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ADVANCING NUTRITIONAL QUALITY OF FOODS THROUGH THE
ASSESSMENT OF LIPID AND CARBOHYDRATE BIOACCESSIBILITY

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Abstract

The relationship between diet and health represents one of the central topics of contemporary scientific research. Numerous epidemiological and experimental studies have demonstrated that the quality and composition of the diet play a decisive role in the onset and progression of major chronic-degenerative diseases, such as type 2 diabetes, obesity, cardiovascular diseases, and certain types of cancer. Although it is the diet as a whole, and not the single food, that determines a possible health risk or, on the contrary, represents an important prevention factor, it is clear that the nutritional quality of food products is important. In this perspective, the food industry is increasingly attentive to the nutritional improvement of its products, also in light of the consumer demand for healthy products. The nutritional evaluation of a food product is almost always based on the chemical composition. However, the chemical composition of a food does not necessarily reflect the amount of nutrients that can actually be utilized by the human body. To properly assess the real impact of nutrients on health, it is therefore essential to consider bioaccessibility, defined as the fraction of a compound that, after digestion, becomes available for intestinal absorption. This PhD thesis aimed to investigate the *in vitro* bioaccessibility of lipids and carbohydrates in various foods and nutritional supplements, with particular attention to the influence of the food matrix, processing level, and chemical composition. In the first experimental approach, an analytical protocol for the assessment of lipid bioaccessibility was optimized and applied to four commercial foods with different degrees of processing (shelled walnuts, canned mackerel, spreadable cheese, and biscuits). The results showed that the degree of processing significantly affects the proportion of lipids effectively released and potentially absorbable, with bioaccessibility values decreasing from mackerel to spreadable cheese, walnuts, and biscuits. The approach was then extended to marine-based dietary supplements (fish oil, krill oil, and *Calanus finmarchicus* oil), demonstrating that both the chemical nature of lipids (triacylglycerols, phospholipids, wax esters) and the encapsulation form influence the bioaccessibility of n-3 fatty acids. For carbohydrates, a simplified protocol was developed to evaluate glucose release after *in vitro* digestion by integrating the final enzymatic hydrolysis phase into the INFOGEST model through the addition of amyloglucosidase. Applied to starchy products (flour, bread, crackers, durum wheat pasta, and gluten-free pasta), this method allowed

comparison between standard and high-fiber formulations, demonstrating that fiber decreases the rate of glucose release, thus modulating the potential glycemic impact of foods. In parallel, during a research stay at Ghent University (Belgium), two natural iminosugars and some of their synthetic derivatives were studied in intestinal carcinoma cell lines (Caco-2, representing an early stage, and HCT-116, representing an advanced stage) under both 2D culture (with and without glucose) and 3D spheroid conditions. The study aimed to assess their antitumor potential and interactions with carbohydrate metabolism. The results showed that these compounds modulate cellular metabolism and viability in both 2D and 3D models.

Overall, the findings obtained in this PhD project demonstrate that bioaccessibility represents a key parameter for understanding the true nutritional and functional value of foods and for developing health-oriented reformulation strategies. The combination of *in vitro* models, chemical-nutritional analyses, and cell-based studies enables progress toward an integrated approach to nutritional science, in which food quality is no longer defined solely by composition, but also by the bioavailability and physiological interaction of nutrients within the organism.

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CHAPTER 1

Introduction

In contemporary society, the quality of nutrition has become a topic of growing interest both within the scientific community and in public health policy. Several studies have highlighted that an unbalanced diet is one of the main risk factors for the development of numerous chronic-degenerative diseases [1]. In a 2019 study, Afshin et al. [2] estimated that over 11 million deaths worldwide are attributable to poor dietary habits, emphasizing the urgency of a structural shift in global eating patterns. Maintaining a balanced diet is a keystone in achieving and preserving optimal metabolic health. An eating pattern that provides the right proportions of macronutrients (carbohydrates, proteins and fats) together with essential micronutrients supports energy homeostasis and overall well-being [3]. Yet, contemporary lifestyles have profoundly altered dietary habits, making it increasingly difficult to adhere to a healthy and balanced regimen [4].

Over the past decades, the prevalence of unbalanced diets has risen markedly, often characterized by excessive or insufficient intake of specific nutrients, and strongly associated with the onset of obesity and multiple metabolic disturbances. These include insulin resistance, dyslipidemia, hypertension and systemic inflammation, which together increase the risk of chronic conditions such as type 2 diabetes, cardiovascular diseases, obesity, cognitive decline and certain forms of cancer [5]. The Western diet (WD), named for its resemblance to the increasingly prevalent dietary pattern in industrialized countries, is characterized by a high intake of fat, sugar and calories. Consumption of a WD has been linked to the development of metabolic syndrome [6]. A specific form of WD is the cafeteria diet (CAFD), which combines high amounts of carbohydrates and fats. This diet is highly palatable and promotes voluntary hyperphagia, ultimately leading to obesity [7]. High fat intake has been consistently associated with excessive fat accumulation and impaired homeostasis in both animal and human studies, contributing to the onset of several disorders [8]. Similarly, excessive sugar consumption has been related to obesity, coronary heart disease, type 2 diabetes, metabolic syndrome and non-alcoholic fatty liver disease. Moreover, sugar can activate reward pathways in the brain, favoring overeating [9]. Another issue that has emerged in recent years is the increased production and consumption of ultra-processed foods (UPFs). According to the NOVA (REF) classification, UPFs are

industrially produced food products composed of various ingredients (formulations) including sugar, oils, fats, and salt (generally in combination and in greater quantities than processed foods) and food substances with little or no culinary use (such as high-fructose corn syrup, hydrogenated oils, modified starches, and protein isolates). UPFs have become central to the debate on diet and public health. The global increase in their availability and consumption has been associated with a deterioration in overall diet quality and an increase in obesity and chronic non-communicable diseases [10,11], particularly diabetes. A recent meta-analysis of prospective studies highlighted a positive dose-response relationship between the percentage of UPF in the diet and the risk of diabetes, demonstrating that every 10% increase in UPF intake corresponds to a 15% increase in risk [12]. Consistent findings were also reported by the prospective French NutriNet-Santé study, in which an equivalent increase in UPF consumption was associated with a 15% increase in the risk of developing type 2 diabetes, independent of other dietary and lifestyle factors [13].

In contrast, a balanced diet rich in plant-based foods and low in simple sugars, trans fats, and sodium is associated with a significant reduction in the risk of chronic diseases and an increase in longevity [14].

A balanced diet provides the body with all the essential nutrients required for proper functioning. It should include a wide variety of foods from different groups, ensuring an adequate intake of macronutrients (carbohydrates, proteins and fats) as well as micronutrients (vitamins and minerals). Since nutritional needs are expressed in terms of nutrients rather than foods, it becomes necessary to translate these requirements into the appropriate quantities of foods that make up a correct diet. A balanced diet does not mean strict calorie counting or excluding entire food groups; rather, it consists of consuming the right proportions of different foods in order to supply the necessary nutrients to maintain health and well-being. Nevertheless, designing a balanced diet is not straightforward, even when considering only the three macronutrients because foods are complex matrices that contain nutrients in different amounts and proportions. In addition, carbohydrates and lipids are not homogeneous categories but encompass different molecules with distinct nutritional properties.

Carbohydrates represent the main source of energy for the human body, providing approximately 45–65% of the recommended daily caloric intake [15]. The quality of carbohydrates consumed, even more than the total quantity, has proven to be a critical determinant of health [16]. Starches represent one of the main energy sources in the human diet and constitute the primary carbohydrate storage form in plants. Chemically, they are

polysaccharides composed exclusively of glucose units linked by linear $\alpha(1\rightarrow4)$ glycosidic bonds and branched $\alpha(1\rightarrow6)$ linkages at branching points [17]. These two types of linkages give rise to two structurally distinct components: amylose and amylopectin. Amylose is a linear polysaccharide composed of glucose units linked by $\alpha(1\rightarrow4)$ glycosidic bonds, with a variable degree of polymerization that can reach several thousand glucose residues, depending on the botanical source. It is generally poorly soluble in water and can adopt a helical conformation, a feature that enables it to form complexes with substances such as iodine or lipids. Amylopectin, by contrast, has a highly branched structure consisting of short glucose chains linked by $\alpha(1\rightarrow4)$ bonds with numerous $\alpha(1\rightarrow6)$ branch points, giving it a more compact and branched architecture. In various plant starches, the ratio between these two components varies, but generally, amylopectin accounts for approximately 70–85% of the total mass, while amylose constitutes about 15–30%. The different botanical origin of starch can lead to variations in the relative content of amylose and amylopectin, as well as in granule size, shape, and overall molecular structure [17,18]. During digestion, starch undergoes enzymatic degradation that begins in the oral cavity through salivary α -amylase, and continues in the duodenum via pancreatic α -amylase and the brush border glucoamylases of the intestinal epithelium, namely maltase-glucoamylase and sucrase-isomaltase [19].

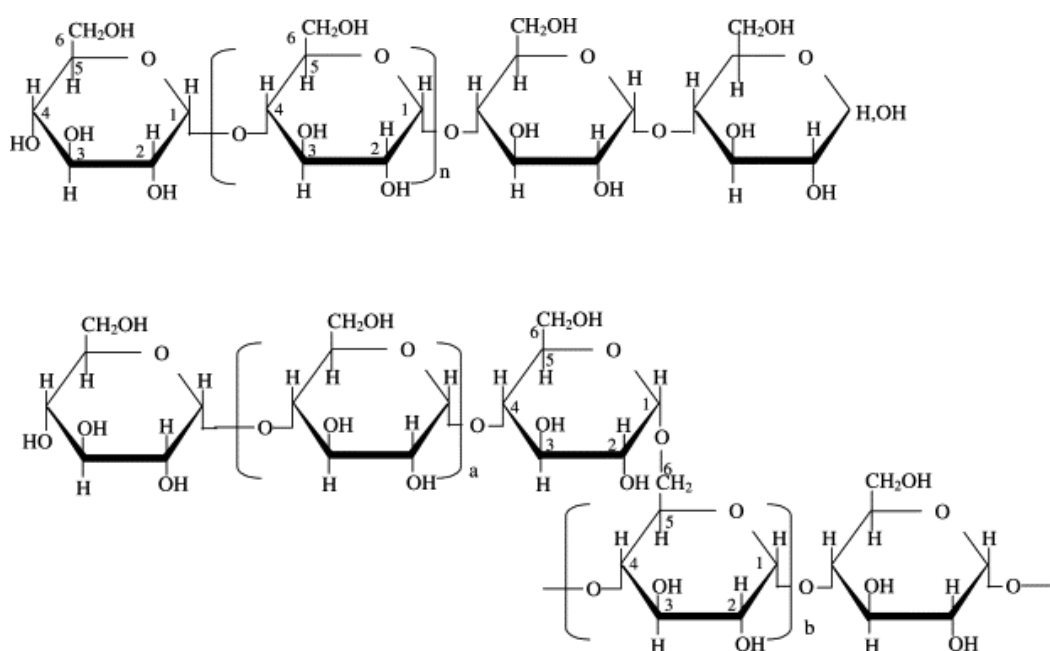


Figure 1. Structure of the two main starch polysaccharides

The efficiency of starch digestion is affected by its molecular structure, the type of thermal processing it has undergone (e.g., gelatinization, cooling), the presence of other nutrients, and the complexity of the food matrix. Based on its physiological digestibility, starch is functionally classified into three main categories: Rapidly Digestible Starch (RDS), which is completely hydrolyzed in the proximal small intestine and leads to a rapid and marked postprandial glycemic response; Slowly Digestible Starch (SDS), which is digested more gradually, releasing glucose steadily and contributing to improved postprandial glycemic control; and Resistant Starch (RS), which escapes digestion in the small intestine and reaches the colon, where it is fermented by the gut microbiota to produce short-chain fatty acids (SCFAs) such as butyrate, acetate, and propionate, compounds known to exert beneficial effects on metabolic and intestinal health [20,21]. Numerous studies demonstrate that colonic fermentation of starch and other fermentable carbohydrates produces not only SCFAs such as acetate, propionate and butyrate, but also lactate. In batch culture experiments with human fecal inocula, the production of both SCFAs and D- and L-lactate varied substantially with substrate type, reflecting differences in microbial metabolism. Furthermore, in *in vitro* fermentations digestible starch has been shown to be rapidly fermented to acetate and lactate, whereas resistant starch is fermented more slowly with greater butyrate formation. These findings indicate that lactate can be a quantitatively relevant product of starch fermentation in the colon, particularly in early phases or with specific substrates and microbial communities [22]. From a nutritional and functional perspective, RS have attracted considerable interest for their potential applications as natural prebiotics and functional agents in the management of type 2 diabetes, digestive tract function, gut microbiota composition, and blood cholesterol levels. Several studies have shown that regular consumption of resistant starch is associated with improved insulin sensitivity and reduced fasting blood glucose levels [23]. Finally, starch also plays a fundamental role in the food and pharmaceutical industries, where it is used not only for its gelling, stabilizing, and texturizing properties, but also as a vehicle for controlled drug release, thanks to its physicochemical modifiability, which allows for improved bioavailability and ensures controlled release of the active ingredient [24].

Simple sugars, or rapidly absorbed sugars, such as glucose, fructose, and sucrose are naturally found in foods like fruit, milk, and honey, but are present in much higher amounts in processed food products, including packaged sweets, sugary beverages, refined cereals, and industrial snacks [25]. While the consumption of simple sugars from natural sources is generally balanced by a high content of fiber, antioxidants, and micronutrients, added

sugars in industrial food products lack nutritional value and are absorbed very rapidly, causing a sharp spike in glycemic and insulinemic responses [26]. Chronic and excessive intake of simple sugars is associated with multiple negative effects on the body. Primarily, it contributes to the development of insulin resistance, promotes hepatic lipogenesis, and may lead to the accumulation of visceral fat all of which are risk factors for type 2 diabetes, obesity, and metabolic dysfunction–associated steatotic liver disease (MASLD) [27]. Another relevant aspect concerns industrial fructose, which is often used in the form of high-fructose corn syrup (HFCS) in many sugar-sweetened beverages. Fructose, primarily metabolized in the liver, can interfere with leptin signaling and contribute to increased plasma triglyceride levels and insulin resistance [28]. In light of this evidence, international guidelines recommend limiting the intake of free sugars —defined as all monosaccharides and disaccharides added to foods by the manufacturer, cook, or consumer, as well as those naturally present in honey, syrups, fruit juices, and fruit juice concentrates— to less than 10% of total daily energy intake, with an ideal target below 5% according to the World Health Organization [29].

Dietary fiber is a heterogeneous group of plant-derived compounds that resist digestion and absorption in the human small intestine and undergo partial or complete fermentation in the large intestine. It includes non-starch polysaccharides such as cellulose, hemicellulose, and pectin, as well as resistant starches, β -glucans, and non-carbohydrate components like lignin [30]. Dietary fiber is commonly classified as either soluble or insoluble, based on its ability to dissolve in water. Soluble fibers, such as viscous or fermentable types (e.g., pectin), dissolve to form gel-like solutions and are fermented in the colon by the intestinal microbiota. Insoluble fibers, such as wheat bran, primarily exert bulking effects and promote intestinal transit, and are only partially fermentable in the colon [31]. A higher intake of dietary fiber has been consistently associated with a lower incidence of coronary heart disease, stroke, and certain cancers. Soluble fibers, in particular, can contribute to lowering blood cholesterol by limiting the reabsorption of bile acids and through fermentation by colonic microbiota, which generates SCFAs capable of inhibiting cholesterol synthesis in the liver. In addition, fibers may play a protective role against cancer through multiple pathways, including the mechanical clearance of damaged epithelial cells from the gastrointestinal tract, promotion of greater stool volume, dilution of potential carcinogens, shortening of intestinal transit time, modulation of gut microbial composition, and binding of estrogens in the colon, which enhances their elimination via the feces and reduces circulating estrogen levels [32]. Whole foods, particularly whole

grains, fruits, vegetables, legumes, nuts, and seeds are naturally rich in dietary fiber and also provide essential micronutrients, antioxidants, phytochemicals, vitamins, and minerals. In whole grains, the bran and germ fractions contain the majority of these bioactive components, while the starchy endosperm primarily provides carbohydrates and protein. Consumption of whole foods has been associated with various health benefits, including antioxidant, antihyperlipidemic, hypocholesterolemic and anticancer effects [33,34]. In contrast, refined foods undergo extensive processing that removes the bran and germ components, significantly reducing their natural fiber and nutrient content. Such alterations often contribute to an increased risk of type 2 diabetes, obesity, and various diseases [5]. The growing availability of industrial products rich in simple sugars, refined flours, and highly processed carbohydrates has contributed, in recent decades, to the rising incidence of diet-related chronic diseases.

Lipids are essential for numerous biological functions, including the formation of cell membranes, energy storage, cell signaling, transport of fat-soluble nutrients, synthesis of enzyme cofactors, vitamins, and pigments [35]. From a chemical standpoint, lipids are compounds that are insoluble in water but soluble in organic solvents, and they include several structural classes such as fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides, as proposed in the systematic classification by [36]. Fatty acids are the fundamental building blocks of lipids. In both the diet and body stores, they are mainly present as triacylglycerols (TAG), i.e., glycerol molecules esterified with three fatty acids, often of different types. Each fatty acid can occupy one of the three positions on the glycerol backbone, designated sn-1, sn-2, and sn-3. The specific composition and arrangement of fatty acids in these positions determine the physicochemical and biological properties of the molecule, influencing, for example, its melting point and digestibility. Fatty acids consist of a chain of carbon atoms, with a methyl group (CH_3) at one end, called the omega (ω) or n terminal, and a carboxyl group (COOH) at the other end, known as the delta (Δ) terminal. Hydrogen atoms are bonded along the carbon chain, forming a hydrocarbon structure. A key distinction within fatty acids is that between saturated fatty acids (SFAs) and unsaturated fatty acids, which are further divided into monounsaturated (MUFAs) and polyunsaturated (PUFAs). SFAs are characterized by the absence of double bonds in the carbon chain and are found mainly in animal products. In contrast, unsaturated fatty acids have one or more double bonds and are generally liquid at room temperature. MUFAs are found mainly in vegetable oils such as olive oil, peanuts, and avocados, while PUFAs include omega-6, abundant in seed oils,

and omega-3, found in fish and some plant sources such as flaxseed and walnuts. PUFAs are classified as n-3 (omega-3) or n-6 (omega-6) according to the position of the first double bond from the methyl terminal: between the third and fourth carbon in n-3 and between the sixth and seventh carbon in n-6. N-3s include one essential fatty acid (EFAs), α -linolenic acid (ALA), which is the precursor of longer and more unsaturated fatty acids as eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), known for their anti-inflammatory, neuroprotective, and cardiovascular benefits [37]. Also, n-6s include an EFA, linoleic acid (LA), which is desaturated and elongated in the body to form other fatty acids as arachidonic acid (AA). N-6s are more abundant in the WD and, when consumed in excess of n-3, promote the pathogenesis of many diseases [38]. ALA and LA are defined as essential because the human body is unable to synthesize them *de novo* due to the lack of the necessary enzymes, and they must therefore be obtained through the diet [39]. Although other n-3s and n-6s can be synthesized by humans, this occurs in insufficient amount and there is the need of a dietary intake of longer and more unsaturated PUFAs. Particularly, EPA and DHA are critical nutrients as they are present only in seafood, which consumption is low in the WD. Double bonds in unsaturated fatty acids can occur in either *cis* or *trans* configuration. The *cis* form, which predominates in foods, lowers the melting point of fatty acids and makes them more fluid at room temperature.

Trans fatty acids (TFAs), less common in nature, are found in small amounts in the fat and milk of ruminants, where they are formed in the rumen during digestion, and can also be produced artificially through the hydrogenation of unsaturated oils, a process traditionally used in the manufacture of margarine [40]. TFAs, mainly of industrial origin (as in commercial baked goods, snacks, solid margarines, and fried foods), pose an even greater risk. They alter the lipid profile by increasing LDL and lowering HDL cholesterol, aggravate chronic inflammation, and are associated with a documented increase in the risk of cardiovascular disease, type 2 diabetes, and total mortality. According to some authors, there is no safe threshold for trans fat intake, and their consumption should ideally be eliminated [41]. Since natural trans fat are present in milk and dairy, the Italian LARN [42] recommend an introduction “as low as possible”. The scientific literature indicates that industrial TFAs, such as elaidic and linoelaidic acids, are strongly associated with increased cardiovascular risk, systemic inflammation, and dyslipidemia, whereas natural TFA, such as vaccenic acid and trans-palmitoleic acid, exhibit neutral or sometimes even beneficial effects on lipid profiles and metabolism [43].

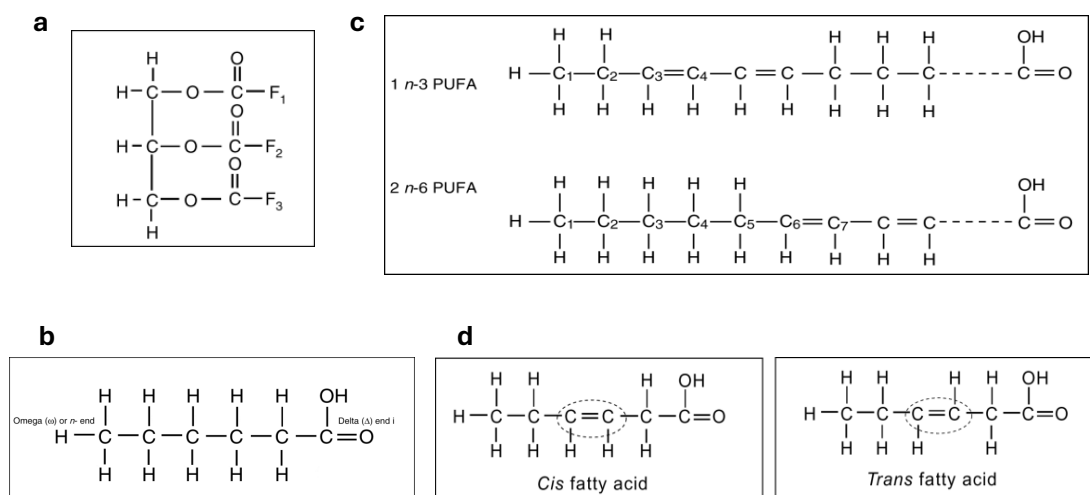


Figure 2. Structure of triacylglycerol and fatty acids

(a) Molecular structure of triacylglycerol, highlighting the fatty acids esterified at the sn-1, sn-2, and sn-3 positions (F₁, F₂, and F₃). (b) Structure of a fatty acid, showing the hydrocarbon chain and the terminal carboxyl group. (c) n-3 and n-6 fatty acids, classified as PUFAs based on the position of the first double bond from the methyl end. (d) Schematic representation of cis and trans configurations of unsaturated fatty acids, highlighting the spatial orientation of hydrogen atoms around the double bond [40].

Another important aspect to consider when formulating a balanced diet is that, to be effective nutrient carriers, foods must be digested. Digestion allows nutrients to be released from the food matrix and hydrolyzed into molecules small enough to be absorbed. Excluding dietary fiber, which exerts its effect at the intestinal level without being absorbed, all other nutrients must cross the intestinal mucosa and enter the circulatory system, that is, they must become bioavailable, in order to exert an effect on the organism, whether positive or negative. The importance of bioavailability is well known, and for carbohydrates the concept of glycemic index (GI) was developed, introduced by Jenkins et al. (1981), which classifies foods based on how quickly and how strongly they raise blood glucose levels compared to a reference carbohydrate, usually pure glucose or white bread [44]. GI reflects the bioavailability of carbohydrates in a specific food, and it is related not only to the concentration of starch and sugars but also the food matrix and its processing. The glycemic load (GL), defined as the product of a food's GI and its carbohydrate content, is an indicator of the overall impact of diet on blood glucose [45]. Jenkins et al. (2002) pointed out that GL has proven particularly useful in epidemiological studies, where it has been associated with an increased risk of coronary heart disease in individuals following

high GI and GL diets [46]. A high dietary GL stimulates excessive insulin release, which, over time, can lead to impaired glucose metabolism and increased systemic inflammation [47]. This pattern, if chronic, can lead to hyperinsulinemia, insulin resistance, and low-grade systemic inflammation, key factors in the development of type 2 diabetes, visceral obesity, and cardiovascular disease [48,49]. Conversely, low GI diets rich in legumes, whole grains, vegetables, fruits, and unrefined wheat products have shown positive effects in the prevention of various chronic diseases, helping to reduce the risk of type 2 diabetes, cardiovascular diseases, and obesity [50,51]. Several nutritional guidelines [52–54] therefore recommend a dietary approach that emphasizes low-GI and high-fiber carbohydrate sources, integrated into a balanced dietary pattern.

Nutrient bioavailability is therefore a parameter to consider when discussing a balanced diet. Since bioavailability depends not only on the type of nutrient, but also on the food matrix in which it is embedded and the transformation processes it has undergone, it is increasingly clear that improving the nutritional value of foods also depends on evaluating the bioavailability/bioaccessibility of their components.

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CHAPTER 2

Aim of the study

Everyday great amounts of food are produced, processed, transported and consumed, and these activities have direct impact on our health and the environment. The current food system has started causing strain on the Earth's natural resources and that is why sustainable food production systems are needed. With the estimate of the world's population reaching to nine billion by 2050, providing our growing planet with safe, sufficient and nutritious food supply is expected to become a challenge. Sustainable diets contribute to food and nutrition security, have low environmental impacts and promote healthy life for present and future generations.

The problem of increasing population put on one side, the present food system produces items containing excess amounts of fat and sugar promoting overweight and obesity. The most important health impacts of excess fat, sugar and sodium include increased risk of heart disease, stroke and cancer, as well as an increased prevalence of obesity and premature death. It is therefore necessary to bring the population back to a healthier and more balanced diet, and to do this, it is essential to formulate, produce, and offer consumers foods with high nutritional value. Although currently the evaluation of a food is based on its chemical composition, the importance of the bioaccessibility/bioavailability of its components is increasingly recognized.

The overall objective of this research was to evaluate *in vitro* the bioaccessibility of lipids and carbohydrates, two macronutrients of fundamental importance in the diet, whose incorrect introduction is frequently associated with the development of chronic degenerative diseases such as diabetes, obesity, and cardiovascular disease. The type of carbohydrates consumed, in terms of chemical complexity, degree of refinement, and total quantity, determines the GI and GL of the diet. These two parameters provide information on the impact of carbohydrates on glucose metabolism and insulin response, and have been associated with an increased risk of chronic degenerative diseases such as type 2 diabetes, cardiovascular disease, obesity, and certain forms of cancer [1–3]. Given the lack of an identifiable safe threshold, EFSA recommends that the consumption of total, added and free sugars be kept as low as possible within the context of a nutritionally adequate diet [3]. As reported by Jankiewicz et al. (2015) , a chronic high-sucrose diet initiated early in life

caused a redistribution of adipose tissue towards the abdominal area in rats, along with dyslipidemia, hypertension, hyperinsulinemia, and signs of insulin resistance, all of which are risk factors for the development of metabolic and chronic degenerative diseases" [4].

As for fats, not only the quantity but also the quality of fat consumed is crucial. SFAs and trans fats are strongly associated with an increased risk of chronic degenerative diseases. SFAs, found in foods such as red meat, butter, cheese, and some tropical oils, are known to increase LDL cholesterol levels and promote systemic inflammation, factors that contribute to the onset of cardiovascular diseases. Replacing SFAs with unsaturated fatty acids, particularly PUFAs, is associated with a significant reduction in coronary risk [5] In the field of nutrition, there is growing recognition of the importance of the bioaccessibility of nutrients—that is, the fraction of a compound present in food that is effectively released during digestion and made available for intestinal absorption [6].

Therefore, it is not sufficient to analyze the chemical composition of foods: the nutrient content does not automatically correspond to the amount that the body can actually utilize. Bioaccessibility is influenced by multiple factors, including the food matrix, the presence of other nutrients or antinutritional compounds and food processing technologies [7]. This variability can lead to either an overestimation or underestimation of the true nutritional value of a food. For example, certain micronutrients, despite being present in high amounts, may be poorly absorbed if they are bound to indigestible components or not properly released in the gastrointestinal tract. Measuring nutrients bioaccessibility and understanding the mechanisms that regulate it allows for a more accurate evaluation of a food's effectiveness in preventing nutritional deficiencies or supporting metabolic health. In this view, as part of my PhD project, I focused on the *in vitro* assessment of the bioaccessibility of fatty acids in commonly consumed foods, with the aim of overcoming the limitations of relying solely on chemical composition and obtaining a more realistic estimate of the fraction of lipids potentially absorbable in the intestine. The work was initially articulated around two main objectives: first, the optimization of an analytical protocol for evaluating lipid bioaccessibility, and second, the application of this protocol to four commercial foods selected for their different lipid composition and matrix structure, and for the degree of processing: shelled nuts (minimally processed food), canned mackerel (processed food), biscuits, and spreadable cheese (ultraprocessed foods). This approach was designed not only to test methodological robustness but also to provide insights into the role of the food matrix and degree of processing in modulating lipid bioaccessibility, thereby offering a more accurate basis for the nutritional classification of foods. In the

second study, already published in Foods (2024), the analytical approach was extended to the evaluation of n-3 bioaccessibility in marine supplements. This work was motivated by the increasing use of marine oil-based supplements as an alternative or complement to fish consumption, aiming to bridge the gap between actual and recommended n-3 intake in the population. In this context, it is not sufficient to merely consider the declared content on the product label; it is essential to assess the fraction that is effectively released and made available during digestion, namely the bioaccessible fraction. For this reason, four commercial supplements were analyzed, selected to represent different marine lipid sources and lipid classes: a traditional fish oil (mainly in the form of TAG), a krill oil (characterized by a high proportion of phospholipids), and two supplements containing oil derived from the copepod *Calanus finmarchicus* (where PUFAs are largely esterified as wax esters). For each product, both the pure oil and the encapsulated form in soft gel capsules were evaluated, with the aim of determining whether and to what extent encapsulation could influence the release and availability of fatty acids during simulated digestion.

