.0±0.0a	15.0±4.2a	< 0.05
Myosin I LC (A1XQT6)	MF	3.0±0.0a	0.0±0.0b	0.0±0.0b	0.0±0.0b	< 0.05	3.0±0.0a	0.0±0.0b	0.0±0.0b	0.0±0.0b	< 0.05
Myosin VII HC (K7GMH0)	MF	1.0±0.0a	0.0±0.0b	0.0±0.0b	0.0±0.0b	< 0.05	1.0±0.0a	0.0±0.0b	0.0±0.0b	0.0±0.0b	< 0.05
Tropomyosin α-1 chain (F2Z5B6)	MF	0.0±0.0a	0.0±0.0a	3.0±2.8a	4.5±2.1a	n.s.	0.0±0.0b	0.0±0.0b	4.0±1.4a	3.5±0.7a	< 0.05
Troponin T (Q75NG6)	MF	1.0±0.0a	0.0±0.0b	0.0±0.0b	0.0±0.0b	< 0.05	1.0±0.0a	0.0±0.0b	0.0±0.0b	0.0±0.0b	< 0.05
PK (F1SHL9)	SP	3.0±0.0a	1.5±0.7a	1.0±0.0a	4.5±2.1a	n.s.	3.0±0.0a	3.0±1.4a	4.0±1.4a	4.0±1.4a	n.s.
Albumin (P008835)	SP	1.0±0.0a	0.5±0.7a	1.0±0.0a	0.5±0.7a	n.s.	1.0±0.0a	1.0±0.0a	0.5±0.7a	0.5±0.7a	n.s.
FBA (F1RJ25)	SP	1.0±0.0a	0.0±0.0b	0.0±0.0b	0.0±0.0b	< 0.05	1.0±0.0a	0.0±0.0b	0.0±0.0b	0.0±0.0b	< 0.05
PBK (F1RP07)	SP	1.0±0.0a	0.0±0.0b	0.0±0.0b	0.0±0.0b	< 0.05	1.0±0.0a	0.0±0.0b	0.0±0.0b	0.0±0.0b	< 0.05
NI		5.0±0.0a	0.0±0.0b	0.0±0.0b	0.0±0.0b	< 0.05	5.0±0.0a	0.0±0.0b	0.0±0.0b	0.0±0.0b	< 0.05
MF peptides		39.0±0.0a	27.5±2.1a	33.0±12.7a	38.0±9.9a	n.s.	39.0±0.0a	29.0±0.0a	36.5±2.1a	43.5±12.0a	n.s.
SP peptides		6.0±0.0a	2.0±0.0a	2.0±0.0a	5.5±2.1a	< 0.05	6.0±0.0a	4.0±1.4a	4.5±0.7a	4.5±0.0a	n.s.
Total peptides		50.0±0.0a	29.5±2.1a	35.0±12.7a	43.5±7.8a	n.s.	50.0±0.0a	33.0±1.4a	41.0±1.4a	48.0±11.3a	n.s.
Average peptides lengths (in AA)		11.0±0.0a	11.0±0.0a	6.0±0.0b	6.0±0.0b	< 0.05	11.0±0.0a	11.5±0.7a	6.0±0.0b	6.0±0.0b	< 0.05

Bioinformatic analysis

The bioactive sequences detected in undigested salami and at different time points of in vitro digestion, their semiquantitative content, and their presumed biological activity are reported in Table 3. No peptides with a documented bioactive sequence were found in undigested salami. Two bioactive sequences (AGDDAPRAVF and FQPSF) were detected in all formulation except SA (containing only FQPSF) at the end of the gastric phase, but were further hydrolyzed during the duodenal phase. Two bioactive sequences were detected in all formulations in the middle and end of duodenal digestion. Among them, at D60, AGDDAPR was more abundant in SA than in other formulations, while at D120, VAPEEHPT was more abundant in SA and CNO_2 (Table S3).

Protein sequence	Protein source	CNO_2 ND	CNO_2 G120	CNO_2 D60	CNO_2 D120	ANOVA p value	C0 ND	C0 G120	C0 D60	C0 D120	ANOVA p value	Reported activity (μM IC_{50})
FQPSF	Actin (P68137)	0.0±0.0b	2.9±0.5a	0.0±0.0b	0.0±0.0b	< 0.05	0.0±0.0b	2.7±0.2a	0.0±0.0b	0.0±0.0b	< 0.05	ACE inhibitor (12.6)
AGDDAPRAVF	Actin (P68137)	0.0±0.0b	2.6±0.3a	0.0±0.0b	0.0±0.0b	< 0.05	0.0±0.0b	2.9±0.1a	0.0±0.0b	0.0±0.0b	< 0.05	Bitterness suppressing (n.r.)
AGDDAPR	Actin (P68137)	0.0±0.0c	0.0±0.0c	2.7±0.0b	3.1±0.2a	< 0.05	0.0±0.0c	0.0±0.0c	3.8±0.0a	2.9±0.3b	< 0.05	Antioxidant (n.r.), ACE (11.9), pancreatic lipase (110.6), and α-amylase (14.7) inhibitor
VAPEEHPT	Actin (P68137)	0.0±0.0b	0.0±0.0b	3.0±0.3a	3.1±0.3a	< 0.05	0.0±0.0c	0.0±0.0c	3.7±0.5a	2.7±0.0b	< 0.05	DPP-IV inhibitor (n.r.)
Protein sequence	**Protein source**	**SA ND**	**SA G120**	**SA D60**	**SA D120**	**ANOVA p value**	**SMA ND**	**SMA G120**	**SMA D60**	**SMA D120**	**ANOVA p value**	**Reported activity (μM IC_{50})**
FQPSF	Actin (P68137)	0.0±0.0b	3.4±0.1a	0.0±0.0b	0.0±0.0b	< 0.05	0.0±0.0b	3.1±0.1a	0.0±0.0b	0.0±0.0b	< 0.05	ACE inhibitor (12.6)
AGDDAPRAVF	Actin (P68137)	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a	n.s.	0.0±0.0b	2.9±0.0a	0.0±0.0b	0.0±0.0b	< 0.05	Bitterness suppressing (n.r.)
AGDDAPR	Actin (P68137)	0.0±0.0c	0.0±0.0c	4.3±0.3a	3.4±0.1b	< 0.05	0.0±0.0c	0.0±0.0c	3.7±0.0a	2.8±0.1b	< 0.05	Antioxidant (n.r.), ACE (11.9), pancreatic lipase (110.6), and α-amylase (14.7) inhibitor
VAPEEHPT	Actin (P68137)	0.0±0.0c	0.0±0.0c	3.6±0.2a	3.2±0.1b	< 0.05	0.0±0.0c	0.0±0.0c	3.1±0.0a	2.7±0.0b	< 0.05	DPP-IV inhibitor (n.r.)

Table 3. Relative bioactive peptide abundance in not digested and digested salami at different digestion time points. Data are means ± SD of not digested or *in vitro* digestion of three independent samples analyzed in duplicate. Statistical analysis was performed by one-way ANOVA with Tukey's post-hoc test comparing each salami before digestion and at the three digestion time points (different letters indicate significant differences). G120: end of gastric phase; D60: 60 minutes of duodenal phase; D120: end of duodenal phase ND: not digested; DPP-IV: dipeptidyl peptidase 4; ACE: angiotensin-converting enzyme; n.r.: not reported; C-NO_2: salami with sodium nitrite, potassium nitrate and with nitrate-reducing microbial starter cultures; C-0: salami containing neither nitrate-reducing microbial starter cultures nor additives (nitrite, polyphenols and ascorbate); SA: salami with nitrate-reducing microbial starter cultures and sodium ascorbate; SMA: salami with nitrate-reducing microbial starter cultures, sodium ascorbate and plant extracts.

Cell proliferation and genotoxicity

The antiproliferative effect of the scalar dilution of the digested salami formulations (D120) is shown in figure 3. To avoid bias due to antiproliferative effect of the digestion fluids, some cells (B) were supplemented with blank digestion samples at the same dilution. The inhibition of cell proliferation by digested salami was concentration dependent. Compared to unsupplemented cells, at the lowest concentration used (dilution 1:200) only C0 showed a significant inhibitory effect; increasing concentration (dilution 1:150), also for SA a significant antiproliferative effect was evident. At the maximum concentration used (dilution 1:100), supplementation of all digested salami formulations caused a decreased in cell proliferation. Blank supplementation caused no effect at any dilution used.

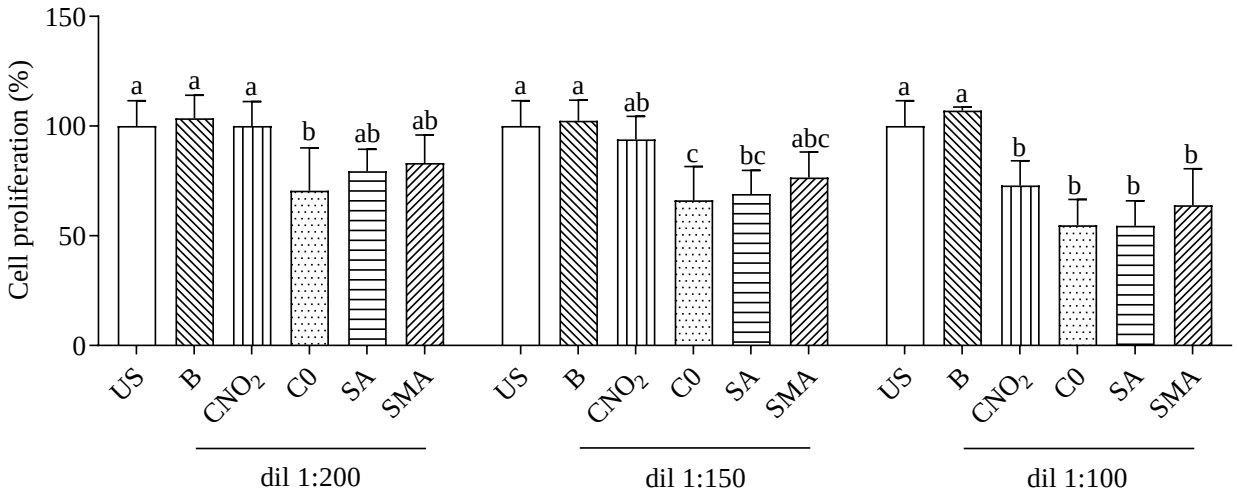


Figure 3. Antiproliferative effect of digested salami formulations. Data are means ± SD of three independent supplementation of in vitro digested, each one analyzed in triplicate. The antiproliferative effect is expressed as percent of cell number in unsupplemented (US) cells (assigned 100%). Statistical analysis was by one-way ANOVA (always $p < 0.05$) with Tukey's post-hoc test comparing US and supplemented cells for each dilution (different letters indicate significant differences). B: "blank" digestion; dil: dilution; C-NO_2: salami with sodium nitrite, potassium nitrate and with nitrate-reducing microbial starter cultures; C-0: salami containing neither nitrate-reducing microbial starter cultures nor additives (nitrite, polyphenols and ascorbate); SA: salami with nitrate-reducing microbial starter cultures and sodium ascorbate; SMA: salami with nitrate-reducing microbial starter cultures, sodium ascorbate and plant extracts.

The genotoxicity of the digested salami formulations (D120) is shown in figure 4. The dilution factor (1:150) for analysis was chosen based on the reduction of about 30% of the proliferation activity. Some cells were supplemented with blank digestion samples (B) (dilution 1:150) or 2 mM ethylmethanesulfonate (EMS). Compared to US cells, blank digestion samples and all salami formulations showed no genotoxicity. Conversely, EMS supplementation had a significant genotoxic effect.

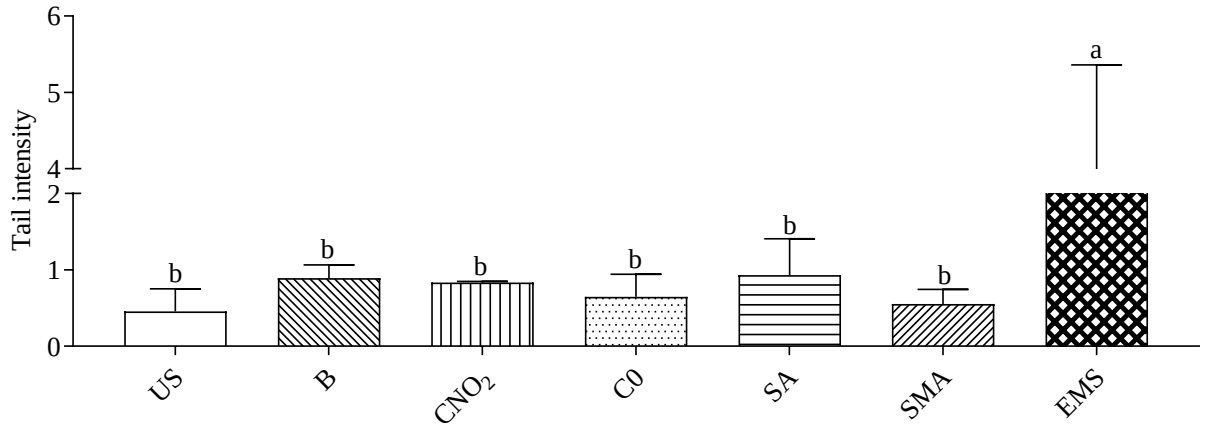


Figure 4. Genotoxic effect of different salami formulations. Data are means ± SD of three independent supplementation of in vitro digested, each one analyzed in triplicate. The genotoxicity is expressed as tail intensity. Statistical analysis was by one-way ANOVA ($p < 0.05$) with Tukey's post-hoc test comparing unsupplemented (US), 2 mM ethylmethanesulfonate (EMS) and digested supplemented cells (different letters indicate significant differences). B: "blank" digestion; C-NO_2: salami with sodium nitrite, potassium nitrate and with nitrate-reducing microbial starter cultures; C-0: salami containing neither nitrate-reducing microbial starter cultures nor additives (nitrite, polyphenols and ascorbate); SA: salami with nitrate-reducing microbial starter cultures and sodium ascorbate; SMA: salami with nitrate-reducing microbial starter cultures, sodium ascorbate and plant extracts.

Discussion

Although nitrates/nitrites are the most widely used food additive in processed meat, their excessive intake is correlated to an increased risk of gastrointestinal cancer [33]. The general concern about nitrate/nitrite dietary intake has stimulated the development of novel approaches to reduce their use in food [34].

In this study, we investigated the effects of replacing synthetic nitrates/nitrites with a combination of natural polyphenols from plant extracts, ascorbate, and ni-trate-reducing microbial starter cultures containing lactic acid bacteria and nitrate-reducing coagulase negative staphylococcaceae. Plant extracts contain various compounds (e.g., phenolics, flavonoids, tannins, and saponins) showing strong antimicrobial activity [35] and preventing lipid peroxidation [36]. Ascorbate plays a part in nitrite reduction and NO formation and it is one of the effective inhibitors in nitrosamine formation [37]. Coagulase-negative staphylococci possess a nitrate-reducing activity that allows the generation of nitrite and NO generation, also favoring the formation of color in meat products without added nitrites/nitrates [38-40]. To allow the replacement of synthetic nitrates/nitrites, a different process was adopted for salami production.

Since formulation and processing can impact on both bioaccessibility and the re-lease/synthesis of bioactive compounds [41,42], and the nutritional value of foods is determined not only by the chemical composition but also by the bioaccessibility of nutrients and the formation of bioactive compounds during digestion [17] we evaluated the bioaccessibility of fatty acids, the release of protein and the formation of peptides during in vitro digestion.

The different formulation/processing slightly modulated the release kinetic of fatty acid from the food matrix, and the bioaccessibility of fatty acids was similar in all salami at the end of in vitro digestion. Although Navarro et al. [43] already showed that nitrite and nitrate per se do not affect the endogenous hydrolysis of the lipids during the fermentation process used to produce dry sausage and Pateiro et al. [44] showed that antioxidants from plant extracts increase the release of fatty acids in Chorizo dry-cured sausage, to the best of our knowledge this is the first study comparing the impact of different formulations and processing on the bioaccessibility of fatty acid during in vitro digestion.

To evaluate protein hydrolysis during in vitro digestion, three complementary spectrophotometric methods were used, which selectively quantify protein concentration with different molecular masses [31,32]. Furthermore, the method based on the NMR spectroscopy also added information on the size and solubility of the digested proteins, being sensitive to all molecules, regardless of the size, which are present in solution. The shape of the signals is informative of the molecular size, the signals be-longing to fragments above 20 kDa being much larger than those belonging to smaller fragment or peptides. The simultaneous use of the four methods allowed to hypothesize that most proteins were hydrolyzed into fragments between 3 KDa and peptides > five amino acids, which are only revealed by the UV assay, at the end of the duodenal phase without any significant difference between the four types of salami. However, a different kinetic of proteolysis was shown by analyzing with OPA and Coomassie blue assays. Indeed, at G120 the OPA assay evidenced a different extent of digestion especially between CNO_2 and SMA, while at D60 the same differences were shown by Coomassie blue. Absorbance at 280 nm is indicative of greater variability in the digestion process within each type of salami, highlighting a different amino acid composition, reflected in the amount of aromatic amino acid detected at 280 nm, between the different fragments released during digestion. Furthermore, differences in the kinetic of proteolysis might be related to the degree of protein oxidation [45], to the modulation of enzymatic activity by phenolic compounds [46], as well as to the food matrix effect [47].

The HR-NMR spectroscopic analysis in the digested sample was conducted to provide qualitative and quantitative information on amino acids and their oligo-/polymers structure such as solute concentration, type of functional groups, and size of the flexibility of the molecules to which the atom is bound [48]. The evident increase in the area of NMR signals during digestion confirmed the formation of hydrolyzed soluble fragments from insoluble proteins, such as myofibrillar ones. It is worth noting here that myofibrillar proteins are not soluble, therefore not detectable by NMR, but only the soluble fragments originate signals visible in the spectra, each amino acid providing signals in different regions of the spectrum, depending on the functional group in which it is bound (aromatic chain, aliphatic moieties or proximity to hydroxyl or charged groups). Furthermore, the simultaneous co-existence of both narrow and wide signals in the spectrum (not shown), confirmed the simultaneous presence of small peptides and larger fragments with a molecular mass compatible with what was

detected by spectrophotometric analysis. This trend was confirmed by the analysis of peptides after in vitro digestion, which gave rise to sequences with different length and molecular weight in the three stages of digestion, ranging from an average number of 11 amino acids in the samples before digestion and at the end of the gastric phase, to an average value of 6 amino acids at the end of the duodenal digestion.

Although no peptides with an already reported bioactivity were identified in un-digested samples, new bioactive sequences were released during digestion. More specifically, a peptide (VAPEEHPT) generated by the cleavage of intact actin and identified as bioactive was released midway through the duodenal phase and found intact at the end of digestion. This peptide, recognized as dipeptidyl peptidase (DPP)-IV inhibitor fragment, was also found by Paolella et al. in dry-cured ham digesta [49], and it is encrypted in the longer sequence LRVAPEEHPTL already identified in beef and trout digesta [50]. These sequences contain the bioactive tripeptide VAP, which has been extensively associated with ACE inhibitory activity [50-52]. VAPEEHPT carries Val and Ala at the N-terminus, a feature playing an important role in ACE-inhibition [53] and associated to an increased antioxidant activity compared to peptides with a prevalence of hydrophilic residues [54]. At D60, the VAPEEHPT sequence was present in the same amount in all salami formulations, while it was significantly more abundant in SA and CNO_2 at the end of the duodenal digestion.

The aromatic peptide AGDDAPRAVF, released during the gastric phase and identified in Spanish, Belgian and Italian dry-fermented sausages [55], was further hydrolyzed during duodenal digestion into the antioxidant, lipase and α-amylase inhibitor sequence AGDDAPR. Both AGDDAPRAVF and AGDDAPR encrypt in their sequence the AG dipeptide, known for its ACE inhibitory activity [55]. At D60, the AGDDAPR sequence was significantly more abundant in SA, while no significant differences were found among the different salami formulations at the end of duodenal digestion (D120).

It should be noted that all bioactive sequences detected contained proline, a feature related to increased resistance to gastrointestinal enzymes and which requires the action of proline-specific peptidases [56].

Since nitrates/nitrites are linked to toxic effects in intestinal cells [57-59], the cytotoxicity and genotoxicity of the different formulations were evaluated in HT-29 cell line. To avoid

misleading results, the potential cytotoxicity of digested salami was evaluated prior to genotoxic experiments. As previously reported [60], supplementation of digested samples to cultured cells caused a concentration-dependent cytotoxic effect. Although there is evidence of a connection between nitrate and nitrite intake and a higher relative risk of different types of cancer [4,61], the salami formulation including these additives showed cytotoxicity only at the lower dilution used (1:100), and no genotoxic effect. This could be explained by the lack of conversion of nitrates to ammonia by enteric bacteria, which normally accounts in vivo. Indeed, several enteric bacteria have shown the ability of catalytic reduction of nitrates to ammonia via nitrites during dissimilatory respiration [62], and chronic exposure to ammonia has been associated with oxidative stress, inflammation, and disbalance of microtubule activity and nutrient transporters in intestinal cells [63]. Before drawing any conclusion, a platform for co-culture of cell lines with anaerobic probiotic bacteria should be mandatory to study their biological effect in the gut.

Conclusions

Based on current results, the replacement of synthetic nitrites and nitrates with nitrate-reducing microbial starter cultures, along with the addition of ascorbate and natural antioxidants from plant sources, appears to be a promising strategy to develop innovative “clean label” salami. In fact, the innovative formulation/processing did not negatively affect the release of fatty acids and the hydrolysis of proteins during digestion. Further studies are needed to evaluate in depth the organoleptic features of the developed products as well their shelf-life.

References

1. Laskowski, W.; Górska-Warsewicz, H.; Kulykovets, O. Meat, Meat Products and Seafood as Sources of Energy and Nutrients in the Average Polish Diet. *Nutrients* 2018, *10*, 1412.

2. Rohrmann, S.; Linseisen, J. Processed meat: the real villain? *Proceedings of the Nutrition Society* 2016, *75*, 233-241, doi:10.1017/S0029665115004255.

3. Crowe, W.; Elliott, C.T.; Green, B.D. A Review of the In Vivo Evidence Investigating the Role of Nitrite Exposure from Processed Meat Consumption in the Development of Colorectal Cancer. *Nutrients* 2019, *11*, 2673.

4. Karwowska, M.; Kononiuk, A. Nitrates/Nitrites in Food—Risk for Nitrosative Stress and Benefits. *Antioxidants* 2020, *9*, 241.

5. Jo, K.; Lee, S.; Yong, H.I.; Choi, Y.-S.; Jung, S. Nitrite sources for cured meat products. *LWT* 2020, *129*, 109583, doi:https://doi.org/10.1016/j.lwt.2020.109583.

6. Balaraman, G.; Sundaram, J.; Mari, A.; Krishnan, P.; Salam, S.; Subramaniam, N.; Sirajduddin, I.; Thiruvengadam, D. Farnesol alleviates diethyl nitrosamine induced inflammation and protects experimental rat hepatocellular carcinoma. *Environmental Toxicology* 2021, *36*, 2467-2474, doi:https://doi.org/10.1002/tox.23359.