As for carbohydrates, a simplified protocol was developed to assess glucose release from starchy products after *in vitro* digestion, with the aim of providing a practical, reproducible, and easily applicable tool for comparing commercial foods. This work stemmed from the need to overcome the limitations of the standard INFOGEST protocol which, although representing the international reference for simulating gastrointestinal digestion, does not include the final stage of starch hydrolysis carried out by brush border glucosidases. *In vivo*, intermediate products of enzymatic digestion such as maltose and dextrins are completely converted into glucose through the action of enzymes such as maltase-glucoamylase and sucrase-isomaltase [8]. The absence of this phase in the *in vitro* model leads to an underestimation of the actual amount of glucose available for absorption. To address this gap, the static INFOGEST protocol was integrated with the addition of amyloglucosidase (AMG), an enzyme that mimics the activity of intestinal α -glucosidases (such as maltase-glucoamylase and sucrase-isomaltase) in the brush border membrane of the small intestine. AMG is an exo-acting enzyme capable of hydrolyzing both α -1,4 and α -1,6 glycosidic linkages at the non-reducing ends of starch oligosaccharides, catalyzing the sequential release of glucose units from maltose, maltotriose, and dextrins. The inclusion of AMG in the digestion protocol ensures a more complete hydrolysis of starch-derived dextrins that would otherwise remain partially undigested after pancreatic α -amylase action alone. This results in a more realistic estimation of glucose release kinetics, better reflecting physiological digestion processes and postprandial glucose availability.

However, it is important to note that AMG does not fully replicate the physiological activity of human brush-border α -glucosidases. Unlike the combined action of maltase-glucoamylase and sucrase-isomaltase—each possessing distinct kinetic properties and substrate specificities—AMG provides a simplified exo-acting activity that does not entirely reflect the complexity of intestinal carbohydrate digestion. Therefore, while the inclusion of AMG improves the estimation of starch hydrolysis *in vitro*, the resulting glucose release should still be interpreted as an approximation of *in vivo* brush-border enzymatic activity. The optimized protocol was applied to five foods representative of the main sources of dietary starch (flour, bread, crackers, durum wheat pasta, and gluten-free pasta), selected also for their nutritional relevance and widespread consumption. For each food, the standard formulation was compared with its corresponding high-fiber version (>6 g/100 g), in order to evaluate whether and to what extent fiber inclusion could modulate the kinetics of glucose release. This experimental approach not only validated the reliability of the protocol but also provided comparative data of practical interest for classifying foods in relation to their potential glycemic impact. In parallel, during my research stay at Ghent University (Belgium), I focused on the study of iminosugars, a class of sugar-mimicking compounds in which a nitrogen atom replaces the oxygen normally present in the sugar ring structure. This structural modification endows iminosugars with unique biological properties, such as the ability to inhibit glycosidases and glycosyltransferases. Iminosugars can occur naturally, being produced by plants and microorganisms, where they often play a defensive role by blocking key enzymes in the carbohydrate metabolism of predators or pathogens. They are naturally found in several foods, including rice, potatoes, buckwheat, stevia, mulberry leaves and roots, as well as in many fermented foods, particularly of Asian origin. Alongside natural compounds, synthetic derivatives have also been developed to improve their stability, potency, or specificity toward particular biological targets. In our study, attention was focused on two natural iminosugars, D-fagomine (FGM) and 1-deoxynojirimycin (DNJ), as well as on several of their synthetic derivatives, including isofagomine (IFGM), miglitol (MGTL), N-(n-Nonyl)-deoxynojirimycin (NN-DNJ), and 2,3,4,6-Tetra-O-benzyl-(+)-1-deoxynojirimycin (TBN-DNJ), with the aim of assessing their potential antitumor effects on intestinal carcinoma cells. To this end, iminosugars were tested on two intestinal cancer cell lines with different degrees of tumor progression: Caco-2 (early stage) and HCT-116 (advanced stage). Cells were cultured under conventional two-dimensional (2D) conditions in DMEM medium, both in the absence and presence of

glucose, as well as in three-dimensional (3D) spheroids, which more realistically reproduce the architectural and metabolic complexity of the tumor microenvironment.

Overall, my research project had four main objectives:

- provide transparent information on the true nutritional value of foods, with potential impact on public health;
- expand knowledge of the influence of different matrices and processing methods on the bioaccessibility of food components;
- produce scientific evidence capable of guiding the food industry toward the production of healthier foods;
- to evaluate the antitumor potential of specific natural molecules and their synthetic derivatives through *in vitro* studies, as a first step toward future preclinical studies.

The outcomes of this research endeavor may thus contribute to the advancement of a more rigorous and comprehensive understanding of the complex relationships between specific foods and human health, by systematically accounting for matrix- and processing-dependent variations in bioaccessibility, and by establishing a robust conceptual framework for future investigations into the antitumor potential of selected natural molecules and their synthetic derivatives.

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CHAPTER 3

Section 1 – Lipids and fatty acids

Lipid digestion begins in the mouth, where lingual lipase partially attacks TAGs, but the core of the metabolic process takes place in the stomach and, above all, in the small intestine. Here, bile salts, secreted by the liver and stored in the gallbladder, emulsify lipids, reducing the size of lipid droplets and increasing the surface area available for the action of digestive enzymes. This emulsification is essential for pancreatic lipase and its auxiliary protein, colipase, to efficiently break down TAGs into free fatty acids (FFAs) and monoglycerides (MAGs). The products of lipid digestion aggregate to form mixed micelles, structures that are essential for transporting lipids through the intestinal lumen to the surface of enterocytes. Micelles are essential because they solubilize lipophilic compounds such as FFAs, MAGs, and cholesterol, facilitating their contact with the apical membrane of intestinal cells. The absorption of lipids through the apical membrane of enterocytes is mediated by specific protein transporters, including CD36, FATP4, and Niemann-Pick C1 like 1 (NPC1L1), which facilitate the entry of FFAs, cholesterol, and other products of lipid digestion [1,2]. After being absorbed into the brush border membrane of enterocytes, lipid portions (such as FFAs and MAGs) must be transported to the endoplasmic reticulum. To this end, specific intracellular transport proteins, such as fatty acid binding proteins 1 and 2 (FABP-1 and FABP-2), bind FFAs and MAGs, while sterol transport proteins such as sterol carrier protein-1 (SCP-1) and sterol carrier protein-2 (SCP-2) transport free cholesterol. Numerous enzymatic reactions are then activated within the endoplasmic reticulum, leading to the de novo synthesis of TAGs, phospholipids, and cholesterol esters. Within the enterocyte, lipids are assembled into chylomicrons within the smooth endoplasmic reticulum, thanks to the action of microsomal transfer protein (MTP), which facilitates the association of apoB-48 with phospholipids, cholesterol, and small amounts of TAGs. In parallel, MTP transports TAGs and cholesterol esters to form larger lipid particles, which associate with apolipoprotein A-IV (apoA-IV). These two types of particles then merge to generate pre-chylomicrons, composed of a neutral lipid core surrounded by an amphipathic monolayer. The assembled particles mature in the Golgi apparatus and are secreted into the lymph, eventually entering the bloodstream. SCFAs can efficiently cross the enterocyte barrier and are directly delivered into the portal

circulation [3]. SCFAs—primarily acetate, propionate, and butyrate—are produced in the colon through bacterial fermentation of undigested carbohydrates. Once formed, these metabolites are absorbed by colonic epithelial cells via facilitated transport and passive diffusion, but their metabolic fate differs depending on the specific molecule. Most butyrate is used locally by colonic epithelial cells (colonocytes) as their main energy source, supporting mucosal health and epithelial barrier integrity. Only small amounts of butyrate reach the portal circulation because the majority is directly oxidized within colonocytes to generate ATP. In contrast, acetate and propionate, after being absorbed by colonic cells, enter the portal circulation and reach the liver and peripheral tissues: acetate is the predominant SCFA in systemic circulation, whereas propionate is largely metabolized in the liver through gluconeogenic pathways. This divergent metabolic fate reflects the distinct functional roles of the three major SCFAs in human metabolism [4]. Chylomicron secretion can be influenced by several factors, including the amount of biliary or dietary phospholipids, the total fat content of the diet, and the composition of dietary fatty acids, particularly the presence of n-3 fatty acids [5].

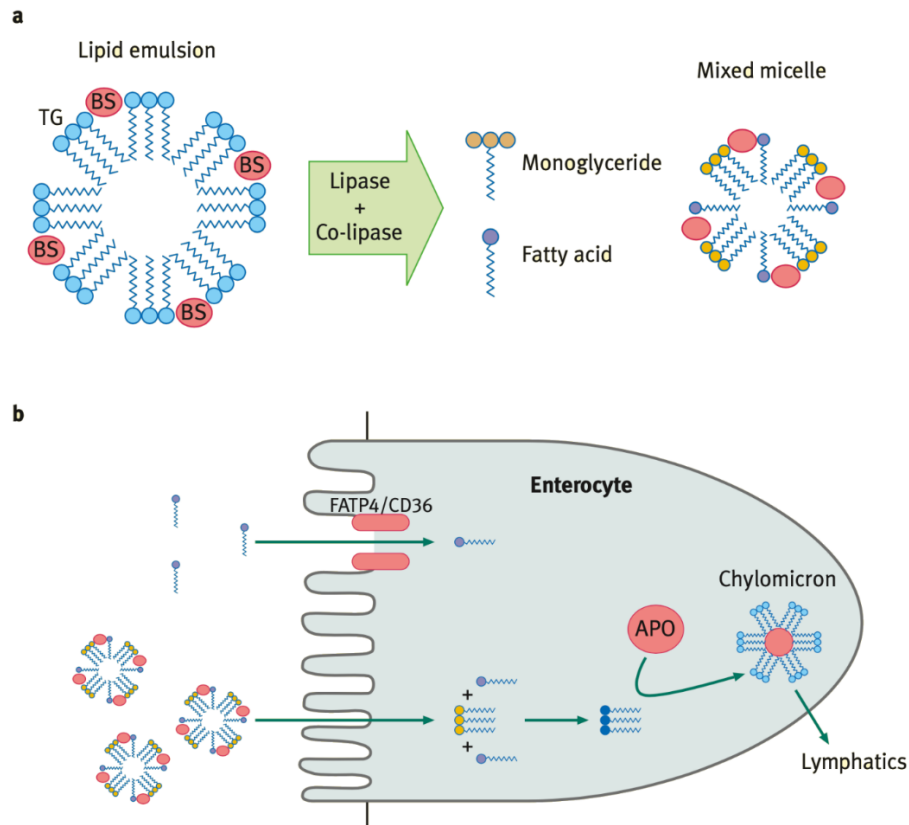


Figure 3. Schematic representation of lipid absorption

(a) Triglycerides in the lipid phase are hydrolyzed by lipases and co-lipases into monoglycerides and FFAs, which organize into mixed micelles with bile salts. (b) Mixed micelles release fatty acids and monoglycerides, which enter enterocytes through specific transporters (e.g., FATP4/CD36). Once in the cytosol, the fatty acids are re-esterified into triglycerides and assembled with apolipoproteins (APO) to form chylomicrons, which are finally secreted into the lymphatic system.

The absorption of dietary lipids and their distribution through the bloodstream is a highly complex mechanism, which determines their bioavailability. However, a fundamental variable that defines bioavailability is bioaccessibility, that is, the extent of release from the matrix and hydrolysis of lipids to chemical forms for which absorption is possible. In turn, bioaccessibility depends on the food matrix and the chemical form of the lipids, and cannot be easily predetermined without direct assessment. The bioaccessibility of lipids in a specific food is rarely verified, and the nutritional assessment is usually based on the chemical composition of the food itself, which must be reported on the label. This can lead to inconsistent nutritional evaluation, because the chemical composition does not take bioaccessibility into account and is therefore only a partial reflection of the possible effect

of the nutrients contained in the food. From a nutritional and health standpoint, differences in lipid bioaccessibility may have important implications for metabolic health. Only the fraction of lipids that becomes bioaccessible during digestion can be absorbed and incorporated into metabolic pathways, influencing plasma lipid profiles, energy homeostasis, and inflammatory balance [6]. Reduced bioaccessibility may limit the uptake of essential fatty acids and other beneficial lipid components, while excessive bioaccessibility of certain lipid classes could contribute to metabolic dysregulation and increased cardiovascular risk. Therefore, understanding and modulating lipid bioaccessibility represents a key step in designing dietary strategies and food products that promote cardiovascular and metabolic well-being. Considering bioaccessibility during food formulation and processing is of paramount importance in the validation and development of innovative food products. Tailoring the structure of the food matrix or the lipid composition can optimize the release, digestion, and absorption of lipids, enhancing their nutritional value and functional properties. Integrating bioaccessibility data into product design allows the food industry and nutrition science to move beyond traditional compositional analysis toward a more physiologically relevant assessment of nutrient quality and potential health effects. The two studies reported in this section aimed to demonstrate how bioaccessibility can vary depending on the type of matrix and the chemical characteristics of the lipids present in the food.

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Impact of food matrix and processing on lipid bioaccessibility: an in vitro comparison across minimally processed, processed, and ultra-processed foods

Introduction

Dietary lipids, commonly called fats, are the major source of energy per unit of weight in the diet (38 kJ/g - 9 kcal/g). They are macronutrients that, in addition to providing energy, serve many other functions in the body; they are components of cell membranes, precursors of bioactive compounds such as prostaglandins, prostacyclins, and thromboxanes, and transporters of fat-soluble nutrients. The main components of dietary fats are triglycerides (TAGs). Mono- (MAGs) and diacylglycerols (DAGs), and phospholipids (PLs) are present in small amounts, and animal fats also contain other lipids such as free cholesterol and cholesterol esters (CE) [1].

The first step in fat processing in the gastrointestinal tract is the release from the food matrix. Then, to be made available for hydrolysis, fats must be emulsified. In the stomach, emulsification occurs through mechanical action, and in adults, only a small portion of dietary fats are ultimately hydrolyzed. Following gastric digestion, in the duodenum, bile produced by the liver acts as a surfactant to produce a fine emulsion that is more readily accessible for hydrolysis by pancreatic enzymes. The hydrolyzed lipid-soluble components (free fatty acids -FFAs-, 2-MAGs, lyso-PLs, free cholesterol) integrate with bile salts in mixed micelles to diffuse between intestinal microvilli and interact with transporters located on the luminal surface of enterocytes [2].

TAGs are molecules composed of a glycerol backbone to which three fatty acids (FAs) are attached, and for nutritional adequacy and health outcomes, the type of esterified fatty acids present in food TAGs is crucial. The importance of the qualitative content of FAs in foods is increasingly evident and confirmed by numerous studies. Saturated fatty acids (SFAs) have been linked to an increased risk of cardiovascular disease when consumed in large quantities, and while there is some controversy about the need to reduce intake of these FAs for cardiovascular prevention [3], EFSA recommendations indicate that SFAs intake should be as low as possible within the context of a nutritionally adequate diet [1]. Conversely, a diet including foods rich in polyunsaturated fatty acids (PUFAs), particularly long-chain omega-3s, is believed to have a positive impact on health [4–6]. In their

systematic review, Schoeneck & Iggman (2021) demonstrated with strong evidence that foods rich in unsaturated fatty acids and low in saturated and TFAs cause at least moderate reductions (i.e., 0.20–0.40 mmol/L) in serum LDL cholesterol [7].

Evidence of the importance of the quantity and quality of FAs consumed through food raises a nontrivial question: is chemical composition a reliable parameter for distinguishing between different foods?

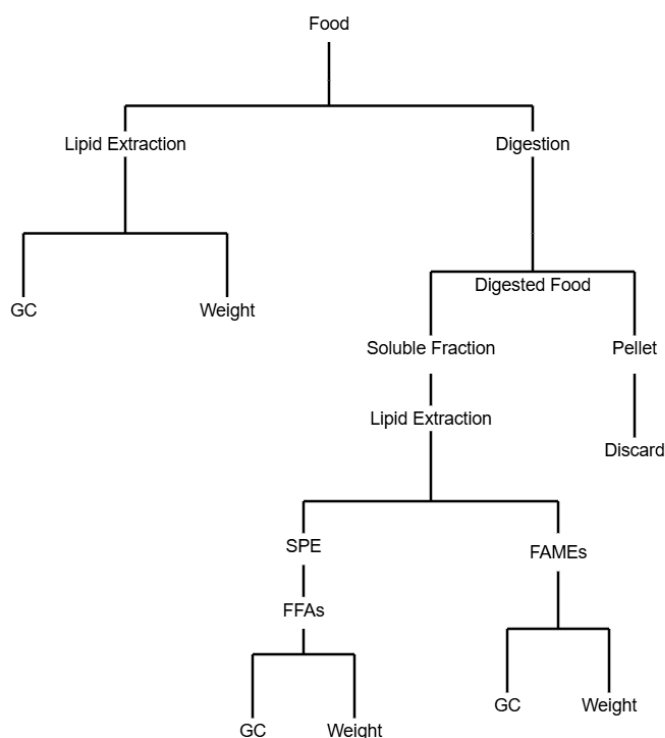
Indeed, to be absorbed in the intestine and thus exert their effects in the body, TAGs must become bioaccessible during digestion, that is, be released from the food matrix and hydrolysed in a suitable form [8]. An assessment of the bioaccessibility of fats could better reflect the role of the food containing them in the diet. Therefore, assessing bioaccessibility is gaining momentum, and *in vitro* protocols are considered an ideal tool for investigating bioaccessibility, at least as a first step in nutritional studies and for guiding the formulation of new foods [9]. From this perspective, since the bioaccessibility of food components in isolation differs from the bioaccessibility of the same components when they are part of foods [10], evaluating the matrix effect, that is, the impact of the food matrix, has become increasingly important. The bioaccessibility of nutrients/food components in general - and of lipids specifically – in a specific matrix is not invariable, as it can be modulated by the processing [11]. In this regard, the potential impact of food processing on nutrient bioaccessibility is one of the proposed mechanisms linking ultra-processed food (UPF) intake to obesity risk, since processing can alter food matrices and nutrient bioaccessibility, influencing metabolism and fuel breakdown [12].

Aim of the study

The aim of this study was to provide further insight on the influence of the food matrix and its processing on the bioaccessibility of lipids. To this purpose, four different commercial foods were digested *in vitro* using the INFOGEST protocol. The four foods chosen had in common that they were ready to eat (RTE) and that they were often evaluated nutritionally based on their quantitative and qualitative fat content, either positively (high in PUFA) or negatively (high in SFA). However, they differed in the degree of processing, as one (shelled walnuts) was minimally processed, one (canned mackerel in brine) was processed and two (cream cheese and biscuits) were UPF, according to the NOVA classification [13]. To evaluate bioaccessible lipids, the digested food obtained at the end of INFOGEST digestion was centrifuged and lipids were extracted from the soluble fraction (SF) [14]. As

the characteristics of the food matrix modulate the effectiveness of extraction methods in the recovery of total lipids, in preliminary experiments lipids were extracted using both the Folch et al (1957) and the Bligh & Dyer (1959) methods. Bioaccessible lipids were quantified by weight and by gas-chromatography, after methyl-esterification and using an internal standard (IS). The evaluation of total lipids and fatty acid methyl-esters (FAMES) concentration and profile was also performed in the corresponding not digested (ND) samples. Finally, FFAs were separated from the total lipids extracted from the SF of the digested food using solid-phase extraction (SPE), and quantified by gas-chromatography. The experimental design is schematized in figure 1. To ensure that the four food items could be directly compared despite their different total lipid contents, all measurements of bioaccessible lipids were normalized to the total lipid content of each individual food matrix (expressed as % of total lipids in the undigested sample). This normalization accounts for the intrinsic differences in fat quantity across foods and allows the comparison to reflect differences in bioaccessibility due solely to matrix structure and processing, rather than absolute fat content. Although INFOGEST was initially developed and validated mainly for protein digestion, it has evolved into a comprehensive framework that also robustly describes lipid digestion. However, as a static model, it cannot reproduce dynamic regulatory mechanisms such as lipid-induced ileal brake responses.

Figure 1. Experimental Design



Material and Methods

Materials and Chemicals

Experiments were performed on four different commercial foods (biscuits, shelled walnuts, canned mackerel in brine, and cream cheese) purchased from local supermarkets. For each food, three different production batches were considered.

Digestive enzymes (α -amylase from human saliva, type IX-A, pepsin from gastric mucosa, pancreatin from porcine pancreas), bile extract porcine, fatty acid methyl esters standard mix and the internal standard (pentadecanoic acid C:15, 10mg/ml) were purchased from Sigma Aldrich (Burlington, MA, USA). All reagents and solvents were purchased from the same company except where specified otherwise.

In Vitro Digestion

The foods were *in vitro* digested according to the INFOGEST protocol [15]. The stock electrolyte solutions making up simulated salivary fluid (SSF), simulated gastric fluid

(SGF) and simulated intestinal fluid (SIF) were formulated following the protocol instructions. *In vitro* digestions of the different foods were performed in triplicates using 5 grams of food for each digestion, as well as a blank weighing 5 grams of distilled water and adding the same enzymes and bile added in the digestion of the food. At the end of digestion, the resulting digested foods were transferred into 50-mL Falcon® test tube and centrifuged at 4500 rpm for 10 min at 4°C, obtaining a pellet and a SF, which was stored at -20 °C degrees until further analyses.

Lipid extraction and methyl-esterification

Lipids were extracted from 0.1 g ND foods and 800 ml of SF of digested food with two different methods [16,17]. For the Bligh and Dyer method [16], samples were homogenized in 9 ml of methanol:chloroform (2:1), then stored at -20 °C overnight. After overnight storage 3 ml of chloroform was added and the solution was mixed by vortexing. Then, 3 ml of HCl 2.5 N was added and the sample was vortexed again. The solution was then transferred into a conical glass tube with a cap and centrifuged at 450 g for 10 minutes at room temperature, thus forming two separate phases. The upper aqueous phase was removed with a Pasteur pipette, retaining the lower organic phase which was poured over a paper filter filled with a spatula of Na₂SO₄ into Pyrex tubes.

In the Folch method [17], samples were homogenized in 8 ml of 1:1 chloroform:methanol solution and incubated at 60°C for 20 minutes. Then the sample was cooled to room temperature and 4 ml of chloroform was added. The solution was vortexed, 4 ml of 0.1 M KCl was added and the sample was incubated at 4 °C overnight. The next day, the top aqueous phase was removed and the bottom organic phase was poured over a paper filter filled with a spatula of Na₂SO₄ into Pyrex tubes.

The Pyrex tubes from both extractions were placed into a thermo-block at 37 °C under nitrogen flow, bringing the sample to complete dryness without oxidizing the lipids, then the lipids were quantified by weighing using an analytical balance.

After weighing the lipids, the same methyl-esterification procedure [18] was used for all samples. After addition of 100 ml of IS (pentadecanoic acid, C:15), samples were put in a thermo-block at 37 °C under nitrogen flow and brought to dryness. Then, 500 ml of methanolic HCl 3N was added, and after vortexing the capped Pyrex tubes were placed in an oven at 100 °C for 1 hour. After allowing the samples to cool to room temperature, 2 ml of hexane and 2 ml of distilled water were added. The upper organic phase was taken out,

transferred into glass truncated conical tubes and stored at -20°C until analysis. Just before gas chromatographic analysis, the conical tubes were placed in a thermo-block at 37° C under nitrogen flow until the organic phase completely evaporated. The sample was then resuspended in hexane via percolating it along the walls of the tube. The tube was closed with a cap and stored at -20 °C until injection, after taking the aliquot to make the injection the sample was again stored at -20 °C.

GC-FID

A 30 m x 0.20 µm x 0.20 mm MEGA 10 fused silica capillary column was used for FAMES analysis at the gas chromatograph (GC), with an initial temperature of 50 °C, ramp 10 °C/min to 250 °C, hold 3 min, and the injector temperature was 250 °C. The detector was a flame ionization detector (FID) with hydrogen flow 32.0 ml/min and air flow 200 ml/min with a temperature of 255 °C and Set split ratio as 5:1 was used. The peaks were identified by comparing them to the Supelco 37 Component FAME Mix peaks, pentadecanoic acid C:15 was used as the internal standard, and the area of each peak was determined with the specific software (LabSolutions version 5.99).

FFA separation

An adapted method based on the work of Kaluzny was developed for solid phase extraction of lipid samples [19–21]. For SPE, Strata[®] NH₂ cartridges (55 µm, 70 Å, 500 mg/6 mL, Phenomenex, Torrance, CA, USA) were conditioned with 6 mL of hexane and kept at atmospheric pressure. Total lipids from the SF of digested food, previously resuspended in 3 mL of hexane, were then loaded onto the cartridge. To elute the glyceride fraction, 6 mL of chloroform were added. Following this step, a collection tube was placed under the cartridge, and FFAs were eluted using 6 mL of a solution prepared by mixing 50 mL of chloroform with 1 mL of glacial acetic acid and 1 mL of methanol. The FFAs fraction was then methyl-esterified and analyzed via gas chromatography as described above.

Statistical analysis

Statistical analysis was performed by using the Student's t test, considering $p < 0.05$ as statistically significant. All statistical tests were performed with Graphpad Prism version 8.0.2 for Windows (GraphPad Software, San Diego, California, USA).

Results

In preliminary experiments, two different methods were used for lipid extraction, which produced comparable results for mackerel and biscuits, while the Bligh & Dyer method showed slightly but significantly higher yields for cream cheese and shelled walnuts (Table 1). Therefore, this method was used in subsequent experiments.

Table 1. Lipid content of the four foods (g/100 g) after extraction using the two different methods

	Lipids (g/100 g) – Folch et al	Lipids (g/100 g) – Bligh & Dyer	p value
Canned mackerel	5.77 ± 1.88	6.93 ± 0.21	n.s.
Cream cheese	18.89 ± 0.63	20.86 ± 0.56	<0.05
Biscuits	15.52 ± 0.97	16.80 ± 0.24	n.s.
Shelled walnuts	44.16 ± 0.32	48.70 ± 0.31	<0.05

In each food, lipid extraction was performed on 3 biological replicates from different production batches. Data are means + SD. Statistical analysis was by the Students' t test.

Each food was divided into two aliquots, one of which was used for lipid extraction and the other for *in vitro* digestion. Two slightly different *in vitro* digestion protocols were performed on walnuts, which differed from one another other in the oral phase, simulating rapid, and more thorough chewing. After *in vitro* digestion, lipids were extracted from the SF and quantified by weight. The results were normalized to 100 g of corresponding ND food and are shown in Figure 1. We tested both the Folch and the Bligh & Dyer extraction protocols to assess whether extraction efficiency differed across food matrices. Bligh & Dyer generally yielded higher lipid recoveries, which is consistent with its higher proportion of aqueous phase relative to Folch. This facilitates the disruption of lipid–protein and lipid–carbohydrate interactions and improves the solubilization of polar lipids. In contrast, the Folch method, with a higher chloroform content, is more efficient for non-polar lipids but can be less effective when lipids are embedded in hydrated or protein-rich matrices. This explains the matrix effect observed in our results: foods differ in moisture content, protein and carbohydrate structure, and the physical state of fats, all of which

influence how efficiently each solvent system can disrupt the matrix and extract lipids. Therefore, the differences in yields between the two methods reflect the chemical nature of the solvent systems and the specific composition and structure of each food matrix. In all foods, the amount of lipids recovered in the SF of the digesta was significantly lower than in the corresponding ND food, being about 70% for cream cheese and biscuits and approximately 55% in canned mackerel. In shelled walnuts, the degree of chewing had a significant impact on lipid recovery after digestion, which was less than 20% after rapid chewing and more than 40% after thorough chewing ($p < 0.0001$).

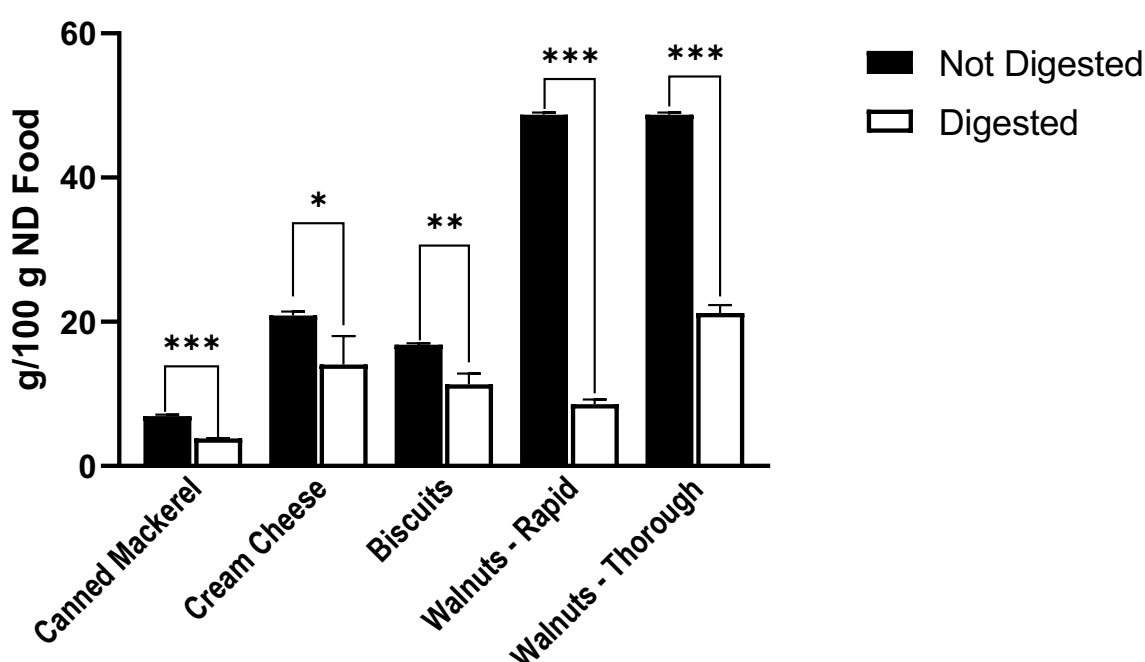


Figure 2. Lipid content in ND and in the SF of digested foods

Lipids were extracted according to Bligh & Dyer [16], and lipid content was quantified by weighing. Data are expressed as g/100 of ND food, and are means $\pm$ SD of 3 biological replicates. Statistical analysis was by the Student's t test, comparing ND food and the corresponding SF: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

An aliquot of lipids extracted from ND foods and corresponding SF was methyl-esterified and gas chromatographed, and FAMES were quantified using pentadecanoic acid as IS. The total amount of FAMES was normalized to 100 g of ND food. The recovery of FAMES in the SF confirmed that the release from the food matrix after digestion was higher in cream cheese and biscuits (about 70% of the corresponding ND food) than in canned mackerel

(about 40%). In walnuts, release from the food matrix depended on the degree of chewing, and it was about 15% and 50% after rapid and thorough chewing, respectively ($p<0.0001$) (Figure 3).

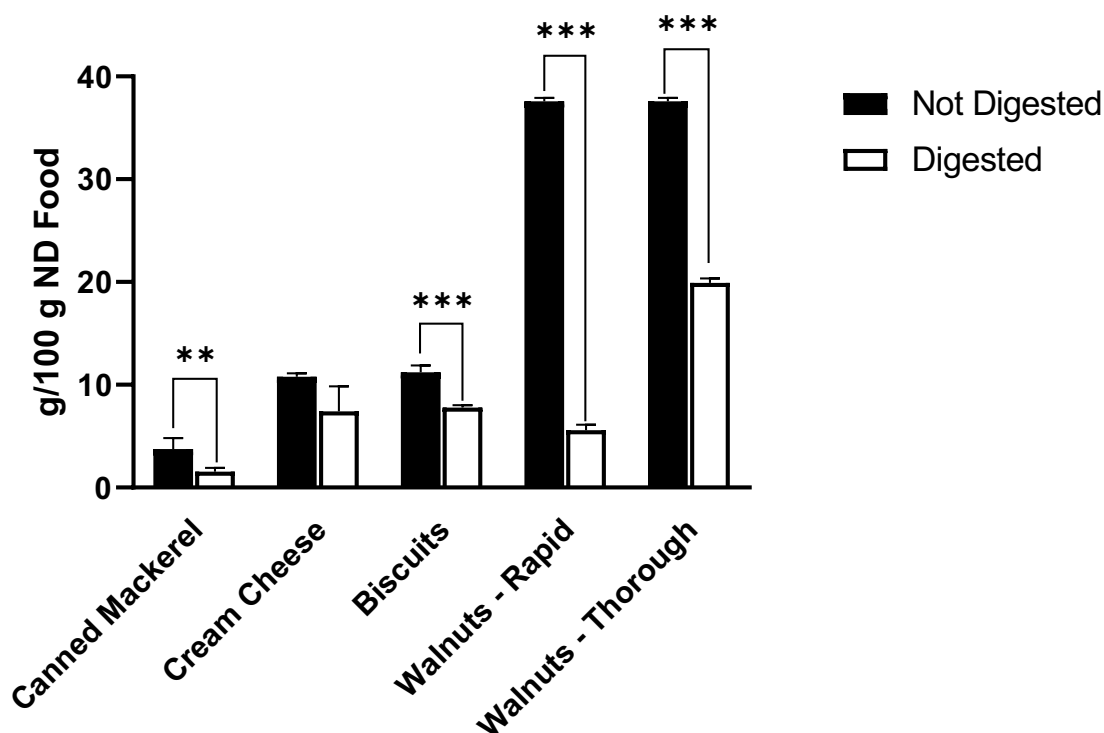


Figure 3. Total FAME content in ND and in the SF of digested foods

Lipids were extracted, methyl-esterified and gas-chromatographed as reported in the Methods section. Data are expressed as g/100 of ND food, and are means $\pm$ SD of 3 biological replicates. Statistical analysis was by the Student's t test, comparing the content of FAMES in ND food and the corresponding SF of the digesta: ** $p<0,01$; *** $p<0,001$.

FFAs were separated from another aliquot of lipids extracted from the SF, and were quantified by weight—comparing them to the total lipids in the corresponding SF—and by gas chromatography, after methyl-esterification—comparing them to the total FAMES in the corresponding SF. For nuts, the SF obtained after extensive chewing was used. In all cases, to facilitate comparison, the results were normalized to 100 g of the corresponding ND food. Regardless of the type of quantification and comparison, it appeared clear that in canned mackerel the TAGs released from the matrix during digestion were almost hydrolysed into FFAs, while in other foods the degree of hydrolysis was lower and the

recovery of FFAs was about 50% in walnut and even lower in cheese and biscuits (Figure 4).

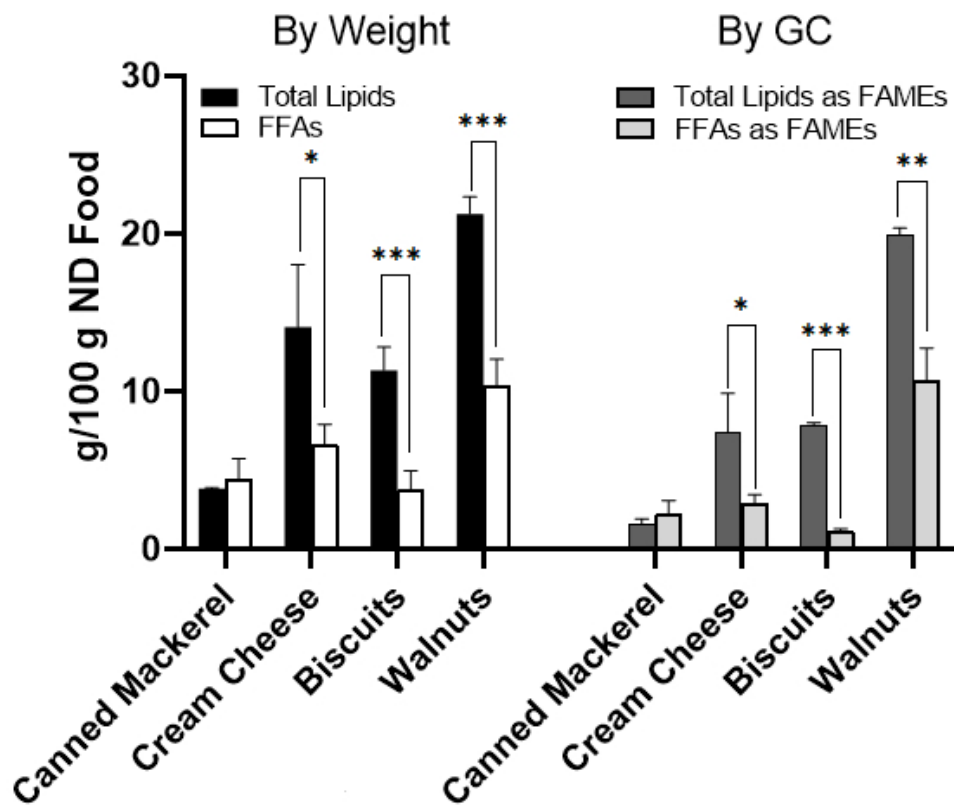


Figure 4. Quantification of FFAs in the SF of digested foods

FFAs were separated by SPE as described in the Methods section, and quantified by weigh (left panel) or by gas-chromatography after methyl-esterification (right panel). Data are expressed as g/100 of ND food, and are means $\pm$ SD of 3 biological replicates. Statistical analysis was by the Student's t test: * $p<0.02$; ** $p<0.01$; *** $p<0.001$.

Bioaccessibility was then calculated as

$$\frac{\text{FFAs (as Methyl Esters) in digesta}}{\text{Total FAMES in ND food}} \times 100$$

and it was in the order mackerel > cheese = walnuts > biscuits (Table 1)

Table 2. Lipid bioaccessibility

Sample	Bioaccessibility
Canned mackerel	67.60 % ± 7.4
Cream cheese	29.67% ± 6.27
Biscuits	9.94% ± 1.50
Shelled walnuts	25.16% ± 5.79

Lipid bioaccessibility was calculated as the percentage of FFA (as methyl esters) in the digested food compared to the total FAMES in the corresponding ND food. Data are means ± SD of 3 biological replicates.

Given that the bioaccessibility was far from 100% and different in the examined foods, we wondered whether it might vary among individual FAs leading to a different pattern of bioaccessible FA. Therefore, the total FA composition (as methyl-esters) of the ND samples and of the corresponding FFA fraction in the SF obtained after digestion were evaluated. The composition of ND canned mackerel was consistent with the Food Composition Database [22] and showed a very high concentration of n-3 fatty acids, mainly docosahexaenoic acid (DHA). No significant differences in the percentage composition were detected between ND mackerel and the FFA fraction obtained after *in vitro* digestion (Table 3), and the n-6/n-3 PUFA ratio remained 0.1:1.

Table 3. Fatty acid (as methyl esters) percent composition of ND canned mackerel and of the corresponding FFA fraction obtained after *in vitro* digestion.

	Not digested (mol/100 mol)	FFA fraction (mol/100 mol)
SFA	37.33 ± 1.17	41.62 ± 2.42
Myristic	4.26 ± 0.09	3.97 ± 0.2
Palmitic	24.61 ± 0.58	26.09 ± 1.8
Stearic	8.46 ± 0.53	11.57 ± 1.91
MUFA	20.06 ± 0.58	19.26 ± 2.39
Palmitoleic	4.58 ± 0.32	4.24 ± 0.52
Oleic	13.89 ± 0.38	13.55 ± 1.59
Gadoleic	1.60 ± 0.15	1.47 ± 0.31
PUFA	30.12 ± 0.87	27.41 ± 1.84
Linoleic	2.37 ± 0.1	2.20 ± 0.17
α -Linolenic	1.22 ± 0.08	1.22 ± 0.11
Eicosapentaenoic acid	6.28 ± 0.23	5.81 ± 0.27
Docosapentaenoic acid	1.62 ± 0.09	1.39 ± 0.23
Docosahexaenoic acid	18.72 ± 0.63	16.79 ± 1.45
n-6 PUFA	2.37 ± 0.1	2.20 ± 0.17
n-3 PUFA	27.84 ± 0.90	25.20 ± 1.81

Lipids were extracted, methyl-esterified and gas-chromatographed as reported in the Methods section. Data are expressed as mol/100 mol, and are means $\pm$ SD of 3 biological replicates. Statistical analysis was by the Student's t test, comparing ND mackerel to the FFA fraction obtained after *in vitro* digestion: * $p < 0.02$. SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

The percentage FA composition of the cream cheese (Table 4) was also comparable to that reported in the Food Composition Database [22]. In ND cheese, SFAs were the main components, particularly palmitic acid. In the FFA fraction obtained after *in vitro* digestion, a significant increase in palmitic and stearic acids was detected, which led to an increase in the total SFA content (Table 4). In both ND cheese and the corresponding FFA fraction, the concentration of n-6 PUFA was higher than that of n-3 PUFA, with a ratio ranging from 5.9:1 (ND cheese) to 5.6:1 (FFA fraction).

Table 4. Fatty acid (as methyl esters) percent composition of ND cream cheese and of the corresponding FFA fraction obtained after *in vitro* digestion.

	Not digested (mol/100 mol)	FFA fraction (mol/100 mol)
SFA	60.49 ± 0.43	63.22 ± 0.68**
Lauric	3.13 ± 0.03	2.58 ± 0.23*
Myristic	11.6 ± 0.03	12.21 ± 0.51
Palmitic	35.97 ± 0.24	37.82 ± 0.28**
Stearic	9.79 ± 0.01	10.53 ± 0.29*
MUFA	29.37 ± 0.54	28.74 ± 0.42
Myristoleic	1.55 ± 0.03	1.54 ± 0.05
Palmitoleic	2.49 ± 0.005	2.29 ± 0.46
Oleic	25.33 ± 0.4	24.91 ± 0.26
PUFA	3.33 ± 0.08	3.19 ± 0.25
Linoleic	2.85 ± 0.05	2.71 ± 0.18
α-Linolenic	0.48 ± 0.01	0.48 ± 0.03
n-6 PUFA	2.85 ± 0.05	2.71 ± 0.18
n-3 PUFA	0.48 ± 0.01	0.48 ± 0.03

Lipids were extracted, methyl-esterified and gas-chromatographed as reported in the Methods section. Data are expressed as mol/100 mol, and are means ± SD of 3 biological replicates. Statistical analysis was by the Student's t test, comparing ND cream cheese to the corresponding FFA fraction obtained after *in vitro* digestion: *p<0.05; **p<0.01. SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

Lipids were extracted, methyl-esterified and gas-chromatographed as reported in the Similarly, the percentage content of myristic and stearic acids was significantly higher in the FFA fraction than in ND biscuits, but the content of SFA did not significantly change (Table 5). A significant increase in α-linolenic acid was also observed in the FFA fraction, which reduced the n-6/n-3 PUFA ratio from 9.1:1 (ND biscuits) to 7.7:1.

Table 5. Fatty acid (as methyl esters) percent composition of ND biscuits and of the corresponding FFA fraction obtained after *in vitro* digestion

	Not digested (mol/100 mol)	FFA fraction (mol/100 mol)
SFA	41.85 ± 0.48	43.89 ± 2.43
Myristic	6.79 ± 0.1	5.9 ± 0.17**
Palmitic	24.35 ± 0.32	25.51 ± 1.21
Stearic	10.71 ± 0.4	12.48 ± 0.63*
MUFA	41.76 ± 0.13	43.56 ± 1.24
Oleic	41.76 ± 0.13	43.56 ± 1.24
PUFA	7.56 ± 0.12	7.50 ± 0.09
Linoleic	6.81 ± 0.1	6.64 ± 0.1
α-Linolenic	0.75 ± 0.01	0.86 ± 0.02***
n-6 PUFA	6.81 ± 0.1	6.64 ± 0.1
n-3 PUFA	0.75 ± 0.01	0.86 ± 0.02***

Lipids were extracted, methyl-esterified and gas-chromatographed as reported in the Methods section. Data are expressed as mol/100 mol, and are means ± SD of 3 biological replicates. Statistical analysis was by the Student's t test, comparing ND biscuits to the corresponding FFA fraction obtained after *in vitro* digestion: *p<0.02; ** p<0.01; ***p<0.001. SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

Finally, the percentage FAs composition of ND walnuts was similar to that reported in the Food Composition Database [22], and confirmed the prevalence of polyunsaturated fatty acids, particularly linoleic acid (Table 6). A significant increase in the SFAs was observed, for both palmitic and stearic acids, while the total amount of PUFAs acids was slightly reduced. Among individual PUFAs, linoleic acid showed a significant decrease, whereas the level of α-linolenic remained substantially unchanged. The n-6/n-3 ratio remained nearly constant (around 4:1), indicating that digestion did not markedly alter the balance between the two classes of PUFAs.

Table 6. Fatty acid (as methyl esters) percent composition of ND walnuts and of the corresponding FFA fraction obtained after *in vitro* digestion

	Not digested (mol/100 mol)	FFA fraction (mol/100 mol)
SFA	10.45 ± 0.78	14.1 ± 1.11*
Palmitic	7.13 ± 0.56	9.40 ± 0.55*
Stearic	3.33 ± 0.22	4.70 ± 0.37*
MUFA	12.96 ± 0.39	12.62 ± 0.16
Oleic	12.96 ± 0.39	12.62 ± 0.16
PUFA	73.75 ± 0.48	70.21 ± 1.04*
Linoleic	58.99 ± 0.97	55.45 ± 1.23*
α-Linolenic	14.77 ± 0.55	14.76 ± 0.38
n-6 PUFA	58.99 ± 0.79	55.45 ± 1.23*
n-3 PUFA	14.77 ± 0.45	14.76 ± 0.38

Lipids were extracted, methyl-esterified and gas-chromatographed as reported in the Methods section. Data are expressed as mol/100 mol, and are means ± SD of 3 biological replicates. Statistical analysis was by the Student's t test, comparing ND walnuts to the corresponding FFA fraction obtained after *in vitro* digestion: *p<0.05. SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids

Discussion

The relationship between food, diet, and human health is now recognized not only by the scientific community but also by consumers. This leads to an ever-increasing demand for information on the products we consume and has led to important policy decisions, such as making nutrition declaration mandatory and regulating the use of nutrition and health claims. In the food industry, there has been a growing focus on product formulation to ensure that foods with the desired nutritional characteristics are available on the market, which consumers can appreciate through the information provided on the product.

As for lipids, the nutrition declaration on the food label must contain information on the fat and saturated fat content per 100 g of food – and per portion in specific cases – and the presence of low amounts of SFA or high amounts of PUFA (total or specific PUFA) can

be highlighted by specific claims. Unfortunately, both nutritional declarations and the conditions of use of nutritional claims are based on the chemical composition of the food, without taking into account that the digestion and absorption processes are important steps in defining the possible health effects. The digestive process, which is essential for releasing the various components from the matrix and hydrolyzing them to the chemical forms that can be absorbed, substantially modifies the food, and its chemical composition before digestion does not reflect the composition of the bioaccessible fraction, i.e., the fraction that is available to be absorbed and transported into the bloodstream. If at all possible, the problem is further complicated by the fact that the food matrix can influence the bioaccessibility of food components [23,24]. Since the multiple interactions between the components that form the food matrix alter the observed behavior of the isolated components [25], it is absolutely necessary to evaluate the food in its entirety to understand the bioaccessibility of its components. Equally important is to consider the extent of matrix processing, as this can also have a strong impact on bioavailability. In many UPFs, the original structures of the ingredients have been disrupted [26] and macronutrients can be digested rapidly because digestive enzymes can easily access and hydrolyse them, thus increasing their bioaccessibility [27]. For proteins, this can be an advantage because it would increase the amount of essential amino acids absorbed, while it can be a disadvantage for carbohydrates because it would lead to a more rapid and significant increase in glycemia. For lipids, the advantage or disadvantage of increasing bioaccessibility depends on the nature of the fatty acids (saturated or unsaturated) present. However, in other UPFs, modifications to the matrix may have also determined interactions between the components that lead to a reduction in bioaccessibility, resulting in effects opposite to those described above on the nutritional value.

To our knowledge, there are no studies in the literature comparing the bioaccessibility of lipids in commercial foods with different degrees of processing. Therefore, we evaluated it in four different RTE commercial foods nutritionally characterized by the qualitative and quantitative presence of fats and classified differently according to NOVA [13]: (1) shelled walnuts, rich in PUFAs, minimally processed; (2) canned mackerel in brine, source of omega-3 fatty acids, processed; (3) cream cheese and (4) biscuits, rich in SFAs, UPF.

The weight of total lipids released from the food matrix after *in vitro* digestion ranged from about 70% in cream cheese and biscuits to less than 20% in walnuts after rapid chewing. These release percentages were also confirmed when evaluating the total amount of FAMES after methyl-esterification. Clearly, in all foods the amount of FAME was lower

than that of total lipids, but the percentage release from the matrix still varied from about 70% in cream cheese and biscuits to about 15% in nuts after rapid chewing. These results seemed to confirm that lipid bioaccessibility is significantly higher in UPFs, and reiterated the importance of chewing in determining lipid bioaccessibility in nuts, as already reported [23]

Although matrix disruption is one of the key events that influences the degree of lipid digestion [28], when evaluating the bioaccessibility of lipids one cannot consider only their release from the matrix but also, as regards TAGs, their hydrolysis into MAG and FFAs [29], which are the chemical forms suitable for absorption [8]. Some previous studies [30–32] have assessed the bioaccessibility of FAs in a food by quantifying FAMES in the SF obtained after *in vitro* digestion, thus assuming that the hydrolysis of TAGs - and other lipids containing FAs - is complete. In fact, methyl-esterification transforms all FAs present in the sample into FAMES, regardless of whether they are esterified in more complex lipids or already in free form, and therefore the assessment of bioaccessibility as a concentration of total FAMES leads to an overestimation. To avoid it, in this work the FFA fraction was separated and quantified by weighing and, after methyl-esterification, by gas-chromatography, highlighting that only a portion of TAGs and other complex lipids were hydrolysed into FFAs, and this portion varied among the tested foods. When bioaccessibility was calculated based on the release of FFA, it appeared significantly higher in mackerel than in other tested foods, with biscuits having the lowest value. Evaluating the various factors that led to such a significant difference in the bioaccessibility of the foods examined was beyond the scope of this study. Our objective was to determine whether the degree of processing is a reliable parameter for assessing lipid bioaccessibility. Based on our results, it is clear that it is not possible to state that the lipids contained in UPFs are more bioaccessible, or that there is a unequivocal relationship between the degree of processing and the bioaccessibility of lipids. Certainly, there are many factors that can interfere, and it is therefore not possible to express an *a priori* judgment based on the degree of processing.

The enormous variability caused by differences in the food matrix and processing, together with the use of different methodologies, makes any comparison between the bioaccessibility results obtained in this study and previous research very difficult. In a very general comparison, we can say that the percent bioaccessibility observed in mackerel was comparable to the results obtained in pork burgers and sausages [31], while in walnuts it was higher than that previously reported in almonds, which was about 10% [32].

The qualitative composition of the bioaccessible FFAs was also evaluated, as the bioaccessibility of specific FAs could also be affected by the food matrix and FA position on the TAG [33], thus becoming a determining factor of the nutritional value of a food. The composition of the FFA fraction resembled that of the corresponding ND food in mackerel, while an increase in SFAs and a decrease in monounsaturated fatty acids were observed in cheese and biscuits, respectively.

Limitations of the study. This study used the original INFOGEST protocol for *in vitro* digestion, which does not include the addition of gastric lipase. This may have underestimated the results, but the underestimation is minimal because in adults, only a small percentage of TAGs are hydrolysed in the stomach. As well, it could be argued that assessing bioaccessibility by considering only FFAs could underestimate it, since MAGs are also absorbed by enterocytes, but underestimation is small as the MAG concentration in the bioaccessible fraction has been reported to be very low [34,35]. A further limitation relates to the experimental design used to investigate the effect of food processing. An alternative approach would have been to apply different processing steps to a single minimally processed food (e.g. walnuts), thereby keeping the basic matrix composition constant while varying only the intensity or type of processing. Such a design would allow a clearer mechanistic separation of processing effects, but would be less representative of the diversity of foods consumed in real diets. In contrast, the present design captures how processing level and food matrix characteristics co-occur in commercially available foods. Future studies combining both controlled processing of a single matrix and comparisons across different food matrices could help more precisely disentangle the relative contribution of matrix structure and processing level to lipid bioaccessibility.

Conclusions

In Western countries, the intake of lipids exceeds the daily dietary reference intake (DRI). This constitutes a health risk, particularly when excessive intake is related to saturated fats. To encourage consumers to make correct choices, the nutritional declaration must contain information on the total and saturated fat content per 100 g of food, but it is increasingly clear that chemical composition is not a reliable parameter for discriminating between different foods. While the evaluation of the bioaccessibility/bioavailability of nutrients is gaining importance, the results of this study highlight that bioaccessibility cannot be deduced based on stereotypes, and that the degree of processing does not necessarily

increase lipid bioaccessibility. We recognize that a more mechanistic assessment of the effects of processing on lipid bioavailability would have required a different experimental design, applying different degrees of processing to a single minimally processed food (e.g., walnuts). This would have kept the base matrix composition constant, varying only the intensity/type of processing. However, this approach would have been less representative of the diversity of foods actually consumed. Our experimental design, however, captures how processing and matrix coexist and interact to modulate lipid bioaccessibility in commercially available foods. Future studies combining both approaches may help to more precisely distinguish the relative contribution of matrix structure and level of processing to lipid bioaccessibility.

Nonetheless, our results further confirm that bioaccessibility should be a parameter to consider in the nutritional evaluation of a food, and bioaccessibility studies should guide the food industry when formulating new foods, so as to modulate their impact on health.

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Comparative Analysis of Fatty Acid Bioaccessibility in Commercial Marine Oil Supplements: An In Vitro Integrated Analytical Study

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The text presented in this section is based on the corresponding published article and has been reproduced with minor additions and clarifications aimed at enhancing the description of the methodological framework and the interpretation of the results, while preserving the original scientific content.

Introduction

It is widely accepted that the omega-3 (n-3) long-chain polyunsaturated fatty acids (n-3 LC-PUFAs) eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) have health benefits for several human diseases, such as cardiovascular diseases [1,2], multiple sclerosis [3] and neurological disorders [4,5]. Major available sources of EPA and DHA include seafoods, and the consumption of fatty fishes twice per week is sufficient to provide 250–300 mg/day of EPA and DHA [6], which corresponds to the recommended daily intake [7]. Although the consumption of these n-3 LC-PUFAs is strongly advised, current daily dietary intakes of EPA and DHA are below the recommended levels in Europe [8], probably due to dietary preferences, geographic reasons, economic status and other reasons [9]. Dietary supplements could overcome low seafood consumption and help consumers to reach the recommended amount of n-3 LC-PUFAs. Various fish oil (FO) supplements like cod liver oil, whole fish body oils, etc., are available on the market to meet such needs. Due to the restrictions on conventional fisheries, the annual production of FO is capped at approximately 1 million metric tons [10], of which about 75% is used by the aquaculture industry to make fish feed [11]. Therefore, there is a need for new and sustainable sources of marine lipids, as well as an improvement in the optimal use of the raw material that is already available. Some companies have started to harvest lower in the marine food web, also called “fishing down the food web”. Zooplankton, such as copepods and krill, are major primary consumers of

plankton in the marine environment [12–14] and are currently being utilized for the production of marine oil supplements. The copepod *Calanus finmarchicus* is present in large quantities in the North Atlantic [15] and has a lipid-rich overwintering stage [16]. While the oil extracted from *C. finmarchicus* has been presented as an alternative to marine fishes because of the presence of high concentration of n-3 LC-PUFAs, this practice is highly controversial and not considered sustainable for the marine ecosystem, as these organisms are of great importance as food for commercial fish [17–19] and are highly important organisms in ecosystem functions such as promoting biological carbon flux and carbon sequestration [16,20]. Recent research has, however, investigated the potential of utilizing zooplankton as a by-product from pelagic fishing [21,22]. Therefore, it is of high importance to evaluate the bioaccessibility of lipids and fatty acids (FAs) in zooplankton oils to add this nutrition- and health-related information to the sustainability evaluation in the ever-increasing demand for harvesting this resource. Oils from lower trophic levels often differ from traditional FO in the distribution of lipids into different classes. In first-generation FO supplements, EPA and DHA are bound to a glycerol backbone forming triacylglycerols (TAGs), while in second-generation FO supplements, EPA and DHA are either in the form of ethyl esters or are re-esterified TAGs. Krill oil (KO) has a high content of phospholipids (PLs) in addition to TAGs [23], and in *C. finmarchicus* oil (CO), most of the FAs esterify with long-chain fatty alcohols, forming the lipid class known as wax esters (WEs) [24]. The bioaccessibility of FAs—i.e., the percentage of FAs that are released in the intestine in a form that can be absorbed by enterocytes—depends on the enzymes required to hydrolyze complex lipids in the oils [25,26]; therefore, the distribution of FAs in different lipid classes can influence it. Particularly, in mammals, WE hydrolysis is assumed to be a slow process because these lipids are poor substrates for lipolytic enzymes, especially pancreatic lipase [27]. The evaluation of the release of FAs in supplements is not trivial, as it modulates bioavailability, i.e., the quantity of FAs that can be absorbed and exert an effect on the human body. Supplements containing similar amounts of LC-PUFAs may therefore have different efficacy due to different bioaccessibility. In addition, n-3 LC-PUFA supplements are commonly found in capsules, usually made from a soft layer of gelatin. Many manufacturers also use an enteric coating that helps keep the capsule from dissolving until it reaches the small intestine. However, how encapsulation modulates the release of FAs in supplements has not yet been fully clarified [28].

Aim of the study

The aim of the present study was to evaluate whether and to what extent the release of FAs from marine oils during digestion is modulated by the form in which they are esterified and by the encapsulation of the oils. To this end, four different commercial n-3 LC-PUFA supplements containing FO, KO or CO were considered, and two different analytical approaches to quantify the different lipid classes before and after *in vitro* digestion were used. The first one was based on thin-layer chromatography (TLC) with a flame ionization detector (FID); the second one was based on ¹H nuclear magnetic resonance (¹H-NMR) spectroscopy. Since, from a chemical point of view, digestion consists of a series of transformations which, at the lipid level, involve the hydrolysis of esters of various nature, ¹H-NMR spectroscopy is particularly effective. In a single acquisition, the spectrum allows for a complete overview of all the molecules dissolved in the sample, provided that they contain at least one hydrogen atom [29]. As a further advantage, preliminary separation/purification steps are not required, and standards are not needed for quantification. In fact, every signal, regardless of the type of molecule to which the hydrogen atom belongs, has an area proportional to the concentration of the molecule, depending only on the number of equivalent hydrogen atoms corresponding to the signal (e.g., three hydrogen atoms for a methyl group signal and two hydrogen atoms for a methylene group). The peculiarity of NMR spectroscopy is that each hydrogen atom resonates at its specific frequency in the spectrum, defined as a chemical shift, and is expressed as ppm of the carrier frequency. This frequency depends on the chemical environment. For instance, the hydrogen atoms in α of a FA chain resonate at different frequencies depending on whether the FA is in the free (2.378–2.369 ppm) or esterified (2.319–2.250 ppm) form. Moreover, the presence of an adjacent double bond to the same atom group (i.e., DHA) causes an additional shift in their frequencies at higher values (2.400–2.378 ppm for the esterified form or 2.420–2.400 ppm for the free acid) [30]. Changes of a similar extent occur on the alcoholic moieties of esters and glycerides, allowing for the direct observation of chemical transformations such as ester hydrolysis.

Materials and methods

Samples

One commercial FO supplement, one commercial Antarctic KO supplement and two commercial CO supplements from different manufacturers (CO-1 and CO-2) were considered. The characteristics of the supplements reported by the manufacturers are listed in Table 1.

Table 1. Characteristics of the commercial supplements reported by the manufacturers.

	FO	KO	CO-1	CO-2
Serving ¹	3 capsules (1500 mg oil)	2 capsules (1000 mg oil)	1 capsule (500 mg oil)	4 capsules (2100 mg oil)
Total n-3 FA/serving (mg)	505	220	90	442
EPA/serving (mg)	240	120	15	136
DHA/serving (mg)	160	55	20	116
n-3 LC-PUFA source	Fish oil	Antarctic krill (<i>Euphasia superba</i>) oil	Calanus (<i>C. finmarchicus</i>) oil	Calanus (<i>C. finmarchicus</i>) oil
Other ingredients	Gelatin (bovine), glycerol, vitamin E	Gelatin (bovine), glycerol	Gelatin (fish), vegetable glycerin, sorbitol	Gelatin (fish), glycerol, vitamin D3

¹ When serving was indicated as a range, the maximum value was considered.

In Vitro Digestion

In vitro digestion was performed using the method developed by the European COST action INFOGEST [31]. According to the protocol developed for lipid-rich samples, 0.17 mM lecithin was added to the simulated gastric juice. The addition of lecithin is necessary to simulate the formation of gastric emulsions that physiologically occur during a meal, allowing lipases to adhere to the surface of lipid droplets to initiate their hydrolysis. This ensures that the results on fatty acid bioaccessibility obtained *in vitro* correlate with actual digestive processes observed *in vivo*. Lecithin is essential to ensure the physiological relevance of the model and to correctly simulate the interaction between enzymes and fats that occurs in the human body. For each supplement, digestion was performed in duplicate on 5 g of pure oil or 5 g of encapsulated oil. At the end of duodenal digestion, enzyme activity was stopped by drop-wise HCl addition to pH 3; then, samples were centrifuged at 4500 rpm for 5 min at 4 °C to obtain a soluble fraction (SF) and a pellet. The SF containing the bioaccessible lipids was filtered with a cellulose acetate filter (pore size: 0.2 µm) and stored at −20 °C after adjusting the pH to 7.