7. Kalaycıoğlu, Z.; Erim, F.B. Nitrate and Nitrites in Foods: Worldwide Regional Distribution in View of Their Risks and Benefits. *Journal of Agricultural and Food Chemistry* 2019, *67*, 7205-7222, doi:10.1021/acs.jafc.9b01194.

8. Zhao, C.; Zhang, H.; Zhou, J.; Lu, Q.; Zhang, Y.; Yu, X.; Wang, S.; Liu, R.; Pu, Y.; Yin, L. Metabolomics-based molecular signatures reveal the toxic effect of co-exposure to nitrosamines in drinking water. *Environmental Research* 2022, *204*, 111997, doi:https://doi.org/10.1016/j.envres.2021.111997.

9. Asioli, D.; Aschemann-Witzel, J.; Caputo, V.; Vecchio, R.; Annunziata, A.; Næs, T.; Varela, P. Making sense of the “clean label” trends: A review of consumer food choice

behavior and discussion of industry implications. *Food Research International* 2017, *99*, 58-71, doi:https://doi.org/10.1016/j.foodres.2017.07.022.

10. Bedale, W.; Sindelar, J.J.; Milkowski, A.L. Dietary nitrate and nitrite: Benefits, risks, and evolving perceptions. *Meat Sci* 2016, *120*, 85-92, doi:10.1016/j.meatsci.2016.03.009.

11. Fraqueza, M.J.; Laranjo, M.; Elias, M.; Patarata, L. Microbiological hazards associated with salt and nitrite reduction in cured meat products: control strategies based on antimicrobial effect of natural ingredients and protective microbiota. *Current Opinion in Food Science* 2021, *38*, 32-39, doi:https://doi.org/10.1016/j.cofs.2020.10.027.

12. Munekata, P.E.S.; Pateiro, M.; Domínguez, R.; Pollonio, M.A.R.; Sepúlveda, N.; Andres, S.C.; Reyes, J.; Santos, E.M.; Lorenzo, J.M. Beta vulgaris as a Natural Nitrate Source for Meat Products: A Review. *Foods* 2021, *10*, 2094.

13. Jaiswal, A.; Jyothi Lakshmi, A. Maximising the bioaccessibility of iron and zinc of a complementary food mix through multiple strategies. *Food Chemistry* 2022, *372*, 131286, doi:https://doi.org/10.1016/j.foodchem.2021.131286.

14. Mashitoa, F.M.; Akinola, S.A.; Manhevi, V.E.; Garcia, C.; Remize, F.; Slabbert, R.M.; Sivakumar, D. Influence of Fermentation of Pasteurised Papaya Puree with Different Lactic Acid Bacterial Strains on Quality and Bioaccessibility of Phenolic Compounds during In Vitro Digestion. *Foods* 2021, *10*, 962.

15. Santos, D.I.; Saraiva, J.M.A.; Vicente, A.A.; Moldão-Martins, M. 2 - Methods for determining bioavailability and bioaccessibility of bioactive compounds and nutrients. In *Innovative Thermal and Non-Thermal Processing, Bioaccessibility and Bioavailability of Nutrients and Bioactive Compounds*, Barba, F.J., Saraiva, J.M.A., Cravotto, G., Lorenzo, J.M., Eds.; Woodhead Publishing: 2019; pp. 23-54.

16. Shahidi, F.; Pan, Y. Influence of food matrix and food processing on the chemical interaction and bioaccessibility of dietary phytochemicals: A review. *Critical Reviews*

in Food Science and Nutrition 2022, *62*, 6421-6445, doi:10.1080/10408398.2021.1901650.

17. Di Nunzio, M.; Loffi, C.; Chiarello, E.; Dellafiora, L.; Picone, G.; Antonelli, G.; Di Gregorio, C.; Capozzi, F.; Tedeschi, T.; Galaverna, G.; et al. Impact of a Shorter Brine Soaking Time on Nutrient Bioaccessibility and Peptide Formation in 30-Months-Ripened Parmigiano Reggiano Cheese. *Molecules* 2022, *27*, 664.

18. Ponce de León-Rodríguez, M.d.C.; Guyot, J.-P.; Laurent-Babot, C. Intestinal in vitro cell culture models and their potential to study the effect of food components on intestinal inflammation. *Critical Reviews in Food Science and Nutrition* 2019, *59*, 3648-3666, doi:10.1080/10408398.2018.1506734.

19. Saccani, G.; Bergamaschi, M.; Schivazappa, C.; Cirlini, M.; Galaverna, G.; Virgili, R. Evaluation of the antioxidant effect of a phytocomplex addition in clean label pork salami enriched in n-3 PUFA. *Food Chemistry* 2023, *399*, 133963, doi:https://doi.org/10.1016/j.foodchem.2022.133963.

20. Minekus, M.; Alminger, M.; Alvito, P.; Ballance, S.; Bohn, T.; Bourlieu, C.; Carrière, F.; Boutrou, R.; Corredig, M.; Dupont, D.; et al. A standardised static in vitro digestion method suitable for food – an international consensus. *Food & Function* 2014, *5*, 1113-1124, doi:10.1039/C3FO60702J.

21. Folch, J.; Lees, M.; Stanley, G.H.S. A SIMPLE METHOD FOR THE ISOLATION AND PURIFICATION OF TOTAL LIPIDES FROM ANIMAL TISSUES. *Journal of Biological Chemistry* 1957, *226*, 497-509, doi:https://doi.org/10.1016/S0021-9258(18)64849-5.

22. Stoffel, W.; Chu, F.; Ahrens, E.H. Analysis of Long-Chain Fatty Acids by Gas-Liquid Chromatography. *Analytical Chemistry* 1959, *31*, 307-308, doi:10.1021/ac60146a047.

23. Dima, C.; Assadpour, E.; Dima, S.; Jafari, S.M. Bioavailability and bioaccessibility of food bioactive compounds; overview and assessment by in vitro methods.

Comprehensive Reviews in Food Science and Food Safety 2020, *19*, 2862-2884, doi:https://doi.org/10.1111/1541-4337.12623.

24. Bradstreet, R.B. Kjeldahl Method for Organic Nitrogen. *Analytical Chemistry* 1954, *26*, 185-187, doi:10.1021/ac60085a028.

25. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976, *72*, 248-254, doi:10.1006/abio.1976.9999.

26. Church, F.C.; Porter, D.H.; Catignani, G.L.; Swaisgood, H.E. An o-phthalaldehyde spectrophotometric assay for proteinases. *Anal Biochem* 1985, *146*, 343-348, doi:10.1016/0003-2697(85)90549-4.

27. Minkiewicz, P.; Iwaniak, A.; Darewicz, M. BIOPEP-UWM Database of Bioactive Peptides: Current Opportunities. *International Journal of Molecular Sciences* 2019, *20*, 5978.

28. Kumar, R.; Chaudhary, K.; Sharma, M.; Nagpal, G.; Chauhan, J.S.; Singh, S.; Gautam, A.; Raghava, G.P.S. AHTPDB: a comprehensive platform for analysis and presentation of antihypertensive peptides. *Nucleic Acids Research* 2014, *43*, D956-D962, doi:10.1093/nar/gku1141.

29. Cordelli, E.; Bignami, M.; Pacchierotti, F. Comet assay: a versatile but complex tool in genotoxicity testing. *Toxicology Research* 2021, *10*, 68-78, doi:10.1093/toxres/tfaa093.

30. Herranz, B.; Ordóñez, J.A.; De La Hoz, L.; Hierro, E.; Soto, E.; Cambero, M.I. Fatty acid composition of salami from different countries and their nutritional implications. *International Journal of Food Sciences and Nutrition* 2008, *59*, 607-618, doi:10.1080/09513590701550270.

31. Egger, L.; Schlegel, P.; Baumann, C.; Stoffers, H.; Guggisberg, D.; Brügger, C.; Dürr, D.; Stoll, P.; Vergères, G.; Portmann, R. Physiological comparability of the harmonized INFOGEST in vitro digestion method to in vivo pig digestion. *Food*

Research International 2017, *102*, 567-574, doi:https://doi.org/10.1016/j.foodres.2017.09.047.

32. Urbinati, E.; Di Nunzio, M.; Picone, G.; Chiarello, E.; Bordoni, A.; Capozzi, F. The Effect of Balsamic Vinegar Dressing on Protein and Carbohydrate Digestibility is Dependent on the Food Matrix. *Foods* 2021, *10*, 411.

33. Kotopoulou, S.; Zampelas, A.; Magriplis, E. Dietary nitrate and nitrite and human health: a narrative review by intake source. *Nutr Rev* 2022, *80*, 762-773, doi:10.1093/nutrit/nuab113.

34. Stoica, M.; Antohi, V.M.; Alexe, P.; Ivan, A.S.; Stanciu, S.; Stoica, D.; Zlati, M.L.; Stuparu-Cretu, M. New Strategies for the Total/Partial Replacement of Conventional Sodium Nitrite in Meat Products: a Review. *Food and Bioprocess Technology* 2022, *15*, 514-538, doi:10.1007/s11947-021-02744-6.

35. Kramer, B.; Thielmann, J.; Hickisch, A.; Muranyi, P.; Wunderlich, J.; Hauser, C. Antimicrobial activity of hop extracts against foodborne pathogens for meat applications. *Journal of Applied Microbiology* 2015, *118*, 648-657, doi:https://doi.org/10.1111/jam.12717.

36. Burri, S.C.M.; Ekholm, A.; Bleive, U.; Püssa, T.; Jensen, M.; Hellström, J.; Mäkinen, S.; Korpinen, R.; Mattila, P.H.; Radenkovs, V.; et al. Lipid oxidation inhibition capacity of plant extracts and powders in a processed meat model system. *Meat Science* 2020, *162*, 108033, doi:https://doi.org/10.1016/j.meatsci.2019.108033.

37. Sallan, S.; Kaban, G.; Şişik Oğraş, Ş.; Çelik, M.; Kaya, M. Nitrosamine formation in a semi-dry fermented sausage: Effects of nitrite, ascorbate and starter culture and role of cooking. *Meat Science* 2020, *159*, 107917, doi:https://doi.org/10.1016/j.meatsci.2019.107917.

38. Gøtterup, J.; Olsen, K.; Knøchel, S.; Tjener, K.; Stahnke, L.H.; Møller, J.K.S. Colour formation in fermented sausages by meat-associated staphylococci with different

nitrite- and nitrate-reductase activities. *Meat Science* 2008, *78*, 492-501, doi:https://doi.org/10.1016/j.meatsci.2007.07.023.

39. Sánchez Mainar, M.; Leroy, F. Process-driven bacterial community dynamics are key to cured meat colour formation by coagulase-negative staphylococci via nitrate reductase or nitric oxide synthase activities. *International Journal of Food Microbiology* 2015, *212*, 60-66, doi:https://doi.org/10.1016/j.ijfoodmicro.2015.03.009.

40. Sánchez Mainar, M.; Stavropoulou, D.A.; Leroy, F. Exploring the metabolic heterogeneity of coagulase-negative staphylococci to improve the quality and safety of fermented meats: a review. *International Journal of Food Microbiology* 2017, *247*, 24-37, doi:https://doi.org/10.1016/j.ijfoodmicro.2016.05.021.

41. Lavelli, V.; D'Incecco, P.; Pellegrino, L. Vitamin D Incorporation in Foods: Formulation Strategies, Stability, and Bioaccessibility as Affected by the Food Matrix. *Foods* 2021, *10*, 1989.

42. Rousseau, S.; Kyomugasho, C.; Celus, M.; Hendrickx, M.E.G.; Grauwet, T. Barriers impairing mineral bioaccessibility and bioavailability in plant-based foods and the perspectives for food processing. *Critical Reviews in Food Science and Nutrition* 2020, *60*, 826-843, doi:10.1080/10408398.2018.1552243.

43. Navarro, J.L.; Nadal, M.I.; Nieto, P.; Flores, J. Effect of nitrate and nitrite curing salts on lipolysis in dry sausages produced using a rapid fermentation process. *Zeitschrift für Lebensmitteluntersuchung und -Forschung A* 1998, *206*, 217-221, doi:10.1007/s002170050246.

44. Pateiro, M.; Bermúdez, R.; Lorenzo, J.M.; Franco, D. Effect of Addition of Natural Antioxidants on the Shelf-Life of "Chorizo", a Spanish Dry-Cured Sausage. *Antioxidants* 2015, *4*, 42-67.

45. Rysman, T.; Van Hecke, T.; Van Poucke, C.; De Smet, S.; Van Royen, G. Protein oxidation and proteolysis during storage and in vitro digestion of pork and beef patties.

Food Chemistry 2016, *209*, 177-184, doi:https://doi.org/10.1016/j.foodchem.2016.04.027.

46. Cirkovic Velickovic, T.D.; Stanic-Vucinic, D.J. The Role of Dietary Phenolic Compounds in Protein Digestion and Processing Technologies to Improve Their Antinutritive Properties. *Comprehensive Reviews in Food Science and Food Safety* 2018, *17*, 82-103, doi:https://doi.org/10.1111/1541-4337.12320.

47. Donadio, G.; Santoro, V.; Dal Piaz, F.; De Tommasi, N. Food Matrices Affect the Peptides Produced during the Digestion of Arthrospira platensis-Based Functional Aliments. *Nutrients* 2021, *13*, doi:10.3390/nu13113919.

48. Di Nunzio, M.; Picone, G.; Pasini, F.; Caboni, M.F.; Gianotti, A.; Bordoni, A.; Capozzi, F. Olive oil industry by-products. Effects of a polyphenol-rich extract on the metabolome and response to inflammation in cultured intestinal cell. *Food Research International* 2018, *113*, 392-400, doi:https://doi.org/10.1016/j.foodres.2018.07.025.

49. Paolella, S.; Falavigna, C.; Faccini, A.; Virgili, R.; Sforza, S.; Dall'Asta, C.; Dossena, A.; Galaverna, G. Effect of dry-cured ham maturation time on simulated gastrointestinal digestion: Characterization of the released peptide fraction. *Food Research International* 2015, *67*, 136-144, doi:https://doi.org/10.1016/j.foodres.2014.10.026.

50. Bauchart, C.; Morzel, M.; Chambon, C.; Mirand, P.P.; Reynès, C.; Buffière, C.; Rémond, D. Peptides reproducibly released by in vivo digestion of beef meat and trout flesh in pigs. *British Journal of Nutrition* 2007, *98*, 1187-1195, doi:10.1017/S0007114507761810.

51. Dziuba, J.; Minkiewicz, P.; Nałecz, D.; Iwaniak, A. Database of biologically active peptide sequences. *Food / Nahrung* 1999, *43*, 190-195, doi:https://doi.org/10.1002/(SICI)1521-3803(19990601)43:3<190::AID-FOOD190>3.0.CO;2-A.

52. Escudero, E.; Sentandreu, M.A.; Arihara, K.; Toldrá, F. Angiotensin I-Converting Enzyme Inhibitory Peptides Generated from in Vitro Gastrointestinal Digestion of Pork Meat. *Journal of Agricultural and Food Chemistry* 2010, *58*, 2895-2901, doi:10.1021/jf904204n.

53. Li, G.-H.; Le, G.-W.; Shi, Y.-H.; Shrestha, S. Angiotensin I–converting enzyme inhibitory peptides derived from food proteins and their physiological and pharmacological effects. *Nutrition Research* 2004, *24*, 469-486, doi:https://doi.org/10.1016/j.nutres.2003.10.014.

54. Chai, K.F.; Voo, A.Y.H.; Chen, W.N. Bioactive peptides from food fermentation: A comprehensive review of their sources, bioactivities, applications, and future development. *Comprehensive Reviews in Food Science and Food Safety* 2020, *19*, 3825-3885, doi:https://doi.org/10.1111/1541-4337.12651.

55. Gallego, M.; Mora, L.; Escudero, E.; Toldrá, F. Bioactive peptides and free amino acids profiles in different types of European dry-fermented sausages. *International Journal of Food Microbiology* 2018, *276*, 71-78, doi:https://doi.org/10.1016/j.ijfoodmicro.2018.04.009.

56. Simpson, D.J. Proteolytic degradation of cereal prolamins—the problem with proline. *Plant Science* 2001, *161*, 825-838, doi:https://doi.org/10.1016/S0168-9452(01)00482-4.

57. Ansari, F.A.; Ali, S.N.; Arif, H.; Khan, A.A.; Mahmood, R. Acute oral dose of sodium nitrite induces redox imbalance, DNA damage, metabolic and histological changes in rat intestine. *PLOS ONE* 2017, *12*, e0175196, doi:10.1371/journal.pone.0175196.

58. Grudziński, I. Studies on the mechanism of the toxic action of sodium nitrite on intestinal absorption in rats. *Archives of Environmental Contamination and Toxicology* 1991, *21*, 475-479, doi:10.1007/BF01060373.

59. Yu, J.; Wang, Y.; Xiao, Y.; Li, X.; Xu, X.; Zhao, H.; Wu, L.; Li, J. Effects of chronic nitrate exposure on the intestinal morphology, immune status, barrier function, and

microbiota of juvenile turbot (Scophthalmus maximus). *Ecotoxicology and Environmental Safety* 2021, *207*, 111287, doi:https://doi.org/10.1016/j.ecoenv.2020.111287.

60. Di Nunzio, M.; Picone, G.; Pasini, F.; Chiarello, E.; Caboni, M.F.; Capozzi, F.; Gianotti, A.; Bordoni, A. Olive oil by-product as functional ingredient in bakery products. Influence of processing and evaluation of biological effects. *Food Research International* 2020, *131*, 108940, doi:https://doi.org/10.1016/j.foodres.2019.108940.

61. Hosseini, F.; Majdi, M.; Naghshi, S.; Sheikhhossein, F.; Djafarian, K.; Shab-Bidar, S. Nitrate-nitrite exposure through drinking water and diet and risk of colorectal cancer: A systematic review and meta-analysis of observational studies. *Clin Nutr* 2021, *40*, 3073-3081, doi:10.1016/j.clnu.2020.11.010.

62. Tiso, M.; Schechter, A.N. Nitrate reduction to nitrite, nitric oxide and ammonia by gut bacteria under physiological conditions. *PLoS One* 2015, *10*, e0119712, doi:10.1371/journal.pone.0119712.

63. Wang, S.; Li, X.; Wang, W.; Zhang, H.; Xu, S. Application of transcriptome analysis: Oxidative stress, inflammation and microtubule activity disorder caused by ammonia exposure may be the primary factors of intestinal microvilli deficiency in chicken. *Science of The Total Environment* 2019, *696*, 134035, doi:https://doi.org/10.1016/j.scitotenv.2019.134035.

Supplementary

	CNO_2	C0	SA	SMA
14:0	1.6±0.0a	1.5±0.0b	1.6±0.0a	1.6±0.0a
16:0	25.4±0.5a	24.9±0.2a	25.4±0.4a	24.9±0.2a
16:1 n-7	2.5±0.02a	2.5±0.0a	2.4±0.1a	2.4±0.0a
18:0	13.8±0.2a	13.5±0.1a	13.8±0.20a	13.5±0.1a
18:1 n-9	43.4±0.0a	43.5±0.3a	43.9±1.0a	43.5±0.5a
18:2 n-6	11.2±0.5a	11.9±0.3a	10.6±1.5a	11.6±0.2a
18:3 n-3	0.5±0.0b	0.6±0.0a	0.6±0.03a	0.6±0.0a
20:1 n-9	1.0±0.0a	0.9±0.0b	0.9±0.02b	1.0±0.0a
20:4 n-6	0.5±0.1a	0.6±0.1a	0.6±0.09a	0.7±0.0a

Table S1. Fatty acid methyl esters of undigested salami. Data are means ± SD of three different samples each analyzed in duplicate and are expressed as mol/100mol. Statistical analysis was by the one-way ANOVA (14:0, 18:3 n-3, and 20:1 n-9: $p < 0.05$) with Tukey's post-hoc test (different letters indicate significant differences). C-NO_2: salami with sodium nitrite, potassium nitrate and with nitrate-reducing microbial starter cultures; C-0: salami containing neither nitrate-reducing microbial starter cultures nor additives (nitrite, polyphenols and ascorbate); SA: salami with nitrate-reducing microbial starter cultures and sodium ascorbate; SMA: salami with nitrate-reducing microbial starter cultures, sodium ascorbate and plant extracts.

	G120				D60				D120			
	CNO_2	**C0**	**SA**	**SMA**	**CNO_2**	**C0**	**SA**	**SMA**	**CNO_2**	**C0**	**SA**	**SMA**
14:0	0.5±0.7a	0.3±0.2a	0.4±0.1a	0.7± 0.4a	30.6±4.2a	38.0±6.5a	37.7±1.8a	38.6±2.4a	37.3±0.2a	43.6±6.4a	42.1±3.1a	40.0±0.6a
16:0	0.8±0.4a	0.4±0.2a	1.0±0.1a	0.9±0.5a	29.2±3.0a	39.2±8.3a	32.3±1.1a	40.2±2.2a	37.4±2.7a	45.6±7.9a	43.9±4.2a	37.5±0.0a
16:1 n-7	0.9±0.5a	0.0±0.0a	0.0±0.0a	0.1±0.1a	27.0±4.7a	32.6±5.7a	29.8±3.1a	35.0±2.8a	31.7±0.3a	37.1±5.7a	37.9±4.8a	38.2±2.5a
18:0	0.9±0.6a	0.6±0.1a	1.2±0.1a	1.0±0.3a	28.2±1.2b	40.2±8.9ab	30.8±1.0ab	40.3±1.8a	38.4±8.5a	47.3±10.8a	43.5±4.6a	30.4±2.2a
18:1 n-9	0.6±0.4a	0.2±0.1a	0.8±0.0a	0.6±0.5a	31.0±4.7a	37.2±7.9a	33.0±2.1a	40.0±2.4a	37.5±0.6a	43.1±6.6a	42.4±4.0a	41.8±2.8a
18:2 n-6	0.6±0.5a	0.3±0.1a	0.4±0.1a	0.6±0.4a	36.6±3.8a	39.9±8.7a	42.4±5.8a	44.0±3.5a	45.2±3.0a	47.2±6.3a	54.4±6.2a	48.1±2.1a
18:3 n-3	0.0±0.0a	0.6±1.1a	0.0±0.0a	0.0±0.0a	40.3±14.0a	38.7±19.7a	41.2±8.6a	44.3±6.7a	45.2±4.5a	51.2±6.1a	51.2±4.1a	49.8±1.1a
20:1 n-9	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a	22.1±7.5a	30.1±7.3a	25.0±2.5a	30.4±0.8a	29.3±0.8a	38.1±5.1a	35.6±5.8a	33.4±3.6a
20:4 n-6	0.0± 0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a	46.3±3.2a	53.0±16.7a	49.5±12.3a	45.0±2.3a	70.3±17.3a	70.0±14.4a	70.3±14.6a	50.8±3.2a
Total	0.6±0.4a	0.3±0.1a	0.8±0.0a	0.7±0.4a	30.6±3.8a	38.3±8.2a	33.3±1.3a	39.9±2.4a	38.3±2.5a	44.7±7.4a	44.1±3.7a	39.8±1.3a

Table S2. Fatty acid bioaccessibility of the different salami formulations at each digestion time. Data are means ± SD of in vitro digestion of three independent samples analyzed in duplicate. Fatty acid bioaccessibility is expressed as % and was calculated as fatty acid methyl ester concentration in digested/fatty acid methyl ester concentration in salami before digestion x 100. Statistical analysis was by the one-way ANOVA (18:0 at D60: $p<0.05$) with Tukey's post-hoc test comparing the different experimental salami at each digestion time (different letters indicate significant differences). G120: end of gastric phase; D60: 60 min of duodenal phase; D120: end of duodenal phase. C-NO_2: salami with sodium nitrite, potassium nitrate and with nitrate-reducing microbial starter cultures; C-0: salami containing neither nitrate-reducing microbial starter cultures nor additives (nitrite, polyphenols and ascorbate); SA: salami with nitrate-reducing microbial starter cultures and sodium ascorbate; SMA: salami with nitrate-reducing microbial starter cultures, sodium ascorbate and plant extracts.

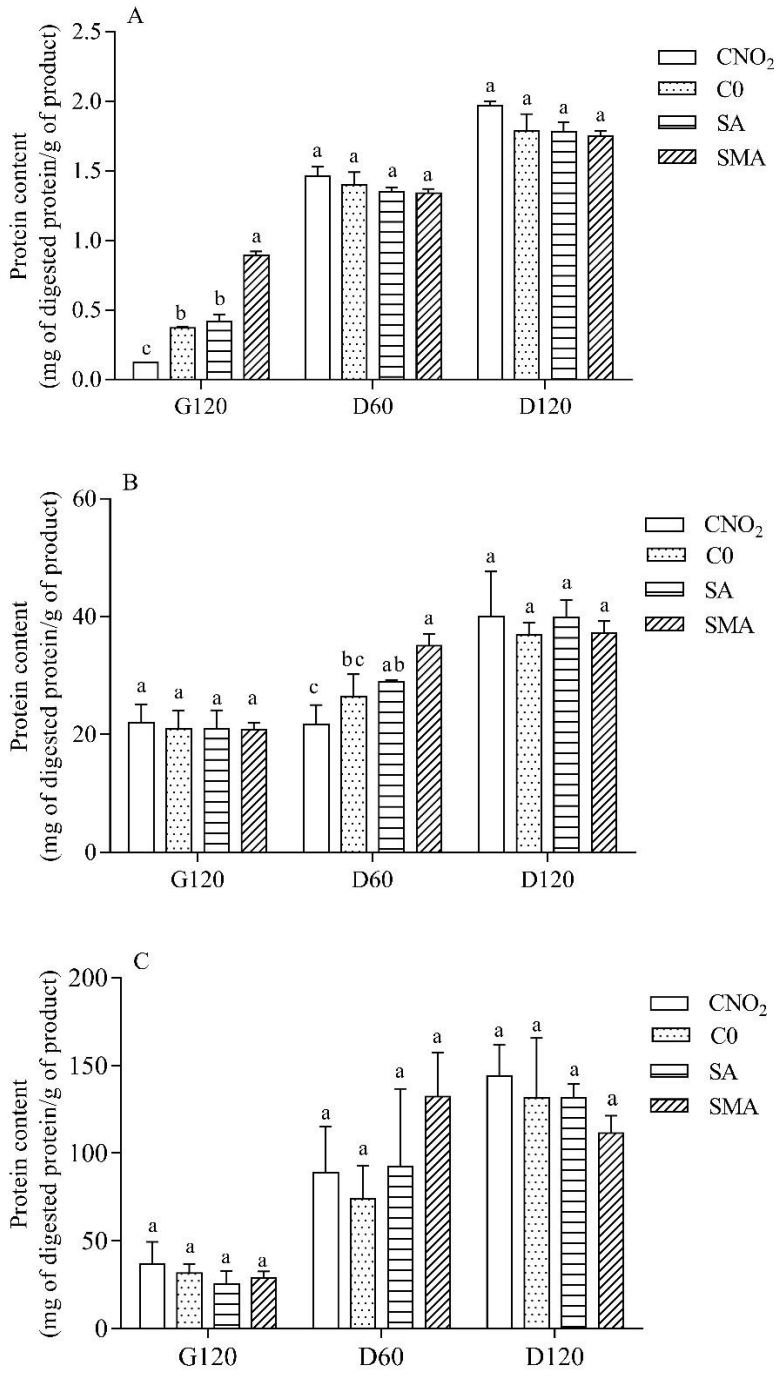


Figure S1. Protein hydrolysys of the different salami formulations at each digestion time. Protein hydrolysis was measured by OPA assay (A), Coomassie assay (B), and by measuring the absorbance at 280 nm (C). Data are means ± SD of *in vitro* digestion of three independent samples analyzed in triplicate. Protein content is expressed as mg of digested protein/g of product. Statistical analysis was by the one-way ANOVA (OPA at G120, and Coomassie at D60 $p < 0.05$) with Tukey's post-hoc test comparing the different formulations at each digestion time (different letters indicate statistical significance). G120: end of gastric phase; D60: 60 min of duodenal phase; D120: end of duodenal phase. C-NO_2: salami with sodium nitrite, potassium nitrate and with nitrate-reducing microbial starter cultures; C-0: salami containing neither nitrate-reducing microbial starter cultures nor additives (nitrite, polyphenols and ascorbate); SA: salami with nitrate-reducing microbial starter cultures and sodium ascorbate; SMA: salami with nitrate-reducing microbial starter cultures, sodium ascorbate and plant extracts.

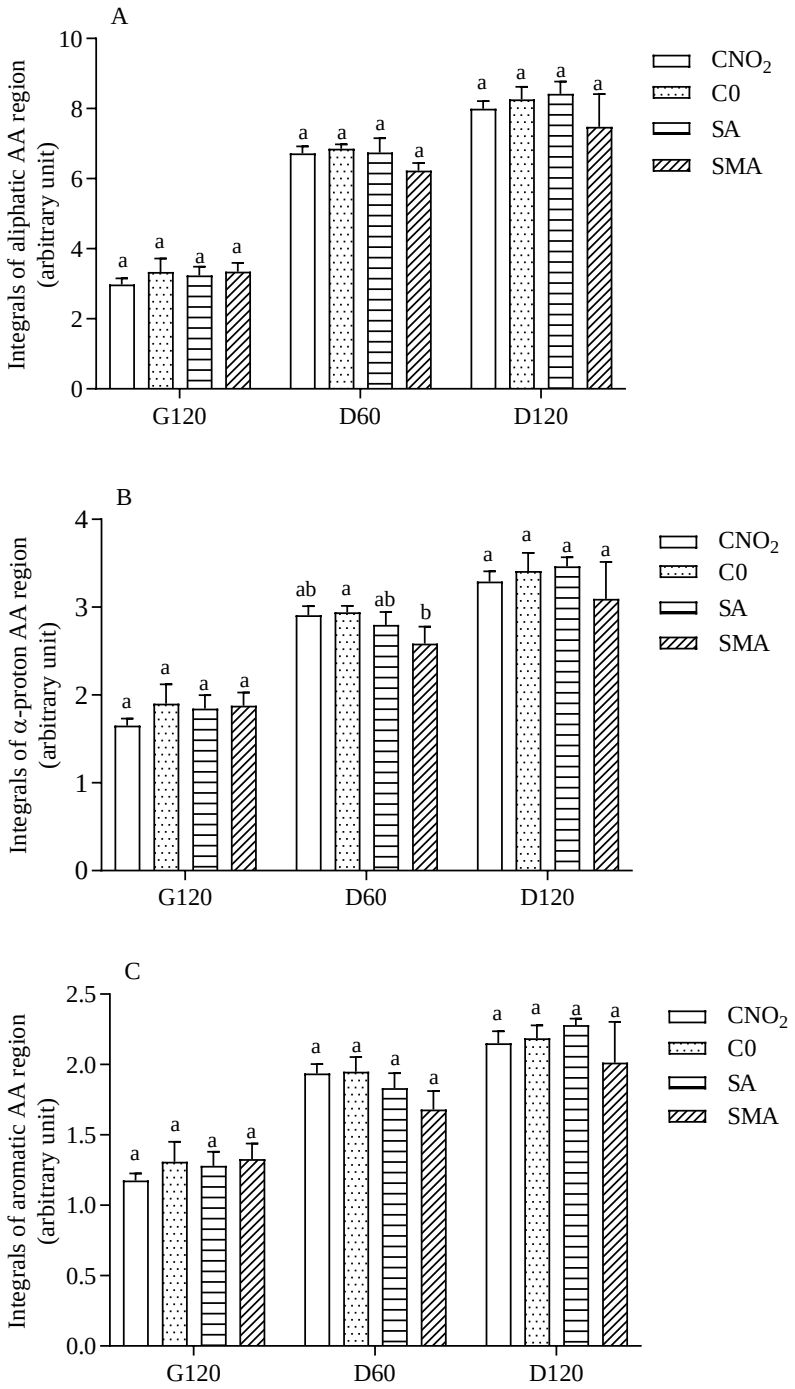


Figure S2. Integral area of aliphatic, α-proton, and aromatic amino acid region of distinct salami at different digestion times. Data are means ± SD of *in vitro* digestion of three independent samples analyzed in duplicate. Integrals of aliphatic (A), α-proton (B), and aromatic (C) amino acid region are expressed as signal area. Statistical analysis was by the one-way ANOVA (B at D60: $p < 0.05$) with Tukey's post-hoc test comparing the different experimental salami in each digestion times (different letters indicate significant differences). G120: end of gastric phase; D60: 60 minutes of duodenal phase; D120: end of duodenal phase; AA: amino acids. C-NO_2: salami with sodium nitrite, potassium nitrate and with nitrate-reducing microbial starter cultures; C-0: salami containing neither nitrate-reducing microbial starter cultures nor additives (nitrite, polyphenols and ascorbate); SA: salami with nitrate-reducing microbial starter cultures and sodium ascorbate; SMA: salami with nitrate-reducing microbial starter cultures, sodium ascorbate and plant extracts.

Peptides sequence	Protein source	ND				ANOVA p value	G120				ANOVA p value	Reported activity (μM IC_{50})
		CNO_2	C0	SA	SMA		CNO_2	C0	SA	SMA		
FQPSF	Actin (P68137)	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a	n.s.	2.9±0.5a	2.7±0.2a	3.4±0.1a	3.1±0.1a	n.s.	ACE inhibitor (12.6)
AGDDAPRAVF	Actin (P68137)	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a	n.s.	2.6±0.3a	2.9±0.1a	0.0±0.0b	2.9±0.0a	< 0.05	Bitterness suppressing (n.r.)
AGDDAPR	Actin (P68137)	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a	n.s.	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a	n.s.	Antioxidant (n.r.); ACE (11.9), pancreatic lipase (110.6), and α-amylase (14.7) inhibitor
VAPEHPT	Actin (P68137)	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a	n.s.	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a	n.s.	DPP-IV inhibitor (n.r.)
Peptides sequence	**Protein source**	**D60**				**ANOVA p value**	**D120**				**ANOVA p value**	**Reported activity (μM IC_{50})**
		CNO_2	C0	SA	SMA		CNO_2	C0	SA	SMA		
FQPSF	Actin (P68137)	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a	n.s.	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a	n.s.	ACE inhibitor (12.6)
AGDDAPRAVF	Actin (P68137)	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a	n.s.	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a	n.s.	Bitterness suppressing (n.r.)
AGDDAPR	Actin (P68137)	2.7±0.0c	3.8±0.0b	4.3±0.3a	3.7±0.0b	< 0.05	3.1±0.2a	2.9±0.3a	3.4±0.1a	2.8±0.1a	n.s.	Antioxidant (n.r.); ACE (11.9), pancreatic lipase (110.6), and α-amylase (14.7) inhibitor
VAPEHPT	Actin (P68137)	3.0±0.3a	3.7±0.5a	3.6±0.2a	3.1±0.0a	n.s.	3.1±0.3a	2.7±0.0b	3.2±0.1a	2.7±0.0b	< 0.05	DPP-IV inhibitor (n.r.)

Table S3. Relative bioactive peptide abundance of salami at different digestion times. Data are means ± SD of not digested or in vitro digestion of three independent samples analyzed in duplicate. Statistical analysis was performed by one-way ANOVA with Tukey's post-hoc test comparing the different formulations before digestion and at each digestion time (different letters indicate significant differences). G120: end of gastric phase; D60: 60 minutes of duodenal phase; D120: end of duodenal phase ND: not digested; DPP-IV: dipeptidyl peptidase 4; ACE: angiotensin-converting enzyme. C-NO_2: salami with sodium nitrite, potassium nitrate and with nitrate-reducing microbial starter cultures; C-0: salami containing neither nitrate-reducing microbial starter cultures nor additives (nitrite, polyphenols and ascorbate); SA: salami with nitrate-reducing microbial starter cultures and sodium ascorbate; SMA: salami with nitrate-reducing microbial starter cultures, sodium ascorbate and plant extracts.

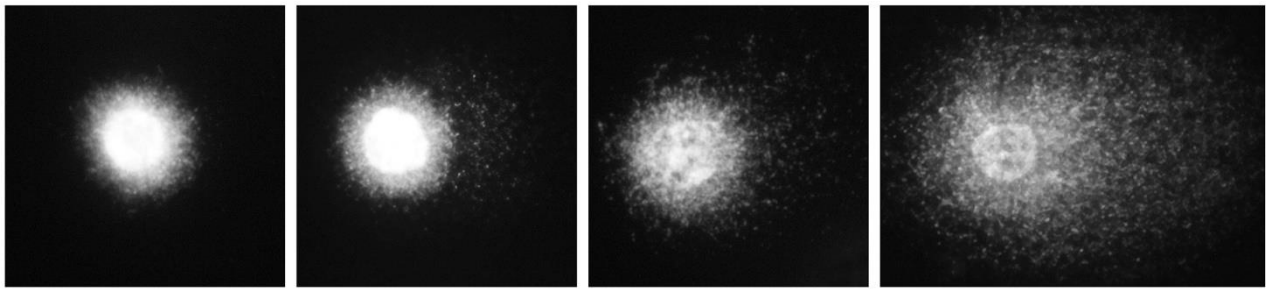


Figure S3. Microscopy images of HT-29 cells were acquired using a Leica DMLS fluorescence microscope (excitation filter BP 515–560 nm, barrier filter LP 580 nm) and an automatic image analysis system (Comet Assay IV—Perceptive Instruments Ltd., Bury St Edmunds, UK). Images provide different levels of DNA damage in HT-29 cancer cells after Comet assay. From left to right we reported an increasing of DNA damage.

CHAPTER-5

Insight on Glucose and Fructose Absorption and Relevance in the Enterocyte Milieu

Already published in *"Nutrients"* 2022, *14*(3), 517; https://doi.org/10.3390/nu14030517

Introduction

Glucose (GLU) and fructose (FRU) are the main monosaccharides in the human diet. FRU and GLU share the same molecular formula ($C_6H_{12}O_6$) and energy value (4 kcal/g), but have different sweetening power and glycaemic index [1], satiating capacity [2], absorption mechanism [3], and metabolism not only once absorbed, but also within the intestinal cell [4]. Sucrose (SUC), a disaccharide composed of GLU and FRU, is one of the highest dietary sources of FRU besides corn/maple syrup, honey, molasses, fruit and fruit juices. GLU is the main catabolic and anabolic cell substrate that controls energy homeostasis in the human body. The classical mechanism for intestinal glucose absorption involves uptake across the apical membrane of enterocytes via sodium-glucose co-transporter 1 (SGLT1). However, it has been recognised that the apical uptake phase has two distinct elements: a saturable, phloridzin-sensitive fraction (SGLT1) and a diffusive component, suggesting that more than one transporter may be involved. Studies have revealed that glucose transporter 2 (GLUT2) is also expressed at the apical membrane of enterocytes during the digestive phase and can contribute significantly to glucose absorption [5]. Release of glucose into the circulation is mediated by GLUT2 located on the basolateral surface of enterocytes [6]. GLUT2 is also responsible for the efflux of FRU, which enters the intestinal cells mainly through GLUT5 (SLC2A5) transporter. Although epidemiological studies indicate a strong correlation between high sugar intake and metabolic diseases, the biological mechanisms underlying this link are still controversial [7], and the role of the gastro-enteric tract often neglected. To exert their effect in the human body, sugars must be released from the food matrix, eventually hydrolysed in monosaccharides, and absorbed. Despite the modulation of sugar transporters has been deeply investigated [8,9], the metabolic modifications occurring in the enterocyte during absorption and the impact of the monosaccharides on the biochemical events taking place within the intestinal cell are still poorly understood.

Aim of the study

The aim of the present study was to further examine the crosstalk occurring in enterocyte during sugar absorption, giving a dynamic, untargeted, and omni-comprehensive vision of the events. We used Caco-2 cells grown on trans well inserts as model system. Caco-2 cells, a human intestinal cell line derived from colon cancer, differentiate on the membrane filter in trans well culture into cells that exhibit both morphological and biochemical features characteristic of intestinal epithelial cells. When grown on filter supports, after reaching confluence the Caco-2 cells spontaneously start to differentiate into a polarized cell layer with apical microvilli and intercellular tight junction complexes [10]. The Caco-2 cell model has been extensively utilised for studies on sugar transport as a model for the small intestine, since the expression and membrane location of GLUTs and SGLT1 are well known under a wide variety of conditions. In addition, the pathways for utilisation of FRU are intact since fructose can be readily used as an energy source by Caco-2 cells [11,12]. Cells were supplemented with equimolar concentration of GLU, FRU or SUC. Concentration chosen for supplementation corresponds to the physiological luminal GLU concentration in the small intestine under normal condition [13]. To avoid bias, possible cytotoxicity of supplemented sugars was also evaluated. Changes in metabolite concentration occurring in the apical (AC) and basolateral chambers (BLC) were evaluated by ^{1}H-NMR spectroscopy and gas-chromatography (GC), allowing us to follow in an 8 h timeframe the overall metabolic response of enterocytes to the exposure to GLU, FRU and SUC.

Materials and methods

Chemicals

Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, and Dulbecco's phosphate-buffered saline (DPBS) were purchased from Lonza (Basel, Switzerland). All other chemicals and solvents were of the highest analytical grade from Sigma-Aldrich Co. (St. Louis, MO, USA).

Methods

Cell Culture and Supplementation

Experiments were carried out using Caco-2 cells (European Collection of Authenticated Cell Cultures (ECACC) 86010202, passage number between 50–60) seeded on a polyester (PET) membrane insert (Trans well; 0.4 µm pore size, 2×10^6 pores/cm^2, 4.2 cm^2 insert membrane growth area) (Corning Incorporated, New York, NY, USA). Cells were seeded at the density of 2×10^5 cells/well and grown for 21 days in DMEM supplemented with fetal bovine serum (FBS) (10% v/v), non-essential amino acids (1% v/v), 100 U/ml penicillin and 100 µg/ml streptomycin. The Caco-2 cells appeared completely differentiated and polarized after 21 days of incubation, exhibiting the morphological and functional characteristics of mature enterocytes. The monolayer integrity was tested before the experiments by measuring the cell monolayer's trans-epithelial electric resistance with the Millicell ERS device (Millipore, Burlington, MA, USA). TEER values between 1000 and 2000 W*cm^2 were considered acceptable for the transport assay. Before sugar supplementation, the apical and basal compartments were washed twice with 1 ml phosphate buffer solution (PBS), then filled with DMEM (2 ml) containing 25 mM GLU, FRU, or SUC, without PBS and no essential amino acid (apical chamber) or the same volume of DPBS (basal chamber) (basolateral chamber). Cell cytotoxicity was measured after 0.5, 1, 2, 4, 6, or 8 hours of incubation, and media was collected from both chambers for ^{1}H-NMR and GC analysis.

Cytotoxicity Evaluation

Cytotoxicity was evaluated by the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay and microscopic examination. Viability was assessed using a Tecan Infinite M200 microplate reader (Tecan, Männedorf, Switzerland) to detect MTT salt conversion to its formazan product at 560 nm. Cell viability was measured as a percentage of the viability of control cells, which was set to 100%. Cell morphology and monolayer integrity were examined by light microscopy using an inverted confocal light microscopy model IB (Exacta Optech, Modena, Italy) at magnifications of 10 ×, 25 ×, and 40 ×.

Lipid Extraction and Fatty Acid Composition Analysis

Total lipids in Caco-2 cells, apical chamber and basolateral chamber were extracted, and relative fatty acids methylated as previously described [18]. Before methylation, pentadecanoic acid was added as internal standard. The qualitative and quantitative content of fatty acid methyl esters (FAMEs) was determined by fast-GC (GC-2030AF; Shimadzu, Kyoto, Japan) using a capillary column (30 mt, 0.2 µm film thickness) with a programmed temperature gradient (50–250 °C, 10 °C/min). The gas chromatographic peaks were identified based on their retention time ratios relative to methyl stearate and predetermined by use of authentic samples [19]. Gas chromatographic traces and quantitative evaluations were obtained using a Lab Solution software (Shimadzu, Kyoto, Japan).

Statistical Analysis

All data are means ± standard deviation (SD) of at least 3 samples from independent experiments. In each experimental condition, metabolite concentration at different time points was statistically evaluated by the one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. To compare the effect of GLU and FRU supplementation at the same time point, we used an unpaired t-test considering $p < 0.05$ as significant.

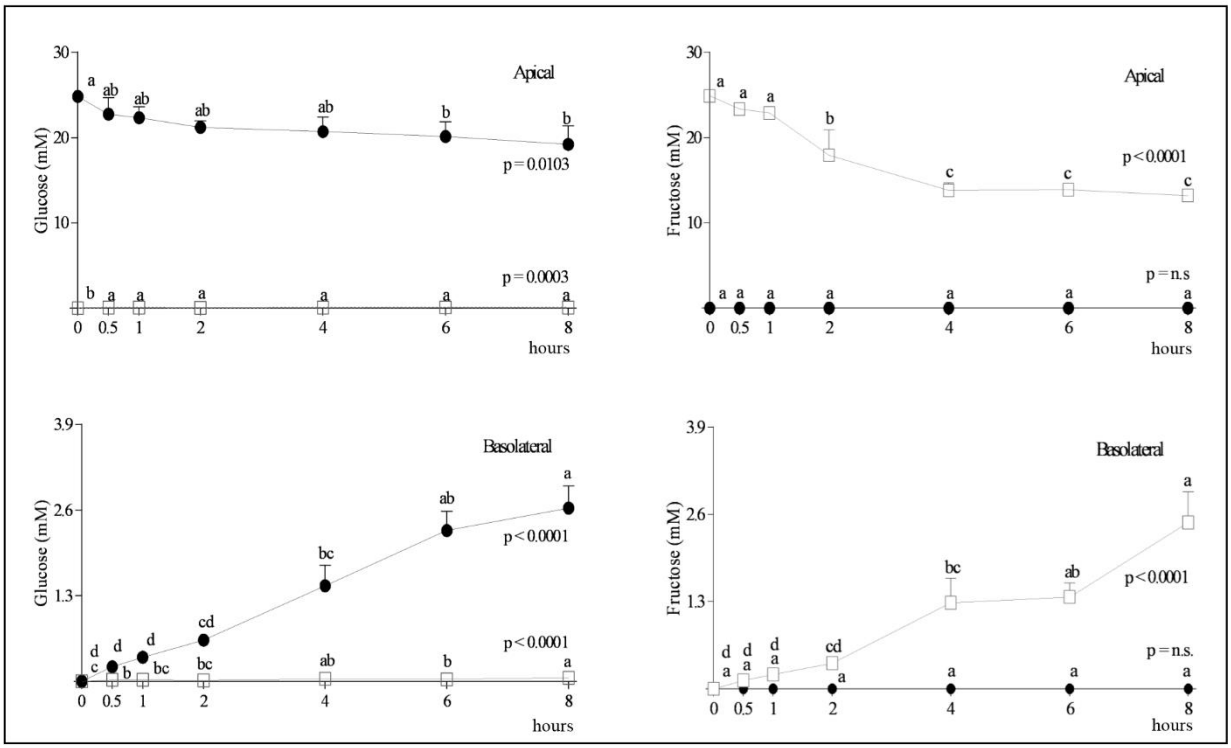
Results

Cytotoxicity

In all conditions, MTT assay and light microscopy examination did not evidence either modification in cell viability or in monolayer integrity at any time point (data not shown).