Lipid Extraction and Separation by TLC-FID

Total lipids were extracted from not-digested and digested FO, KO and CO according to Bligh and Dyer (1959) [32]. Extraction was performed on 100 mg of not-digested oil or SF obtained after digestion, either without or with capsules. After extraction, the lipids were dried under nitrogen flow and quantified by weighing using an analytical balance. Separation of the different lipid classes was performed using an IATROSCAN MK-5 TLC-FID. (SES GmbH—Analytical Systems, Bechenheim Germany). The total lipid sample was resolved in chloroform/methanol at a ratio of 2:1, and lipids in chloroform were drawn off and filtered through a phase-separating filter paper (grade 920) and collected in 15 mL vials. The chloroform was left to evaporate under a stream of nitrogen gas. A sub-sample of the lipid was weighted into pre-weighted vials to make a concentration of 15–20 mg oil mL⁻¹ hexane. Individual lipid classes were separated by thin-layer chromatography (TLC). A lipid sample was spotted onto silica-coated quartz rods (chromarods) using an automated sample spotter (Model SES 3200/IS-01; both from SES GmbH—Analytical Systems, Bechenheim Germany). Two samples of each oil were analyzed, each in triplicate, to test the instrument's reproducibility. The lipid classes were separated on the silica rods by development in two solvent systems: hexane/diethyl ether/formic acid (81.5:17.5:1, by volume) followed by hexane/diethyl ether (96:4 by volume). The rods were humidified for 10 min in a constant humidity chamber before developments. The Iatroscan was set to have 20 L min⁻¹ air flow, 160 mL min⁻¹ hydrogen flow and a scan rate of 30 s. The peaks obtained for each sample were quantified using SES-i-ChromStar 6.4 software (SES GmbH—Analytical Systems, Bechenheim Germany). The peaks were identified by comparison to the retention time of known standards of wax esters (from the copepod *C. finmarchicus*), triacylglycerol (tripalmitin) and cholesterol.

Sample Preparation for Spectroscopic Analysis by 1H-NMR

Six drops of the oils were collected from a capsule, pierced with a needle into a micro-tube and weighted to determine their actual mass (about 200 mg). After the addition of deuteriochloroform (CDCl₃, 500 µL), the solution was mixed vigorously and centrifuged at 14,000 rpm for 5 min at 4 °C. The supernatant was diluted again with CDCl₃ (1:4 by volume) and the centrifugation step was repeated. Part of the solution (700 µL) was transferred into a 5 mm diameter NMR tube and stored at 4 °C in the autosampler until analysis. For each oil, 3 samples were prepared from different capsules to test the

reproducibility. The SF of the digested oils was weighed, and the pH was adjusted to 2.5–3.0 by adding HCl to convert all the organic acids into their neutral form; this allowed for the partitioning of the molecules released by digestion in favor of chloroform to ensure their complete extraction from the aqueous SF, with the exception of glycerol, short-chain alcohols and glycerophosphocholine, which remain in the aqueous phase. Then, 350 μ L of CDCL₃ were added to the sample and the mixture was mixed vigorously and centrifuged at 14,000 rpm for 5 min at 4 °C. The chloroform phase (bottom) was transferred into an NMR tube, and 350 μ L of CDCL₃ were added to the tube to reach a final volume of 700 μ L. For each digested sample, 3 replicates were analyzed. All samples were analyzed using an AV600 UltrashieldTM Plus AVANCE IIITM Spectrometer (Bruker, Karlsruhe, Germany) operating at 600.13 MHz. The acquisition parameters for analysis were as follows: 1D zg without pre-saturation sequence; correct solvent CDCL₃; number of time-domain data points TD = 64 k; spectral width SW = 25 ppm; number of scans NS = 8, TD0 = 32 (for a total of $32 \times 8 = 256$ scans); dummy scans DS = 4; acquisition time AQ = 2.18 s; relaxation delay RD = 8.0 s. The total acquisition time was about 45 min for each sample. All NMR spectra were manually phased and baseline-corrected using Topspin 3.2 (Bruker Biospin, Rheinstetten, Germany).

Lipid Quantification by ¹H-NMR

The quantitative determination of the molecules in the samples was performed by integrating specific signals belonging to selected hydrogen atoms of each identified molecule, opportunely free from interferences. The assignment of the signals to the corresponding protons of the different lipid classes (Table 2) was made based on data from the literature for high-resolution ¹H-NMR spectra of oils from different sources analyzed in CDCL₃ solvent [33–38]. The results of the quantitative evaluation are reported as the molar fraction distribution of the different species and as the ponderal composition (% w/w) of the different molecules, assuming an average molar mass of 300 g/mol for the FA chains.

Table 2 Assignment of the ^1H -NMR signals of selected lipid classes.

Signal No.	ppm1	ppm2	Assignment	Molecule
1	5.286	5.233	$\text{ROCH}_{a1}\text{H}_{a2}\text{-CH}_b\text{OR}'\text{-CH}_{c1}\text{H}_{c2}\text{OR}''$	TAG
2	5.230	5.170	$\text{ROCH}_{a1}\text{H}_{a2}\text{-CH}_b\text{OR}'\text{-CH}_{c1}\text{H}_{c2}\text{OPOOR}$	1,2-DAG-P
3	5.106	5.050	$\text{ROCH}_{a1}\text{H}_{a2}\text{-CH}_b\text{OR}'\text{-CH}_{c1}\text{H}_{c2}\text{OH}$	1,2-DAG
4	4.931	4.894	$\text{HOCH}_{a1}\text{H}_{a2}\text{-CH}_b\text{OR-CH}_{c1}\text{H}_{c2}\text{OH}$	2-MAG
5	4.250	4.200	$\text{ROCH}_{a1}\text{H}_{a2}\text{-CH}_b\text{OH-CH}_{c1}\text{H}_{c2}\text{OR}''$	1,3-DAG
6	4.070	4.020	$\text{RCH}_2\text{O-COR}$	wax ester
7	3.970	3.877	$\text{ROCH}_{a1}\text{H}_{a2}\text{-CH}_b\text{OH-CH}_{c1}\text{H}_{c2}\text{OH}$	1-MAG
8	3.670	3.620	$\text{R-CH}_2\text{OH}$	primary alcohol
9	2.420	2.400	$\text{R-CH}_2\text{-COOH}$	DHA free
10	2.378	2.369	$\text{R-CH}_2\text{-COOH}$	FFA (no DHA)
11	2.400	2.378	$\text{R-CH}_2\text{-COOR}$	DHA bound
12	2.319	2.250	$\text{R-CH}_2\text{-COOR}$	BFA (no DHA)
13	0.920	0.840	$\text{CH}_3\text{-R}$	all FA except n-3
14	0.995	0.950	$\text{CH}_3\text{-R}$	n-3 FA
15	3.330	3.220	$\text{R-N(CH}_3)_3$	phosphocholine

The extremes of the intervals where signals resonate are indicated as left (ppm1) and right (ppm2) limits. Assignments refer to the hydrogen atom groups (in bold) associated with the signal.

Statistical Analysis

To facilitate comparison between not-digested and digested oils, in the SF of digested oil, the content of the different lipid classes was normalized and expressed in mg/100 mg of the corresponding not-digested oil. Statistical analysis was performed via one-way analysis of variance using Tukey's test as post-test. *p* values lower than 0.05 were considered significant.

Results

The initial stage of fat digestion occurs in the stomach, where gastric lipase begins the hydrolysis of TAGs into diglycerides (DAGs) and free fatty acids (FFAs). Gastric lipase hydrolyses TAGs mainly at position sn-3, containing short- and medium-chain FAs (<C12) that are directly transferred to the blood [39]. However, most fat digestion takes place in the small intestine via pancreatic enzymes and bile salts. The emulsification of fat by bile acids facilitates enzymatic action to further break down fats into FFAs and monoglycerides (MAGs) [40]. After digestion, the hydrolyzed lipid-soluble components (long-chain FAs, 2-MAGs, lysophospholipids, free cholesterol) integrate with bile salts into mixed micelles to diffuse between the intestinal microvilli to interact with the luminal surface of enterocytes [41]. The hydrolysis of more complex lipids into FFAs and MAGs represents a crucial step. Indeed, since the absorption of FAs and MAGs is highly efficient [42], it is presumably the extent of their formation from more complex lipids, as well as the extent

of lipid release from the food matrix, that plays a major role in determining the extent of absorption. It is therefore of fundamental importance to evaluate the quantities of FFAs and MAGs that are formed after digestion, particularly in foods or supplements that are consumed because they are sources of specific FAs. In this work, using two analytical techniques—TLC-FID and ¹H-NMR spectroscopy—we first evaluated the partitioning of lipids in the different classes in not-digested n-3 LC-PUFA supplements. We then verified the formation of FFAs and MAGs after *in vitro* digestion. As shown in Table 3, TLC-FID analysis evidenced that TAGs were more abundant in FO than in other oils, and the concentration of wax esters (WEs) was much higher in CO than in FO and KO, as already reported by Cholewski et al. (2018) [43] and Pedersen et al. (2014) [44], respectively. In KO, which is widely reported to be rich in phospholipids (PLs) [45], TLC-FID analysis evidenced acetone-mobile polar lipids (AMPLs) as the most represented lipid class. Antarctic krill may accumulate large amounts of glycerophospholipids that, together with triacylglycerols, occur as depot lipids [46], and polar lipids, i.e., AMPLs—comprising glycerophospholipids—and PLs are the most represented lipids in microalgae [47], which are consumed by krill [48]. In all not-digested samples, TLC-FID detected small amounts of FFAs.

Table 3. Partition of lipids in the different classes in not-digested oils, evaluated by TLC-FID.

	FO	KO	CO-1	CO-2	<i>p</i> -Value
WEs	4.19 ± 3.84 ^b	2.98 ± 2.60 ^b	86.49 ± 1.45 ^a	78.82 ± 7.13 ^a	<0.001
TAGs	88.84 ± 3.32 ^a	8.97 ± 8.66 ^b	5.10 ± 3.46 ^b	5.84 ± 6.12 ^b	<0.001
FFAs	2.00 ± 2.83 ^a	4.11 ± 3.56 ^a	3.27 ± 1.04 ^a	4.01 ± 3.99 ^a	0.863
ALCs	1.90 ± 2.68 ^a	1.00 ± 1.72 ^a	2.73 ± 0.64 ^a	2.26 ± 2.44 ^a	0.853
DAGs	1.12 ± 1.58 ^a	n.d.	n.d.	0.37 ± 0.83 ^a	0.394
AMPLs	1.59 ± 0.57 ^b	74.22 ± 7.41 ^a	2.41 ± 0.34 ^b	5.76 ± 1.58 ^b	<0.001
PLs	0.36 ± 0.50 ^a	8.72 ± 9.45 ^a	n.d.	2.94 ± 5.65 ^a	0.411

Data are expressed as m% (w/w) and are means ± standard deviation (SD) of 2 replicates. Statistical analysis was performed by Student's t-test when comparing two values and by ANOVA (one-way analysis of variance) using Tukey's test as post-test when comparing three or more values. *p* < 0.05 was considered significant. Different letters in the same row indicate statistical significance. WEs = sterol esters and wax esters; TAGs = triglycerides; FFAs = free fatty acids; ALC = free aliphatic alcohols; DAGs = diglycerides; AMPLs = acetone-mobile polar lipids; PLs = phospholipids and other acetone immobile polar lipids; FO = fish oil; KO = krill oil; CO = Calanus oil; n.d. = not detectable.

The complete ^1H -NMR spectra from 0 to 6 ppm of the four not-digested oils are re-ported in Figure 1, and some spectral regions (0.80–1.40 ppm; 1.40–2.50 ppm; 2.50–3.50 ppm; 3.50–4.50 ppm; 4.50–6.00 ppm; 6.00–10.00 ppm) are enlarged in Figure S1 (Supplementary material) to outline the most significant differences among them. ^1H -NMR analysis (Table 4) confirmed the TLC-FID results. It is important to clarify that TLC-FID is a chromatographic separation technique that provides an estimate of the weight of different fractions compared to that of three standard compounds mentioned in the Materials and Methods section. This does not allow for a fine subdivision of the chromatographic classes in the different molecular species. NMR, on the other hand, produces precise and accurate information on the molar concentration of the molecular classes present in the mixture, without fractionation, each characterized by a specific chemical functional group. Therefore, the two techniques are not comparable, but it is possible to check the compatibility between the overall data obtained as relative differential quantities before and after digestion. The ^1H -NMR spectrum of the FO (Figure 1) presented the typical signals of the protons of the glyceryl backbone of TAGs (signal from 5.286 to 5.233 ppm, referring to the hydrogen bound to the glycerol C2), confirming TAGs as the main component. The ^1H -NMR spectra of CO-1 and CO-2 (Figure 1) significantly differed from the FO spectrum, mainly due to the presence of the signals referring to WEs (primary hydrogens of esterified fatty alcohols, from 4.070 to 4.020 ppm [38]), the latter indicating an even higher proportion due to a separation step which, on the contrary, does not affect the NMR results. The KO spectra (Figure 1) showed a more complex distribution of signals than FO and CO, highlighting a more complex variety of lipid classes. In addition to TAG, the presence of PLs was recognizable by the signals of the glycerophospholipid backbone (5.230–5.170 ppm) and of the choline head (3.330–3.220 ppm) of phosphatidylcholine, the most abundant PLs in krill [49]. In all samples, the amount of FFAs detected by ^1H -NMR was higher than that detected by TLC-FID. However, the NMR data are particularly robust because the LC-FAs were quantified by integrating signals from both the ω -methyl and β -methylene groups of the FA molecule. The latter resonates at frequencies completely different from those of any other alkyl hydrogen (around 2.2–2.4 ppm vs. 1.1–1.5 ppm). A ratio of 2:3 in the corresponding signal areas confirms that all methyl hydrogen atoms found in the mixture are balanced by the proper number of FA β -methylene hydrogen atoms.

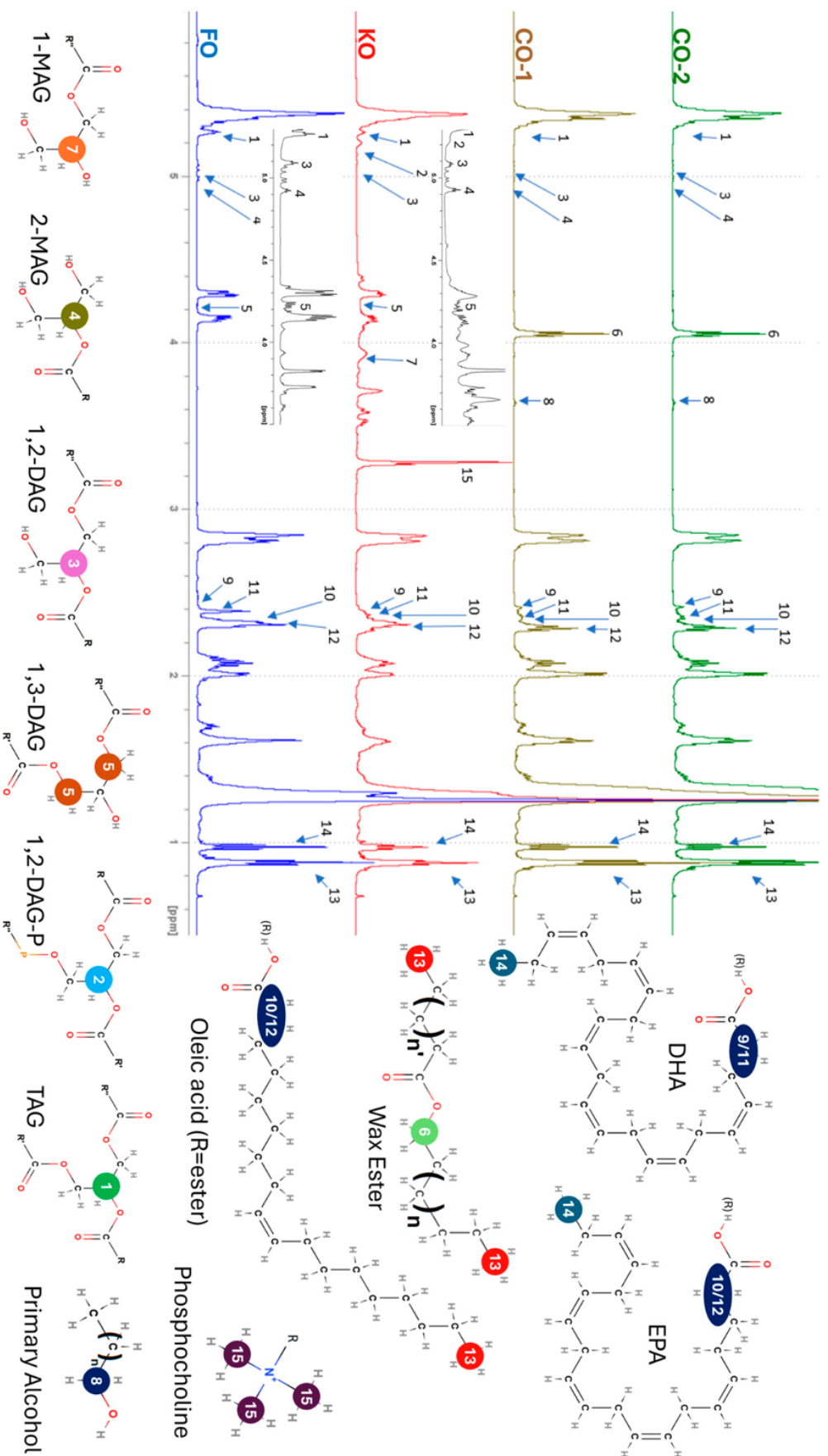


Figure 1. ¹H-NMR spectra (between 0.40 and 6.00 ppm) of not-digested fish oil (FO), krill oil (KO) and Calanus oil (CO-1 and CO-2) supplements in CDCL₃ solvent.

Signal assignment is provided by reporting the label number placed on the spectra also on the corresponding chemical group indicated in the molecular structural formulas. The FO and KO spectra have insets showing the glyceride region after digestion to better appreciate the signals of di- and mono-glycerides that are formed during hydrolysis. The extremes of the integration areas are reported in Table 2.

Table 4. Partition of lipids in the different classes in not-digested oils, evaluated by ¹H-NMR

	FO	KO	CO-1	CO-2	<i>p</i> -Value
TAGs	68.74 ± 0.16 ^a	26.24 ± 0.22 ^b	0.91 ± 0.06 ^c	1.26 ± 0.06 ^c	<0.001
1,3-DAGs	1.43 ± 0.01 ^a	0.94 ± 0.34 ^b	0.06 ± 0.01 ^c	0.44 ± 0.03 ^c	<0.001
1,2-DAGs	2.61 ± 0.02 ^a	2.97 ± 0.75 ^a	1.16 ± 0.30 ^b	0.17 ± 0.004 ^b	<0.001
1,2-DAG-P	n.d.	5.80 ± 0.15	n.d.	n.d.	
2-MAGs	0.40 ± 0.02 ^a	0.10 ± 0.04 ^b	0.02 ± 0.00 ^c	0.05 ± 0.01 _{b,c}	<0.001
1-MAGs	0.50 ± 0.02 ^c	2.79 ± 0.21 ^a	1.18 ± 0.01 ^b	0.14 ± 0.004 _d	<0.001
WEs	0.02 ± 0.00 ^c	0.38 ± 0.16 ^c	63.86 ± 0.59 ^b	70.30 ± 0.03 ^a	<0.001
Primary alcohol	0.11 ± 0.01 ^c	0.21 ± 0.02 ^c	3.04 ± 0.45 ^a	2.12 ± 0.03 ^b	<0.001
total	0.04 ± 0.004	21.83 ± 0.31	n.d.	n.d.	<0.0001
P-choline	****				
FFAs (except DHA)	21.04 ± 0.14 ^c	36.09 ± 0.60 ^a	24.25 ± 0.15 ^b	19.06 ± 0.08 _d	<0.001
Free DHA	5.13 ± 0.05 ^b	2.67 ± 0.04 ^d	5.52 ± 0.03 ^a	3.70 ± 0.06 ^c	<0.001

Data are expressed as % (w/w) and are means ± SD of 3 replicates. Statistical analysis was performed by Student's t-test when comparing two values and by ANOVA (one-way analysis of variance) using Tukey's test as post-test when comparing three or more values. *p* < 0.05 was considered significant. **** means *p* ≤ 0.0001. Different letters in the same row indicate statistical significance. TAGs = triglycerides; DAGs = diglycerides; MAGs = monoglycerides; WEs = wax esters; FFAs = free fatty acids; FO = fish oil; KO = krill oil; CO = Calanus oil. n.d. = not detectable.

After digestion, the lipid content in the SF was lower than in corresponding not-digested oil regardless of the presence of the capsule (Table 5). We hypothesize that this was mainly related to the different types and levels of emulsifiers used, which can regulate lipid digestibility, since the oil–water interface plays a critical role in modulating the digestive behavior of lipid droplets [50]. Encapsulation played a role in the KO supplement; in fact, when the digestion was performed with capsules, the quantity of lipids in the SF was

significantly lower than during digestion without the capsule. Information on the type and amount of emulsifiers present in the commercial capsules was not available, as it was not specified by the manufacturers. Therefore, a specific evaluation of the impact of emulsifiers on lipid digestibility was not possible within the scope of this study.

Table 5. Total lipids recovered in the SF of the different oils after *in vitro* digestion without or with the capsule.

	FO	KO	CO-1	CO-2	<i>p</i> -Value
Without capsule	61.51 ± 1.54 ^a	44.00 ± 0.54 ^c	61.99 ± 0.34 ^a	53.24 ± 2.76 ^b	0.001
With capsule	63.55 ± 2.90 ^a	19.21 ± 0.31 ^{c ***}	53.95 ± 4.03 ^{a,b}	53.20 ± 0.44 ^b	<0.001

Data are expressed in mg/100 mg not-digested oil and are means ± SD (n = 2). Statistical analysis was performed by one-way ANOVA using Tukey's test as post-test, and *p* < 0.05 was considered significant. Different letters in the same row indicate statistical significance. In each oil, differences related to the presence of the capsule during digestion were evaluated using Student's t-test (***) *p* < 0.001). FO = fish oil; KO = krill oil; CO = Calanus oil.

Due to the activity of hydrolytic enzymes during digestion, lipid partition between classes was different in SF than in not-digested oil. In FO (Table 6), regardless of the presence of the capsule, TLC-FID evidenced a significant decrease in TAG content and an increase in FFAs, DAGs and AMPLs, the latter also including MAGs [51]. Statistical significance was sometimes obscured by within-group variability, which was greater when digestion was performed with capsules. This was probably due to the different dissolution times of the capsules during digestion, which caused different hydrolytic cleavage by the digestive enzymes.

Table 6. Partition of lipids in the different classes in SF of FO after digestion with or without capsules, evaluated by TLC-FID.

	Not Digested	SF (Digested w/o Capsule)	SF (Digested with Capsule)	<i>p</i> -Value
WEs	4.19 ± 3.84 ^a	0.85 ± 0.81 ^a (1.38%)	3.49 ± 4.93 ^a (5.32%)	0.55
TAGs	88.84 ± 3.32 ^a	15.57 ± 1.49 ^b (25.31%)	12.22 ± 1.55 ^b (19.20%)	<0.001
FFAs	2.00 ± 2.83 ^a	10.95 ± 0.90 ^a (17.80%)	26.26 ± 19.94 ^a (42.08%)	0.25
ALCs	1.90 ± 2.68	n.d.	n.d.	
DAGs	1.12 ± 1.58 ^a	9.53 ± 1.60 ^a (15.49%)	7.13 ± 4.21 ^a (11.08%)	0.10
AMPLs	1.59 ± 0.57 ^a	21.58 ± 3.05 ^a (35.08%)	10.08 ± 9.58 ^a (15.54%)	0.09
PLs	0.36 ± 0.50 ^a	3.02 ± 1.50 ^a (4.91%)	4.36 ± 2.57 ^a (6.78%)	0.19

Data are expressed as mg/100 mg of not-digested oil and are means $\pm$ SD of 2 replicates. Statistical analysis was performed by Student's t-test when comparing two values and by one-way ANOVA with Tukey's test as post-test when comparing three values. $p < 0.05$ was considered significant. Different letters in the same row indicate statistical significance. The mean percentage contribution of each class to total lipids in SF is reported in parentheses. SF = soluble fraction; WEs = sterol esters and wax esters; TAGs = triglycerides; FFAs = free fatty acids; ALCs = free aliphatic alcohols; DAGs = diglycerides; AMPLs = acetone-mobile polar lipids; PLs = phospholipids and other acetone immobile polar lipids; n.d. = not detectable.

After digestion, the significant decrease in TAGs and the parallel increase in FFAs were confirmed by $^1\text{H-NMR}$ (Table 7). DAGs and MAGs also increased after digestion, suggesting the progressive hydrolysis of TAGs. Modifications were more relevant when digestion was performed without the capsule. The $^1\text{H-NMR}$ spectra of digested FO in comparison to not-digested oil are reported in Figure S2.

Table 7. Partition of lipids in the different classes in SF of FO after digestion with or without capsules, evaluated by $^1\text{H-NMR}$.

	Not Digested	SF (Digested w/o Capsule)	SF (Digested with Capsule)	<i>p</i> -Value
TAGs	68.74 $\pm$ 0.16 ^a	19.02 $\pm$ 7.76 ^b (19.02%)	15.45 $\pm$ 0.05 ^b (24.35%)	<0.001
1,3-DAGs	1.43 $\pm$ 0.01 ^a	2.21 $\pm$ 0.81 ^a (2.2%)	1.07 $\pm$ 0.42 ^a (1.7%)	0.14
1,2-DAGs	2.61 $\pm$ 0.02 ^b	7.75 $\pm$ 2.22 ^a (7.75%)	5.41 $\pm$ 0.66 ^{a,b} (8.54%)	0.02
1,2-DAGs-P	n.d.	n.d.	n.d.	
2-MAGs	0.40 $\pm$ 0.02 ^b	3.62 $\pm$ 1.21 ^a (3.62%)	2.31 $\pm$ 0.71 ^{a,b} (3.67%)	0.02
1-MAGs	0.50 $\pm$ 0.02 ^b	2.90 $\pm$ 1.39 ^a (2.09%)	2.13 $\pm$ 0.45 ^{a,b} (3.35%)	0.05
WEs	0.02 $\pm$ 0.001 ^{**}	n.d.	0.01 $\pm$ 0.002 (0.02%)	0.0045
Primary alcohol	0.11 $\pm$ 0.01 ^a	0.12 $\pm$ 0.08 ^a (0.12%)	0.04 $\pm$ 0.03 ^a (0.07%)	0.24
Total P-choline	0.04 $\pm$ 0.005	n.d.	n.d.	
FFAs (except DHA)	21.04 $\pm$ 0.14 ^b	54.27 $\pm$ 11.92 ^a (54.27%)	30.35 $\pm$ 5.38 ^b (47.62%)	0.01
Free DHA	5.13 $\pm$ 0.05 ^b	10.10 $\pm$ 1.15 ^a (10.10%)	6.79 $\pm$ 0.28 ^b (10.68%)	0.002

Data are expressed as mg/100 mg of not-digested oil and are means $\pm$ SD of two biological replicates, except for the not-digested oil, which was analyzed in three biological replicates. Each biological replicate was evaluated in triplicate. Statistical analysis was performed by Student's t-test when comparing two values and by ANOVA (one-way analysis of variance) using Tukey's test as post-test when comparing three values. $p < 0.05$ was considered significant. ^{**} means $p \leq 0.01$. Different letters in the same row indicate statistical significance. The mean percentage contribution of each class to total lipids in SF is reported in parentheses. TAGs = triglycerides; DAGs = diglycerides; MAGs = monoglycerides; WEs = wax esters; FFAs = free fatty acids; DHA = docosahexaenoic acid; FO = fish oil. n.d. = not detectable.

Comparing the SF of KO to the not-digested oil (Table 8), the TLC-FID analysis highlighted the disappearance of PLs, presumably related to their hydrolysis by pancreatic phospholipases. The absolute content of AMPLS significantly decreased after digestion, particularly when it was performed with the capsule; however, considering the percentage distribution, this class remained the most represented one. No significant modification in absolute FFA content was observed after *in vitro* digestion of KO.

Table 8. Partition of lipids in the different classes in SF of KO after digestion with or without capsules, evaluated by TLC-FID.

	Not Digested	SF (Digested w/o Capsule)	SF (Digested with Capsule)	<i>p</i> -Value
WEs	2.98 ± 2.60	n.d.	0.12 ± 0.16 (0.62%)	0.26
TAGs	8.97 ± 8.66 ^a	5.89 ± 1.98 ^a (13.39%)	1.21 ± 0.58 ^a (6.30%)	0.42
FFAs	4.11 ± 3.56 ^a	2.08 ± 0.65 ^a (4.73%)	2.45 ± 0.14 ^a (12.75%)	0.63
ALCs	1.00 ± 1.72 ^a	0.60 ± 0.85 ^a (1.36%)	0.31 ± 0.44 ^a (1.61%)	0.84
DAGs	n.d.	0.10 ± 0.14 (0.23%)	0.72 ± 1.01 (3.75%)	0.48
AMPLs	74.22 ± 7.41 ^a	35.32 ± 2.59 ^b (80.27%)	12.44 ± 0.77 ^c (64.76%)	0.002
PLs	8.72 ± 9.45	n.d.	n.d.	

Data are expressed as mg/100 mg of not-digested oil and are means ± SD of two biological replicates, except for the not-digested oil, which was analyzed in three biological replicates. Each biological replicate was evaluated in triplicate. Statistical analysis was performed by Student's *t*-test when comparing two values and by ANOVA (one-way analysis of variance) using Tukey's test as post-test when comparing three values. *p* < 0.05 was considered significant. ** means *p* ≤ 0.01. Different letters in the same row indicate statistical significance. The mean percentage contribution of each class to total lipids in SF is reported in parentheses. TAGs = triglycerides; DAGs = diglycerides; MAGs = monoglycerides; WEs = wax esters; FFAs = free fatty acids; DHA = docosahexaenoic acid; FO = fish oil. n.d. = not detectable

After digestion, ¹H-NMR analysis confirmed the decrease in PL content, indicated by the reduction in total P-choline (Table 9). In addition, it allowed for the verification of the almost complete hydrolysis of TAGs. Although the absolute quantity of FFAs was lower in the SF, due to the lower amount of total lipids recovered in this fraction, FFAs accounted for about 61% of the total lipids.

Table 9. Partition of lipids in the different classes in SF of KO after digestion with or without capsules, evaluated by ¹H-NMR.