Metabolite diffusion

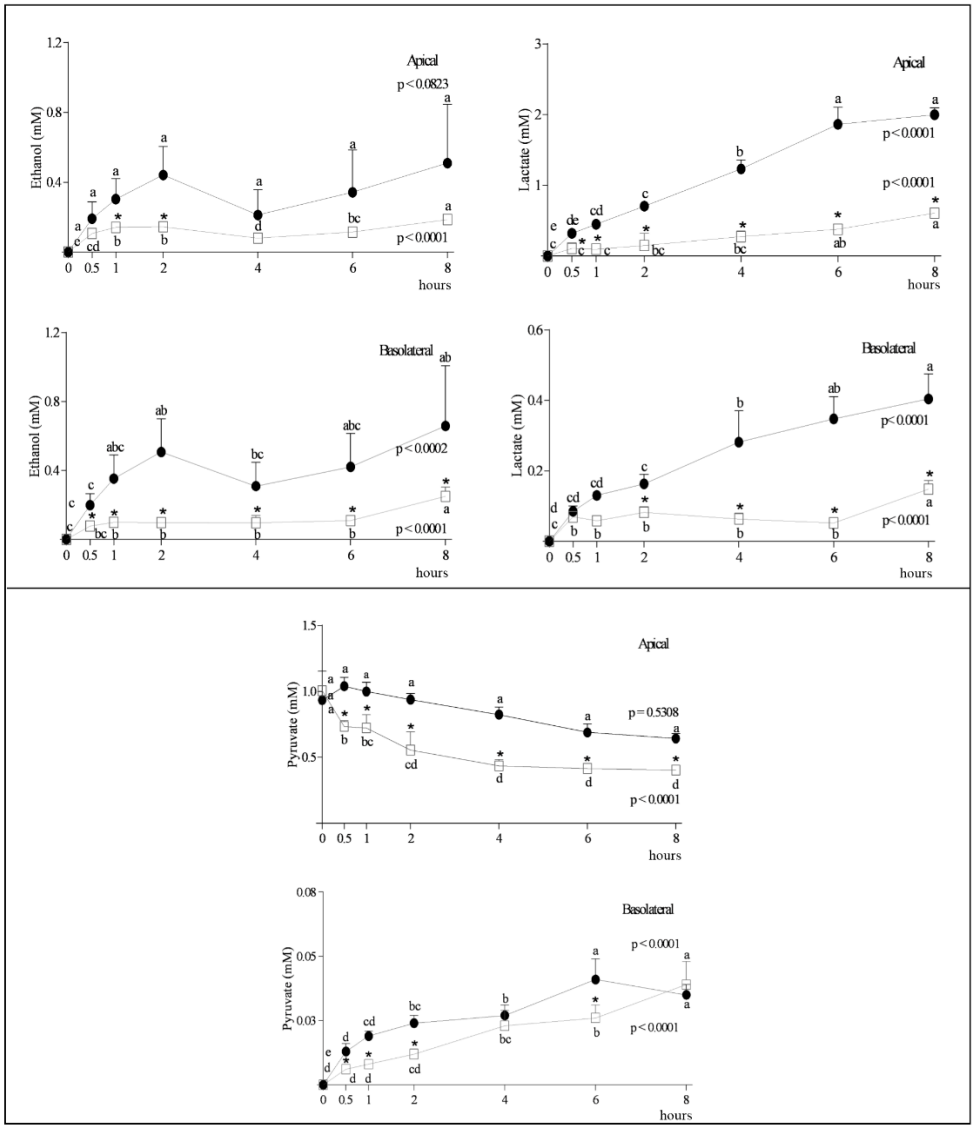
The time course and extent of GLU and FRU diffusion from the AC to the BLC in GLU and FRU-supplemented cells is reported in figure 1. Although in both conditions the reduction of the concentration of the monosaccharide supplemented in the AC was concomitant to its increase in the BLC, in GLU-supplemented cells the total amount (AC + BLC) of GLU was similar at T0 and T8, while in FRU-supplemented cells the total amount of FRU was significantly lower at T8 than T0, this indicating an active metabolization of the supplemented ketohexose, including its conversion in small amount to aldohexose.



GLU-supplemented cells							
	T0			T8			
	AC	BLC	**Total**	AC	BLC	**Total**	P
GLU	24.8±0.22	0	**24.8±0.22**	19.2±2.18	2.52±0.41	**23.2±1.90**	0.14
FRU	0	0	**0**	0	0	**0**	n.d.
FRU-supplemented cells							
	T0			T8			P
	AC	BC	**Total**	AC	BC	**Total**	
GLU	0	0	**0**	0.06±0.01	0.05±0.01	**0.12±0.02**	<0.0001
FRU	24.9±0.11	0	**24.9±0.11**	13.2±0.79	2.48±0.46	**15.6±0.99**	<0.0001

Figure 1. Changes in GLU and FRU concentration in the AC and BLC of GLU- and FRU-supplemented cells. Data are means ± SD of at least 3 samples from independent experiments. In graphs, GLU (left panel) and FRU (right panel) concentration (mM) at different time points is indicated by black circles (GLU-supplemented cells) or white squares (FRU-supplemented cells). In each experimental condition, differences between different time points were evaluated by the one-way ANOVA with Tukey's post-hoc test (different letters indicate significant differences). In the table, the total concentration of the monosaccharides at T0 and T8 in the same experimental condition is reported and compared by unpaired t test. In each condition, statistical analysis was by unpaired t-test to evaluate differences between T0 and T8.

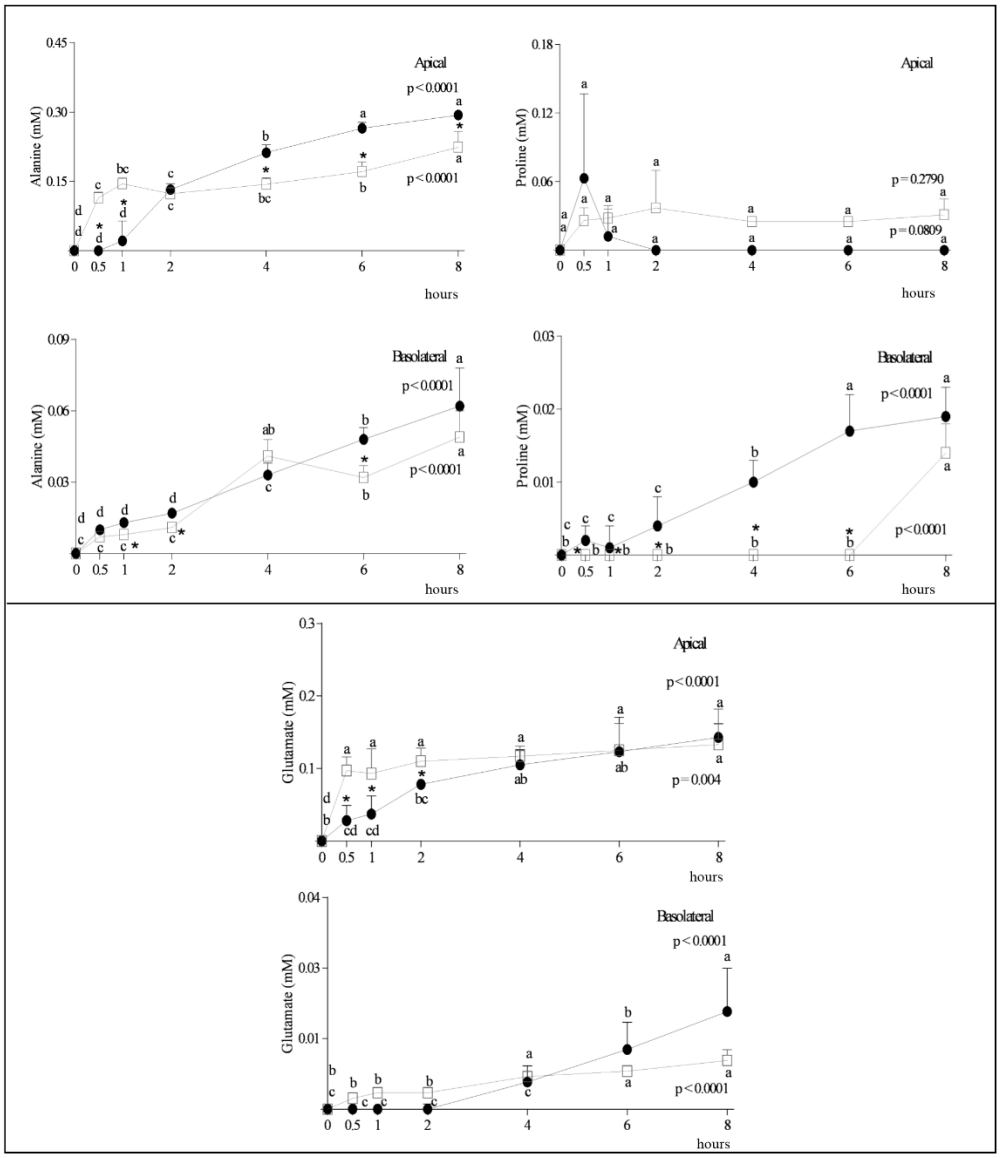
In SUC-supplemented cells, we detected very low amounts of GLU or FRU (< 0.65 mM) either in the AC or BLC at any time after supplementation, indicating that the disaccharide was only minimally hydrolysed. At longer incubation time, SUC slightly diffused from the AC to the BLC, probably via paracellular transport (Supplementary figure 1). Since SUC-supplemented cells did not reproduce a physiological condition, we did not focus on this condition. Anyway, results obtained in SUC-supplemented cells are provided as supplementary material (Supplementary Figures 1-6). The time-course of changes in carbonyl metabolites (ethanol – ETH, lactate – LAC, pyruvate – PYR) concentration in the AC and BLC of GLU- and FRU-supplemented cells, and their total concentration (AC + BLC) at T0 and T8, is reported in figure 2. Although ETH and LAC were not present in the supplemented media, their concentration significantly increased with time in both chambers, more evidently in GLU-supplemented cells. In these cells, PYR concentration in the AC was almost constant over time, while it significantly decreased in FRU supplemented cells. Notwithstanding, at T8 PYR concentration in the BLC was similar in both conditions. The total amount (AC+BLC) of PYR was significantly lower at T8 than T0, indicating its active metabolization particularly in FRU-supplemented cells.



GLU-supplemented cells							
		T0			T8		
	AC	BLC	**Total**	AC	BLC	**Total**	P
ETH	0	0	**0**	0.51±0.34	0.66± .35	**1.11±0.83**	0.037
LAC	0	0	**0**	2.00±0.1	0.37±0.07	**2.37±0.15**	<0.001
PYR	0.94±0.09	0	**0.94±0.09**	0.65±0.05	0.03±0.00	**0.68±0.05**	0.002
FRU-supplemented cells							
		T0			T8		
	AC	BC	**Total**	AC	BC	**Total**	P
ETH	0	0	**0**	0.19±0.01	0.25±0.06	**0.43±0.02**	<0.001
LAC	0	0	**0**	0.61±0.07	0.15±0.02	**0.75±0.09***	<0.001
PYR	1.01±0.01	0	**1.01±0.01**	0.40±0.04	0.04±0.01	**0.43±0.04***	<0.001

Figure 2. Changes in ETH, LAC and PYR concentration in the AC and BLC of GLU- and FRU-supplemented cells. Data are means ± SD of at least 3 samples from independent experiments. In graphs, carbonyl metabolites concentration (mM) at different time points is indicated by black circles (GLU-supplemented cells) or white squares (FRU-supplemented cells). In each experimental condition, differences between different time points were evaluated by the one-way ANOVA with Tukey's post-hoc test (different letters indicate significant differences). At each time point, differences between GLU- and FRU-supplemented cells were evaluated by unpaired t-test (* at least $p<0.05$). In the table, the total concentration of metabolites at T0 and T8 in the same experimental condition is reported and compared by unpaired t test. Statistical differences between the two experimental conditions in the metabolite total concentration at T8 were calculated by unpaired t-test (* p at least <0.05).

At T0, alanine (ALA), proline (PRO) and glutamic acid (GLA) were not present either in the AC or in the BLC medium. Over time, their concentration significantly increased in both chambers (Figure 3).



GLU-supplemented cells							
		T0			T8		
	AC	BLC	**Total**	AC	BLC	**Total**	P
ALA	0	0	**0**	0.29±0.01	0.06±0.02	**0.36±0.02**	<0.001
PRO	0	0	**0**	0	0.02±0.00	**0.02±0.00**	<0.001
GLA	0	0	**0**	0.14±0.02	0.02±0.01	**0.16±0.02**	<0.001
FRU-supplemented cells							
		T0			T8		
	AC	BC	**Total**	AC	BC	**Total**	P
ALA	0	0	**0**	0.22±0.03	0.05±0.01	**0.27±0.03***	<0.001
PRO	0	0	**0**	0.03±0.01	0.01±0.00	**0.04±0.02**	0.009
GLA	0	0	**0**	0.13±0.06	0.01±0.00	**0.14±0.06**	0.016

Figure 3. Changes in ALA, PRO and GLA concentration in the AC and BLC of GLU- and FRU-supplemented cells. Data are means ± SD of at least 3 samples from independent experiments. In graphs, amino acid concentration (mM) at different time points is indicated by black circles (GLU-supplemented cells) or white squares (FRU-supplemented cells). In each experimental condition, differences between different time points were evaluated by the one-way ANOVA with Tukey's post-hoc test (different letters indicate significant differences). At each time point, differences between GLU- and FRU-supplemented cells were evaluated by unpaired t-test (* at least $p<0.05$). In the table, the total concentration of metabolites at T0 and T8 in the same experimental condition is reported and compared by unpaired t test. Statistical differences between the two experimental conditions in the metabolite total concentration at T8 were calculated by unpaired t-test (* p at least <0.05).

Glutamine (GLN), phenylalanine (PHE), leucine (LEU), isoleucine (ILE), valine (VAL), methionine (MET), threonine (THR), lysine (LYS), arginine (ARG), serine (SER) and tyrosine (TYR) were already present in the media supplemented in the AC at con-centration ranging from about 1.8 mM (GLN) to 0.2 mM (MET), while their concentration was null in the BLC medium. In GLU-supplemented cells, these amino acids diffused to the BLC, and the total (AC+BLC) amount of the most of them was similar at T0 and T8. Opposite, in FRU-supplemented cells the total concentration of all the above listed amino acids except GLN and THR was significantly lower at T8 than T0, suggesting that the overtime decrease in their concentration in the AC was due to a metabolic conversion rather than simply a diffusion toward the BLC (Supplementary figures 7 and 8).

Finally, to verify whether and to which extent supplemented GLU and FRU, and eventually other substrates, were converted to acetyl-CoA providing carbon inputs in-to fatty acids, we compared the fatty acid content in cells, AC and BC at T0 and T8 in the two experimental conditions. At T8, cell total FAME content was higher than at T0, mainly due to an increase in saturated and monounsaturated fatty acid concentration, without any significant difference between GLU and FRU supplementation (Table 1).

Table 1. Fatty acid methyl esters (FAME) content in Caco-2 cells at T0 and T8.

		GLU-supplemented	FRU-supplemented	ANOVA
FAME	**T0**	**T8**		
14:0	3.2±0.3b	4.0±0.3a	4.7±0.3a	P=0.001
16:0	40.7±3.5b	47.5±6.3ab	55.0±8.5a	P=0.0504
16:1 n-7	7.8±0.9b	9.9±0.7a	11.4±1.1a	P=0.0038
18:0	39.3±4.6a	45.0±7.4a	52.0±10.1a	P=0.1457
18:1 n-9	44.8±5.0b	55.6±3.6a	61.3±3.5a	P=0.0037
18:1 n-7	11.6±1.6b	14.1±0.8ab	16.0±1.4a	P=0.0109
18:2 n-6	1.6±0.3b	1.9±0.1ab	2.1±0.1a	P=0.0446
18:3 n-3	2.8±0.2a	2.7±1.2a	3.8±0.4a	P=0.1584
20:4 n-6	3.7±0.6b	4.3±0.2ab	4.8±0.3a	P=0.0366
20:5 n-3	0.7±0.8a	1.2±0.2a	1.3±0.2a	P=0.3442
22:6 n-3	2.7±0.5a	3.7±0.7a	3.8±0.6a	P=0.1298
Total	**158.9±11.3b**	**189.7±15.5a**	**216.0±10.8a**	P=0.0017

Table 1. Fatty acid methyl esters (FAME) content in Caco-2 cells at T0 and T8. Data are expressed as μg/well and are means ± SD of at least 3 samples from independent experiments. Statistical analysis was by one-way ANOVA with Tukey's post hoc test. Different letters in the same row indicate significant difference (at least $p < 0.05$).

At T0, media in the AC and BLC did not contain any fatty acid. After 8 h supple-mentation, myristic, palmitic, stearic and oleic acids were detected in both chambers, palmitic and stearic acid concentration being higher in the BLC of FRU than GLU supplemented cells (Table 2). Summing the fatty acid content in cells, AC, and BLC at T8 a significant difference was detected between GLU- (total FAME = 228.8±7.7 μg/well) and FRU-supplemented cells (total FAME = 265.2±18.7 μg/well; $p = 0.0356$).

	GLU-supplemented		FRU-supplemented	
FAME	**AC**	**BLC**	**AC**	**BLC**
14:0	0.5±0.6	0.23±0.06	0.50±0.13	0.32±0.05
16:0	11.8±2.8	5.80±0.91	13.58±6.19	7.40±0.25*
18:0	11.1±2.5	5.31±0.81	13.95±5.45	7.13±0.19*
18:1 n-9	4.3±3.9	0.0±0.0	3.86±3.08	2.39±3.28
Total	**27.76±6.12**	**11.34±1.76**	**31.89±9.27**	**17.24±3.61**

Table 2. Fatty acid methyl esters (FAME) content in the AC and BLC at T8. Data are expressed as μg/well and are means ± SD of at least 3 samples from independent experiments. Statistical analysis was by unpaired t-test to evaluate differences between and GLU and FRU-supplemented cells at T8 (* p at least <0.05).

Discussion

After supplementation of Caco2 cells with equimolar amount of GLU or FRU, we observed a faster reduction of the ketohexose than the aldohexose sugar concentration in the AC. This more efficient uptake could be related to the increased GLUT5 mRNA stability, already observed in human Caco-2 cells after FRU exposure [20], or to the overexpression of GLUT5 mRNA and protein by dietary FRU [3]. Notwithstanding the more efficient diffusion of FRU than GLU, at T8 the BLC concentration of the supplemented monosaccharide was similar in the two experimental conditions, suggesting that FRU was only partially released in the BLC in its parent form, and was it actively metabolized within the cell. Indeed, in GLU-supplemented cells the total concentration (AC + BLC) of GLU was similar at T0 and T8, while in FRU-supplemented cells FRU total concentration at T8 was significantly lower than at T0.

SLGT1 functionality was reported as different in Caco-2 cells obtained from 3 different cell banks, being lower in cells from ECACC than DSMZ [21]. In that work, values obtained in Caco2 cells were not compared to in vivo SLGT1 functionality, so observed differences did not mirror the reliability of the different cell lines but simply a biological variability. In addition, Steffenson et al. evaluated the functionality of SGLT1 and not of sodium-independent transporters such as GLUT2, and Alzaid et al. [22], using 3H-D-glucose as tracer, evidenced that the diffusive component accounts for more than 40% of total glucose uptake in Caco2 cells. Therefore, a lower SLGT1 functionality in our cell line, if any, had limited effect on total GLU transport.

As reported above, we supplemented cells with the same concentration (25 mM) of GLU and FRU. This concentration corresponds to the physiological luminal GLU concentration in the small intestine under normal condition. Although in the literature there are not specific data on intestinal FRU concentration under normal condition, we speculated that a 25 mM concentration of FRU could correspond to a high FRU intake. Intestinal FRU catabolism is dose dependent, and at low doses the 90% of FRU is cleared by the intestine [23]. Opposite, Patel et al. [24] estimated that as much as 10%–30% of an absorbed FRU load may undergo catabolism within enterocytes. In our study, about 40% of the supplemented ketohexose sugar

was actively catabolized by Caco2 cells, confirming that we were reproducing a condition that is considered risky for the development of metabolic diseases.

Fructolysis leads to increased glyceraldehyde, dihydroxy-acetone-phosphate, and glyceraldehyde-3-phosphate production, which are the source of gluconeogenic and lipogenic substrates. In FRU-supplemented cells a small amount of GLU was detected, in agreement with the reported existence of a conversion of FRU to GLU in human jejunum [25]. In GLU-supplemented cells, the conversion of GLU to FRU was not evidenced, confirming that FRU biosynthesis from GLU is mostly inactive under physio-logical conditions [4].

In agreement with results already obtained in mice [23], in our experimental conditions LAC formation was greater for GLU than FRU, which was poorly converted. LAC was detected in both chambers, consistent with the presence of the main intestinal LAC transporter both in the apical and basolateral membrane of the enterocyte [26]. Interestingly, a small amount of ETH was detected in both chambers. The ETH formation inside human tissues was considered in the past - the so-called ‘au-to-brewery’ syndrome - [27], and it was mainly ascribed to the microbial fermentation of the carbohydrates in the gastro-intestinal tract. Besides the synthesis of ETH by intestinal flora, some researchers have considered the possibility of ETH formation inside human tissues [28]. Our study indicated that a metabolic pathway in which ETH is formed as an intermediate does exist in Caco-2 cells. Although endogenous production of ETH was relatively minute, and probably far too low to have any forensic or medical significance, its possible biochemical interactions (i.e., with some carboxylic acids) deserve future attention.

During incubation, small amounts of ALA, GLA and PRO were detected in both the AC and BLC of GLU- and FRU-supplemented cells, confirming that a bidirectional transport of amino acids took place in this intestinal epithelial cell layer model, as suggested by Sakai et al. [29]. We speculate ALA was produced by reductive amination of PYR, which concentration decreased over time.

Since enterocytes express the complete enzymatic machinery required for de novo lipogenesis (DNL) and secretion of triglyceride-rich lipoprotein particles [30], and differences in the metabolism of FRU, mainly the lack of regulation of fructokinase, as compared to GLU may mean it is a more potent precursor for DNL, we verified the existence and entity of this

pathway upon supplementation. In our experimental conditions, an active DNL was observed in both GLU- and FRU-supplemented cells. Within the enterocytes, not only saturated but mainly unsaturated fatty acid concentration increased upon supplementation, without significant difference related to the supplemented monosaccharide. Although an increased intestinal expression of lipogenic and lipoprotein genes, including the stearoyl-CoA desaturase 1 gene, was already demonstrated in the mouse intestine and organoids upon FRU supplementation [31], to our knowledge there are no previous report about an increased synthesis of unsaturated fatty acids mediated by GLU.