	Not Digested	SF (Digested w/o Capsule)	SF (Digested with Capsule)	<i>p</i> -Value
TAGs	26.24 ± 0.22 ^a	0.35 ± 0.16 ^b (0.80%)	0.48 ± 0.14 ^b (2.51%)	<0.001
1,3-DAGs	0.94 ± 0.34 ^a	0.23 ± 0.06 ^{a,b} (0.52%)	0.01 ± 0.01 ^b (0.05%)	0.03
1,2-DAGs	2.97 ± 0.75 ^a	0.51 ± 0.10 ^b (1.15)	0.47 ± 0.12 ^b (2.43%)	0.010
1,2-DAGs-P	5.80 ± 0.15 ^a	0.87 ± 0.08 ^b (1.97%)	0.36 ± 0.02 ^c (1.89%)	<0.001
2-MAGs	0.10 ± 0.04 ^b	0.62 ± 0.28 ^{a,b} (1.41%)	0.69 ± 0.17 ^a (3.61%)	0.03
1-MAGs	2.79 ± 0.21 ^a	0.70 ± 0.03 ^b (1.59%)	0.35 ± 0.02 ^b (1.81%)	<0.001
WEs	0.38 ± 0.16 ^a	0.05 ± 0.04 ^a (0.11%)	0.06 ± 0.08 ^a (0.34%)	0.06
Primary alcohol	0.21 ± 0.02 ^a	3.03 ± 3.35 ^a (6.93%)	1.19 ± 1.65 ^a (6.28%)	0.35
Total P-choline	21.83 ± 0.31 ^a	5.72 ± 0.32 ^b (12.99%)	2.32 ± 0.20 ^c (12.05%)	<0.001
FFAs (except DHA)	36.09 ± 0.60 ^a	27.07 ± 1.94 ^b (61.59%)	11.87 ± 1.88 ^c (61.74%)	<0.001
Free DHA	2.67 ± 0.04 ^b	4.86 ± 1.12 ^a (11.03%)	1.40 ± 0.47 ^b (7.28%)	0.01

Data are expressed as mg/100 mg of not-digested oil and are means ± SD of two biological replicates, except for the not-digested oil, which was analyzed in three biological replicates. Each biological replicate was evaluated in triplicate. Statistical analysis was performed by one-way ANOVA using Tukey's test as post-test, and *p* < 0.05 was considered significant. Different letters in the same row indicate statistical significance. The mean percentage contribution of each class to total lipids in SF is reported in parentheses. TAGs = triglycerides; DAGs = diglycerides; MAGs = monoglycerides; WEs = wax esters; FFAs = free fatty acids; DHA = docosahexaenoic acid; KO = krill oil.

The presence of primary alcohols in the SF of KO may be associated with the formation of lysophospholipids, which possess a free primary alcoholic function at position 1 of the glycerol moiety. As we were not able to assign the other hydrogen atoms of the glycerol moiety to identified signals, for example, those in position 3 (esterified with phosphatidylcholine) or in position 2 (which remains esterified with a FA), we are forced to assign any hydrogen atoms in position 1 (non-esterified) of the glycerol moiety in lysophospholipids as a generic primary alcohol, as it resonates in the typical alcoholic region anyway. The ¹H-NMR spectra of digested KO in comparison to the not-digested oil are reported in Figure S3. Regardless of the presence of the capsule, the TLC-FID analysis showed a lower absolute content of WEs in the SF of CO than in the corresponding not-digested oil (Table 10). This decrease was mainly related to the lower lipid content of the SF compared to the not-digested oils, and only partially to WE hydrolysis. Indeed, in the SF, WEs still represented more than 70% of the total lipids, except in CO-2 when the digestion was performed without capsule. The poor hydrolysis of WEs was confirmed by the low amount of FFAs in the SF. Of note, an increase in FFAs was only observed after the digestion of CO-2 without the capsule, concomitant to the increased hydrolysis of WEs.

Currently, it is hard to explain why WE hydrolysis and FFA release were significantly different in the two CO supplements; we are sure that the presence of the capsule had an impact on it.

Table 10. Partition of lipids in the different classes in SF of COs after digestion with or without capsules, evaluated by TLC-FID.

CO-1	Not Digested	SF (Digested w/o Capsule)	SF (Digested with Capsule)	p-Value
WEs	86.49 ± 1.45 ^a	45.76 ± 1.53 ^b (73.82%)	41.95 ± 7.03 ^b (77.76%)	0.003
TAGs	5.10 ± 3.46 ^a	2.66 ± 1.55 ^a (4.29%)	0.21 ± 0.29 ^a (0.39%)	0.23
FFAs	3.27 ± 1.04	n.d.	1.11 ± 1.01 (2.06%)	0.16
ALCs	2.73 ± 0.64	n.d.	0.89 ± 0.40 (1.65%)	0.07
DAGs	n.d.	n.d.	0.29 ± 0.41 (0.54%)	
AMPLs	2.41 ± 0.34 ^a	12.66 ± 4.04 ^a (20.42%)	8.98 ± 4.21 ^a (16.65%)	0.12
PLs	n.d.	0.50 ± 0.71 ^b (0.81%)	0.59 ± 0.31 ^b (1.09%)	0.88
CO-2	Not Digested	SF (Digested w/o Capsule)	SF (Digested with Capsule)	p-Value
WEs	78.82 ± 7.13 ^a	24.05 ± 4.54 ^b (45.17%)	44.20 ± 0.96 ^b (83.08%)	0.003
TAGs	5.84 ± 6.12	0.66 ± 0.68 (1.24%)	tr	0.36
FFAs	4.01 ± 3.99 ^a	16.19 ± 8.97 ^a (30.41%)	2.25 ± 1.77 ^a (4.23%)	0.17
ALCs	2.26 ± 2.44 ^a	0.26 ± 0.10 ^a (0.49%)	0.38 ± 0.53 ^a (0.71%)	0.41
DAGs	0.37 ± 0.83	0.65 ± 0.92 (1.22%)	n.d.	0.78
AMPLs	5.76 ± 1.58 ^a	10.24 ± 3.55 ^a (19.23%)	5.71 ± 1.40 ^a (10.73%)	0.24
PLs	2.94 ± 5.65	1.29 ± 1.83 (2.42%)	n.d.	0.73

Data are expressed as mg/100 mg not-digested oil and are means ± SD of 2 replicates. Statistical analysis was performed by Student's t-test when comparing two values and by one-way ANOVA with Tukey's test as post-test when comparing three values. $p < 0.05$ was considered significant. Different letters in the same row indicate statistical significance. The mean percentage contribution of each class to total lipids in SF is reported in parentheses. WEs = sterol esters and wax esters; TAGs = triglycerides; FFAs = free fatty acids; ALC = free aliphatic alcohols; DAG = diglycerides; AMPLs = acetone-mobile polar lipids; PLs = phospholipids and other acetone immobile polar lipids; n.d. = not detectable.

¹H-NMR analysis confirmed the absence of any hydrolysis of WEs in the CO-1 supplements (Table 11). The observed decrease in absolute WE content was related to the reduced total lipid content in the SF. The mean percentage contribution of each class to the total lipids in the SF was almost the same as in the not-digested sample, indicating that CO-1 did not undergo any hydrolysis. Conversely, some small modification occurred in CO-2, in which a partial hydrolysis of WEs and TAGs was observed after digestion, coupled to a small increase in the contribution of FFAs to total lipids. The ¹H-NMR spectra of the digested CO in comparison to the not-digested oil are given in Figures S4 and S5. TAGs,

PLs and cholesterol esters are the predominant dietary lipids [52]. During intestinal digestion, pancreatic triacylglycerol lipase and phospholipase hydrolyze TAGs and PLs, respectively [53]. Other digestive enzymes may hydrolyze TAGs, cholesterol esters (CEs), PLs and galactolipids with reaction rates varying accordingly to the substrates, releasing FFAs together with monoacylglycerols (MAGs), lysophospholipids and free cholesterol [54]. As the reaction rates of lipolytic enzymes vary according to their substrates, the bioaccessibility of fatty acids, including n-3 LC-PUFAs, depends on the lipid classes they are bound to. The rate of lipid hydrolysis in the intestine is not a trivial concern, as most lipid classes are too large to be absorbed into the intestinal epithelial cells [53]. It is therefore clear that the n-3 LC-PUFA concentration in supplements is not a reliable reflection of their possible effectiveness, as it does not consider the chemical form of lipids and consequently the bioaccessibility of FAs.

Table 11. Partition of lipids in the different classes in SF of COs after digestion with or without capsules, evaluated by ¹H-NMR.

CO-1	Not Digested	SF (Digested w/o Capsule)	SF (Digested with Capsule)	p-Value
TAGs	0.91 ± 0.06 ^a	0.51 ± 0.09 ^b (0.82%)	0.50 ± 0.05 ^b (0.92%)	0.004
1,3-DAGs	0.06 ± 0.01 ^a	0.03 ± 0.004 ^b (0.06%)	0.03 ± 0.004 ^b (0.05%)	0.02
1,2-DAGs	1.16 ± 0.30 ^a	1.14 ± 0.60 ^a (1.84%)	0.63 ± 0.05 ^a (1.17%)	0.34
1,2-DAGs-P	n.d.	n.d.	n.d.	
2-MAGs	<0.01	<0.01	<0.01	
1-MAGs	1.18 ± 0.01 ^a	0.74 ± 0.03 ^b (1.20%)	0.65 ± 0.06 ^b (1.20%)	<0.001
WEs	63.86 ± 0.59 ^a	38.83 ± 0.33 ^b (62.64%)	34.61 ± 2.79 ^b (64.13%)	<0.001
Primary alcohol	3.04 ± 0.45 ^a	2.28 ± 0.24 ^{a,b} (3.67%)	1.54 ± 0.03 ^b (2.86%)	0.02
Total P-choline	n.d.	n.d.	n.d.	
FFAs (except DHA)	24.25 ± 0.15 ^a	14.95 ± 0.24 ^b (24.12%)	12.99 ± 0.85 ^c (24.09%)	<0.001
Free DHA	5.52 ± 0.03 ^a	3.49 ± 0.08 ^b (5.63%)	3.00 ± 0.26 ^b (5.56%)	<0.001
CO-2	Not Digested	SF (Digested w/o Capsule)	SF (Digested with Capsule)	p-Value
TAGs	1.26 ± 0.06 ^a	0.25 ± 0.07 ^b (0.48%)	0.35 ± 0.02 ^b (0.66%)	<0.001
1,3-DAGs	0.44 ± 0.03 ^a	0.03 ± 0.01 ^b (0.05%)	0.03 ± 0.01 ^b (0.05%)	<0.001
1,2-DAGs	0.17 ± 0.10 ^a	0.43 ± 0.21 ^a (0.81%)	0.34 ± 0.21 ^a (0.63%)	0.25
1,2-DAGs-P	n.d.	n.d.	n.d.	
2-MAGs	0.05 ± 0.01	n.d.	n.d.	
1-MAGs	0.14 ± 0.002 ^b	0.58 ± 0.07 ^a (1.08%)	0.37 ± 0.26 ^{a,b} (0.70%)	0.06
WEs	70.30 ± 0.03 ^a	34.42 ± 0.59 ^b (64.71%)	34.95 ± 0.09 ^b (65.70%)	<0.001
Primary alcohol	2.12 ± 0.03 ^a	1.87 ± 0.26 ^a (3.50%)	1.75 ± 0.14 ^a (3.29%)	0.11
Total P-choline	n.d.	n.d.	n.d.	
FFAs (except DHA)	19.06 ± 0.08 ^a	12.84 ± 1.50 ^b (23.94%)	12.60 ± 0.69 ^b (23.68%)	0.001
Free DHA	6.45 ± 0.10 ^a	2.82 ± 0.17 ^b (5.30%)	2.81 ± 0.03 ^b (5.29%)	<0.001

Data are expressed as mg/100 mg of not-digested oil and are means ± SD of two biological replicates, except for the not-digested oil, which was analyzed in three biological replicates. Each biological replicate was evaluated in triplicate. Statistical analysis was by one-way ANOVA using Tukey's test as post-test, and $p < 0.05$ was considered significant. Different letters in the same row indicate statistical significance. The mean percentage contribution of each class to total lipids in SF

is reported in parentheses. TAGs = triglycerides; DAGs = diglycerides; MAGs = monoglycerides; WEs = wax esters; FFAs = free fatty acids; DHA = docosahexaenoic acid; KO = krill oil. n.d. = not detectable.

Although some *in vitro* studies have been carried out to investigate the digestibility of the oils usually used as n-3 LC-PUFA sources in supplements [55–57], the different methodologies used for analyzing it make it difficult to compare the results. In addition, to our knowledge, the modulation of digestibility related to the presence of capsules has never been investigated. To our knowledge, this is the first study comparing FA bioaccessibility in commercial n-3 LC-PUFA supplements using the validated INFOGEST method [31], which was performed without and with the capsule. We did not include gastric lipase in the digestion protocol since in adults, most of the lipid digestion occurs in the intestine. Although this could be considered a limitation, and we are aware that FA bioaccessibility could be higher *in vivo*, our aim was to compare different n-3 LC-PUFA supplements to evidence whether they have a different release of FAs. It is known that porcine pancreatin does not provide a complete replacement of the enzymes and lipolytic activities present in human pancreatic juice, since the activity of phospholipase, galactolipase and cholesterol esterase is lower [58]. Usually, this does not have a significant impact on the overall release of FAs, most of which are esterified into TAGs, but in samples containing high amounts of PL or CE, this may represent a limitation of the *in vitro* digestion method. However, in our study, PLs almost completely disappeared in the digested KO samples, and if lower cholesterol esterase activity had an impact on FA release in COs, the impact was small, as CEs account for 1.2–3.2% of total lipids [59]. The release of FFAs after digestion was quantified using two analytical techniques, TLC-FID and ¹H-NMR, which gave consistent results, except for some differences arising from the fact that TLC-FID determines the quantities of classes of molecules, which are grouped according to their behavior during the chromatographic elution (e.g., high mobility in acetone) rather than according to their different molecular structure, as is the case of NMR. Consequently, ¹H-NMR appeared more specific and suitable for quantifying FFAs while also allowing us to discriminate free DHA from all other FAs. Results clearly indicate that the release of FFAs after simulated digestion significantly depends on the oil source, and it is related to the partition of lipids in the different classes. The lowest FFA release after digestion was detected in COs, which contain high amounts of WEs. In mammals, WEs hydrolysis is assumed to be a slow process, primarily because they are poor substrates for lipolytic enzymes, especially

pancreatic lipase [27]. The poor digestibility of WEs is also suggested by the outbreaks of keriorrhea (oily diarrhea) that are associated with the consumption of large portions of WE-rich fish [60]. Although some studies indicate that mammals can digest moderate amounts of WEs [61] and absorb the liberated FAs and alcohols [62], our results confirmed that CO is not a good source of bioaccessible FAs. That, in addition to the ecological and ethical concerns of directly catching the zooplankton species studied, puts a serious question mark on any sustainability and health claims associated with CO products. In this study, the highest concentration of FFAs and free DHA in the SF of digested oils was found in KO, confirming that FAs in the form of PLs have higher bioavailability than fatty acids in the form of TAGs [63]. The higher bioaccessibility of phospholipids compared to triacylglycerols observed in this study can be attributed to their amphipathic properties and their greater tendency to remain solubilized in the aqueous phase during digestion. Phospholipids facilitate micelle formation in the presence of bile salts and are therefore more efficiently recovered in the soluble fraction, whereas non-hydrolyzed or partially hydrolyzed TAGs remain associated with the insoluble phase. In nature, n-3 PUFAs are prevalent as neutral lipids (triglycerides, esters, etc.) and, to a lesser extent, in the form of polar lipids (PL, glycerophospholipids, glycolipids and sphingolipids). The chemical form modulates the release of FAs by digestive enzymes, making them absorbable by enterocytes and increasing their concentration in the blood stream. Therefore, the bioaccessibility of n-3 LC-PUFA is a determining factor of their efficacy. Based on the ¹H-NMR results reported here, we calculated the amounts of total FFA and free DHA available after the digestion of a recommended serving size of the supplements (Figure 2), and they varied significantly between supplements and were in most cases lower than what was reported on the label (Table 1). Although the results reported in Figure 2 refer to specific supplements and cannot be generalized, and although they underestimate bioaccessibility since FAs can also be absorbed as MAGs, they highlight an important issue in the use and testing of n-3 LC-PUFA supplements. Indeed, although the evidence suggests that the consumption of foods or supplements containing marine oils may affect chronic diseases and complications of metabolic dysfunctions, the literature on n-3 LC-PUFA supplementation is highly conflicting [64,65], and large trials and meta-analyses have yielded inconsistent findings [1,66,67]. Supplement oxidation is a potential explanation [68], but our results clearly indicate that the source of the marine oil could play a role.

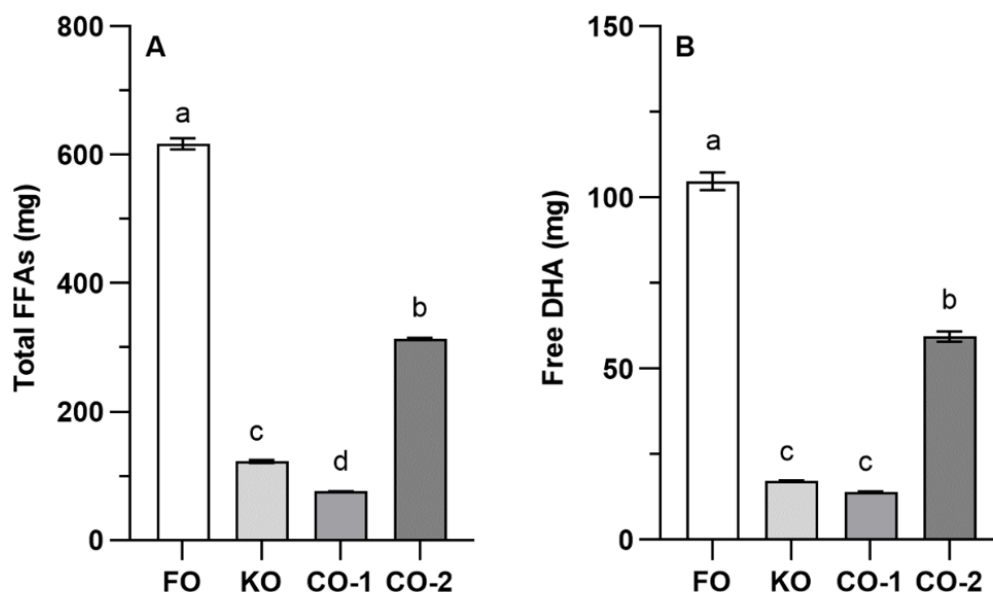


Figure 2. Theoretical amount of (A) total FFAs and (B) free DHA released after the digestion of the recommended serving size of the different supplements. Free FFAs and DHA were calculated based on serving size as indicated on the product label. Statistical analysis was performed using one-way ANOVA ($p < 0.001$ for both lipid classes) with Tukey's post-hoc test. Different letters above columns indicate statistical significance at least at $p < 0.05$. FO = fish oil; KO = krill oil; CO = Clanus oil; FFAs = free fatty acids; DHA = decosahexaenoic acid.

Conclusions

Although evidence suggests that n-3 LC-PUFAs may affect chronic diseases, the evidence regarding n-3 LC-PUFA supplementation is not as straightforward, and there are some inconsistencies regarding the role of marine oil supplements in both the primary and secondary prevention of cardiovascular diseases. Our results clearly indicate that the source of the marine oil and its encapsulation have a significant impact on the bioaccessibility of fatty acids, thus likely modulating their effectiveness. The potential discrepancy between the theoretical and the actual intake of n-3 LC-PUFAs should be carefully evaluated prior to clinical trials, and our study highlights that *in vitro* digestion studies coupled with ^1H -NMR detection represent an effective tool for rapid and cost-effective product screening.

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Supplementary data

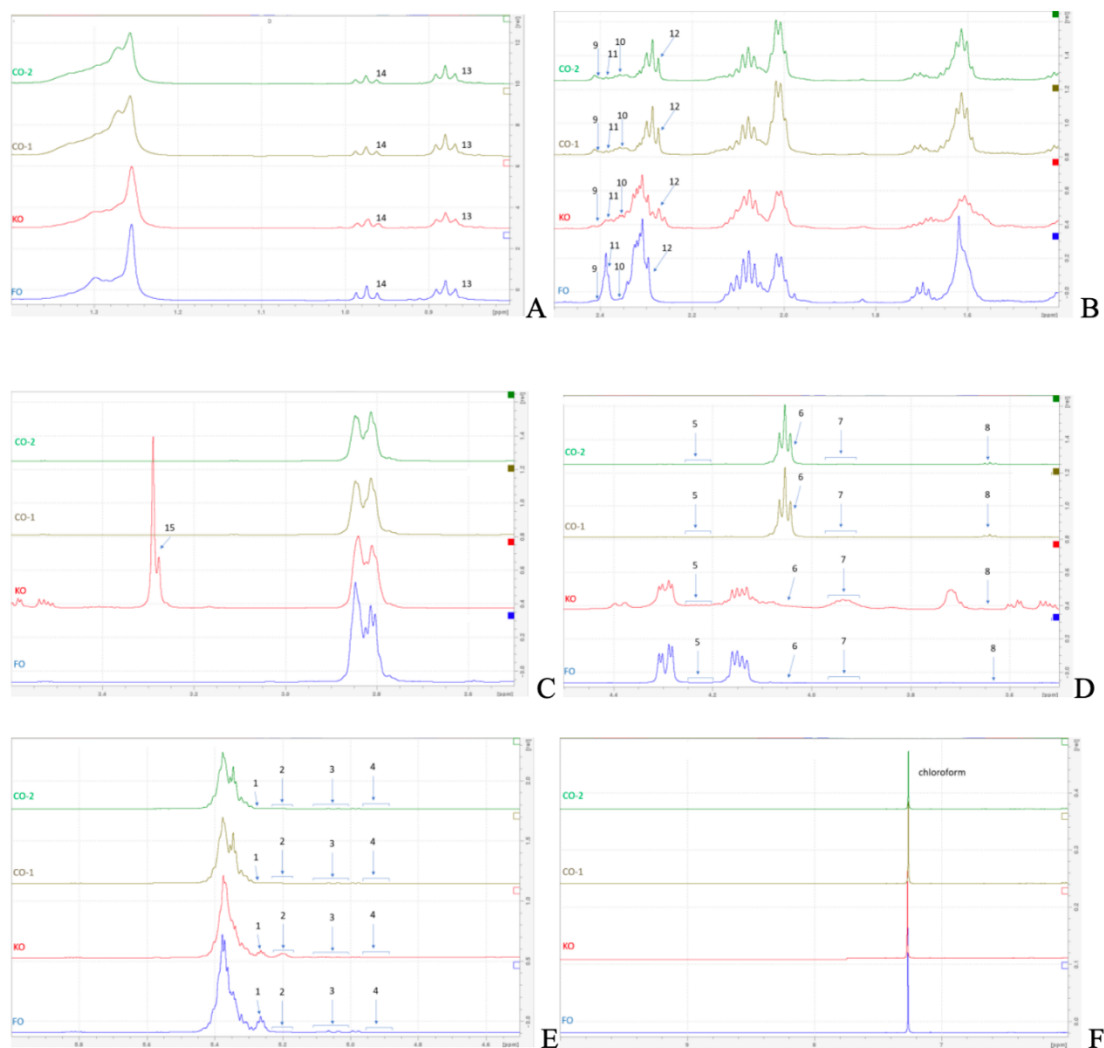


Figure S1. Expansions of specific regions ^1H -NMR spectra recorded in CDCl_3 solvent on fish oil (FO, in blue), krill oil (KO, in red), Calanus oils (CO-1 in brown and CO-2 in green) from not-digested supplements, in the regions of: A (0.84-1.40 ppm) terminal CH_3 of all FAs; B (1.40-2.50 ppm) β -methylene groups of DHA and other FA free and bound; C (2.50-3.50 ppm) phosphocholine signal - visible only in the KO spectrum; D (3.50-4.50 ppm) esterified 1° alcohol (4.05 ppm) typical of CO-1 and CO-2 oil; E (4.50-6.00 ppm) MAG, 1,2-DAG, 1,3-DAG and 1,2-DAG-P; F (6.00-10.00 ppm), the downfield region with the chloroform signal.

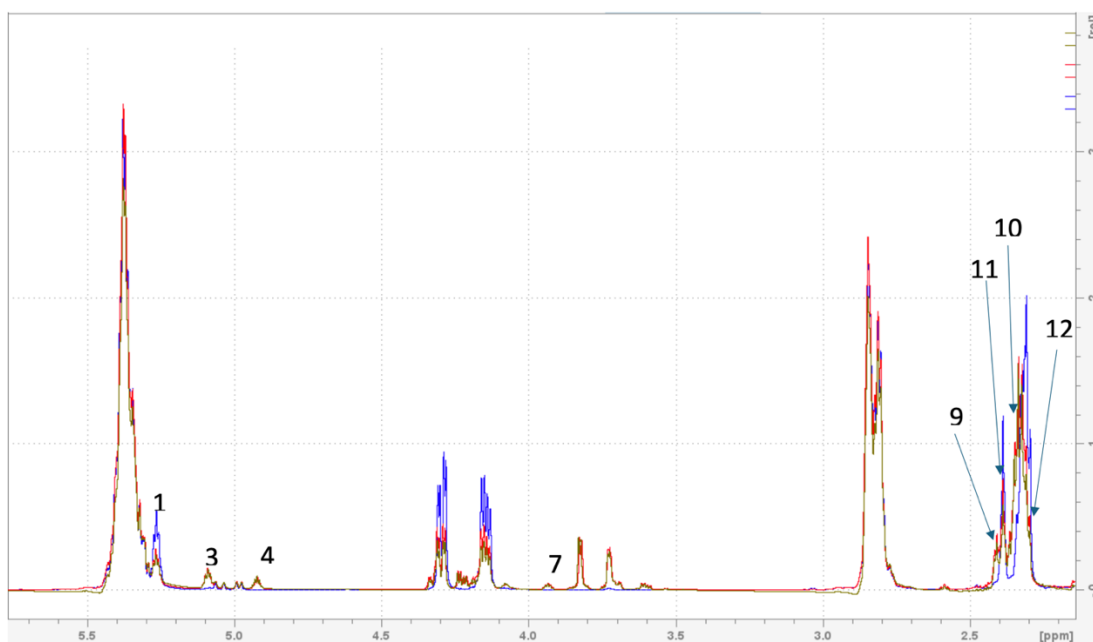


Figure S2. Zoom of the glyceride region (2.20-5.80 ppm) of ^1H -NMR spectra recorded on not-digested FO (blue), and on digestate of encapsulated (red) or non-encapsulated (brown) supplements. Peak assignment is provided in Table 2.

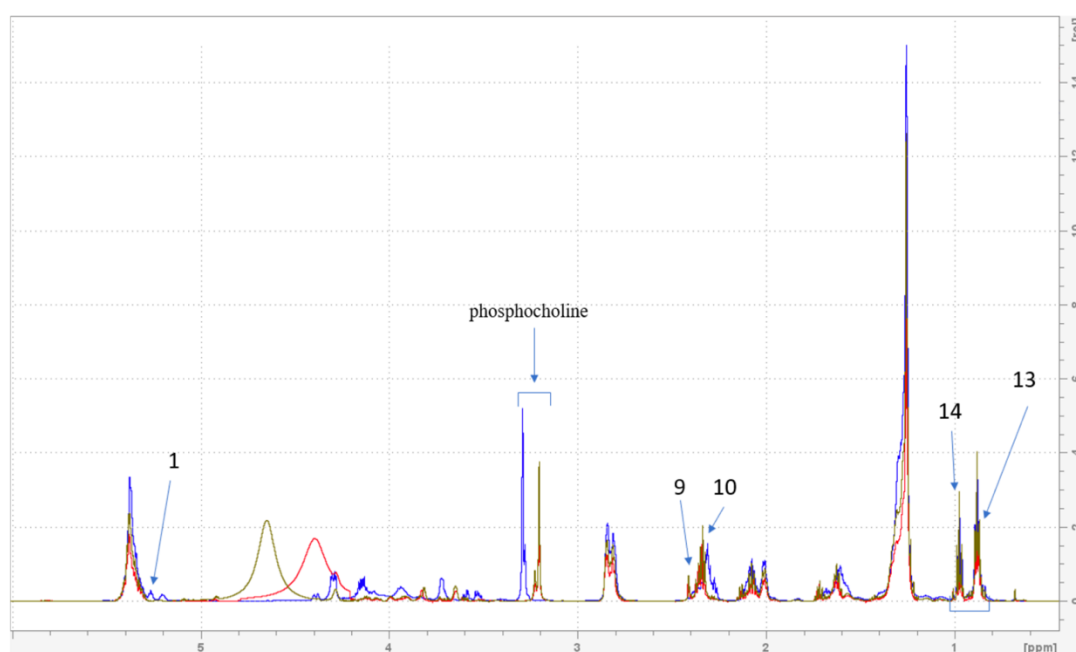


Figure S3. ^1H -NMR spectra recorded on not-digested KO (blue), and on digestate of encapsulated (red) or non-encapsulated (brown) KO supplements. Peak assignment is provided in Table 2.

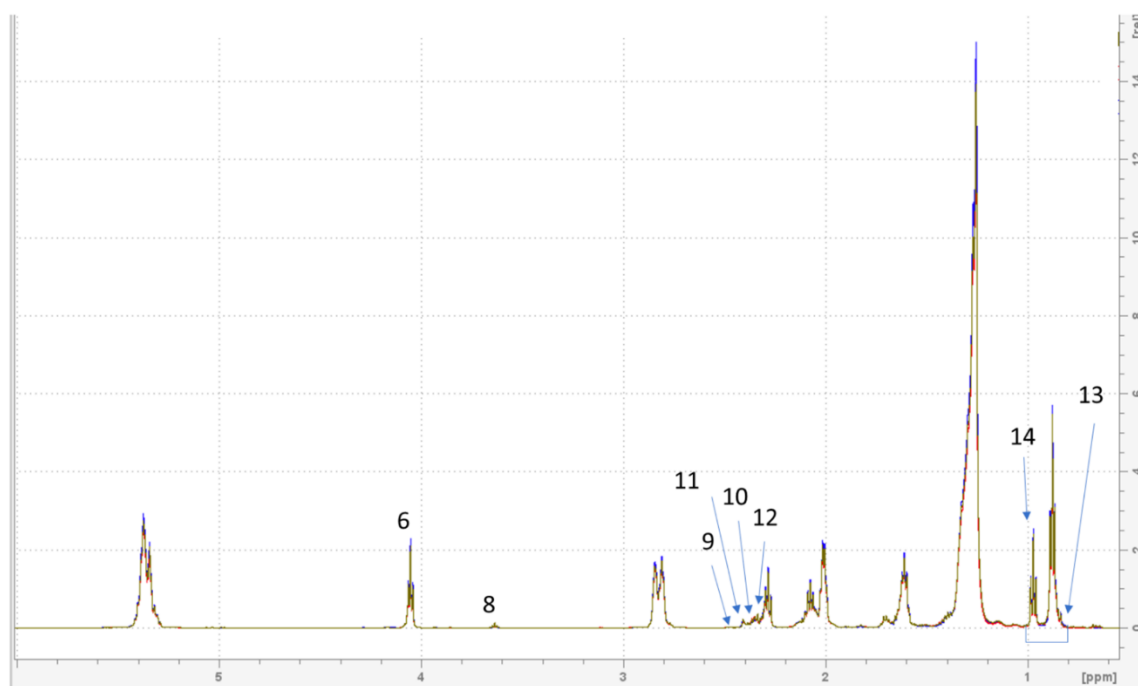


Figure S4. ^1H -NMR spectra recorded on not-digested CO-1 (blue), and on digestate of encapsulated (red) or non-encapsulated (brown) CO-1 supplements. Peak assignment is provided in Table 2.