At T8, saturated and monounsaturated fatty acids were detected in the AC. Although the protein-mediated fatty acid uptake system is now believed to be the dominant means by which fatty acids are absorbed by enterocytes, the fatty acid transport across the plasma membrane also occurs by simple, passive diffusion [32], and the absence of lipids in the medium could have favored their efflux in the AC. Fatty acids were detected in the BLC as well, palmitic and stearic acid concentration being higher in FRU- than GLU-supplemented cells. In addition, the total amount of de novo synthetized fatty acids was higher in FRU than GLU-supplemented cells, confirming that FRU specifically stimulates expression of enterocyte genes for lipid and apolipoprotein synthesis, as already observed in mice [31]. Since a de-novo synthesis of linoleic acid is not possible in mammalian cells, we speculate that the small increase in 18:2 n-6 concentration observed in FRU-supplemented cells was related to retro conversion of longer/more unsaturated n-6 fatty acids. Regarding the more abundant fatty acids, the composition of Caco-2 cells observed in our study closely resembled data from another study, which detected minor n-6 fatty acids [33]. In our study, the amount of total fatty acids recovered from each well was low, so it was difficult to discriminate the peaks of minor n-6 fatty acids from the background noise.

Over 8 h of culture, the concentration of individual amino acids already included in the AC medium changed to different extents in GLU- and FRU-supplemented cells, suggesting an already reported role of monosaccharides in the regulation of amino ac-id entry in the circulation. Indeed, a 24% to 57% higher transport of LEU, ILE, VAL, ALA, PHE, TRP and HIS in the presence of 10 mM FRU than 10mM GLU was already observed in epithelial cells isolated from the small intestine of rats [34]. Furthermore, in GLU-supplemented cells the

total amount (AC + BLC) of amino acids supplemented in the AC medium was similar at T0 and T8 (T0 = 5.58 ± 0.21 mM; T8 = 6.01 ± 0.45 mM; p = 0.286), while in FRU-supplemented ones it significantly decreased over time (T0 = 5.77 ± 0.01 mM; T8 = 4.13 ± 0.46 mM; p = 0.0089), suggesting that a remarkable amount of amino acids taken up from AC was metabolized. Amino acids are primary carbon sources for DNL, and in vivo tracing studies confirmed as dietary amino acids are twice more efficient than GLU in labelling the hepatic acetyl-CoA and fatty acid pool [35]. Thus, in FRU-supplemented cells the observed increased DNL could be related not only to FRU but also amino acid metabolization.

Conclusions

Sugar consumption appears to be causal or contributory in the development of metabolic diseases, and sugar in the form of FRU is clearly the bad guy. Based on the results of 34 stable isotope tracer studies, Sun et al. [36] provided a summary of the quantitative disposal routes of FRU in the body. It was concluded that the mean oxidation rate of dietary FRU is about 45%, and a small percentage of FRU (<1%) is directly converted to plasma TG while conversion to GLU and LAC occurs to a great extent. The use of Caco2 cells and NMR plus GC analysis allowed us to further understand the contribution of intestinal cells to FRU systemic metabolization, and the differential effects of GLU and FRU on cell metabolism. Our results confirmed the prominent role of intestinal cells in FRU metabolism and clearance after absorption. Enterocytes shield hepatocytes from FRU exposure, and FRU oxidation seems to occur already in the intestine. Intestinal conversion of FRU to fatty acids was small, although higher than GLU conversion, and enterocyte also contributed to gluconeogenesis. Conversely, enterocytes did not significantly contribute to the systemic conversion of FRU to LAC observed by Sun et al. [36]. Although in future research the use of labelled substrates will be important to confirm GLU and FRU conversion into other metabolites, in the present study NMR metabolomics provided a powerful tool for studying the different impact of the two sugars on the uptake and metabolism of other molecules. In our experimental condition differences were small, but they suggest a possible stronger effect in more complex condition, i.e. after a meal, suggesting a further link between sugar consumption and the onset of metabolic diseases.

The lack of data on expression/activity of transporters/enzymes that could be involved in the observed modifications could be considered a limitation of the present study, which was an untargeted one. It is worth noting that untargeted approaches provide the most appropriate route to detect unexpected changes in metabolite concentrations, and usually precedes targeted ones. In the future, a targeted analysis will be fundamental to further clarify the events occurring in the enterocytes upon GLU and FRU absorption.

It has been well documented that polarized Caco-2 monolayers represent a reliable correlate for studies on the absorption of compounds after oral intake in humans. Several studies have compared Caco-2 permeability coefficients with absorption data in humans and found high

correlation [10]. Obviously, data analysis of experiments in the Caco-2 cell model, as well as in vivo experiments on animal models, cannot be directly compared with the human situation, but findings from cell culture models can be considered as tracers to facilitate and reduce the complexity of human studies.

References

1. Grembecka, M. Natural sweeteners in a human diet. *Roczniki Panstwowego Zakladu Higieny* 2015, *66*, 195-202.

2. Page, K.; Chan, O.; Arora, J.; Belfort-Deaguiar, R.; Dzuira, J.; Roehmholdt, B.; Cline, G.; Naik, S.; Sinha, R.; Constable, R., et al. Effects of Fructose vs Glucose on Regional Cerebral Blood Flow in Brain Regions Involved With Appetite and Reward Pathways. *JAMA : the journal of the American Medical Association* 2013, *309*, 63-70, doi:10.1001/jama.2012.116975.

3. Ferraris, R.P.; Choe, J.-y.; Patel, C.R. Intestinal Absorption of Fructose. *Annual Review of Nutrition* 2018, *38*, 41-67, doi:10.1146/annurev-nutr-082117-051707.

4. Merino, B.; Fernández-Díaz, C.M.; Cózar-Castellano, I.; Perdomo, G. Intestinal Fructose and Glucose Metabolism in Health and Disease. *Nutrients* 2020, *12*, 94.

5. Kellett, G. The facilitated component of intestinal glucose absorption. *The Journal of physiology* 2001, *531*, 585-595, doi:10.1111/j.1469-7793.2001.0585h.x.

6. Koepsell, H. Glucose transporters in the small intestine in health and disease. *Pflugers Arch* 2020, *472*, 1207-1248, doi:10.1007/s00424-020-02439-5.

7. Moore, J.B.; Fielding, B.A. Sugar and metabolic health: is there still a debate? *Curr Opin Clin Nutr Metab Care* 2016, *19*, 303-309, doi:10.1097/mco.0000000000000289.

8. FERRARIS, R.P. Dietary and developmental regulation of intestinal sugar transport. *Biochemical Journal* 2001, *360*, 265-276, doi:10.1042/bj3600265.

9. Schreck, K.; Melzig, M.F. Intestinal Saturated Long-Chain Fatty Acid, Glucose and Fructose Transporters and Their Inhibition by Natural Plant Extracts in Caco-2 Cells. *Molecules* 2018, *23*, 2544.

10. Verhoeckx, K.; Cotter, P.; Lopez-Exposito, I.; Lea, T.; Mackie, A.; Requena, T.; Swiatecka, D.; Wichers, H. The Impact of Food Bioactives on Health: in vitro and ex vivo models. 2015.

11. Fanjul, C.; Barrenetxe, J.; Iñigo, C.; Sakar, Y.; Ducroc, R.; Barber, A.; Lostao, M.P. Leptin regulates sugar and amino acids transport in the human intestinal cell line Caco-2. *Acta Physiol (Oxf)* 2012, *205*, 82-91, doi:10.1111/j.1748-1716.2012.02412.x.

12. O'Brien, P.; Corpe, C.P. Acute Effects of Sugars and Artificial Sweeteners on Small Intestinal Sugar Transport: A Study Using CaCo-2 Cells As an In Vitro Model of the Human Enterocyte. *PLoS One* 2016, *11*, e0167785, doi:10.1371/journal.pone.0167785.

13. Ferraris, R.P.; Yasharpour, S.; Lloyd, K.C.; Mirzayan, R.; Diamond, J.M. Luminal glucose concentrations in the gut under normal conditions. *American Journal of Physiology-Gastrointestinal and Liver Physiology* 1990, *259*, G822-G837, doi:10.1152/ajpgi.1990.259.5.G822.

14. Zhang, R.; Zhang, Q.; Ma, L.Q.; Cui, X. Effects of Food Constituents on Absorption and Bioaccessibility of Dietary Synthetic Phenolic Antioxidant by Caco-2 Cells. *Journal of Agricultural and Food Chemistry* 2020, *68*, 4670-4677, doi:10.1021/acs.jafc.9b07315.

15. Valli, V.; Taccari, A.; Di Nunzio, M.; Danesi, F.; Bordoni, A. Health benefits of ancient grains. Comparison among bread made with ancient, heritage and modern grain flours in human cultured cells. *Food Research International* 2018, *107*, 206-215, doi:https://doi.org/10.1016/j.foodres.2018.02.032.

16. Di Nunzio, M.; Picone, G.; Pasini, F.; Chiarello, E.; Caboni, M.F.; Capozzi, F.; Gianotti, A.; Bordoni, A. Olive oil by-product as functional ingredient in bakery products. Influence of processing and evaluation of biological effects. *Food Research International* 2020, *131*, 108940, doi:https://doi.org/10.1016/j.foodres.2019.108940.

17. Di Nunzio, M.; Picone, G.; Pasini, F.; Caboni, M.F.; Gianotti, A.; Bordoni, A.; Capozzi, F. Olive oil industry by-products. Effects of a polyphenol-rich extract on the metabolome and response to inflammation in cultured intestinal cell. *Food Research International* 2018, *113*, 392-400, doi:https://doi.org/10.1016/j.foodres.2018.07.025.

18. Di Nunzio, M.; van Deursen, D.; Verhoeven, A.J.; Bordoni, A. n-3 and n-6 Polyunsaturated fatty acids suppress sterol regulatory element binding protein activity and increase flow of non-esterified cholesterol in HepG2 cells. *Br J Nutr* 2010, *103*, 161-167, doi:10.1017/s000711450999167x.

19. Bub, A.; Malpuech-Brugère, C.; Orfila, C.; Amat, J.; Arianna, A.; Blot, A.; Di Nunzio, M.; Holmes, M.; Kertész, Z.; Marshall, L., et al. A Dietary Intervention of Bioactive Enriched Foods Aimed at Adults at Risk of Metabolic Syndrome: Protocol and Results from PATHWAY-27 Pilot Study. *Nutrients* 2019, *11*, 1814.

20. Gouyon, F.; Onesto, C.; Dalet, V.; Pages, G.; Leturque, A.; Brot-Laroche, E. Fructose modulates GLUT5 mRNA stability in differentiated Caco-2 cells: role of cAMP-signalling pathway and PABP (polyadenylated-binding protein)-interacting protein (Paip) 2. *Biochem J* 2003, *375*, 167-174, doi:10.1042/bj20030661.

21. Steffansen, B.; Pedersen, M.D.L.; Laghmoch, A.M.; Nielsen, C.U. SGLT1-Mediated Transport in Caco-2 Cells Is Highly Dependent on Cell Bank Origin. *Journal of Pharmaceutical Sciences* 2017, *106*, 2664-2670, doi:https://doi.org/10.1016/j.xphs.2017.04.033.

22. Alzaid, F.; Cheung, H.-M.; Preedy, V.R.; Sharp, P.A. Regulation of glucose transporter expression in human intestinal Caco-2 cells following exposure to an anthocyanin-rich berry extract. *PloS one* 2013, *8*, e78932-e78932, doi:10.1371/journal.pone.0078932.

23. Jang, C.; Hui, S.; Lu, W.; Cowan, A.J.; Morscher, R.J.; Lee, G.; Liu, W.; Tesz, G.J.; Birnbaum, M.J.; Rabinowitz, J.D. The Small Intestine Converts Dietary Fructose into Glucose and Organic Acids. *Cell Metabolism* 2018, *27*, 351-361.e353, doi:https://doi.org/10.1016/j.cmet.2017.12.016.

24. Patel, C.; Douard, V.; Yu, S.; Tharabenjasin, P.; Gao, N.; Ferraris, R.P. Fructose-induced increases in expression of intestinal fructolytic and gluconeogenic genes are regulated by GLUT5 and KHK. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 2015, *309*, R499-R509, doi:10.1152/ajpregu.00128.2015.

25. Öckerman, P.A.; Lundborg, H. Conversion of fructose to glucose by human jejunum absence of galactose-to-glucose conversion. *Biochimica et Biophysica Acta (BBA) - Enzymology and Biological Oxidation* 1965, *105*, 34-42, doi:https://doi.org/10.1016/S0926-6593(65)80173-4.

26. Felmlee, M.A.; Jones, R.S.; Rodriguez-Cruz, V.; Follman, K.E.; Morris, M.E. Monocarboxylate Transporters (SLC16): Function, Regulation, and Role in Health and Disease. *Pharmacological Reviews* 2020, *72*, 466, doi:10.1124/pr.119.018762.

27. Logan, B.K.; Jones, A.W. Endogenous Ethanol 'Auto-Brewery Syndrome' as a Drunk-Driving Defence Challenge. *Medicine, Science and the Law* 2000, *40*, 206-215, doi:10.1177/002580240004000304.

28. Antoshechkin, A.G. On intracellular formation of ethanol and its possible role in energy metabolism. *Alcohol Alcohol* 2001, *36*, 608, doi:10.1093/alcalc/36.6.608.

29. Sakai, R.; Ooba, Y.; Watanabe, A.; Nakamura, H.; Kawamata, Y.; Shimada, T.; Takumi, A.; van Goudoever, J.B.; Narita, T. Glutamate metabolism in a human intestinal epithelial cell layer model. *Amino Acids* 2020, *52*, 1505-1519, doi:10.1007/s00726-020-02908-2.

30. Steenson, S.; Umpleby, A.M.; Lovegrove, J.A.; Jackson, K.G.; Fielding, B.A. Role of the Enterocyte in Fructose-Induced Hypertriglyceridaemia. *Nutrients* 2017, *9*, 349.

31. Al-Jawadi, A.; Patel, C.R.; Shiarella, R.J.; Romelus, E.; Auvinen, M.; Guardia, J.; Pearce, S.C.; Kishida, K.; Yu, S.; Gao, N., et al. Cell-Type–Specific, Ketohexokinase-Dependent Induction by Fructose of Lipogenic Gene Expression in Mouse Small Intestine. *The Journal of Nutrition* 2020, *150*, 1722-1730, doi:10.1093/jn/nxaa113.

32. Schwenk, R.W.; Holloway, G.P.; Luiken, J.J.; Bonen, A.; Glatz, J.F. Fatty acid transport across the cell membrane: regulation by fatty acid transporters. *Prostaglandins Leukot Essent Fatty Acids* 2010, *82*, 149-154, doi:10.1016/j.plefa.2010.02.029.

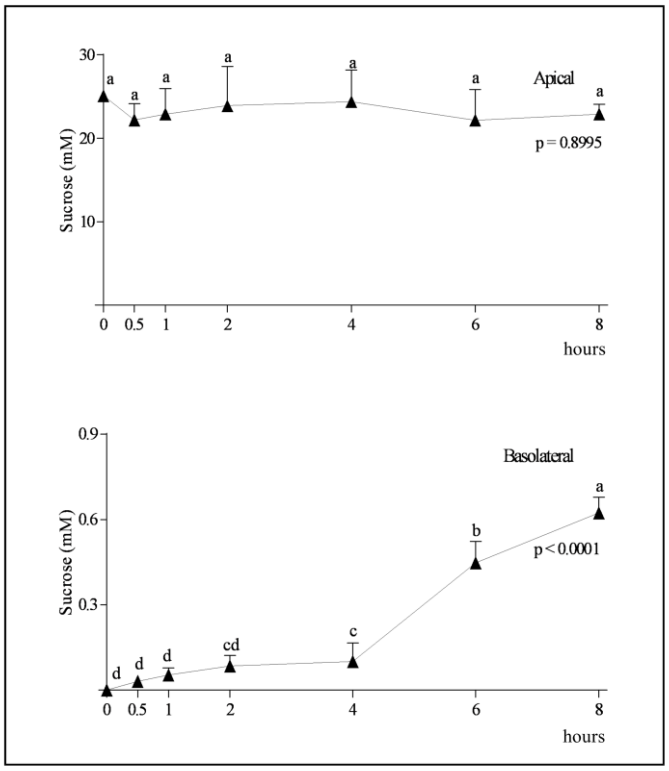
33. Rosa, A.; Rescigno, A.; Piras, A.; Atzeri, A.; Scano, P.; Porcedda, S.; Zucca, P.; Assunta Dessì, M. Chemical composition and effect on intestinal Caco-2 cell viability and lipid profile of fixed oil from Cynomorium coccineum L. *Food and Chemical Toxicology* 2012, *50*, 3799-3807, doi:https://doi.org/10.1016/j.fct.2012.07.003.

34. Reiser, S.; Hallfrisch, J. Stimulation of Neutral Amino Acid Transport by Fructose in Epithelial Cells Isolated from Rat Intestine. *The Journal of Nutrition* 1977, *107*, 767-774, doi:10.1093/jn/107.5.767.

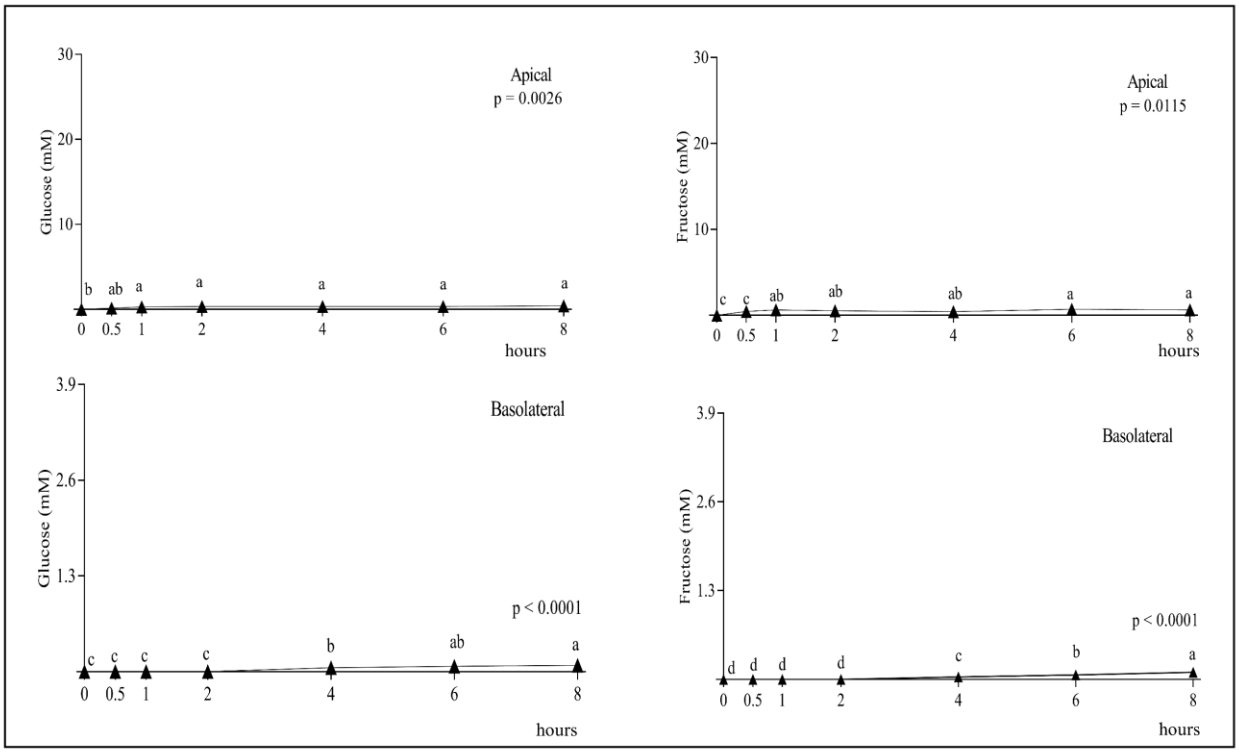
35. Liao, Y.; Liu, L.; Li, H.; Bai, X.; Sun, F.; Xiao, X.; Fu, S. Metabolic Complementation between Glucose and Amino Acid Drives Hepatic De Novo Lipogenesis and Steatosis. *bioRxiv* 2021, 10.1101/2021.05.08.443229, 2021.2005.2008.443229, doi:10.1101/2021.05.08.443229.

36. Sun, S.Z.; Empie, M.W. Fructose metabolism in humans - what isotopic tracer studies tell us. *Nutr Metab (Lond)* 2012, *9*, 89, doi:10.1186/1743-7075-9-89.

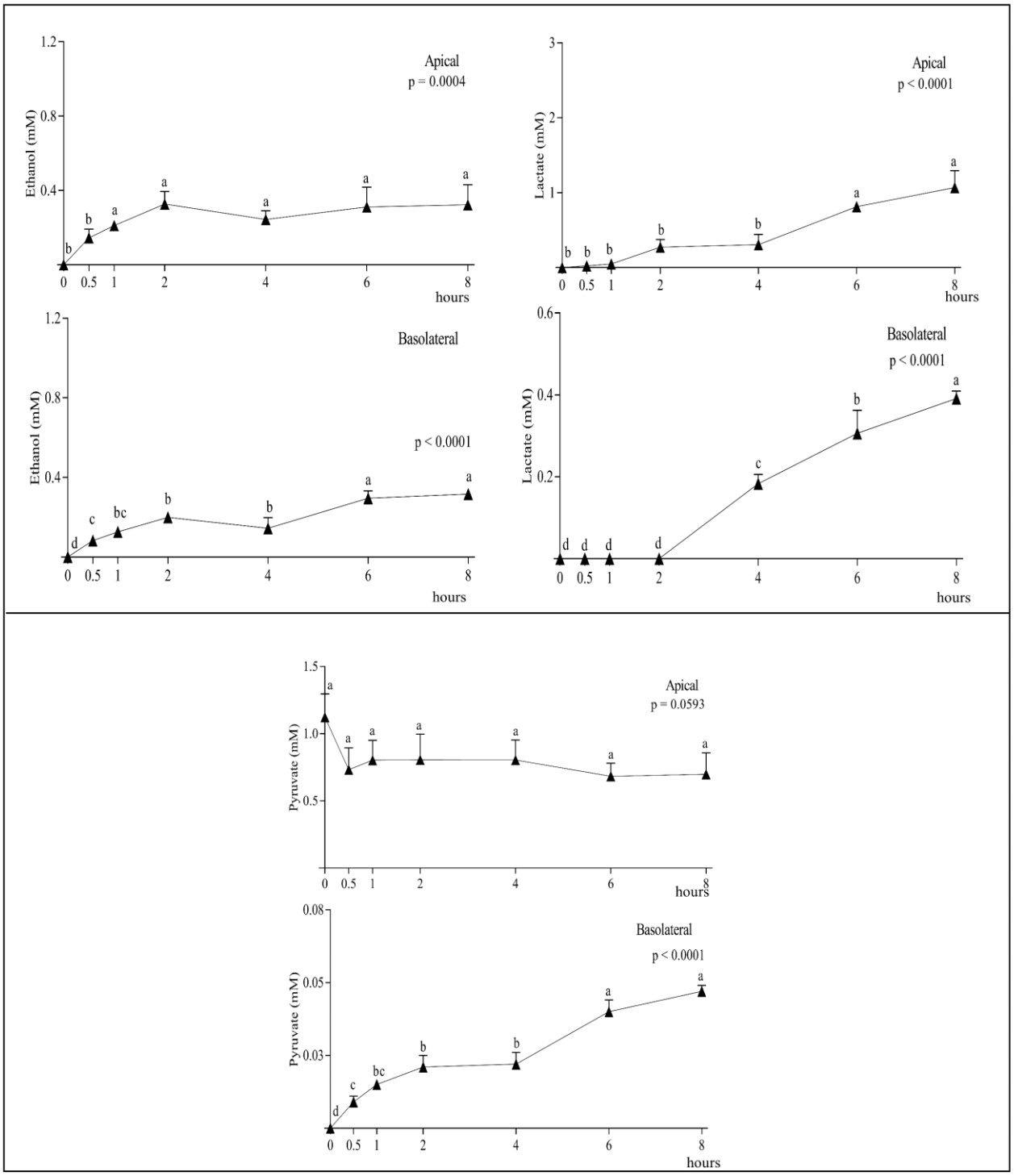
Supplementary



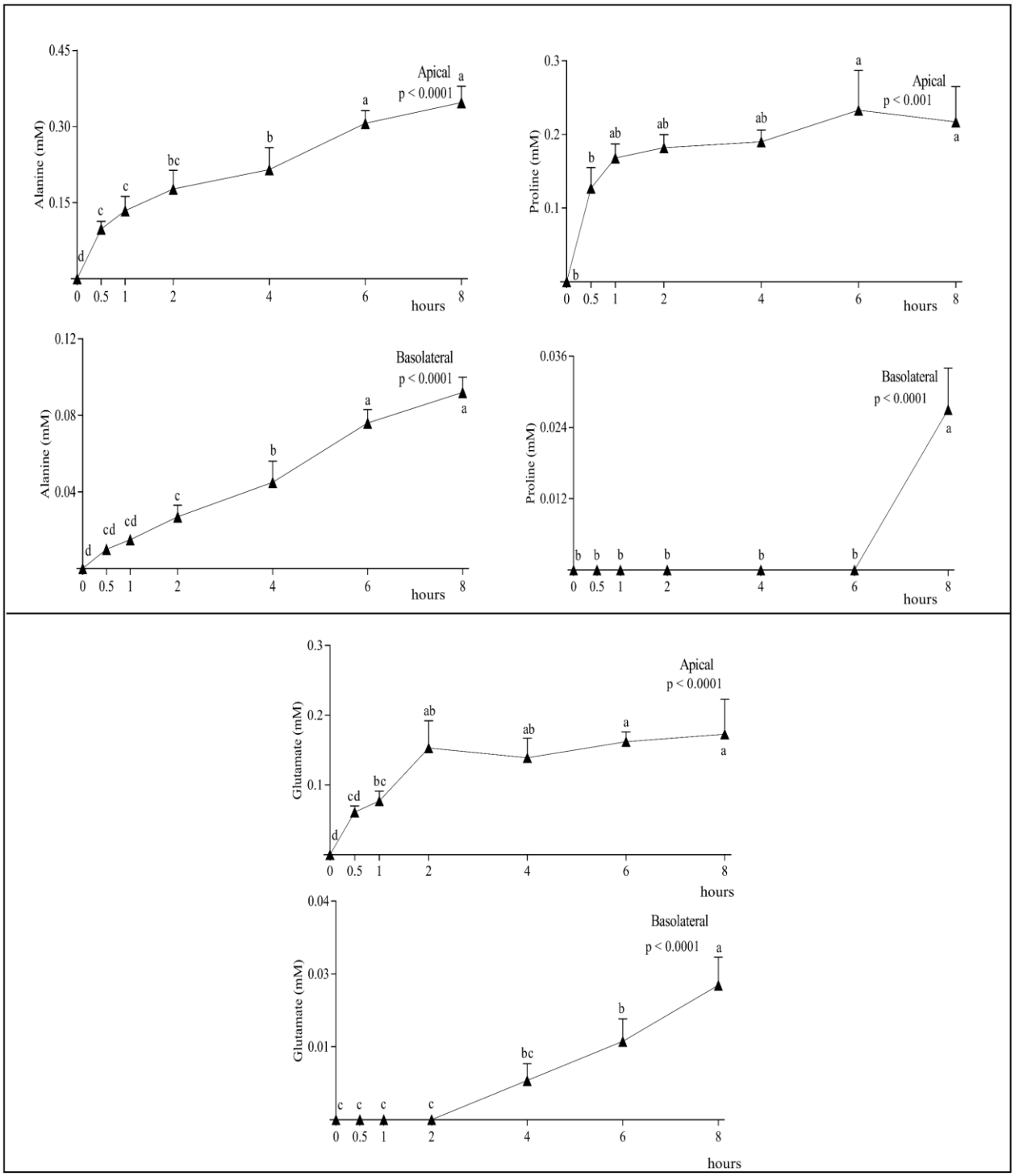
Supplementary figure 1. Time course of SUC diffusion from the apical to the basolateral chamber in SUC-supplemented cells. SUC concentration (mM) at different time points is indicated by black triangle. Data are means ± SD of at least 3 samples from independent experiments. Statistical analysis was by the one-way ANOVA with Tukey's post-hoc test to compare SUC concentration at different time points (different letters indicate significant differences).



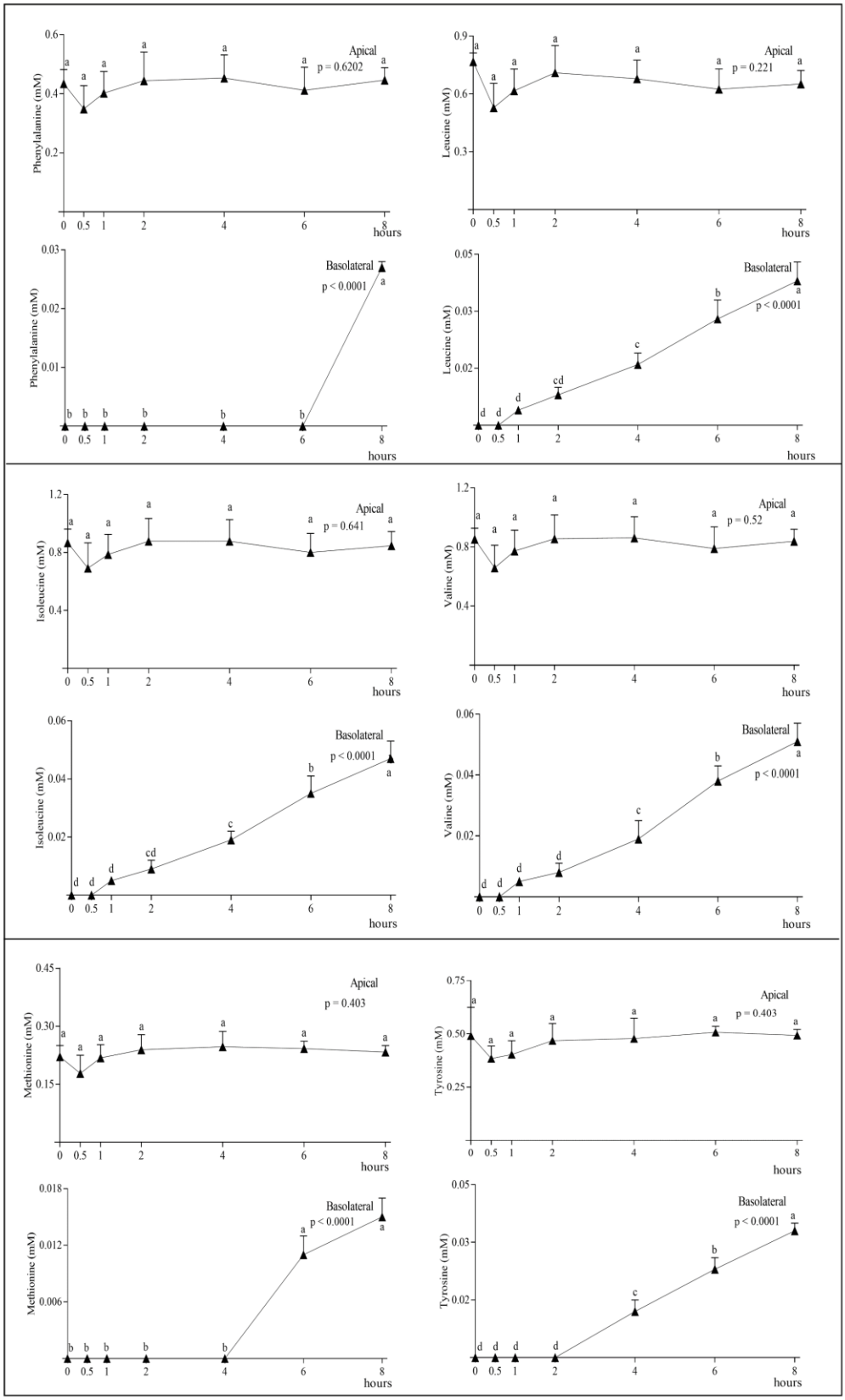
Supplementary figure 2. GLU, FRU concentration in apical and basolateral chambers at different time points in SUC-supplemented cells. Data are means ± SD of at least 3 samples from independent experiments. Statistical analysis was by the one-way ANOVA with Tukey's post-hoc test to compare metabolite concentration at different time points (different letters indicate significant differences).



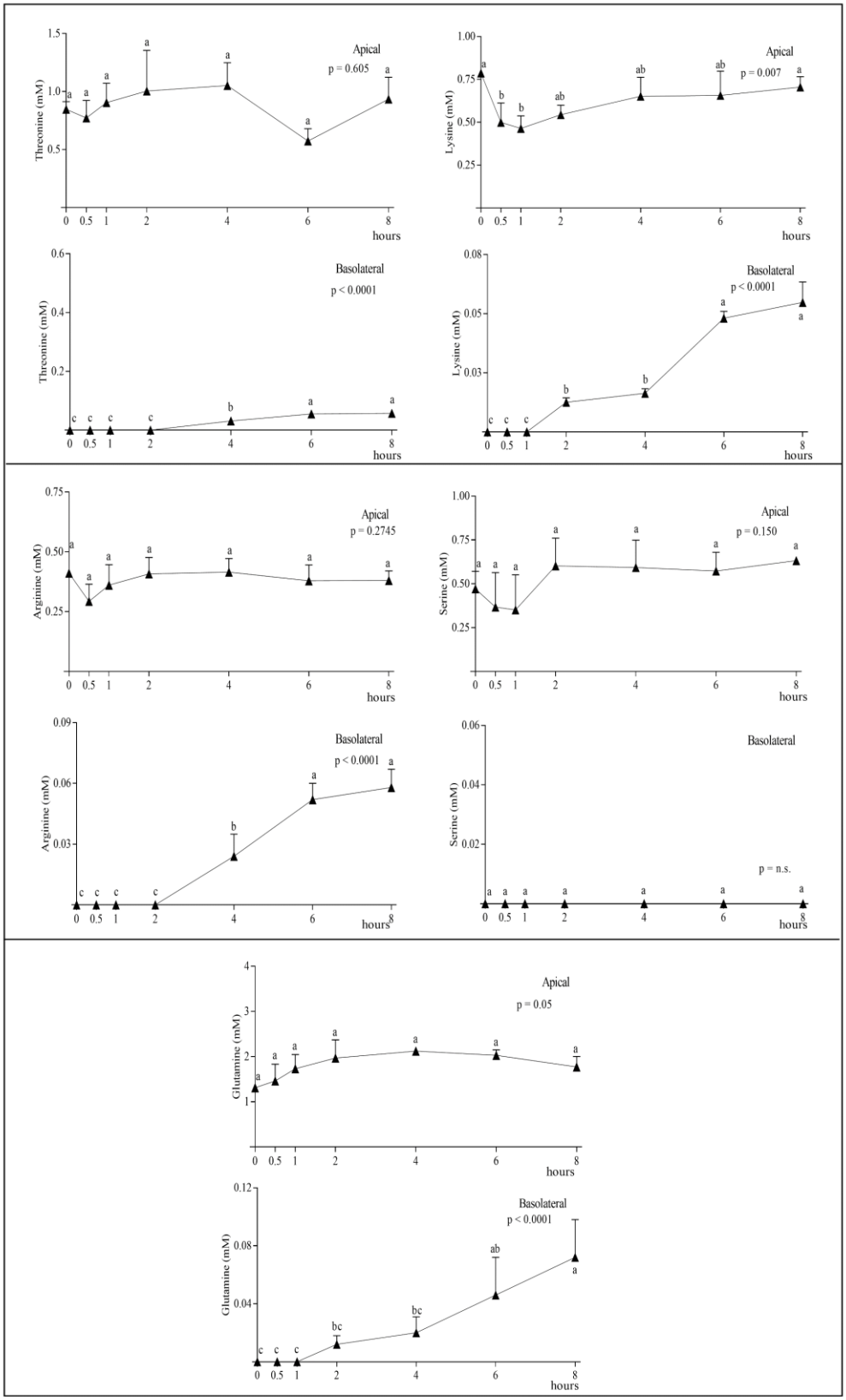
Supplementary figure 3. ETH, LAC, PYR concentration in apical and basolateral chambers at different time points in SUC-supplemented cells. Data are means ± SD of at least 3 samples from independent experiments. Statistical analysis was by the one-way ANOVA with Tukey's post-hoc test to compare metabolite concentration at different time points (different letters indicate significant differences).



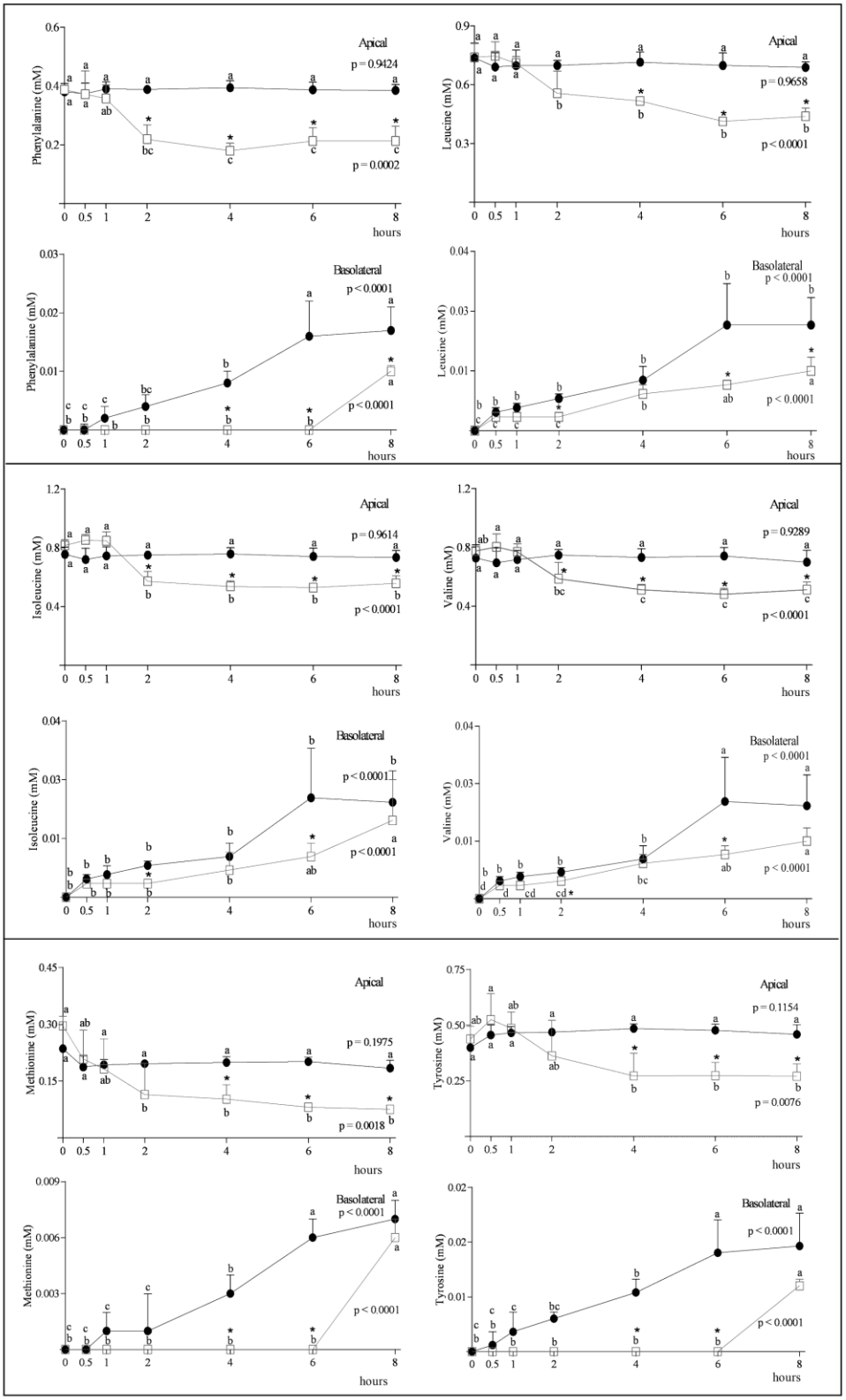
Supplementary figure 4. ALA, PRO, GLA concentration in apical and basolateral chambers at different time points in SUC-supplemented cells. Data are means ± SD of at least 3 samples from independent experiments. Statistical analysis was by the one-way ANOVA with Tukey's post-hoc test to compare metabolite concentration at different time points (different letters indicate significant differences).



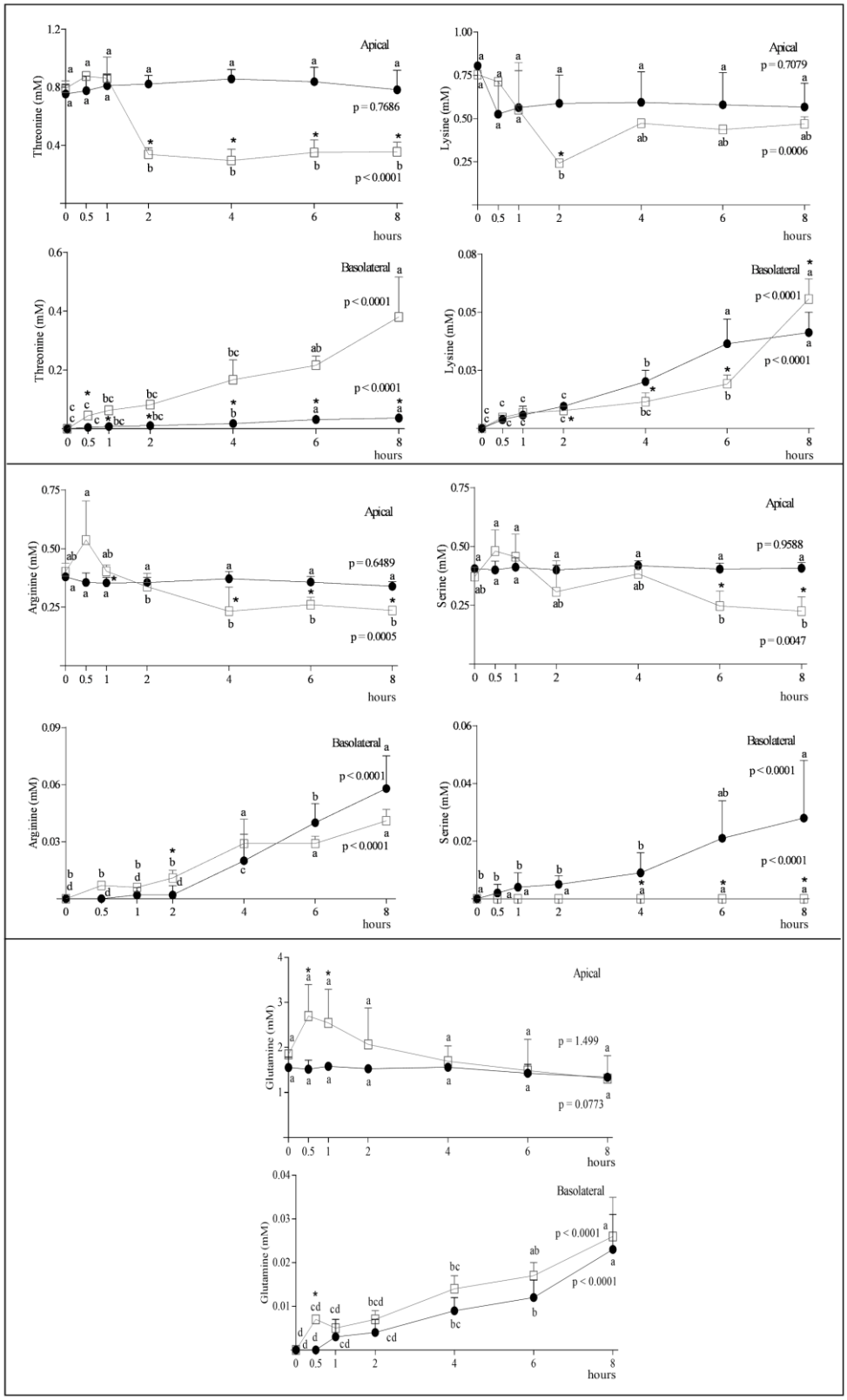
Supplementary figure 5. PHE, LEU, ILE, VAL, MET, TYR concentration in apical and basolateral chambers at different time points in SUC-supplemented cells. Data are means ± SD of at least 3 samples from independent experiments. Statistical analysis was by the one-way ANOVA with Tukey's post-hoc test to compare metabolite concentration at different time points (different letters indicate significant differences).



Supplementary figure 6. THR, LYS, ARG, SER, GLN concentration in apical and basolateral chambers at different time points in SUC-supplemented cells. Data are means ± SD of at least 3 samples from independent experiments. Statistical analysis was by the one-way ANOVA with Tukey's post-hoc test to compare metabolite concentration at different time points (different letters indicate significant differences).



Supplementary figure 7. PHE, LEU, ILE, VAL, MET, TYR concentration in apical and basolateral chambers at different time points. Aminoacid concentration at different time points is indicated by black circles (GLU-supplemented cells) or white squares (FRU-supplemented cells). Statistical analysis was by the one-way ANOVA with Tukey's post-hoc test to compare the metabolites concentration at different time points (different letters indicate significant differences) and by the Student's t-test to evaluate differences between GLU and FRU-supplemented cells at each time point (* at least $p<0.05$).



Supplementary Figure 8. THR, LYS, ARG, SER, GLN concentration in apical and basolateral chambers at different time points. Aminoacid concentration at different time points is indicated by black circles (GLU-supplemented cells) or white squares (FRU-supplemented cells). Statistical analysis was by the one-way ANOVA with Tukey's post-hoc test to compare the metabolites concentration at different time points (different letters indicate significant differences) and by the Student's t-test to evaluate differences between GLU and FRU-supplemented cells at each time point (* at least $p<0.05$).