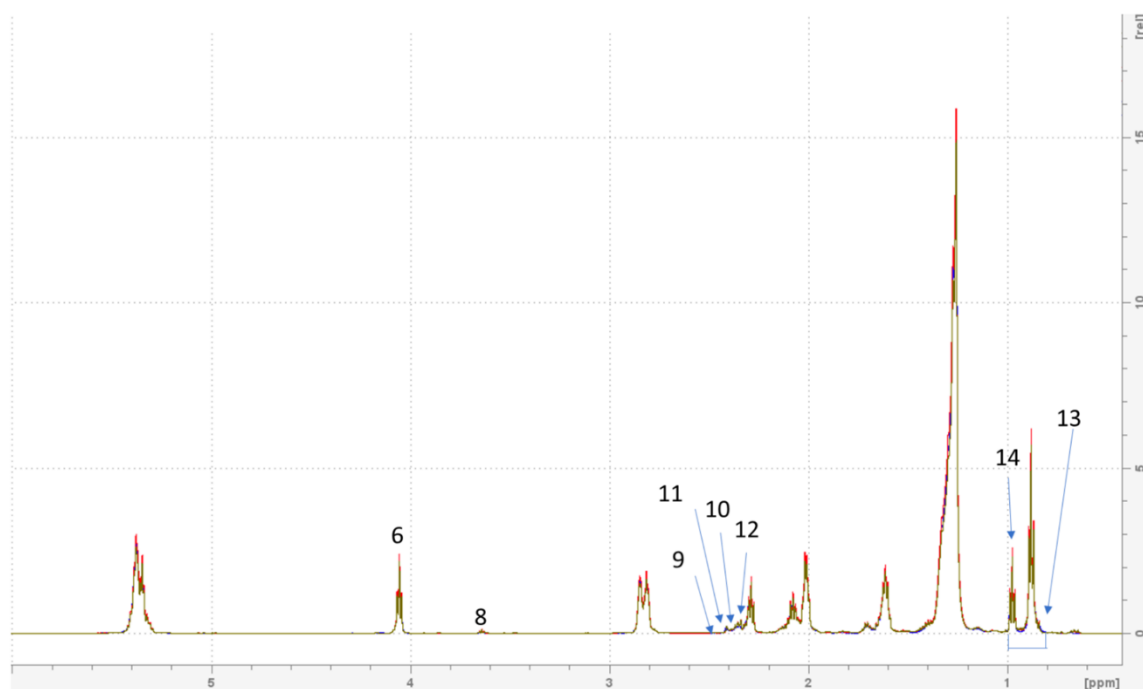


Figure S5. ^1H -NMR spectra recorded on undigested CO-2 (blue), and on digestate of encapsulated (red) or non-encapsulated (brown) CO-2 supplements. Peak assignment is provided in Table 2.

Section 2. Sugars

During digestion, salivary α -amylase initiates starch breakdown in the mouth, but it is rapidly inactivated by the acidic pH of the stomach. However, a portion of the enzyme may resist degradation and remain active in the duodenum. Pancreatic α -amylase is the main enzyme responsible for the intraluminal hydrolysis of starch. It cleaves α -1,4 glycosidic bonds, producing maltose and maltotriose from amylose. In amylopectin, it cannot act on the α -1,6 bonds, and therefore generates limit dextrins, which are branched oligosaccharides. The products of starch digestion (oligosaccharides) are further broken down by brush border enzymes located on the apical membrane of enterocytes. These include glucoamylase (or maltase), sucrase-isomaltase, and isomaltase. Glucoamylase removes glucose residues one at a time; sucrase cleaves maltose and maltotriose; and isomaltase is the only enzyme capable of hydrolyzing the α -1,6 bonds found in branch points. The most relevant disaccharides in the human diet are lactose, sucrose, and maltose, all of which must be digested at the brush border of enterocytes before they can be absorbed as monosaccharides.

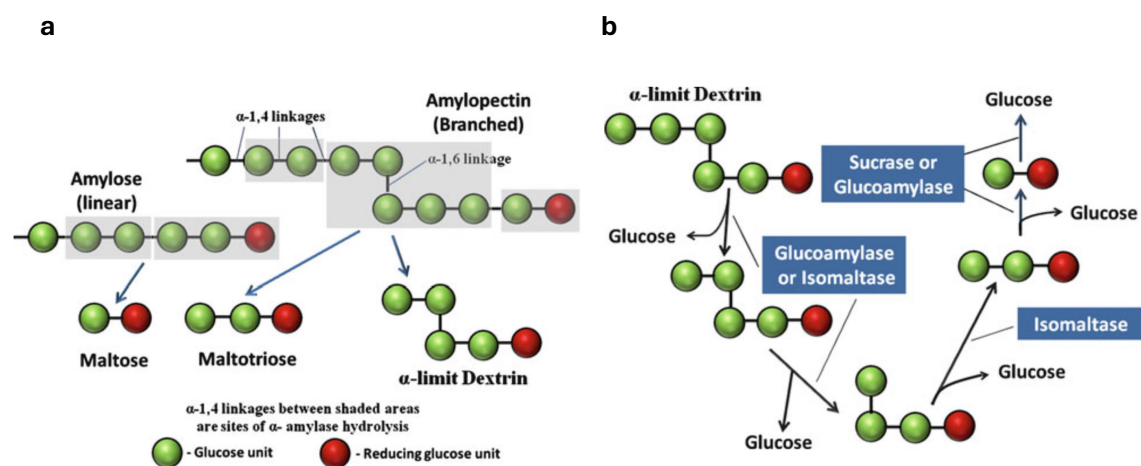


Figure 4. Carbohydrate digestion

(a) Pancreatic α -amylase is involved in the breakdown of starch, acting specifically on amylose and amylopectin, the two main polysaccharide structures of starch. (b) The limited dextrins

produced by initial digestion are further degraded by oligosaccharidases located on the surface of the brush border of intestinal cells.

Lactose is hydrolyzed by the enzyme lactase into glucose and galactose. Lactase activity is the rate-limiting step for efficient lactose absorption and plays a key role in determining an individual's tolerance to this sugar. Sucrose is split by sucrase into glucose and fructose, whereas maltose is degraded by maltase into two glucose molecules. Lactase activity is physiologically high in newborns, as milk constitutes their primary food source, but it typically declines after weaning. In individuals with adult-type hypolactasia, that is, a persistent reduction in lactase expression, undigested lactose reaches the colon, where it is fermented by the gut microbiota, leading to the production of gases and metabolites that may cause gastrointestinal symptoms such as diarrhea, bloating, and flatulence. Significant genetic variation exists among populations in terms of the persistence of lactase activity [1]. The absorption of monosaccharides occurs through highly specialized mechanisms. Glucose and galactose are absorbed into the apical membrane of enterocytes through a sodium-dependent transporter known as SGLT1 (Sodium-Glucose Linked Transporter 1). This cotransport requires energy derived from the electrochemical gradient of sodium, maintained by the Na^+/K^+ -ATPase pump located on the basolateral membrane. Fructose, on the other hand, uses a facilitated diffusion mechanism mediated by the GLUT5 transporter. Once inside the enterocyte, all three monosaccharides are transferred into the portal blood via the GLUT2 transporter. GLUT2 can also be detected in the brush border membrane of enterocytes. This mechanism is particularly relevant for glucose absorption in the enterocyte, when luminal glucose increases SGLT1 promotes the rapid insertion of GLUT2 into the brush border membrane through a signal transduction pathway involving protein kinase C β II (PKC β II). Under conditions of high fructose load, GLUT2 may assist in the absorption of excess luminal fructose [2].

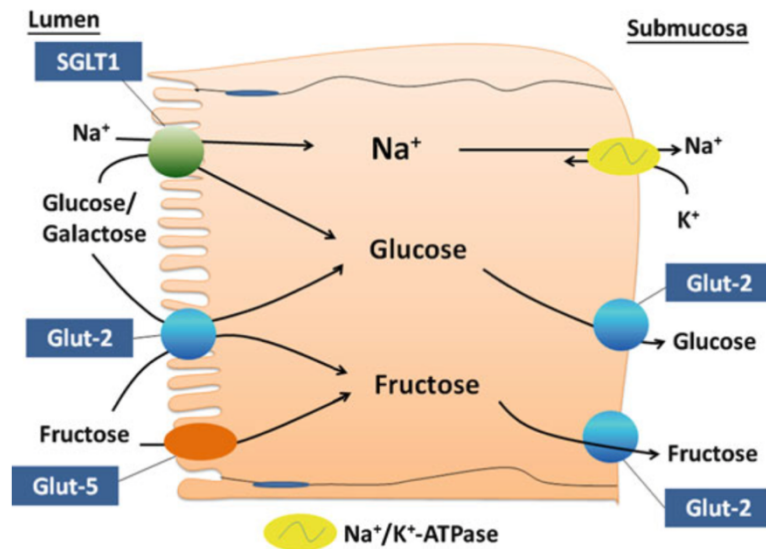


Figure 5. Transport of monosaccharides from the brush border (apical membrane) to the basolateral membrane across the enterocyte

Regardless of whether it derives from the digestion of starch or simple sugars, glucose is the main monosaccharide absorbed. Its concentration in the blood (glycemia) depends on dietary intake and is finely regulated by the secretion of two hormones, insulin and glucagon. The extent of the increase in blood sugar after consuming a food defines its glycemic index and load. The GI and GL describe related but distinct concepts. GI reflects the intrinsic ability of a food to raise blood glucose when a fixed amount of available carbohydrates is consumed, and is mainly influenced by food structure, matrix and processing. In contrast, GL combines GI with the amount of carbohydrates consumed in a typical portion and is therefore more relevant for dietary exposure and epidemiological studies. In the present work, glucose release after *in vitro* digestion is conceptually closer to GI than to GL, as it focuses on carbohydrate digestibility and matrix effects under standardized conditions and does not account for portion size or dietary intake.

Since excessively high blood glucose levels are associated with various pathologies, a low glycemic index diet is strongly recommended. However, assessing the glycemic index *in vivo* is very complicated and expensive, so having an *in vitro* system that can measure glucose release after digestion of a food is important. The first study reported in this section describes the development of a method for assessing glucose release from foods *in vitro*. Although the amount of glucose release *in vitro* is not equivalent to the glycemic index *in vivo*, as it does not take into account the absorption and action of insulin, it is still an

important parameter for the nutritional evaluation of a food as it represents the first necessary and indispensable step for subsequent absorption.

The second study reported in this section concerns a particular type of sugar, iminosugars. Although present in small quantities in foods, these compounds are attracting considerable interest due to their diverse biological activities.

From a nutritional and health perspective, the rate and extent of glucose release during digestion are closely linked to postprandial glycemic responses and, consequently, to metabolic health. Foods characterized by slower glucose release may help prevent large glycemic excursions, improving insulin sensitivity and reducing the risk of developing obesity, type 2 diabetes, and cardiovascular diseases [3]. In this context, *in vitro* models capable of predicting the glycemic impact of foods provide essential tools for both nutrition science and the food industry, as they can guide the formulation of products with controlled starch digestibility and lower glycemic potential.

Moreover, some particular sugars may be functional components. In fact, iminosugars naturally occurring in some foods and their synthetic derivatives show promising modulatory effects on carbohydrate metabolism, including inhibition of intestinal glycosidases, attenuation of postprandial hyperglycemia, and potential anti-inflammatory and anticancer activities through the regulation of cellular energy metabolism. These properties highlight the possibility of exploiting iminosugars and their analogues not only for therapeutic purposes but also as bioactive ingredients for the development of innovative functional foods aimed at promoting metabolic health. Therefore, integrating *in vitro* digestion approaches with the study of bioactive molecules such as iminosugars represents a key step in the validation, optimization, and innovation of food products that combine nutritional quality with beneficial health effects.

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Development of a Simple Protocol to Assess Glucose Release After In Vitro Digestion, Allowing Comparision of Starchy Foods

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The text presented in this section is based on the corresponding published article and has been reproduced with minor additions and clarifications aimed at enhancing the description of the methodological framework and the interpretation of the results, while preserving the original scientific content.

Introduction

The incidence of obesity and type 2 diabetes (T2D) is increasing worldwide, and nutritional management plays a crucial role in their prevention and treatment [1]. Diets incorporating foods with low glycemic index (GI) have been proposed as useful means for managing glucose response [2]. The GI is the increase in blood glucose concentration in response to a test food consumed under standard conditions and is expressed as a percentage of the area under the curve (AUC) following the consumption of a reference food that the same individual consumes on a different day [3]. Since the GI value of a food is not based on the characteristics of the individual who consumed it but depends on the characteristics of the food itself [4], it serves as a systematic classification system. Carbohydrates account for 45%–60% of total energy requirements in the human diet [5], and are among the main determinants of postprandial glycemia. They are directly related to GI, and the form and quantity of carbohydrate-rich foods in the diet play a fundamental role in the onset of T2D [6]. The GI of a food is considered an indicator of health choices and can offer useful guidance on which foods to choose [7]. Therefore, obtaining information relating carbohydrate-rich foods and their GI is crucial. Unfortunately, the GI of many staple foods, such as bread, pasta, and potatoes, is high [8]. Several strategies for producing foods with low GI have been proposed, mainly focused on slowing the process of starch digestion [9,10]. In fact, the rate of starch digestion is useful for predicting the glycemic response to starchy foods as it is directly related to the amount of glucose absorbed into the

bloodstream. However, the complex relationship between the chemical properties of starch-rich foods and the digestibility of starch has not yet been fully elucidated. It is known that the digestibility of starch, that is, its conversion into free glucose, is linked to its morphological characteristics, its organization within the crystalline lamellae, the proportion of amylose and amylopectin, and the formation of amylose-fat inclusion complexes [11]. Non-starchy components (proteins, fats, and polysaccharide related substances) can interact with starch, changing its structure and making it inaccessible to digestive enzymes [12], and the processing of starchy foods can affect digestibility by reorganizing the starch structure [13]. Consequently, the solely chemical composition of a food is not sufficient to predict its GI, and a more direct measurement is needed. The *in vivo* determination of GI is very complicated as it involves the supervision of human subjects with intraindividual variations and is both expensive and cumbersome [14]. To address these issues, several *in vitro* digestion methods have been developed to study the digestive behavior of starch-rich foods [10]. Despite being simple, convenient, and cost-effective, these models have rarely been validated with *in vivo* results. The *in vitro* static digestion method developed under the COST action INFOGEST has high intra- and inter-laboratory reproducibility, cost-effectiveness, robustness, allows for easy evaluation of results at each digestion stage [15], and it was validated with *in vivo* results for digestibility of dietary proteins [16]. Although the INFOGEST protocol has been validated *in vivo* mainly for protein digestion, such validation cannot be directly extrapolated to carbohydrates because of differences in digestive enzymes and mechanisms. However, the protocol offers standardized conditions that are suitable for comparative assessment of carbohydrate digestibility and matrix effects *in vitro*. Despite all these advantages, this *in vitro* digestion method does not consider the last phase of starch hydrolysis. *In vivo*, disaccharides such as maltose and limit dextrin, which are the products of starch hydrolysis by salivary and pancreatic α -amylases, are hydrolyzed into glucose by the dual action of two brush border enzymes (BBEs), namely maltase-glucoamylase and sucrase-isomaltase [17]. BBEs are not included in the INFOGEST protocol [15], which only yields a partial release of free glucose from available starch. Amyloglucosidase is not a physiological equivalent of the BBEs α -glucosidases maltase-glucoamylase and sucrase-isomaltase, which are membrane-bound enzymes with distinct substrate specificities and regulatory properties. However, in static *in vitro* digestion models, amyloglucosidase is commonly used as a functional surrogate to ensure the complete hydrolysis of starch-derived oligosaccharides into glucose. By cleaving both α -1,4 and α -1,6 glycosidic linkages from

non-reducing ends, amyloglucosidase allows estimation of the maximal amount of glucose potentially available for absorption, thereby preventing underestimation of carbohydrate bioaccessibility. Its use should therefore be interpreted as a methodological simplification rather than a direct replication of *in vivo* brush-border digestion.

Aim of the study

The aim of the present study was to develop a simple and reliable protocol to evaluate *in vitro* glucose release from starchy foods by including in the INFOGEST protocol a final starch digestion step that mimics BBE digestion by adding the enzyme amyloglucosidase (AMG). To verify the reliability of the protocol, glucose release was evaluated in five commercial starchy foods (flour, bread, crackers, and durum wheat pasta and gluten-free pasta). The foods were selected based on their different processing and their availability on the market in two forms produced by the same company, one of which was rich in fiber (over 6g/100g, according to the Regulation [EC] 1924, European Commission, 2006). In fact, it has been reported that adding dietary fiber to starchy foods can lower the rate of starch digestion [18]. Although other studies have combined the INFOGEST protocol and the addition of AMG to mimic the last phase of starch digestion [19–21], to our knowledge this is the first one comparing different commercial starchy foods, obtaining results consistent with previous assessment of GI *in vivo*.

Materials and Methods

Materials

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

Food Samples

Five different commercial starchy foods were considered: wheat flour, frozen wheat bread, wheat crackers, and durum wheat pasta, and gluten-free corn pasta. Two different types—control and high-fiber (HF)—of each food, produced by the same company, were studied. All food samples were purchased at a local market. Wheat flour and crackers were digested without any further processing; frozen bread was defrosted at room temperature, cut into

1cm slices, and the 5g to be digested were taken from the central slice, taking care to maintain the same proportion between crust and crumb. The durum wheat semolina pasta (fusilli) and the gluten-free pasta (fusilli) were cooked according to the ISO 7304-1:2016(E) method, following the cooking time indicated by the manufacturer. After the addition of extra virgin olive oil (1g of oil/8g of pasta), the cooked pasta was immediately digested to avoid starch retrogradation.

In Vitro Digestion

Five grams of food were *in vitro* digested according to the INFOGEST protocol (Minekus et al. 2014) with some modifications. Digestion lasted for 2min (oral digestion with simulated salivary fluid at pH7) plus 120min (gastric digestion with simulated gastric juice containing 2000U/mL pepsin at pH3) plus 60 min (duodenal digestion with simulated pancreatic juice containing 10mM bile and an amount of pancreatin such that the trypsin activity in the final mixture was 100U/mL at pH7) at 37°C. At the end of duodenal digestion, samples were centrifuged at 4500×g for 10min at 4°C. The supernatant was aliquoted in 1mL vials, and 150µL AMG (230U/mL in glycerol, final concentration 30 U/mL) or water (T0) were added, as reported by Li et al. (2022). Samples were incubated in a shaking bath at 37°C. Incubation was stopped after 30 (T0 and T30), 60 (T60), or 120 (T120) min by inactivating the enzymes at 100°C for 10min. Samples were cooled, centrifuged at 10,000×g for 5min at 4°C, and the supernatant was used for glucose determination.

Determination of Amylase Activity

Amylase activity was assessed using the Amylase Activity Assay kit (Sigma-Aldrich, MO, USA) following the manufacturer's instructions and using ethylenedipNP-G7 as the substrate. One unit is the amount of the enzyme that cleaves ethylenedipNP-G7 to generate 1.0 mmol of p-nitrophenol per min, which is measured colorimetrically at 405 nm.

Determination of Amyloglucosidase Activity

Amyloglucosidase activity was determined using the Amyloglucosidase Activity Assay kit (Megazyme, Bray, Ireland) following the manufacturer's instructions and using p-nitrophenyl beta-maltoside plus thermostable beta-glucosidase as substrate. One unit is the

amount of enzyme that releases 1.0mmol of p-nitrophenol per min from p-nitrophenyl β -maltoside, which is measured colorimetrically at 400 nm.

Determination of Glucose Content

Glucose concentration was measured with the D-Glucose Assay Kit using GOPOD reagent (Megazyme, Bray, Ireland) following the manufacturer's instructions. Based on the matrix that was digested, a dilution factor (with water) was chosen for the samples, which were analyzed in duplicate. One hundred microliters of water were used as the blank, and 100 μ L D-Glucose solution as standard. Three milliliters of the GOPOD reagent were added to each tube, and after vortexing the tubes were incubated in a hot water bath at 45°C for 20 min. The colorimetric change was measured at 510 nm. Relative to the absorbances of the standard, the content of D-Glucose was determined using the calculation files downloadable from Megazyme's website (<https://www.megazyme.com/d-glucose-assay-kit>) and the following equation.

$$\text{D - Glucose } (\mu\text{g}/0.1\text{mL}) = \Delta \text{ Sample} / \Delta \text{ D - Glucose}$$

Statistical Analysis

Statistical analysis was by the one-way analysis of variance (ANOVA) with Tukey's as post-test, and by the Student's t-test, considering $p < 0.05$ as significant.

Results and Discussion

Several intestinal enzymes are needed for starch digestion [22]: two luminal endo-glucosidases called alpha-amylases and different maltases. These maltases are exo-glucosidases bound to the luminal surface of enterocytes that hydrolyze linear starch oligosaccharides to glucose. In this study, the addition of AMG after 1h of the duodenal phase of the INFOGEST *in vitro* digestion facilitated the hydrolysis of terminal α -1,4 and α -1,6 D-glucose residues successively from non-reducing ends of maltodextrins, thus breaking down available starch completely into glucose. Notably, AMG was added without stopping the pancreatic amylase activity, thus better mimicking the mucosal digestion *in vivo*. In the INFOGEST method [23], the amount of pancreatin to be added during digestion is calculated based on the activity of the trypsin enzyme. Since our aim was to evaluate the

release of glucose from starch, in preliminary experiments we adjusted the pancreatin amount based on amylase activity (200 U/mL), then compared results obtained in the same sample by including in the duodenal digestion an amount of pancreatin calculated based on the activity of the enzyme trypsin or amylase. Glucose release was evaluated at T0, T30, T60, and T120 of AMG digestion. As shown in Figure S1, no differences were detected at any time point. Based on this, in subsequent experiments the amount of pancreatin to be added during duodenal digestion was calculated according to the INFOGEST protocol. The nutritional composition of the commercial products considered in the study is shown in Table 1 according to the producer labeling. Ingredients in analyzed foods are reported in Table S1. Using the described protocol, we evaluated the time course of glucose release during INFOGEST + AMG digestion. *In vitro* digestion was performed on 5g of different starchy foods, and results were normalized for 100 g of undigested product (raw, in the case of flour and pasta) (Figure 1). In flour (Figure 1a), glucose release at T0 was very low and similar in the two products, confirming that starch digestion by amylases is incomplete [24]. In both control and HF flour, glucose release increased significantly after 30min of AMG digestion and continued to increase for the next 30min, with no significant differences between the two samples. After 2h, glucose release was significantly greater in control than in HF flour; however, it was lower than in other products. It is conceivable that the low glucose release was linked to the high quantity of non-gelatinized starch, since starch gelatinization, that is, the heat- and moisture-mediated breakdown of ordered structures in granular starch, is considered a prerequisite for its use. During the starch gelatinization process, also called cooking [25], the high temperature and water cause the starch granules to absorb water and swell, softening. This causes the linear and branched chains of starch to be released into the cooking medium and become gelatinized, making starch more susceptible to enzymatic hydrolysis [26].

Table 1. Nutritional composition of the commercial products according to the producer label.

Content (g/100 g)	Control	High-fiber
<i>Wheat flour</i>		
Starch	68.7 (75.3 g glucose ^a)	49.5 (54.9 g glucose ^a)
Sugars	1.5	0.5
Fiber	1.6	20
Fats	1.6	1.7
Proteins	12	17
<i>Frozen bread</i>		
Starch	45.6 (50.6 g glucose ^a)	38.6 (42.5 g glucose ^a)
Sugars	1.4	0.3
Fiber	4.4	11.3
Fats	1.6	2.5
Proteins	9.7	10
<i>Crackers</i>		
Starch	67.7 (74.15 g glucose ^a)	59 (65.5 g glucose ^a)
Sugars	1.3	2
Fibers	2.7	6.1
Fats	15	16
Proteins	9.6	10
<i>Durum wheat pasta (fusilli)</i>		
Starch	67.5 (74.3 g glucose ^a)	60.5 (66.6 g glucose ^a)
Sugars	3.5	3.5
Fibers	3	8
Fats	3	2.5
Proteins	13	13
<i>Gluten-free pasta (fusilli)</i>		
Starch	79.4 (88.1 g glucose ^a)	62.1 (68.3 g glucose ^a)
Sugars	0.6	0.7
Fibers	1.7	7.8
Fats	0.8	1.2
Proteins	7.1	12.1

Note: Data are expressed as g/100 g. ^aStarch weight was converted to glucose weight using 1.11 as the conversion factor (ratio between the weight of anhydrous D-glucose in starch and the weight of free D-glucose).

At T0, glucose release was low and similar in the two types of bread (Figure 1b); however, it was higher than that of flour (control flour = 2.16 ± 0.16 g/100 g flour; control bread = 6.48 ± 0.32 g/100 g bread), suggesting that starch gelatinization during bread cooking facilitated the release of glucose even during *in vitro* oro-gastric-duodenal digestion. Glucose release significantly increased after the first 30min of mucosal digestion, without any further significant increase, and was significantly higher in the control compared to HF bread at all time points. It is noteworthy that resistant starch (RS) was included as an

ingredient in both HF flour (17%) and HF bread (9.3%). RS, a linear molecule of α -1,4-D-glucan obtained mainly from retrogradation of the amylose fraction [27], cannot be hydrolyzed by digestive enzymes due to its compact molecular structure [27,28], and previous reports indicated that the presence of RS has a significant impact on the GI of starchy foods [29]. At T0, glucose release was low in control crackers. HF crackers showed a different kinetic of glucose release; indeed, glucose concentration was higher than in control at T0 and lower at further time points (Figure 1c).

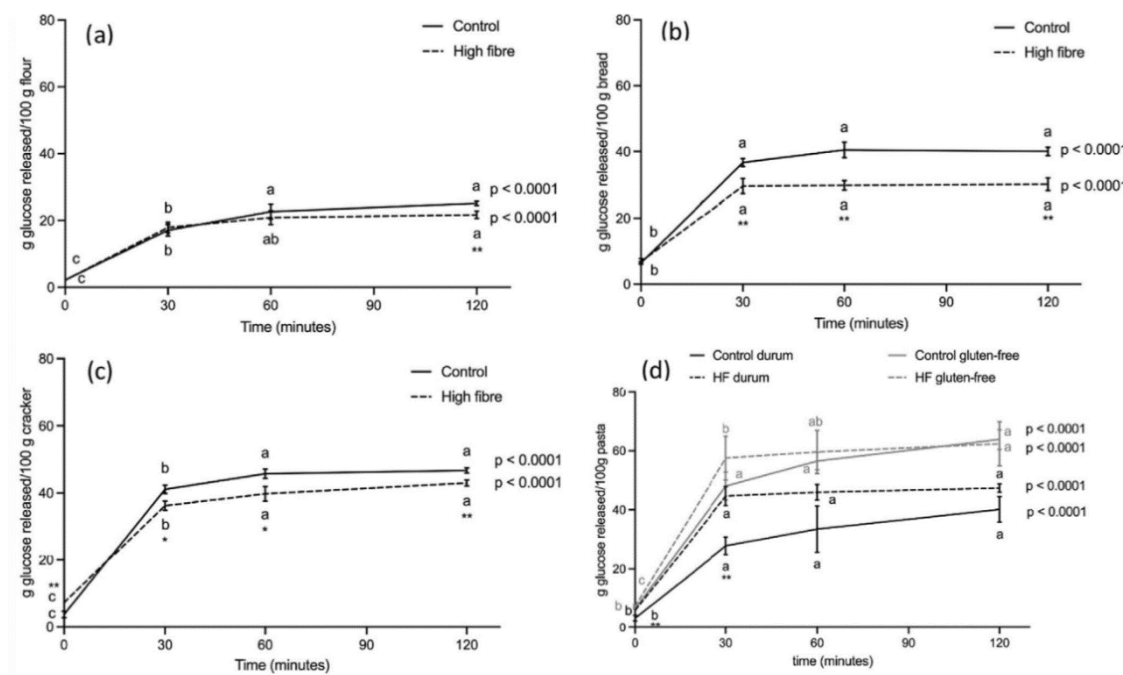


Figure 1. Glucose release after INFOGEST + AMG *in vitro* digestion of the starchy foods. (a) Flour, (b) bread, (c) crackers, and (d) pasta. Data are expressed as g released glucose/100g not digested food (raw, in the case of flour and pasta) and are means of three samples coming from different production batches. Statistical analysis was by the Student's t-test comparing the two different samples (control and high-fiber) at the same time point (*p<0.05; **p<0.01) and by the one-way ANOVA with Tukey's post-test comparing the same product at the different time points of AMG digestion (different letters indicate statistical significance, at least p < 0.05).