CHAPTER-6

Comparison of the stability and intestinal cell uptake of cholecalciferol and retinol solubilized in ethanol or mixed micelles

Introduction

Vitamins are a very heterogeneous group of molecules that regulate a wide range of metabolic reactions. Vitamins are present in minute amounts in natural foodstuffs and are classified as water-soluble and fat-soluble. Among the fat-soluble vitamins, vitamin A and vitamin D are characterised by the existence of the corresponding provitamins, i.e. substances that may be converted within the body to vitamins.

Indeed, vitamin A is present in food of animal origin as retinol and retinoids (vitamin A) and in food of plant origin as provitamin A (carotenoids).

Carotenoids are a wide group of terpenoid pigments consisting of a long chain of carbon atoms with a terminal ring. Among these, β-carotene; α-carotene and β-cryptoxanthin are the main pro-vitamin A carotenoids [1]. These molecules can act as antioxidants, as free radical scavengers, in lipophilic environments such as membranes and lipoproteins [2].

Pro-vitamin A carotenoids are absorbed, and bio converted in the intestine. Incorporated with bile salts, cholesterol, fatty acids, monoacylglycerides and phospholipids, carotenoids contribute to create mixed micelles [2]. Mixed micelles are important structures that make fat-molecules more bioavailable and easily absorbed by enterocytes. After intestinal absorption, pro-vitamin A compounds are converted into retinoids and other metabolites. Retinaldehyde, the main conversion product, is processed to form retinoic acid, retinol, and retinyl esters [3]. The retinyl esters are then combined with other dietary lipids to form chylomicrons, which are released into the lymph before entering the bloodstream.

The vitamin A Recommended Dietary Allowance (RDA), equal to 700 µg of retinol-equivalent (RE) per day for adult men [4], is reached through the intake of both vitamin and provitamins A (1 µg RE corresponds to 1 µg of retinol or 6 µg of β-carotene or 12 µg of other provitamin carotenoids). Regardless its dietary origin, retinol plays an important role in the

human body not only for the proper maintenance of night vision, but also through modulation of gene expression and antioxidant activity [5].

Vitamin D is a group of secosteroids. In humans, the most important compounds in this group are vitamin D_3 (cholecalciferol) and vitamin D_2 (ergocalciferol) [6], Cholecalciferol and ergocalciferol can be ingested from the diet and supplements. Cholecalciferol (vitamin D_3) is present only in foods of animal origin as egg yolk (2.5-10 micrograms/100 grams), butter (1-2.7 micro/100 g) and liver (0.25-1.15 micro/100 g), while ergocalciferol (vitamin D_2) is typical of plant food. In addition, cholecalciferol can be synthetized by the human body in the lower layers of epidermis of the skin through a chemical reaction that is dependent on sun exposure (specifically UVB radiation) and requires 7-dehydrocholesterol as substrate. Although endogenous synthesis provides significant amount of vitamin D_3, its intake with food is needed. The vitamin D Recommended Dietary Allowance (RDA) is 10 μg expressed as cholecalciferol (1 μg cholecalciferol = 40 IU vit. D) for adult men [7]. The importance of vitamin D_3 resides mainly in its regulatory action of calcium metabolism. The active vitamin D metabolite calcitriol mediates the biological effects by binding to the vitamin D receptor (VDR), which is principally located in the nuclei of target cells [8]. The binding of calcitriol to the VDR allows the VDR to act as a transcription factor that modulates the gene expression of transport proteins that are involved in calcium absorption in the intestine [8]. Through VDR, vitamin D also regulates cell proliferation and differentiation, and affects the immune system. Indeed, VDR is expressed in several white blood cells [9].

In order to perform their functions in the body, it is necessary that retinol, carotenoids and cholecalciferol in foods are made bioaccessible during digestion and available for intestinal absorption. Retinyl esters are hydrolyzed in the intestinal lumen by pancreatic carboxylic ester hydrolase. Following the hydrolysis, the free retinol is taken up by the mucosal cell and it is re-esterified by acyl-CoA:retinol acyltransferase (LRAT) [10]. The resulting retinyl esters combine with other lipid esters to form chylomicrons, which are transported by the lymphatic system [10]. It has been shown that the co-ingestion of the lipids improves the intestinal absorption of dietary carotenoids and vitamin A [11]. The liver is the main storage site and, in this organ, the retinyl esters are hydrolyzed and re-esterified. Over 95% of the retinol in the liver exists as long-chain fatty acid esters, principally palmitate and stearate [10].

All-trans retinol is released from the liver and transported to the peripheral tissues in plasma bound to a specific carrier protein, retinol-binding protein (RBP). RBP is a monomeric polypeptide (M.W. 21. 000) with a single binding site and it is primarily synthesized in the liver [12]. It is associates with a plasma protein (M.W. 55.000) called transthyretin (TTR), involved in the transport of thyroid hormones. When the complex RBP-TTR encounters the cellular receptor STRA6, the complex dissociates and holo-RBP bind to STRA6 which transports the vitamin into the cell [13]. RBP depleted from retinol has a significantly lower affinity for TTR and it is eliminated from circulation by filtration in the kidneys [14].

Dietary forms of vitamin D are de-esterified before absorption in the small intestine (IOM (Institute of Medicine) 2011). The dietary vitamin D absorption is carried out by emulsification and solubilization in mixed micelles for diffusion and permeabilization through the enterocyte's membrane. First, in the stomach, where the dietary vitamin D is not sensitive to the acidic pH, the pepsin may contribute to release the fraction of the vitamin associated with proteins, and the gastric lipase may partially hydrolyze the esters of vitamin D [15]. In the small intestine, vitamin D absorption requires the presence of fat in the intestinal lumen, which triggers the release of bile acids and pancreatic lipase. In the duodenum, digestive enzymes, such as proteases, amylases, and lipases, continue to release the dietary vitamin D from the food matrix; vitamin D esters are hydrolyzed by bile salt stimulated lipase or carboxyl ester lipase [15]. Within the enterocyte in the intestinal wall, vitamin D and other lipids are packaged together into chylomicrons that reach the systemic circulation through the lymphatic pathway [16]; [15].

The exact absorption mechanism of vitamin A and D is still partially unclear, and some studies have shown how their absorption is affected by interaction with other molecules as lipids [17]. Since vitamin A and D deficiency is widespread even in the European population, the evaluation of the factors that can influence the bioaccessibility and bioavailability of these vitamins is of fundamental importance in order to guarantee the optimal supply of these micronutrients.

Aim of the study

To this aim, in this preliminary study the stability and intestinal absorption of retinol and cholecalciferol incorporated into micelles or solubilized in ethanol was evaluated using Caco-2/TC7 cell line as intestinal model. Caco-2/TC7 model is a feasible alternative to the original cell line, as it is useful for drug transport and biotransformation studies due to its physiological efficiency [18]. This model is widely used to evaluate the intestinal transport of lipids such as fatty acids, cholesterol or lipophilic vitamins [19]. After spontaneously differentiating into polarized absorptive cell monolayers, Caco-2 cells exhibit morphological and biochemical features of human enterocytes.

First, stability tests were performed after incubating the solutions containing 1 µM of retinol or cholecalciferol solubilized in ethanol or incorporated in micelles at 37°C for different times. After the incubation time, samples were extracted and analysed by High Performance Liquid Chromatography (HPLC). The stability test demonstrated that cholecalciferol is stable in both experimental conditions (ethanol and micelles), while retinol is degraded when solubilized in ethanol. Therefore, this condition was excluded in further experiments. Caco2/ TC7 cells were supplemented with cholecalciferol or retinol incorporated in micelles and cholecalciferol solubilized in ethanol for 30, 60 or 120 min. At the end of incubation time, the media in the apical and basolateral chambers and cells were collected and stored at -80°C. Subsequently, the samples were extracted and analysed by HPLC.

Materials and methods

Chemicals

DMEM containing 4.5 g/L glucose and trypsin-EDTA (500 and 200 mg/L, respectively) was purchased from BioWhittaker (Fontenay-sous-Bois, France), fetal bovine serum (FBS) from Biomedia (Issy-les-Moulineaux, France), and non-essential amino acids, penicillin/streptomycin and PBS from Gibco BRL (Cergy-Pontoise, France). All solvents used for HPLC analyse were HPLC grade from Carlo Erba Reagents (Peypin, France). All other chemicals and solvents were from Sigma-Aldrich (Saint-Quentin-Fallavier, France).

Preparation of mixed micelles

Mixed micelles, formed using monoolein (0.3 mM), oleic acid (0.5 mM), phosphatidylcholine (0.04 mM), lysophosphatidylcholine (0.16 mM), and cholesterol (0.1 mM) dissolved in trichloromethane/methanol (2:1, v/v) and 1 μM cholecalciferol or retinol dissolved in ethanol, were transferred to a glass tube and solvent was evaporated under nitrogen. The dried residue was dispersed in DMEM containing 5 mM sodium taurocholate and incubated at 37 °C for 30 min. The solution was mixed by sonication in a bath sonicator (Branson 3510 MT, 40 kHz; Branson Ultrasonics, Danbury, CT, USA) for 30 min, incubated at 37 °C for 1 h, and filtered through cellulose ester membranes (0.22 μm) (Millipore S.A.S., Molsheim, France). The resulting solution of cholecalciferol- or retinol-rich mixed micelles was stored at −20 °C until use.

Stability experiment

Solutions were incubated for up to 120 minutes at 37°C in a cell incubator with 10% CO_2, sheltered from light. At the end of the incubation time, samples were collected and stored at -80 °C before HPLC analysis.

Cell culture and cell supplementation

Experiments were carried out using Caco-2 cells clone TC7 (passage number 68-70) seeded on Millicell® hanging cell culture inserts (1.0 μm pore size, 4.6 cm2 insert membrane growth rea) (Millipore S.A.S., Molsheim, France). Cells were seeded at a density of 2.105 cells/well and grown for 21 days in DMEM supplemented with foetal bovine serum (FBS) (10% v/v), non-essential amino acids (1% v/v), 100 U/mL penicillin and 100 μg/mL streptomycin. After 21 days of culture, Caco-2/TC7 cells were completely differentiated and polarized, resembling the morphological and functional features of mature enterocytes. DMEM without FBS (1 ml) containing 1 μM cholecalciferol or retinol incorporated in mixed micelles or 1 μM cholecalciferol in ethanol was added to apical chamber, whereas the basolateral chamber was filled with 2 ml of DMEM without FBS. After 30, 60 or 120 min of incubation, media from both chambers and cells were collected to perform HPLC analysis.

Cholecalciferol and retinol extraction

Cholecalciferol and retinol were extracted from 500 µl aqueous samples using the following method. Distilled water was added to sample volumes below 500 µl to reach a final volume of 500 µl, and retinyl acetate was used as internal standard. The mixture was extracted twice with 2 ml of hexane. The hexane phase obtained after centrifugation (1200 g, 10 min, 4°C) was evaporated under nitrogen, and the dried residue was dissolved in 200 µl of HPLC mobile phase (60% acetonitrile, 38% methanol and 2% water). A volume of 100 µl was used for HPLC analysis.

Cholecalciferol and retinol HPLC analysis

The HPLC system and method were set up according to previous studies [20]. The HPLC system was a Thermo Scientific Ultimate3000 equipped with a pump LPG-3400SD, an autosampler WPS-3000 TS, and a diode array detector DAD-3000. Cholecalciferol and retonol were detected at the maximum absorption wavelengths of 265 nm and 325 nm, respectively. The vitamins were separated using a 250 × 4.6mm, 5µm Zorbax Eclipse XDB-C18 column (Agilent Technologies, Les Ulis, France) preceded by a guard column, maintained at a constant temperature (40 °C). The mobile phase was 60% acetonitrile, 38% methanol and 2% water and the flow rate was 1.5 mL/min. Cholecalciferol and retinol were identified by spectral analysis and/or on the basis of the retention time and co-injection compared with the pure standards. Quantification was performed using the Chromeleon software (version 7.2, ThermoFisher Scientific, Villebon sur Yvette, France).

Statistical Analysis

All data are means ± standard deviation (SD) of 3 samples from independent experiments. In each experimental condition, vitamins concentration at different time points was statistically evaluated by the one-way analysis of variance (ANOVA) followed by Tukey's post hoc test.

Results

Stability of cholecalciferol and retinol

In Figure 1, the stability of cholecalciferol incorporated in mixed micelles or in ethanol is reported. Data were normalized with respect to initial quantities and expressed as percentage (%). Cholecalciferol incorporated in mixed micelles or solubilized in ethanol was highly stable during the incubation time (0, 30, 60, 120 min), with no statistically significant difference between the two conditions.

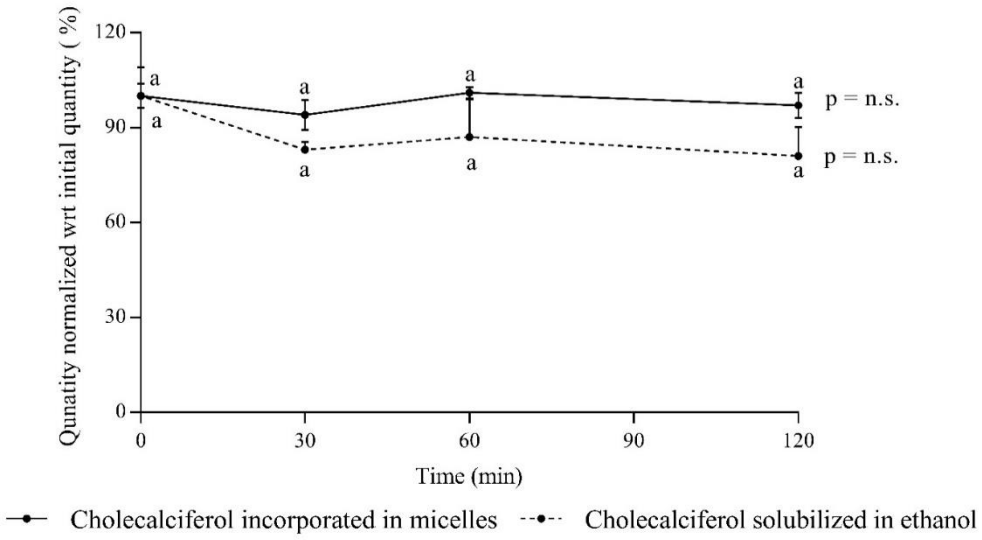


Figure 1. Stability of cholecalciferol incorporated in mixed micelles or in ethanol. In each experimental condition, differences between different time points were evaluated by the one-way ANOVA with Tukey's post-hoc test (different letters indicate significant difference).

In Figure 2 the stability of retinol incorporated in mixed micelles or in ethanol is reported. Data were normalized with respect to initial quantities and expressed as %. Retinol incorporated in mixed micelles was almost stable during the incubation time (0, 30, 60, 120 min), and the % quantity slightly decreased from 100.00 ±1.39 to 94.00 ±3.12. Instead, retinol solubilized in ethanol was completely degraded from initial value 100.00±26.64 to final value 5.00 ± 0.11.

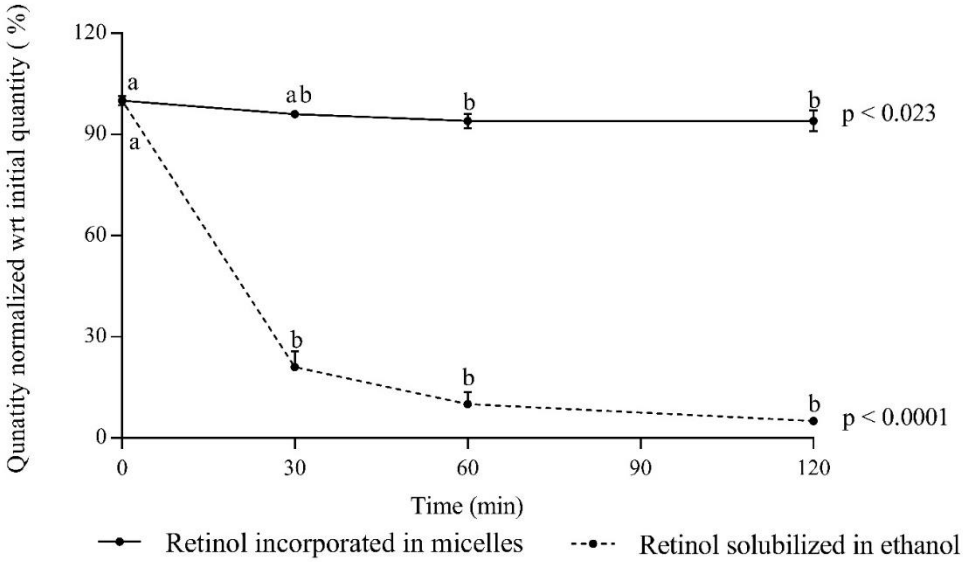


Figure.2 Stability of retinol incorporated in mixed micelles or in ethanol. In each experimental condition, differences between different time points were evaluated by the one-way ANOVA with Tukey's post-hoc test (different letters indicate significant difference).

Cholecalciferol uptake

After supplementation to Caco-2 cells, cholecalciferol concentration inside the cells and in the apical chamber was evaluated at different time points (Figure 3). Regardless the form of supplementation (incorporation in mixed micelles or solubilization in ethanol), vitamin D_3 was not detected in the basolateral chamber.

Supplementing cells with cholecalciferol incorporated in micelles, the increase of vitamin D_3 in the cellular compartment was proportional to its decrease in the apical chamber, with a significant time-dependent correlation (Pearson $r = -0.986$, $r^2 = 0.9723$, $p < 0.05$). On the contrary, when solubilized in ethanol cholecalciferol was minimally taken up by cells. In fact, after 120 min the % quantity of cholecalciferol in the apical chamber slight decreased from 100.00±9.04 to 97.13±2.75, and intracellular cholecalciferol concentration was 5.06±0.29%.

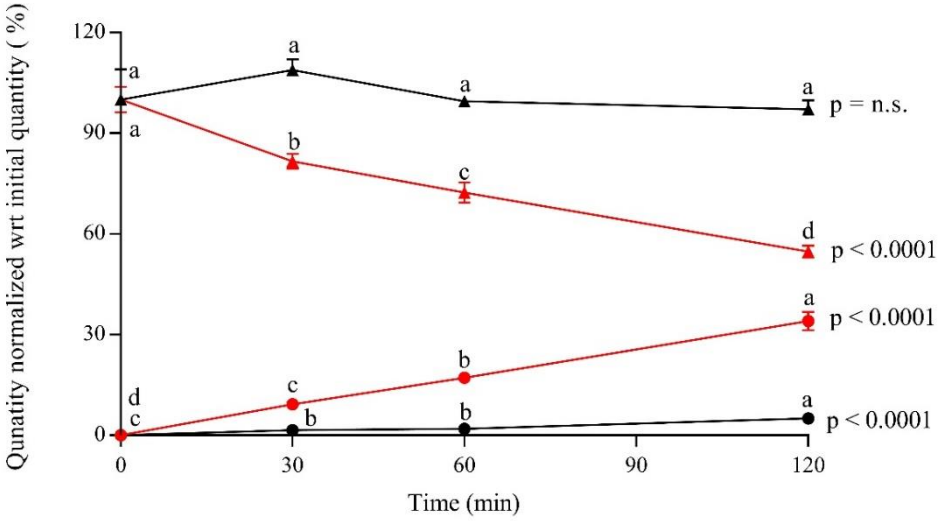


Figure 3. Concentration of cholecalciferol inside cells and in the apical chamber after supplementation as mixed micelles or solubilized in ethanol. Red lines refer to concentration in cells (circles) and apical chamber (triangles) of cholecalciferol supplemented as in mixed micelles. Black lines refer to concentration in cells (circles) and apical chamber (triangles) of cholecalciferol supplemented in ethanol. In each experimental condition, differences between different time points were evaluated by the one-way ANOVA with Tukey's post-hoc test (in each line, different letters indicate significant difference).

The time course of cholecalciferol uptake, estimated as the quantity of cholecalciferol in the harvested cells divided by the sum of the quantity of cholecalciferol remaining in the apical chamber and in the harvested cells, is reported in table 1. In both cases, the uptake increased with time. At each time point, the uptake was significantly higher when cholecalciferol was supplemented as micelles than when solubilized in ethanol. Final uptake was 27.30 ± 2.00 for micelles and 3.00 ± 0.00 for ethanol-solubilized cholecalciferol.

			% cholecalciferol uptake			
Cholecalciferol incorporated in micelles	*t (min)*	0	30	60	120	
	%	0.00±0.00d	6.44±0.00c	12.30±0.00b	27.30±0.03a	p<0.001
Cholecalciferol solubilized in ethanol	*t (min)*	0	30	60	120	
	%	0.00±0.00c	1.00±0.00b**	1.00±0.00b**	3.00±0.00a**	p<0.001

Table 1. Time course of cholecalciferol uptake in the different experimental conditions. In each experimental condition, differences between different time points were evaluated by the one-way ANOVA with Tukey's post-hoc test (different letters indicate significant differences). Differences between the two experimental conditions at each time point were evaluated by the Students' t test (**p< 0.05).

Retinol uptake

After supplementation to Caco2 cells, retinol concentration inside cells and in the apical chamber was evaluated at different time points (Figure 4). Vitamin A was not detected in the basolateral chamber.

As for cholecalciferol, the increase of retinol within cells was coupled to its decrease in the apical chamber but no significant time-dependent correlation was found.

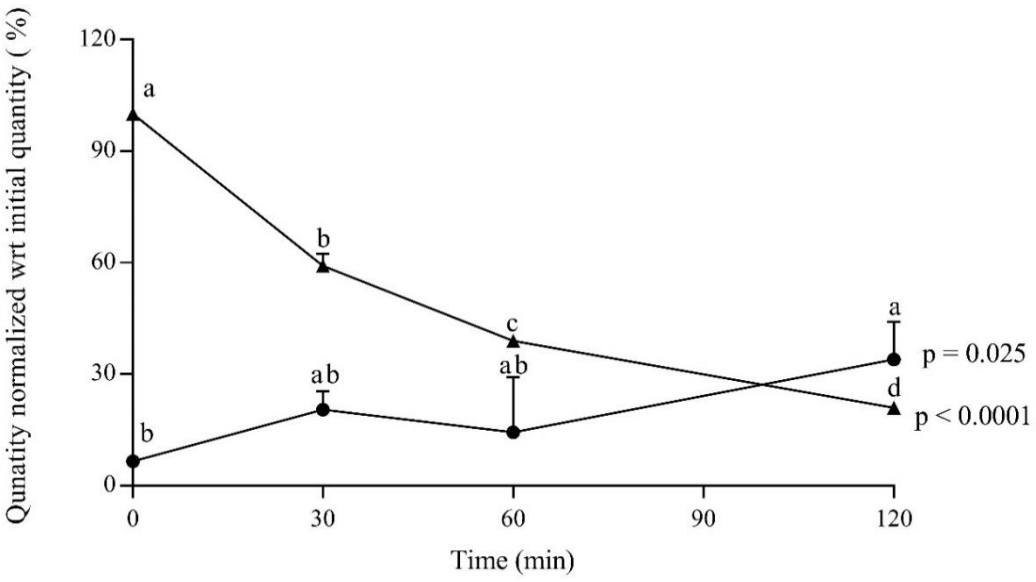


Figure 4. Concentration of retinol within cells and in the apical chamber after supplementation as mixed micelles. Circles and triangles refer to retinol concentration in cells and apical chamber, respectively. In each experimental condition, differences between different time points were evaluated by the one-way ANOVA with Tukey's post-hoc test (in each line, different letters indicate significant difference).