In durum wheat pasta, free glucose content was low at the beginning of AMG digestion (Figure 1d). Then, the time course of glucose release was different in the control and HF product. Indeed, it increased at a slower rate in control pasta than in HF pasta, where it plateaued early at T30. At T0, a low and similar release of glucose was also observed in gluten-free pasta (Figure 1d), and no significant differences were detected at any time of

AMG digestion between the control and HF gluten-free products. When comparing durum wheat and gluten-free pasta, glucose release was greater in the latter, and differences were more evident in control than in the HF variant (control: $p < 0.001$ at any time point; HF: T0 $p < 0.01$; T30 $p = 0.052$; T60 and T120 $p < 0.05$). The described protocol allowed us to compare different starchy foods by evaluating the release of free glucose after *in vitro* digestion. This should not be confused with the GI. In fact, the GI also takes into account the absorption of glucose by enterocytes, which partly release it into the bloodstream and partly convert it into other metabolites [30], and the modulation by insulin secretion. However, since much of the information in the literature comes from clinical studies evaluating the GI of starchy foods, which is defined as the incremental area under the blood glucose response curve 2h after ingestion of a food containing 50g of available carbohydrates, we renormalized glucose release at T120 on the quantity of foods containing 50g available carbohydrates to make our results somewhat comparable with those *in vivo* and to verify their reliability (Figure 2). Glucose release was confirmed to be very low in flour, where the starch was not gelatinized. Among other food products, the lowest glucose release was observed in control durum wheat pasta (28.29 ± 3.73 g/50 g of available carbohydrates). Durum wheat pasta has been reported to induce a lower postprandial glycemic response than bread and other wheat products in both healthy subjects and subjects with T2D, probably by virtue of its dense and compact physical structure (dry pasta) and the gluten network that surrounds its starch granules [31]. In gluten-free products, the absence of gluten affects starch digestibility and raises it, thus increasing the postprandial glycemic response [32]. Accordingly, we observed a higher glucose release in gluten-free pasta than in durum wheat pasta (39.91 ± 2.08 and 49.36 ± 5.96 g/50g of available carbohydrates in control and HF GF pasta, respectively). Of note, when glucose release was expressed as g/50g of available carbohydrate, no significant differences were observed between the control-HF pairs of flour, bread, and crackers, and in HF pasta (both durum and gluten-free) glucose release was higher than in the corresponding control. As it is reported that dietary fibers serve as a physical barrier in digestion, slowing down the interaction between enzymes and substrates [18], our results appear in contrast with the GI-lowering effect of fiber inclusion in foods. This apparent discrepancy may be explained by matrix-driven effects. In pasta products, starch digestibility is largely governed by the integrity of the protein–starch network. Fiber inclusion may interfere with this structure, either by weakening protein–starch interactions in durum wheat pasta or, in maize-based pasta, by the absence of a continuous gluten network. In both cases, reduced structural

constraints may increase enzyme accessibility to starch under static *in vitro* digestion conditions, leading to higher glucose release. The term “fiber” encompasses a wide range of different compounds, usually classified as insoluble and soluble (viscous) fibers. Although they are generically listed on food labels as “fiber,” their effect on GI is different [33]. We were not aware of the type of fiber present in the foods tested, other than the presence of RS in flour and bread. The positive effect of RS on glucose homeostasis has been confirmed by the EFSA [34] approved health claim “replacing digestible starch with RS induces a lower rise in blood glucose after a meal”. However, there are five types of RS, RS1–RS5, and all evidence for EFSA health claims is based on studies evaluating type 2 RS ingredients derived from high-amylose maize starch [35]. To our knowledge, the effects of other types of RS from different plant origins have not yet been evaluated. The type of fiber present in the formulation of the tested foods could explain the lack of any effect on glucose release and further underscore the imperative need to avoid generalizations and carefully consider the type of fiber when reformulating a food to have a lower glycemic impact. Furthermore, it is noteworthy that the mechanism of action of viscous fiber is related to its ability to reduce the rate of diffusion of nutrients from the lumen of the small intestine, thus reducing their absorption. This may have an impact on the GI of the food, but not on the *in vitro* assessment of glucose release.

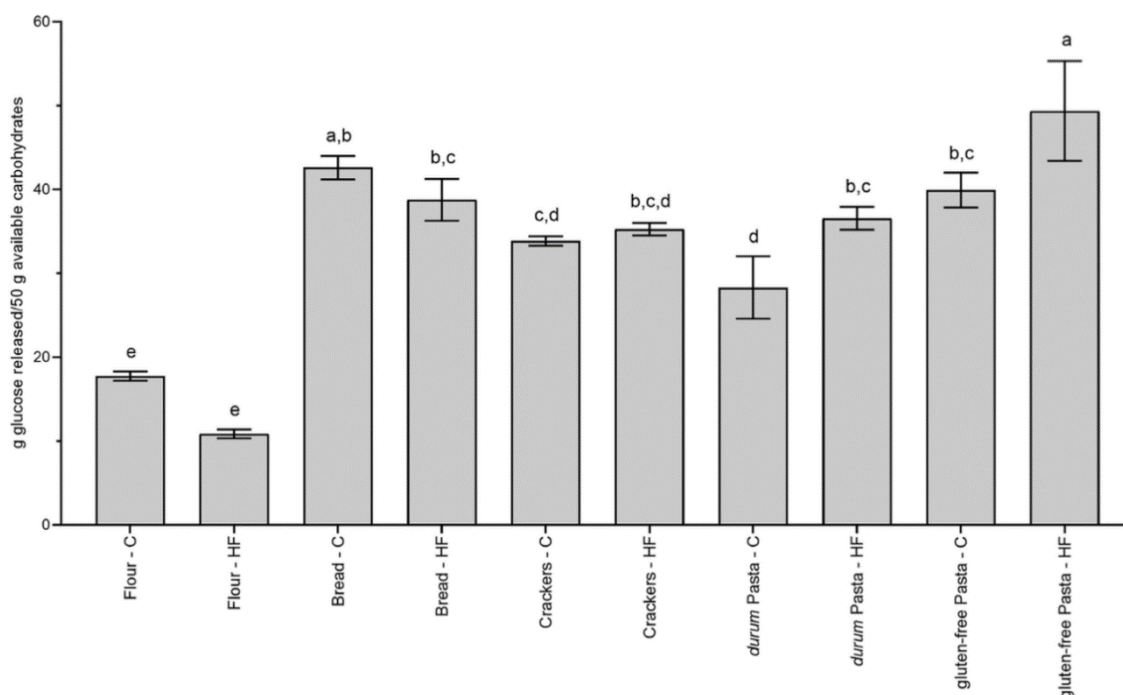


Figure 2. Glucose release after INFOGEST + 120min AMG *in vitro* digestion of the starchy foods. Data are expressed as g released glucose/ quantity of not digested food (raw, in the case of flour and pasta) containing 50g available carbohydrates, and are means of three samples coming from different production batches. Statistical analysis was by the one-way ANOVA with Tukey's post-test (different letters indicate statistical significance, at least $p < 0.05$)

In addition to these possible reasons for the poor effect of the presence of fiber, it should be considered that several studies have not highlighted any changes in postprandial glycemic and insulin responses after the elimination of cereal fiber from foods through refining [36]. In particular, *in vivo* data reported ambiguous results for spaghetti, with some studies showing the same GI regardless of fiber content [33]. It could be argued that the pasta format can modulate the GI and therefore this comparison is difficult, but it is worth noting that Pugnali et al. (2022) [37] highlighted a higher GI in whole meal than non-whole meal fusilli, the same format of pasta considered in this study. However, based on our results, it could be argued that the reduced glucose release observed in some HF products when the evaluation was done on 100g of food was mainly related to the replacement of available carbohydrates with fiber rather than a direct effect of fiber on starch hydrolysis. In fact, the effect disappeared when the results are normalized to 50g of available carbohydrates. Of course, the final effect on glucose release could also have been related to components other than fiber in the tested foods. In a review on the beneficial effects of pulse consumption on blood glucose and insulin levels, it was reported that the

positive effect of lentil consumption is probably due to their complex macronutrient content, and both protein and dietary fiber content were identified as potential factors in their lower glycemic response [38]. However, direct evidence for both remains inconclusive. In agreement, although in our work 3 out of 5 foods were isoproteic in the HF-control pair, in two of them (bread and crackers) the HF version showed a lower glucose release (expressed as g/100g of food), while pasta showed the opposite trend. When glucose release was normalized on 50g of available carbohydrates, no difference was detected between the HF-control pairs. The glucose release from foods can be affected by several factors, including the type and proportion of ingredients, processing technology, and cooking parameters, which act in concert [39]. Evaluating and explaining which of these characteristics have a greater influence on glucose release was not the aim of our work, but results obtained confirm that it cannot be predicted without a direct measurement.

Conclusions

The evaluation of glucose release from starch via the *in vitro* protocol described in this study was easy to perform, cost effective, and had no ethical issues. Using this new protocol, we were able to compare the extent and time course of starch hydrolysis in different commercial foods. Since sucrase and lactase enzymes were not included in the protocol, the foods considered in this study had no or very low sucrose/lactose content. While not equivalent to GI evaluation, this *in vitro* protocol can be useful for classifying starchy foods and, once further validated *in vivo*, it may provide information that will allow consumers to appropriately adapt their food choices. Additionally, since there are many factors that influence GI, including the type of starch, amount and type of fiber, non-starchy ingredients, particle size of the food, pH, and processing, etc., and the formulation of low GI products is challenging for the food industry, the *in vitro* protocol reported here can help in the preliminary steps of prototype development, proving advantageous in terms of time and costs.

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Food Iminosugars and Related Synthetic Derivatives Shift Metabolism and Induce Structural Changes in Colon Cancer Cell Lines

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Introduction

Iminosugars, also known as azasugars, are small organic molecules that mimic carbohydrates. These compounds can be found in nature or synthesized in monocyclic or bicyclic forms. The distinguishing feature of iminosugars compared to common sugars is the presence of nitrogen instead of oxygen in the ring structure. In nature, there are five main classes of iminosugars: indolizidines, piperidines, nortropanes, pyrrolizidines, and pyrrolidines. Synthetic iminosugars, on the other hand, encompass a wide variety of ring structures [1]. Iminosugars act as potent inhibitors of glycosidases, enzymes responsible for breaking glycosidic bonds in oligosaccharides and glycoconjugates. Additionally, they effectively inhibit glycosyltransferases, enzymes that catalyze the formation of glycosidic bonds from sugar donors with a leaving group in the anomeric position [2]. Iminosugars are found in various plants, aiding in their defense against destruction by animals or pathogens, and thus often end up in foods. Once ingested, iminosugars can perform several beneficial actions in the body. For example, the stevia plant contains iminosugars like steviamine, which inhibit glycosidases and show promising antimetastatic activity. Some cucumber varieties possess idoBR1, an iminosugar with potent anti-inflammatory activity. Another example is the Indian Ayurvedic medicinal plant *Gymnema sylvestre*, which may restore insulin responses and beta-cell health over time. Despite these examples, iminosugars have often been overlooked, and many are believed to be yet undiscovered in food plants. For instance, potatoes contain nortropan calistegins, potent inhibitors of glucosidase and galactosidase. Rice and buckwheat contain D-fagomine (FGM), which can inhibit a wide range of enzymes, although the presence of iminosugars in rice may depend on the variety [3]. Iminosugars from rice and potato are stable after cooking and are excreted in urine once ingested. Clinical studies have shown that FGM can control postprandial glycemic response and improve immune responses in the presence of opportunistic microorganisms [4]. FGM was first isolated as a natural product in 1974 from

Japanese buckwheat (*Fagopyrum esculentum Moench*) seeds, leading to the discovery of its inhibitory activities against α -glucosidase and β -galactosidase in mammals. As studies on FGM intensified, a regioisomeric analogue, Isofagomine (IFGM), was synthesized in 1994 and proved to be a potent glucosidase inhibitor. IFGM has been used in the treatment of Gaucher disease as an active-site-specific pharmacological chaperone (ASSPC) strategy [5]. One of the most well-known naturally occurring iminosugars is 1-deoxynojirimycin (DNJ), an analog of D-glucose with a nitrogen atom substituted for oxygen in the ring. DNJ is found in various parts of mulberry trees and is known for its antidiabetic, antiviral, and antitumor activities. It is the basis of the diabetes drug GlysetTM, also known as Miglitol (MGLT), due to its function as a potent inhibitor of various glucosidases. In the intestine, DNJ and MGLT inhibit digestive glucosidases such as maltase, isomaltase, and sucrase. Thus, compounds like DNJ can be used therapeutically in the oral treatment of non-insulin-dependent diabetes mellitus (type II) due to their inhibitory ability against β -glucosidases [3]. Besides mulberries, DNJ is produced by several *Bacillus* and *Streptomyces* strains, such as *Bacillus subtilis* MORI and *B. amyloliquefaciens*, isolated from traditional Korean fermented foods like Chungkookjang. *B. amyloliquefaciens* has also been isolated from traditional Korean fermented cabbage, Kimchi, and several other fermented foods. *B. atrophaeus* has been identified in fermented tofu, *B. subtilis* in the traditional Chinese fermented Okara, Meitauza, and *B. velezensis* in traditional Korean fermented soybean paste, Doenjang. DNJ inhibits glucosidases I and II, which are involved in N-linked glycosylation of secretory proteins [6,7]. N-(n-Nonyl)deoxynojirimycin (NN-DNJ) is a derivative of DNJ with a nonyl group attached to the nitrogen atom, increasing its hydrophobicity. This modification enhances its interaction with biological membranes, altering membrane permeability and structural integrity [8]. Computational analysis by Thirumal Kumar et al. (2019) suggested that NN-DNJ could act as a pharmacological chaperone to enhance β -glucocerebrosidase activity in patients with specific mutations, paving the way for potential applications in the treatment of Gaucher's disease [9]. Another DNJ derivative is 2,3,4,6-Tetra-O-benzyl-(+)-1-deoxynojirimycin (TBN-DNJ), widely employed as a protected intermediate for the synthesis of N-substituted DNJ derivatives, enabling selective functionalization at the nitrogen position [10]. So far, iminosugars have mainly been studied as therapeutics to improve metabolic health by acting on intestinal enzymes. However, another application in which glycosidases play an important role is cancer. Glycosidase activity is increased in tumor cells and may aid in tumor cell shedding, local invasion, and structural changes in cell surface glucoconjugates. These changes

increase attachment to endothelial cells and metastasis [11]. Besides their ability to modulate protein glycosylation, iminosugars may inhibit specific glycosidases involved in cell proliferation and interfere with the altered metabolic processes of cancer cells. While further research is needed to optimize their selectivity and reduce potential side effects, their role in regulating tumor-related biochemical pathways represents a growing area of interest in oncological research [12]. Yet, the effect of iminosugars on these pathways remains relatively unexplored. In this study, we aim to compare the potential of different food-related and synthetic iminosugars on intestinal cell processes that may be related to their anticancer effects (Figure 1). We will focus on naturally derived iminosugars, such as FGM and DNJ, and their synthetic derivatives, IFGM, MGTL, NN-DNJ, and TBN-DNJ.

Aim of study

As a case study, we investigated colon cancer, as the intestine is one of the first exposure sites of food-related compounds. Moreover, *in vivo* studies have shown that intestinal glycosidase activity is implicated in tumor progression in the colon, suggesting that their inhibition may represent a promising therapeutic strategy. This makes intestinal tissue a relevant model for exploring both the local enzymatic effects and potential anticancer mechanisms of iminosugars. *In vitro* experiments were performed under different nutritional conditions (glucose-rich and glucose-depleted medium), in two different cell lines representing different stages of colon cancer (Caco-2 and HCT-116 cells as early and later stages, respectively), and in both 2D monolayer and 3D spheroid morphology, the latter being a better reproduction of the tumor architecture and nutrient gradient [13]. Cell viability was assessed using standard microscopic and biochemical tools, bioenergetic parameters such as mitochondrial health and glycolytic capacity were further explored using the Seahorse Extracellular Flux (XF) Analyzer, and the effect on tissue organization was investigated by the staining of intracellular nutrient stores.

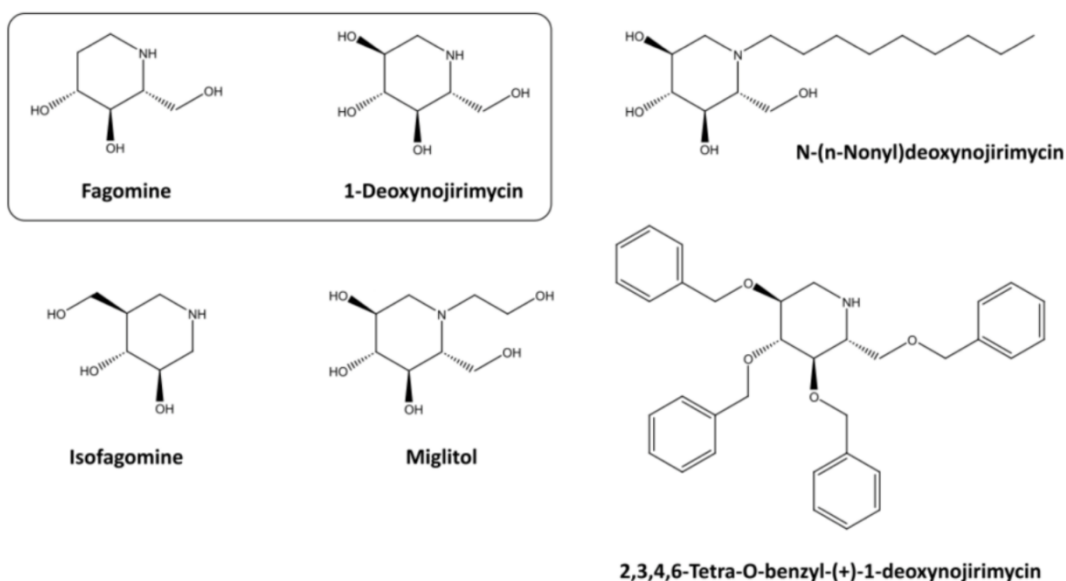


Figure 1. Differences in chemical structure between natural iminosugars (boxed) and their synthetic derivatives. Isofagomine is derived from Fagomine, while 1-Deoxynojirimycin serves as the core for the synthetic derivatives Miglitol (Glyset®), N-(nonyl)deoxynojirimycin, and 2,3,4,6-Tetra-O-benzyl-(+)-1-deoxynojirimycin.

Materials and methods

Chemicals

Iminosugars were purchased from the company Biosynth (Bratislava, Slovakia). Dulbecco's modified Eagle's medium (DMEM), DMEM without glucose, penicillin, streptomycin, and Dulbecco's phosphate-buffered saline (DPBS) were purchased from Gibco (Fisher Scientific, Merelbeke, Belgium). Trypan blue and Fetal Bovine Serum (FBS) were obtained from Thermo Fisher Scientific, whereas XF base medium was purchased from Agilent (Ghent, Belgium). Seahorse plates, Seahorse cartridges, Seahorse calibrant, and Mito Stress assay kits were all obtained from Agilent. Cell culture plates with 96 wells and spheroid plates were purchased from Greiner (Vilvoorde, Belgium) and S-BIO (Neuss, Germany).

Cell Culture and Exposure

Caco-2 and HCT-116 cells (ATCC, Molsheim, France) were cultured in DMEM with 4.5 g·L⁻¹ of glucose, supplemented with 10% heat-inactivated and sterile-filtered FBS, 1% non-essential amino acids, and 1% penicillin/streptomycin, at 37 °C and 10% CO₂. The

medium was refreshed every two or three days. Upon 80% confluency, cells were trypsinized, counted using trypan blue and a Bürker counting chamber, and seeded in plates. For the monolayer experiments, cells were seeded in 96-well plates at 2×10^4 cells per well and cultured in two different media: glucose-rich DMEM ($4.5 \text{ g} \cdot \text{L}^{-1}$ glucose and 10% FBS) or glucose-depleted DMEM (no glucose and 2% FBS). The HCT-116 cell line was used further in spheroid experiments (3D culture), where cells were seeded at 5×10^3 cells per well in a 384-well plate. Cells were treated with different iminosugar concentrations (25–50 μM) for 1 or 13 days.

Resazurin Assay

The activity of cellular dehydrogenases as a measure for mitochondrial respiration was quantified with the resazurin assay, where metabolically active cells reduce resazurin, a non-fluorescent blue dye, into resorufin, which is fluorescent and pink in color. The resazurin stock solution ($1 \text{ mg} \cdot \text{mL}^{-1}$ in distilled water) was diluted 1:100 v/v in DMEM (without FBS), resulting in a final concentration of $10 \mu\text{g} \cdot \text{mL}^{-1}$. The plate was incubated at 37°C for 2 h, after which fluorescence ($\lambda_{\text{exc}}/\lambda_{\text{em}} = 560/590 \text{ nm}$) was measured in a 96-well black plate.

Sulforhodamine B Assay

The sulforhodamine B (SRB) assay was used as an indirect indicator of cell proliferation. The amount of SRB bound to the cells, measured spectrophotometrically, is proportional to the number of cells present in the sample. After the resazurin assay, cells were fixed by adding 50% TCA solution (1:4 v/v) to the medium and incubated at 4°C for at least one hour. Next, cells were washed 5 times with tap water, and 50 μL of SRB solution was added to each well. After 30 min of incubation, the plates were washed 5 times with 1% acetic acid solution. The protein-bound SRB staining was solubilized by adding 200 μL of a 10 mM Tris solution, and the absorbance was measured at 490 nm.

IncuCyte Live Cell Imaging

IncuCyte® is an advanced live-cell imaging system for acquiring and analyzing images directly inside an incubator, thereby providing real-time information on cell growth, metabolism, and other cellular dynamics to ensure optimal conditions for experiments even

over extended times. Plates containing iminosugar-treated cells were placed in a 37 °C incubator, and cell growth was monitored over time. For Caco-2 and HCT-116 cells seeded in 96-well plates (2D culture), 4 images per well were acquired every 4 h. In the case of the spheroid experiment in 384-well plates (3D culture), one image per well was acquired every 12 h.

Seahorse Extracellular Flux Assays

The Agilent Seahorse XFe96 Extracellular Flux Analyzer was used in combination with the Agilent Seahorse XF Cell Mito Stress Test kit to assess mitochondrial function by targeting specific points in the electron transport chain (ETC). Oligomycin (OM), a complex V inhibitor, reduces electron flow through the ETC by blocking ATP synthase, leading to a decreased oxygen consumption rate and a corresponding decline in mitochondrial respiration, and hence mitochondrial ATP production. Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), a protonophore, disrupts the mitochondrial membrane potential by collapsing the proton gradient, thereby allowing unrestricted electron flow through the ETC, resulting in maximal oxygen consumption by complex IV. The oxygen consumption rate stimulated by FCCP is used to calculate spare respiratory capacity, the difference between maximal and basal respiration, providing insights into mitochondrial efficiency and ATP synthesis capacity [14]. By measuring the oxygen consumption rate and extracellular acidification rate, basal respiration, maximal respiration, ATP production, proton leakage, non-mitochondrial respiration, and respiratory spare capacity were calculated according to the manufacturers' protocols. Practically, the day before the experiment, cartridges were hydrated in calibration solution, placed in a sealed plastic bag containing moist paper to prevent dehydration, and incubated at 37 °C for 24 h. On the following day, the 96-well Agilent Seahorse spheroid plates were prepared by adding 30 µL of poly-D-lysine to each well and incubated for 20 min. Each well was then washed twice with 200 µL of water. Next, the wells were rinsed with 200 µL of XF DMEM Seahorse supplemented with pyruvate, glucose, and glutamine in a 100:1:1:1 ratio and subsequently filled with 175 µL of the same medium pre-warmed to 37 °C. Finally, spheroids were individually collected using a pipette and transferred from the 384-well plate to the 96-well Agilent Seahorse plates. Subsequently, 22 µL of OM (1 µM final concentration) was added to injection port A of the cartridge, 22 µL of FCCP (0.5 µM final concentration) to port B, and 25 µL of Rotenone/Antimycin A (R/AM, 0.5 µM final

concentration) to port C. The substrate oxidation test experiment was set up similarly, but 20 μ L of etomoxir (Eto, 4 μ M final concentration) was added to port A of the cartridge. The other ports were loaded as described for the Mito Stress kit, and the analysis was run likewise.

AdipoRed Staining

After 1 and 13 days of incubation and treatment, DMEM was removed, and each well was washed with DPBS (+Ca/Mg). Subsequently, paraformaldehyde was added for cell fixation, and the plates were stored at 4 °C. Next, intracellular lipids were stained with AdipoRed 2.5% solution, diluted in DPBS at a 1:40 ratio for 30 min. Finally, the IncuCyte was used to create phase-contrast and red fluorescence images and to calculate the red fluorescence intensity per surface covered with cells.

Periodic Acid–Schiff (PAS) Staining

Similarly, cells fixed in paraformaldehyde were washed with distilled water and treated with periodic acid (0.5%) for 5 min. Next, the cells were washed three times with distilled water, and Schiff's reagent was added for 15 min. After incubation, the remaining Schiff's reagent was washed away with water, and the cells were imaged using a Zeiss Primovert phase-contrast microscope with an Axiocam color camera.

Statistics

Statistical analysis of the resazurin and SRB cytotoxicity assays, as well as the AdipoRed assay and maximal respiration after Eto injection, was performed using SPSS Statistics 29 software. A two-tailed Student's t-test was applied to compare treated samples with untreated controls. Comparisons between the untreated control and the different iminosugar treatments for each mitochondrial parameter (basal respiration, maximal respiration, ATP production, proton leakage, non-mitochondrial respiration, and spare respiratory capacity) were performed using one-way analysis of variance (ANOVA), followed by Tukey's HSD post hoc test to identify statistically significant differences ($p < 0.05$). The extracellular acidification rate parameter was also statistically analyzed using one-way ANOVA followed by Tukey's HSD post hoc test. These analyses were carried out using GraphPad

Prism version 8. For iminosugar-treated samples, the number of replicates ranged from n = 4 to n = 5; for untreated controls, from n = 7 to n = 31.

Results

Iminosugars Affect Cell Viability of Intestinal Cell Lines

In the first step, cell viability after exposure to iminosugars was investigated in the Caco-2 and HCT-116 cell lines, representing the early and late stages of colon cancer, according to the ATCC product files. Table 1 shows the viability of cell monolayers exposed to iminosugars for the short term (24 h) in either a glucose-rich or glucose-depleted medium

Table 1. The effect of natural iminosugars and synthetic derivatives on the growth and metabolism of Caco-2 and HCT-116 cell lines after 24 h of incubation at a concentration of 50 μ M in DMEM with and without glucose. Cellular parameters were assessed using the resazurin metabolic activity assay (Res.), the protein content assay (SRB), and cell confluency (IncuCyte). Results are expressed as mean $\pm$ standard deviation (SD), with statistical significance indicated by an asterisk (*) relative to untreated controls ($p < 0.05$). Statistical significance was determined using a two-tailed t-test between treated samples and untreated controls.

Iminosugar	Glucose +/-	Caco-2			HCT-116		
		Mitochondrial Activity	Protein Content	Confluence	Mitochondrial Activity	Protein Content	Confluence
DNJ	+	103.8 $\pm$ 15.2	59.1 $\pm$ 14.2 *	89.9 $\pm$ 6.6	114.7 $\pm$ 17.1	86.8 $\pm$ 14.5	44.4 $\pm$ 6.5 *
	-	87.9 $\pm$ 5.9 *	52.3 $\pm$ 7.2 *	58.7 $\pm$ 5.8 *	74.8 $\pm$ 17.3 *	79.4 $\pm$ 17.1 *	88.8 $\pm$ 3.9
NN-DNJ	+	91 $\pm$ 14.9	65.3 $\pm$ 11.8 *	65.2 $\pm$ 11 *	89.3 $\pm$ 21.6	72.9 $\pm$ 15.9 *	58.9 $\pm$ 4.5 *
	-	86.4 $\pm$ 11.5 *	54.6 $\pm$ 6.1 *	50.8 $\pm$ 12.3 *	83.9 $\pm$ 15.5	87.3 $\pm$ 7.4	78.2 $\pm$ 10.7
TBN-DNJ	+	82.9 $\pm$ 7.9	68.28 $\pm$ 16.1 *	56.5 $\pm$ 12.6 *	66.5 $\pm$ 21 *	61.56 $\pm$ 8.8 *	54 $\pm$ 9.5 *
	-	22.1 $\pm$ 5.4 *	40.65 $\pm$ 15.2 *	36.2 $\pm$ 5.1 *	49.9 $\pm$ 3.7 *	65.21 $\pm$ 3.7 *	56 $\pm$ 9.8 *
MGTL	+	87.4 $\pm$ 7.5 *	109.15 $\pm$ 21.3	79.6 $\pm$ 17.6	77.9 $\pm$ 14.2	107.3 $\pm$ 18	93.6 $\pm$ 18.7
	-	93 $\pm$ 4.4 *	67.9 $\pm$ 9.6 *	72.4 $\pm$ 14.7 *	74.8 $\pm$ 10.1 *	74.71 $\pm$ 13.2 *	88.6 $\pm$ 13.4
FGM	+	126.2 $\pm$ 24.3 *	79.7 $\pm$ 21.5	100.2 $\pm$ 9.7	104.3 $\pm$ 15.1	99.4 $\pm$ 6.7	91.1 $\pm$ 9.4
	-	126.8 $\pm$ 27	72.6 $\pm$ 17.3 *	82.1 $\pm$ 14.2 *	92.8 $\pm$ 10.7	97.3 $\pm$ 21.4	121.6 $\pm$ 15.2
IFGM	+	94.4 $\pm$ 11.6	89.37 $\pm$ 16.5	80.3 $\pm$ 9.9 *	85.3 $\pm$ 13.9	93.5 $\pm$ 9.1	92.4 $\pm$ 10.4
	-	110.6 $\pm$ 13.4	71.26 $\pm$ 19	69.1 $\pm$ 10.4 *	76.2 $\pm$ 9.9 *	80 $\pm$ 21.5	70.7 $\pm$ 11.5 *

Generally, treatment of cells with iminosugars resulted in a lower mitochondrial activity (resazurin), protein content (SRB), and confluency compared to untreated cells. The effects varied depending on the type of iminosugar, cell type, and growth conditions (presence or absence of glucose). Particularly in the Caco-2 cell line, treatment with DNJ derivatives in the absence of glucose generally showed lower values compared to treatments with glucose, indicating a possible metabolic dependence on glucose for maintaining cell

viability and proliferation. Notably, in a comparison of the natural iminosugars, FGM appeared less impactful than DNJ. Among the synthetic derivatives, TBN- DNJ and NN-DNJ showed a more negative impact on cell confluence, suggesting potential cytotoxic or inhibitory effects on proliferation. In the next step, the effect of iminosugar treatment on a 3D spheroid model, which better represents tumor architecture, was investigated (Figure 2). HCT-116 cells were used because Caco-2 cells are unable to form such 3D structures, and an exposure time of 13 days was applied to allow sufficient tumor growth and simulate longer-term treatment. Growth curves monitored via the IncuCyte live cell imager showed that NN-DNJ, MGTL, TBN-DNJ, and FGM were more effective in decreasing spheroid diameter compared to 1-DNJ and IFGM.