The time course of retinol uptake, estimated as the quantity of retinol in the harvested cells divided by the sum of the quantity of retinol remaining in the apical chamber and in the harvested cells, is reported in table 2. Even for retinol, the uptake increased significantly with time, and after 120 min it was 47.00±8.00%.

			% retinol uptake (micelles)		
t (min)	0	30	60	120	
%	0.00±0.00c	16.00±3.00bc	24.1±14.00b	47.00±8.00a	p<0.0005

Table 2. Time course of retinol uptake. Differences between different time points were evaluated by the one-way ANOVA with Tukey's post-hoc test (different letters indicate significant difference).

Discussion

Fat-soluble vitamins are a wide family of micronutrients with different vital roles for the human body. Vitamin A deficiency can result in vision disorders, growth retardation and skin diseases, while the lack of vitamin D can induce rickets and insufficient calcification of bones. Therefore, figuring out how to deliver them in the most suitable way is an important step toward creating an effective nutritional approach.

In this preliminary study, the stability and intestinal uptake of cholecalciferol and retinol conveyed in ethanol and in mixed micelles were compared, evidencing the instability of Vitamin A in ethanol. Therefore, Vitamin D was supplemented to cells as mixed micelles or solubilized in ethanol, while Vitamin A as mixed micelles only. Cell supplementation lasted for 120 min since previous studies showed that these vitamins plateaued within 2 hours [21].

Our results showed that the incorporation of cholecalciferol in mixed micelles increases its % uptake by cells. In fact, although Vitamin D was stable in both experimental conditions (mixed micelles or solubilized in ethanol) its % uptake was significantly higher when it was supplemented as mixed micelles than in ethanol. On the contrary, when cholecalciferol solubilized in ethanol was supplemented on intestinal cells, there was a very little % of vitamin uptake.

These results confirm that the interaction of fatty acids with cholecalciferol is a key step of the intestinal absorption of the vitamin. Cholecalciferol diffusion can be mediated by lipids, which act as a hydrophobic phase in which lipophilic molecules can be solubilized. Also, by stimulating the bile juice secretion lipids contribute to produce micelles. This prevents the vitamin D deposition in enterocytes and increases its absorption in the gastrointestinal tract [22]. In a randomized study involving healthy subjects, it was demonstrated that the absorption efficiency of cholecalciferol incorporated in an oil vehicle is more effective than in powder or in ethanol formulation. [23]. In addition, cholecalciferol intestinal absorption can be optimized according to oil FA composition, oleic acid being the main enhancer [24]. Of note, oleic acid was the main fatty acid (0.5mM) present in our micelles.

The significant time-dependent correlation between vitamin D increase in the cellular compartment and its decrease in the apical chamber, coupled with its no detection in the

basolateral chamber, let us hypothesize that cholecalciferol supplemented as micelles was efficiently taken up by cells, and it was not either metabolised or immediately released after absorption.

In the stability assay, retinol incorporated in mixed micelles was highly stable while it underwent 95% degradation when it was solubilized in ethanol. Although ethanol is the suitable solvent for solubilizing retinol, the high sensitivity to exogenous factors could affect its stability. Retinol, which has trans double bonds in the isoprenoid side chain and is unstable to oxygen, heat, and light, degrades quickly through a variety of instability pathways, including isomerization to cis-isomers, molecular fragmentation, and photochemical oxidation-reduction [25]. The cellular absorption of vitamin A is significantly influenced by the phospholipid concentration in micelles, mainly lysophosphatidylcholine (lysoPC), which facilitates the intestinal epithelium's simple diffusion mechanism [26], [27]. Our results confirm that when fat-soluble vitamins are tightly incorporated into micelles, their absorption efficiency is higher [27]. Indeed, the incorporation of retinol into the micelles containing 0.04 mM phosphatidylcholine and 0.16 mM lysophosphatidylcholine made it almost stable and facilitated its cellular uptake.

Comparing % uptake after 2 h supplementation, retinol uptake (about 47%) was higher than cholecalciferol (about 27%). At dietary doses, Vitamin D_3 and A absorption is probably mediated by intestinal proteins, that can be modulated by nutrients. The first nutrients that can likely modulate the expression of proteins involved in absorption of retinol and cholecalciferol are the vitamins themselves. To support this hypothesis, the expression of SR-BI, the transporter involved in apical uptake of several fat-soluble vitamins, is modulated by vitamin A [28]. Since vitamin A and D have a well-known effect on gene expression, further studies assessing whether these vitamins modulate the expression of proteins involved in their own absorption are needed.

Conclusion

It is assumed that during digestion, Vitamin A and D are transferred from the food matrix in which they are embedded to the mixed micelles generated by lipolysis of dietary triacylglycerols [29]. Mixed micelles are a mixture of phospholipids, cholesterol, lipid digestion products (such as free fatty acids, monoacylglycerols and lysophospholipids) and bile salts.

The exact mechanisms of Vitamin A and D_3 absorption are still in part unknown, although it is documented that it is affected by numerous factors, including their dose, food matrix and other nutrients in the meal [29]. As well, other gaps of knowledge to be filled are the metabolism of retinol and cholecalciferol inside the intestinal cells, the efficiency of their incorporation in chylomicrons and transport across the enterocytes, and their efflux into the intestinal lumen.

To increase our knowledge on absorption is of paramount importance to develop suitable and efficient food formulation capable of efficiently delivering fat-soluble vitamins, so counteracting deficiencies. In this view, the high sensitivity and thus instability over time of Vitamin A and D_3 must be carefully considered, as well as the applicability of processing and technologies such as nano and micro-encapsulation in complex food matrices and in the industrial environment [30]. At present, many carrier systems developed for fat-soluble vitamins appear promising, but they have not yet found application in the industrial field.

The present study represents a preliminary step to evaluate new strategies to increase the bioavailability of retinol and cholecalciferol. Recently, it has been shown that Retinol-Binding Protein (RBP) and vitamin D-Binding protein (DBP) are produced by salivary glands under mechanical stimulation [31]. RBP and DBP are medium-molecular-weight proteins present in both the intestine and liver and enable the transport of fat-soluble proteins within the human body [32].

Further studies are being planned to evaluate if salivary retinol- and vitamin D-binding proteins can affect the intestinal absorption of retinol and cholecalciferol.

References

1. Toti, E.; Chen, C.Y.O.; Palmery, M.; Villaño Valencia, D.; Peluso, I. Non-Provitamin A and Provitamin A Carotenoids as Immunomodulators: Recommended Dietary Allowance, Therapeutic Index, or Personalized Nutrition? *Oxidative Medicine and Cellular Longevity* 2018, *2018*, 4637861, doi:10.1155/2018/4637861.

2. von Lintig, J.; Moon, J.; Lee, J.; Ramkumar, S. Carotenoid metabolism at the intestinal barrier. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* 2020, *1865*, 158580, doi:https://doi.org/10.1016/j.bbalip.2019.158580.

3. Kedishvili, N.Y. Retinoic Acid Synthesis and Degradation. In *The Biochemistry of Retinoid Signaling II: The Physiology of Vitamin A - Uptake, Transport, Metabolism and Signaling*, Asson-Batres, M.A., Rochette-Egly, C., Eds. Springer Netherlands: Dordrecht, 2016; 10.1007/978-94-024-0945-1_5pp. 127-161.

4. EFSA Panel on Dietetic Products, N.; Allergies. Scientific Opinion on Dietary Reference Values for vitamin A. *EFSA Journal* 2015, *13*, 4028, doi:https://doi.org/10.2903/j.efsa.2015.4028.

5. Gürbüz, M.; Aktaç, Ş. Understanding the role of vitamin A and its precursors in the immune system. *Nutrition Clinique et Métabolisme* 2022, *36*, 89-98, doi:https://doi.org/10.1016/j.nupar.2021.10.002.

6. Bikle, D.D. Vitamin D metabolism, mechanism of action, and clinical applications. *Chem Biol* 2014, *21*, 319-329, doi:10.1016/j.chembiol.2013.12.016.

7. EFSA Panel on Dietetic Products, N.; Allergies. Dietary reference values for vitamin D. *EFSA Journal* 2016, *14*, e04547, doi:https://doi.org/10.2903/j.efsa.2016.4547.

8. Fleet, J.C.; Shapses, S.A. Chapter 6 - Vitamin D. In *Present Knowledge in Nutrition (Eleventh Edition)*, Marriott, B.P., Birt, D.F., Stallings, V.A., Yates, A.A., Eds. Academic Press: 2020; https://doi.org/10.1016/B978-0-323-66162-1.00006-8pp. 93-114.

9. Watkins, R.R.; Lemonovich, T.L.; Salata, R.A. An update on the association of vitamin D deficiency with common infectious diseases. *Canadian Journal of Physiology and Pharmacology* 2015, *93*, 363-368, doi:10.1139/cjpp-2014-0352 %M 25741906.

10. O'Byrne, S.M.; Blaner, W.S. Retinol and retinyl esters: biochemistry and physiology. *J Lipid Res* 2013, *54*, 1731-1743, doi:10.1194/jlr.R037648.

11. Harrison, E.H. Mechanisms involved in the intestinal absorption of dietary vitamin A and provitamin A carotenoids. *Biochim Biophys Acta* 2012, *1821*, 70-77, doi:10.1016/j.bbalip.2011.06.002.

12. Bhagavan, N.V.; Ha, C.-E. Chapter 36 - Vitamin Metabolism. In *Essentials of Medical Biochemistry (Second Edition)*, Bhagavan, N.V., Ha, C.-E., Eds. Academic Press: San Diego, 2015; https://doi.org/10.1016/B978-0-12-416687-5.00036-1pp. 683-699.

13. Perduca, M.; Nicolis, S.; Mannucci, B.; Galliano, M.; Monaco, H.L. Human plasma retinol-binding protein (RBP4) is also a fatty acid-binding protein. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* 2018, *1863*, 458-466, doi:https://doi.org/10.1016/j.bbalip.2018.01.010.

14. Steinhoff, J.S.; Lass, A.; Schupp, M. Retinoid Homeostasis and Beyond: How Retinol Binding Protein 4 Contributes to Health and Disease. *Nutrients* 2022, *14*, doi:10.3390/nu14061236.

15. Borel, P.; Caillaud, D.; Cano, N.J. Vitamin D Bioavailability: State of the Art. *Critical Reviews in Food Science and Nutrition* 2015, *55*, 1193-1205, doi:10.1080/10408398.2012.688897.

16. Jones, G. Pharmacokinetics of vitamin D toxicity. *The American Journal of Clinical Nutrition* 2008, *88*, 582S-586S, doi:10.1093/ajcn/88.2.582S.

17. Reboul, E. Absorption of Vitamin A and Carotenoids by the Enterocyte: Focus on Transport Proteins. *Nutrients* 2013, *5*, 3563-3581.

18. Sambuy, Y.; De Angelis, I.; Ranaldi, G.; Scarino, M.L.; Stammati, A.; Zucco, F. The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biology and Toxicology* 2005, *21*, 1-26, doi:10.1007/s10565-005-0085-6.

19. Reboul, E.; Goncalves, A.; Comera, C.; Bott, R.; Nowicki, M.; Landrier, J.-F.; Jourdheuil-Rahmani, D.; Dufour, C.; Collet, X.; Borel, P. Vitamin D intestinal absorption is not a simple passive diffusion: Evidences for involvement of cholesterol transporters. *Molecular Nutrition & Food Research* 2011, *55*, 691-702, doi:https://doi.org/10.1002/mnfr.201000553.

20. Kopec, R.E.; Gleize, B.; Borel, P.; Desmarchelier, C.; Caris-Veyrat, C. Are lutein, lycopene, and β-carotene lost through the digestive process? *Food & Function* 2017, *8*, 1494-1503, doi:10.1039/C7FO00021A.

21. Harrison, E.H. Mechanisms of digestion and absorption of dietary vitamin A. *Annual review of nutrition* 2005, *25*, 87.

22. Maurya, V.K.; Aggarwal, M. Factors influencing the absorption of vitamin D in GIT: an overview. *J Food Sci Technol* 2017, *54*, 3753-3765, doi:10.1007/s13197-017-2840-0.

23. Žmitek, K.; Hribar, M.; Hristov, H.; Pravst, I. Efficiency of Vitamin D Supplementation in Healthy Adults is Associated with Body Mass Index and Baseline Serum 25-Hydroxyvitamin D Level. *Nutrients* 2020, *12*, 1268.

24. Goncalves, A.; Gleize, B.; Roi, S.; Nowicki, M.; Dhaussy, A.; Huertas, A.; Amiot, M.-J.; Reboul, E. Fatty acids affect micellar properties and modulate vitamin D uptake and basolateral efflux in Caco-2 cells. *The Journal of Nutritional Biochemistry* 2013, *24*, 1751-1757, doi:https://doi.org/10.1016/j.jnutbio.2013.03.004.

25. Park, H.; Mun, S.; Kim, Y.-R. UV and storage stability of retinol contained in oil-in-water nanoemulsions. *Food Chemistry* 2019, *272*, 404-410, doi:https://doi.org/10.1016/j.foodchem.2018.08.076.

26. Sugawara, T.; Kushiro, M.; Zhang, H.; Nara, E.; Ono, H.; Nagao, A. Lysophosphatidylcholine Enhances Carotenoid Uptake from Mixed Micelles by Caco-2 Human Intestinal Cells. *The Journal of Nutrition* 2001, *131*, 2921-2927, doi:10.1093/jn/131.11.2921.

27. Reboul, E.; Borel, P. Proteins involved in uptake, intracellular transport and basolateral secretion of fat-soluble vitamins and carotenoids by mammalian enterocytes. *Progress in Lipid Research* 2011, *50*, 388-402, doi:https://doi.org/10.1016/j.plipres.2011.07.001.

28. Lobo, G.P.; Hessel, S.; Eichinger, A.; Noy, N.; Moise, A.R.; Wyss, A.; Palczewski, K.; Von Lintig, J. ISX is a retinoic acid-sensitive gatekeeper that controls intestinal β,β-carotene absorption and vitamin A production. *The FASEB Journal* 2010, *24*, 1656-1666, doi:https://doi.org/10.1096/fj.09-150995.

29. Borel, P. Factors Affecting Intestinal Absorption of Highly Lipophilic Food Microconstituents (Fat-Soluble Vitamins, Carotenoids and Phytosterols). 2003, *41*, 979-994, doi:doi:10.1515/CCLM.2003.151.

30. Lavelli, V.; D'Incecco, P.; Pellegrino, L. Vitamin D Incorporation in Foods: Formulation Strategies, Stability, and Bioaccessibility as Affected by the Food Matrix. *Foods* 2021, *10*, 1989.

31. Blakeley, M.; Sobczyńska-Malefora, A.; Carpenter, G. The Origins of Salivary Vitamin A, Vitamin B12 and Vitamin D-Binding Proteins. *Nutrients* 2020, *12*, 3838.

32. Albahrani, A.A.; Greaves, R.F. Fat-Soluble Vitamins: Clinical Indications and Current Challenges for Chromatographic Measurement. *Clin Biochem Rev* 2016, *37*, 27-47.

CHAPTER-7

Conclusion

Today, the increasing focus on healthy eating habits and a balanced lifestyle are driving consumers to require natural and organic foods that are sustainably produced and free of synthetic ingredients [1]. Therefore, the growing demand for "clean-label" foods motivates food technologists, in collaboration with nutrition scientists, to develop innovative production technologies and to increase the nutritional value of foods.

One of the main roles of nutrition science is to relate the composition of the food to its physiological effect, with the final aim of maintaining health and wellbeing and reducing diseases risk. Doing it, nutrition science cannot simply consider the food chemical composition. Indeed, the bioavailability and bioaccessibility of nutrients and bioactives have a role that cannot be neglected. Understanding the dynamics and the factors that influence the nutrient release from food matrix during gastrointestinal digestion fundamentally contributes to the assessment of the real nutritional value of foods. Indeed, not-bioaccessible components cannot be absorbed and cannot exert any effect within the body. Considering that bioaccessibility is influenced by any modification of the food matrix and by applied technological processes, to evaluate it should be considered mandatory before granting a food as healthy. For ethical, practical and economic reasons, bioaccessibility of food components is usually evaluated performing *in vitro* digestion.

Bioaccessible nutrients are considered available for intestinal absorption, but their chemical form or interaction with other components released from the food matrix during digestion can affect their intestinal uptake. Absorbability of nutrients/food bioactives can be assessed using *in vitro* intestinal models that simulate the intestinal lumen and internal milieu. The Caco-2 cell line is often used to study how food matrix affects the transport of molecules through the intestinal epithelial layer, and to determine the relationships between molecules during intestinal epithelial transport.

The research work performed during my Ph.D. focused on some foods which formulation and/or production technology was modified to make them “healthier" than their conventional counterparts. The use of *in vitro* models and high-throughput analytical techniques requiring

limited use of reagents and solvents allowed us to evaluate if the new products actually had an improved nutritional value

Beside the specific results obtained in the different studies, which are described in detail in previous chapters, some general findings deserve attention:

- the concentration of some “perceived as negative” nutrients or synthetic ingredients can be reduced without affecting, or even enhancing, the nutritional value of the food. Of note, some of the tested foods were also evaluated for their sensory characteristics and evidenced a good acceptability;
- the digestive process can lead to the formation of molecules with bioactive activity, such as bioactive peptides, that were not originally present in the food;
- the nutrient content of foods is not enough to define their nutritional value, and studying the factors that can influence the bioaccessibility and bioavailability of nutrients is an integral part of the evaluation.

Emerging food processing technologies play an essential role in in improving the quality and bioavailability of nutrients in foods [2]. To achieve healthy diets from sustainable food systems, require significant changes toward healthy eating patterns and enhancements in food production lines [3]. This can be achieved through the adoption of common scientific goals by all sectors of the food chain. Overall, the studies reported in this PhD thesis emphasize the need for evaluating the bioaccessibility of components of potential healthy foods before they can be properly advocated for consumption to the public. This approach has significantly influenced the work and methods of current food supply systems, leading to the development of new foods with specific functional compounds, reduction or replacement of undesirable ingredients, modification of food composition, and improvement of quality and safety.([4]. A better understanding of the behavior of nutrients within the gastrointestinal tract, by identifying ingredients that could have a positive effect on health and well-being, ensuring their stability and defining their bioavailability, could direct scientific research toward formulating foods with a high nutritional value contributing to the maintenance of good health and to the reduction of disease risk [5].

The research activities carried out during my Ph.D. clearly indicate that the collaboration and the combined intensive work of different public and private institutions, researchers and food industries does contribute to the pursuit of a proper nutrition.

References

1. Chang, M.-Y.; Chen, H.-S. Understanding Consumers’ Intentions to Purchase Clean Label Products: Evidence from Taiwan. *Nutrients* 2022, *14*, 3684.

2. Khajehei, F.; Piatti, C.; Graeff-Hönninger, S. Novel Food Technologies and Their Acceptance. In *Food Tech Transitions: Reconnecting Agri-Food, Technology and Society*, Piatti, C., Graeff-Hönninger, S., Khajehei, F., Eds. Springer International Publishing: Cham, 2019; 10.1007/978-3-030-21059-5_1pp. 3-22.

3. Willett, W.; Rockström, J.; Loken, B.; Springmann, M.; Lang, T.; Vermeulen, S.; Garnett, T.; Tilman, D.; DeClerck, F.; Wood, A., et al. Food in the Anthropocene: the EAT-Lancet Commission on healthy diets from sustainable food systems. *Lancet* 2019, *393*, 447-492, doi:10.1016/s0140-6736(18)31788-4.

4. Kourkoutas, Y.; Chorianopoulos, N.; Nisiotou, A.; Valdramidis, V.P.; Karatzas, K.A.G. Application of Innovative Technologies for Improved Food Quality and Safety. *BioMed Research International* 2016, *2016*, 9160375, doi:10.1155/2016/9160375.

5. John, R.; Singla, A. Functional Foods: Components, health benefits, challenges, and major projects. 2021, *2*, 61-72, doi:10.37281/DRCSF/2.1.7.

Acknowledgements

"We must not let people believe that all scientific progress is reduced to mechanisms, machines, gears, which also have their own beauty. I do not believe that in our world the spirit of adventure is in danger of disappearing. If I see anything vital around me, it is precisely this spirit of adventure that seems to me impossible to eradicate, and which has much in common with curiosity." Marie Curie, Diary, 1934.

Scientific research has always guided humanity toward the search for truth and knowledge of our surroundings. It is the backbone of the progress of modern civilization. Understanding its mechanisms and systems represents a clear and distinguishable light that guides us toward the discovery of important axioms, which are reflected in our daily lives. Over the centuries, the development of scientific research, traversing the meanders of the unknown, has uplifted society from popular beliefs, contributed to the application of physicochemical principles and their translation to a large scale. Advancement has brought about major changes that, while they have improved our living conditions, have also resulted in an imbalance between innovation and surplus. In reversing the direction to correct this imbalance, scientific research is analyzing the impact of progress on human life but also on the environment around us. And it is precisely the noble endeavor of science and the fascination of discovery that has captured the curiosity of scientists, soliciting my interest as well, like a vocation.

In my research field, the opportunity to explore even the surface of the scientific world has been an intense, exciting and arduous journey. And for that I must warmly thank Prof. Alessandra Bordoni for giving me the necessary tools to approach my PhD career, for stimulating my interest in research with sane and genuine challenges, and for supporting me.

Every success achieved is the result of collaboration and sharing of professional and personal knowledge and experience. To the entire Biochemistry of Nutrition team of the Department of Food Science and Technology, to Prof. Francesca Danesi and Dr. Giorgia Antonelli, I extend a great thank you. The work environment becomes like a second home and sharing it with friendly, determined and brilliant people has been a great honor.

To Dr. Mattia Di Nunzio, a colleague, a mentor and a friend, for believing in me from the first moment and who supported and sustained me in every step and choice. I dedicate a special thanks and dedicate this research thesis. I thank him for sharing the joys, fears, and satisfactions during my doctoral studies. For taking me by the hand and teaching me not only how to approach the fascinating world of research but especially for helping me through the most difficult times, both professional and personal. And when someone believes in us so much that he exposes himself so that the road is lined with bright beacons, it is an honor and a fortune that I wish all aspiring researchers.

I express my thanks to my family for their support and for teaching me to observe the world critically, spurring me to look beyond the obvious, sowing the seeds of curiosity. A fundamental tool not only for being a good researcher, but for being free people.

For support, for trust and for a multitude of advice. For reminding me that failure is not a disgrace but an encouragement to continue and that satisfactions, however small, should be celebrated. As a reminder of what we are capable of doing. To Davide, my life partner, thank you for everything.

I also express gratitude to Prof. Patrick Borel and the entire team at the Center for Cardiovascular Research and Human Micronutrition, University of Marseille for hosting me during my research period abroad. To Dr. Donato Vairo, Dr. Angela and all the PhD students, Batoul, Lisa, Mark, Katherine I dedicate special thanks for their support and wonderful collaboration. Sharing not only ideas but also emotions with young researchers, from different parts of the world, is one of the most beautiful and formative experiences in life. And I hope to continue to share them.

Finally, I would like to extend my thanks to all my colleagues from the Department of Food Science and Technology, Cesena, University of Bologna and the Department of Food and Drug Sciences, University of Parma for fruitful collaborations, which led to the publication of scientific papers.