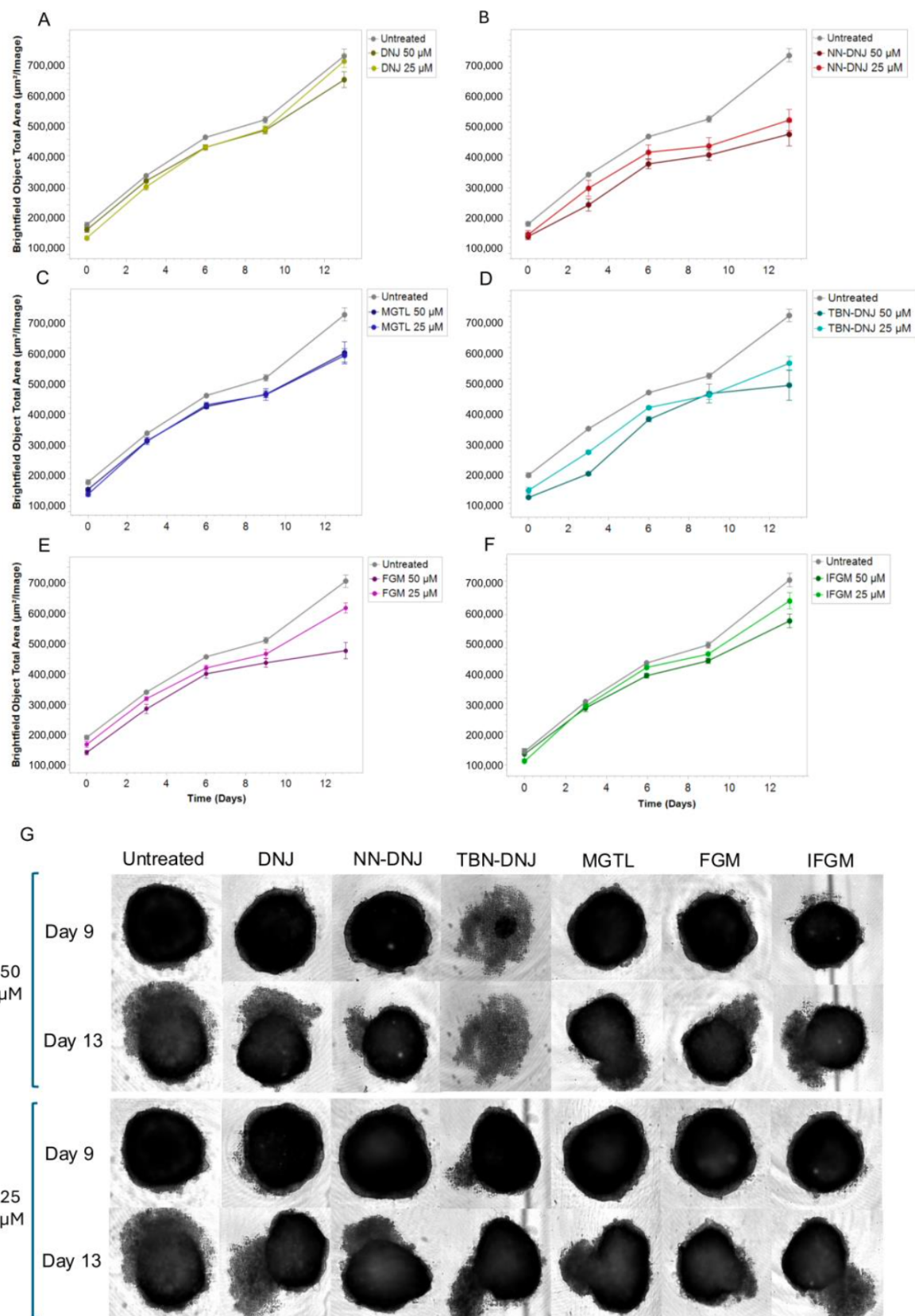


Figure 2. Effects of treating HCT-116 cells with iminosugars on spheroid growth over time. (A–F) The graphs represent growth curves of cells when treated with the iminosugars (A) DNJ; (B) NN-DNJ; (C) TBN-DNJ; (D) MGTL; (E) FGM; and (F) IFGM; or untreated, over a time frame of 0 to 13 days, obtained using the IncuCyte live-cell imaging system, which captures phase-contrast images over time. The images depict spheroids on days 9 and 13 after treatment with either 50 μ M or 25 μ M iminosugar (G). The images illustrate the evolution over time of the morphology and cellular density of the spheroids.

Iminosugars Affect Energy Metabolism in HCT-116-Derived Spheroids

To understand the effect of iminosugars on tumor growth, the energy metabolism was investigated closely as iminosugars are specific inhibitors of glucosidase activity, potentially impacting glucose and other energetic substrate mobilization within cells. To this end, spheroids were transferred to a 96-well spheroid plate, and shifts in energy metabolism were monitored using the XFe96 Efflux Analyzer. Figure 3 shows that basal respiration, reflected by the oxygen consumption rate (OCR) before the addition of mitochondrial stressors, was maintained upon treatment with most iminosugars, except TBN-DNJ at 50 μ M. Despite maintained basal respiration, a higher glycolytic rate, indicated by a higher extracellular acidification rate (ECAR), was observed, suggesting a shift to a less efficient energy metabolism pathway to meet energy demands. Additionally, for all tested conditions, maximal respiratory capacity, visible after FCCP injection, was lower than that in the untreated condition, indicating an impact on mitochondrial fitness. Interestingly, the maximal respiration of cells treated with 25 μ M FGM was higher than that of all other treatments.

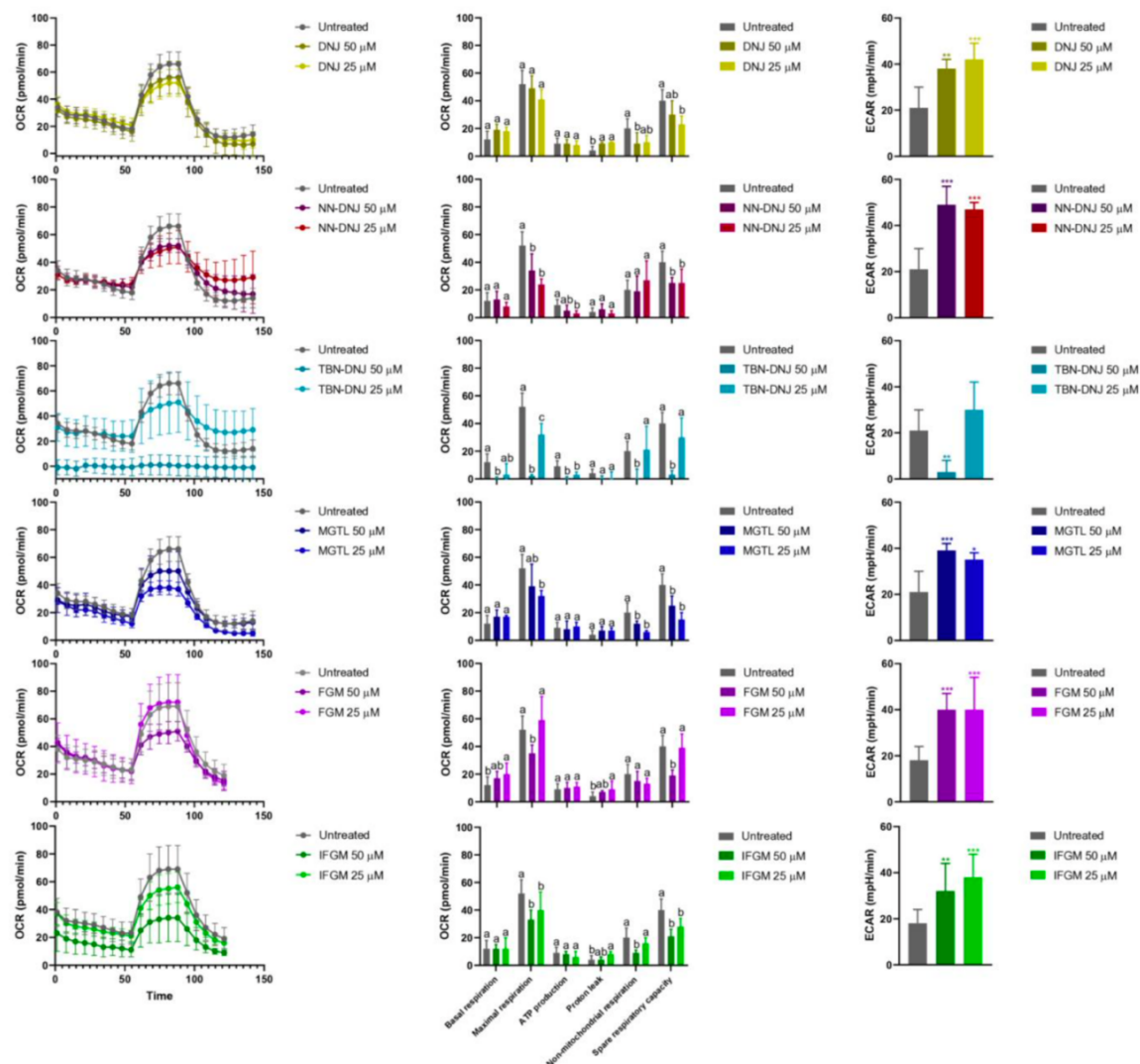


Figure 3. The effect on energy production, as determined by the XFe96 Efflux Analyzer (Agilent Technologies, Diegem, Belgium), is given for the treatment of cells with 50 μ M or 25 μ M iminosugar. Left panels show OCR as a function of time and upon different stressor injections. The middle panels show various mitochondrial parameters. Basal respiration refers to mitochondrial activity at rest. Maximal respiration after the injection of FCCP, an uncoupling agent that forces the electron transport chain to work at its full capacity, indicates mitochondrial reserve capacity. Mitochondrial ATP production is the difference between basal respiration and the OCR after OM inhibition of the ATP synthase. Proton leakage is the residual OCR after OM addition, and it reflects oxygen consumption not linked to ATP production but rather due to proton leakage across the mitochondrial membrane. Non-mitochondrial respiration is the OCR after the complete inhibition of the electron transport chain by R/AM and reflects oxygen consumption by non-mitochondrial sources (e.g., ox- idases or peroxisomes). Spare respiratory capacity is the difference between maximal and basal respiration, representing the highest possible reserve capacity upon elevated energy demand. A low value may indicate that cells are already operating near their respiratory limit, making them more vulnerable to stress. On the right, ECAR in spheroids after 13 days of

treatment is depicted. Data are presented as mean $\pm$ standard deviation. Mean values marked with different letters (i.e., a, b, c) for each mitochondrial parameter indicate significant differences ($p < 0.05$) among treatments, as determined by one-way ANOVA followed by Tukey's HSD post hoc test. Statistical difference is marked by ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$ compared with control by ANOVA followed by Tukey's HSD post hoc test.

As shown in Figure 3, bioenergetic analysis of spheroids treated with DNJ, FGM, and their synthetic derivatives for 13 days showed a decrease in the oxygen consumption rate, most pronounced at 50 μ M iminosugar. Mitochondrial parameters revealed that basal respiration was generally similar between treated and untreated spheroids. However, a decrease in both maximal respiration and spare respiratory capacity, defined as the mitochondria's ability to meet increased energy demands, was observed. Significantly decreased non-mitochondrial respiration for various treatments (except FGM and NN-DNJ), particularly at 50 μ M, may point to a decreased overall viability. In general, no substantial differences were observed for mitochondrial ATP production or proton leakage. Interestingly, the extracellular acidification rate was significantly increased for all iminosugar treatments, indicating a shift to a less efficient compensatory mechanism to meet energy demands. As an exception, TBN-DNJ significantly decreased the extracellular acidification rate at 50 μ M, likely due to a higher cytotoxicity compared to other compounds. Overall, strong shifts in energy metabolism occurred, with many natural and synthetic iminosugars inducing similar effects in terms of maximal respiration and glycolysis, leading to increased lactate production, a key feature of aerobic glycolysis often observed in tumor cells in the presence of oxygen.

Iminosugars Induce Shifts in Substrate Use for Energy Metabolism

To further investigate whether these shifts in metabolism could result from differences in nutrient use for respiration, the major energy stores of the cells, glycogen and lipids, were stained microscopically. Additionally, using Eto as a carnitine palmitoyltransferase-1 inhibitor to inhibit fatty acid transport into the mitochondria, we aimed to further distinguish between the modes of action of natural and synthetic iminosugars. Overall, quantification of the AdipoRed stain as a proxy for intracellular lipid content showed increased values over time (Figure 4A). While no significant differences were observed in the early stages, marked variations emerged by day 13. In Caco-2 cells, a significant decrease in intracellular lipid content was observed upon long-term treatment with NN-DNJ, TBN-DNJ, and FGM compared to the untreated control. Interestingly, such a decrease

was not visible in long-term-treated HCT-116 cells. Figure 4B shows that maximal mitochondrial respiration in HCT-116 spheroids (13 days) was affected by iminosugar treatment and Eto injection. Spheroids treated with MGTL, TBN-DNJ, and IFGM showed increased dependence on lipids for mitochondrial respiration. Microscopic pictures of PAS staining for glycogen in Caco-2 cells treated for 13 days (Figure 4C) showed a strong reduction in glycogen content in cells treated with iminosugars, particularly FGM. This suggests that certain iminosugars influence lipid metabolism and cellular glycogen storage, likely due to their inhibitory effect on α -glycosidases. Additionally, glycogen distribution in different zones was diverse in untreated conditions, whereas iminosugar treatment resulted in either a more diffuse and equally distributed phenotype (NN-DNJ) or more pronounced site-specific localization (IFGM). These effects were less pronounced in HCT-116-treated monolayers (Figure 4C). Similarly, intracellular lipid concentration followed the same distribution pattern in cell monolayers, indicating a role of iminosugars in cell differentiation and epithelial organization.

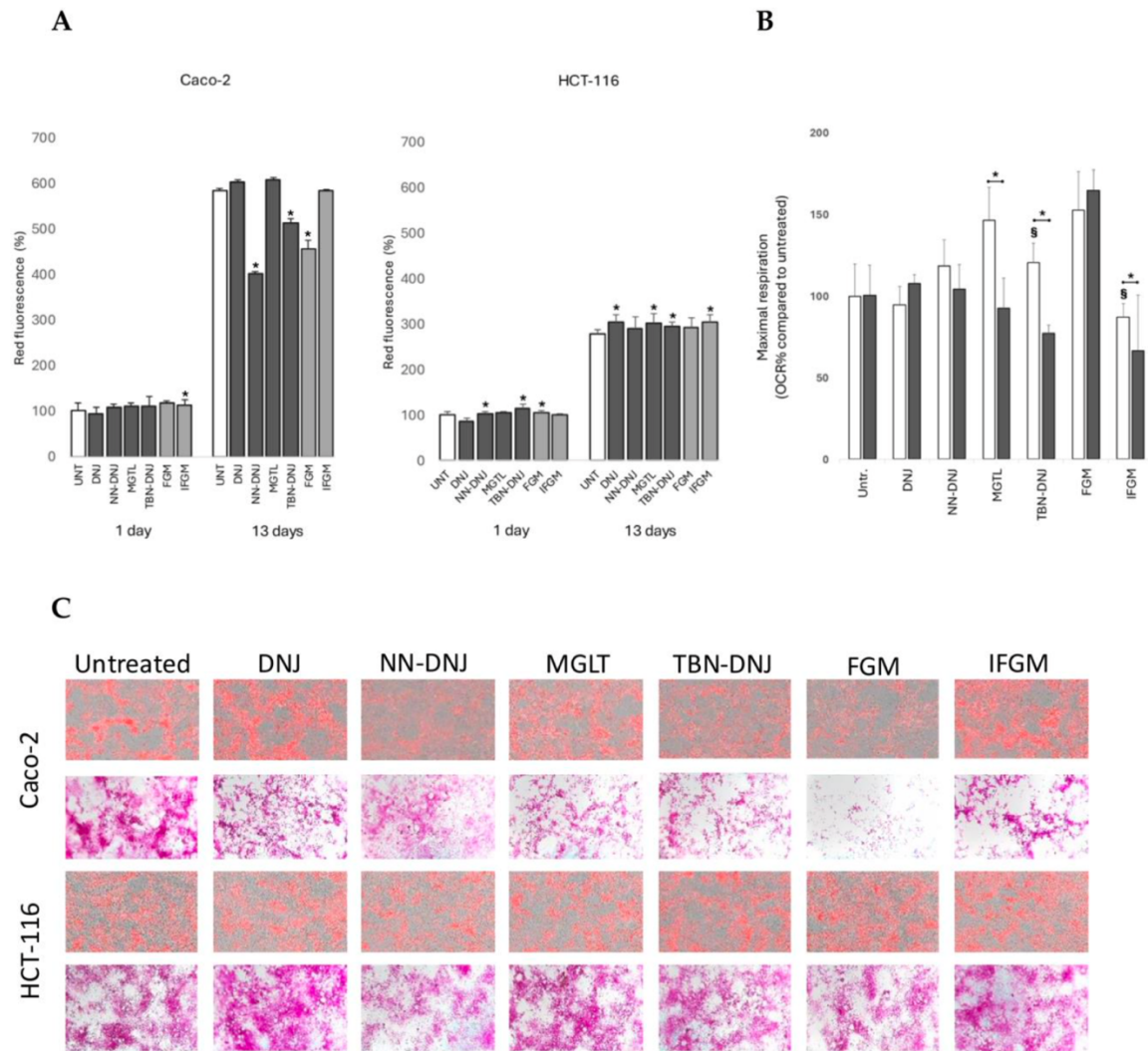


Figure 4. Impact of iminosugars on nutrient use in colon cancer cells. (A) Intracellular lipid content (AU) upon AdipoRed staining of Caco-2 cells. *: indicates significant difference compared to the untreated control ($p < 0.05$). (B) Maximal respiration of HCT-116 spheroids treated for 13 days with iminosugars after injection of the fatty acid transport inhibitor Eto. *: significantly different after Eto injection of the same iminosugar treatment, §: from the untreated condition ($p < 0.05$). (C) Microscopic pictures of Caco-2 and HCT-116 cells treated with iminosugars for 13 days after AdipoRed staining for lipids (upper) and PAS staining for glycogen (lower).

Discussion

This study presents, to our knowledge, the first attempt to investigate the effects of natural iminosugars and synthetic derivatives on two intestinal cell lines by integrating innovative techniques such as three-dimensional culture techniques and bioenergetic phenotyping. Although multiple studies have demonstrated the impact of drug-like iminosugars, such as

MGLT and FGM, on human metabolism, almost no studies compare the effectiveness and compound-specific effects in a single screening. Our results demonstrate that both natural and synthetic iminosugars (i) decrease cell growth depending on the iminosugar, the presence of glucose in the medium, and the cell type and architecture, (ii) decrease maximal respiration and increase glycolysis, indicating a less efficient energy production machinery, (iii) alter substrate preferences to sustain mitochondrial respiration, and (iv) substantially alter lipid and glycogen deposits in intestinal monolayers depending on the cell type and iminosugar.

Iminosugars Decrease Cell Growth Depending on Iminosugar Structure, Presence of Glucose in the Medium, and Cell Type and Architecture

Our findings indicate that iminosugars significantly impact cellular bioenergetics, particularly affecting the cells' ability to cope with metabolic stress and increased energy demands. The strength of this effect varied depending on the type and concentration of the iminosugar, with the most pronounced impairments observed for synthetic derivatives at 50 μ M. Overall, cell growth was inhibited by iminosugars, and this effect was enhanced under glucose-deprived conditions compared to glucose-rich conditions. This aspect is likely attributable to one of the main characteristics of iminosugars, namely their ability to inhibit key enzymes regulating the availability of energy substrates. As iminosugars act as glycosidase inhibitors, the mobilization of glycogen as a backup nutrient in case of glucose depletion is hampered. This feature may be beneficial for targeting cancer cells located at the inner core of the tumor, where nutrient and oxygen depletion is more prevalent than in the outer layer. Hampering glycolysis in cancer cells can also contribute to combating drug resistance by reducing the available energy for multidrug efflux pumps [15]. Additionally, the inhibition of α -glucosidase and mannosidase can lead to a reduction in glycolysis and protein synthesis, as well as increased endoplasmic reticulum stress [16], which may lead to cytotoxic effects. Furthermore, as glycosidases are important enzymes for tumor cell surface glycoconjugate synthesis, their inhibition may alter the 3D architecture of the tumor spheroid, making the cells more loosely connected to each other, as observed with TBN-DNJ. This hypothesis should be confirmed by determining specific cell surface glycoconjugates using more complex analytical techniques such as chromatography (lectin) coupled with mass spectrometry. In the presence of glucose, natural compounds such as FGM showed a positive effect on mitochondrial activity, as reflected by higher

resazurin conversion compared to the control in both Caco-2 and HCT-116 monolayers in the short term, as well as by higher maximal respiration in HCT-116 spheroids in the long term. This effect may suggest a protective and stimulatory capacity for cell metabolism under conditions of sufficient energy availability. However, more pronounced and inhibitory effects on cell viability were observed with synthetic derivatives compared to natural iminosugars, especially at higher concentrations. For instance, the higher effects with TBN-DNJ and NN-DNJ suggest that chemical modifications, i.e., the hydrophobic side chains introduced in synthetic derivatives, may improve the uptake of iminosugars by passive diffusion through cell membranes [17,18]. In addition, the aromatic rings in the structure of TBN-DNJ introduce extra features, possibly improving the iminosugar's affinity for enzymatic targets, enhancing uptake through cellular receptors, or increasing the metabolic stability of the compound.

Iminosugars Decrease Maximal Respiratory Capacity and Increase Glycolysis

This research is, to our knowledge, the first report investigating the effect of iminosugars on cellular bioenergetics. Our initial observation was that treatment with different iminosugars led to decreased basal respiration compared to untreated cells. This decrease was particularly visible at higher concentrations (50 μ M), suggesting that these compounds may interfere with mitochondrial respiratory capacity. Interestingly, natural DNJ and FGM had a less pronounced effect compared to their synthetic derivatives, indicating that structural modifications in the synthetic derivatives may be more effective in targeting energy metabolism or may have better stability and cellular uptake. Similarly, except for DNJ, all iminosugars were able to reduce the spare mitochondrial capacity after FCCP stress. This suggests that either fewer mitochondria were present or that shifts in mitochondrial enzymes and/or structure were induced by the iminosugars. Indeed, the literature reports that iminosugars can inhibit glucocerebrosidase, which may affect lipid metabolism within the mitochondria. This effect was demonstrated for DNJ analogs such as N-butyl-1-deoxynojirimycin (N-Butyl-DNJ), which is investigated for treating Gaucher's disease, and N-dodecyl-1-deoxynojirimycin (N-dodecyl-DNJ) [19,20]. Our results also demonstrated that this decrease in mitochondrial capacity was paralleled by an increase in glycolysis and lactate production for all tested iminosugars, suggesting that cells may become more dependent on alternative energy pathways to sustain their survival and to compensate for their energy deficit apart from oxidative phosphorylation. Whether this

is a beneficial effect is still debatable. On one hand, stimulation of this inefficient pathway for ATP production may limit the outgrowth of cancer cells and hence open opportunities for iminosugars to be used in cancer treatment. On the other hand, the shift towards glycolysis, also called the Warburg effect, is a typical hallmark for cancer progression. This metabolic state provides greater plasticity, allowing cancer cells to adapt to different microenvironments [21]. More specifically, the Warburg effect offers tumor cells higher flexibility in nutrient utilization, enabling them to metabolize various energy substrates to meet the increasing bioenergetic and biosynthetic demands of tumor development across diverse microenvironments. Additionally, intermediate metabolites of glycolysis, such as lactate and pyruvate, can be recycled for the synthesis of essential cellular biomass, thus promoting cell proliferation. Furthermore, the shift towards glycolysis may limit the overproduction of reactive oxygen species (ROS), which may trigger adaptive stress responses that support survival, tumor progression, and mutagenic events promoting tumorigenesis and metastasis, while avoiding the cytotoxic effects associated with excessive ROS levels. Therefore, the hybrid metabolic phenotype has been specifically associated with metastatic potential, supporting the ability of cancer cells to adapt to and colonize distant environments [22]. As we are the first to report this shift towards glycolysis, it is not yet clear whether this effect is beneficial for the cell, although it is now known that lactate is used continuously as fuel by various cells under conditions of complete aerobiosis. Under low-glucose conditions, cancer cells take up and oxidize lactate [23]. Enhanced glycolysis could be the result of endoplasmic stress, as various glycosidases are crucial in protein glycosylation and quality control, or it can be a sign pointing towards autophagy [24,25]. It is likely that the higher glycolytic rate is not really meant to compensate for increased energy demands, but may simply be a compensatory side effect of the cells maintaining the production rate of essential glycoconjugates due to the inhibition of glycosidase activity [26,27]. To support this hypothesis, it would be relevant to examine potential changes in nutrient transporters and mitochondrial and related enzymes, as well as in the glycoconjugate profile of the cells. Overall, we may conclude that the actual effect of iminosugars on the induction of a glycolytic shift has not yet been clearly demonstrated. To date, no studies in the literature have directly shown an association between these compounds and the modulation of glycolytic metabolism. However, since iminosugars inhibit glycosidases, thereby altering the glycosylation profile of numerous proteins, including those involved in the regulation of cellular metabolism, it is plausible

to hypothesize an indirect effect on metabolic pathways, which deserves further experimental investigation.

Iminosugars Alter Lipid and Glycogen Deposits in Intestinal Monolayers and Affect Tissue Organization

Our results clearly show that long-term iminosugar treatment impacts nutrient deposits in cells, depending on the iminosugar structure and cell line. The most pronounced differences were observed in differentiating Caco-2 cells. Microscopic staining of intracellular lipids revealed that cells treated with NN-DNJ, TBN-DNJ, and FGM had significantly lower intracellular lipid content compared to control cells. This suggests that, in response to iminosugars, the cells may exhibit decreased lipogenesis, increased fatty acid consumption as a secondary energy source, and/or effects on other pathways such as the inhibition of glucocerebrosidases. To test whether intracellular fatty acids were used to compensate for energy deficits, etomoxir was added as an inhibitor of fatty acid transport into mitochondria. A significant decrease in maximal respiration was confirmed for the slightly cytotoxic TBN-DNJ but not for NN-DNJ and FGM. Additionally, MGLT and IFGM showed higher dependency on fatty acid oxidation for energy metabolism. We therefore concluded that increased fatty acid oxidation is likely not the primary pathway explaining the lower lipid deposits in cells caused by NN-DNJ and FGM. In the literature, DNJ consumption has been associated with decreased triglyceride content in the liver and increased fatty acid β -oxidation [28]. However, in our intestinal cell models, only synthetic DNJ derivatives showed a similar effect. Whether this is due to the stability of DNJ or differences in DNJ uptake or function in other tissue types remains unclear and warrants further study. DNJ has also been reported to inhibit acetyl- CoA carboxylase and fatty acid synthase, which may directly decrease lipogenesis. Furthermore, in fat tissue, adiponectin and AMPK were upregulated. Together with a decrease in lipogenesis, this may have beneficial effects on atherosclerosis and fatty liver disease. Whether such effects also occur in intestinal cells needs to be established. In the literature, DNJ and FGM analogs, such as N-butyl-DNJ and N-dodecyl-DNJ, are reported to inhibit glucocerebrosidase, thereby affecting (sphingo)lipid metabolism in Gaucher disease. The accumulation of substrates such as glucosylceramide, which impairs mitochondrial function and autophagy and induces the hyperactivation of the mTORC1 pathway in neuronal models, suggests a broader impact on cellular homeostasis beyond lipid storage [29]. It can therefore be

suggested that NN-DNJ, TBN-DNJ, and FGM have a similar mode of action in differentiating cells. Sphingolipids are constituents of the cell membrane, modulators of cellular trafficking, and precursors for mediators and messenger molecules. They play an important role in gut homeostasis, intestinal barrier integrity, immune responses, and composition of the gut microbiome [30]. Additionally, sphingolipids are important mediators of cancer cell growth, migration, transformation, and drug resistance [31,32]. In particular, ceramide, the end product of glucocerebrosidase, has a beneficial effect against cancer by inhibiting many of these processes. Therefore, sphingolipid dynamics should be studied before considering the use of iminosugars to treat intestinal diseases and cancer. Furthermore, staining of intracellular glycogen indicated that iminosugars influence not only lipid but also glycogen metabolism. Glycogen is an intracellular polymer consisting of α -(1,4) and α -(1,6) bound glucose units. Its synthesis requires glucose transport inside the cell and multiple phosphorylation and isomerization reactions to generate UDP- glucose as a donor. On the other hand, glycogen is mobilized by an interplay of glycogen phosphorylases, glycogen detangling enzyme and lysosomal degradation by α -glycosidases, and the glucose-6-phosphatase system [33]. In Caco-2 cells treated for 13 days, a visible reduction in glycogen accumulation was observed in cells treated with all tested iminosugars, especially FGM. The literature reports that FGM can reduce postprandial blood glucose levels upon intake of sucrose and starch by inhibiting disaccharidase activity [34]. Likewise, the antihyperglycemic action of MGLT is caused by a reversible inhibition of membrane-bound intestinal α -glucoside hydrolase enzymes [35]. Membrane-bound intestinal α -glucosidases hydrolyze oligosaccharides and disaccharides to glucose and other monosaccharides in the brush border of the small intestine. Yet, in our cell culture setup, such brush border disaccharidase activity is not needed as monomeric glucose is the only external sugar source. Furthermore, if iminosugars such as FGM and MGTL would inhibit intracellular α -glycosidases responsible for glycogen mobilization, we would expect a higher instead of a lower glycogen content. Such an increase in glycogen content, especially in liver and muscle tissues, was reported upon DNJ administration to a diabetic tilapia model [36]. Although in normal tilapia, no changes in the activity of enzymes involved in glycogen synthesis were observed, DNJ caused a significant increase in hexokinase, glucose-6-phosphate dehydrogenase, glucose-6-phosphatase, and fructose 1,6-biphosphase. The glycogen content and these enzymatic activities were not studied in intestinal tissue, so it remains unknown how iminosugars affect glucose dynamics in the intestinal cells. Microscopic images not only revealed differences in lipid and glycogen

content in Caco-2 cells but also showed three distinct phenotypes in monolayer organization: (i) a high distribution of nutrients in both flat and elongated cells (untreated), (ii) a low distribution in flat cells while a high content was maintained in elongated cells (DNJ, MGLT, TBN-DNJ, IFGM), and (iii) a general ‘flattening’ of the monolayer with relatively low and more equal distribution (NN-DNJ and FGM). These altered phenotypes of epithelial monolayers may indicate important changes in differentiation and tissue architecture. Indeed, DNJ has been shown to inhibit the expression of MMP2 and MMP9, upregulate TIMP-2, and alter cell surface glycan-binding motifs, which are crucial in extracellular matrix remodeling, differentiation, and migration of tumor-derived cells [37]. One important observation was that the more tumorigenic and metastatic HCT-116 cell line was less susceptible to changes in lipid and glycogen accumulation, and the cell monolayer was less differentiated compared to the long-term-treated Caco-2 cell line. This may be due to the fact that cancer cells inherently have higher lipid and glycogen stores to meet the higher demand for substrates for energy production and biosynthesis of rapidly dividing cells, to overcome changing tissue environments, cope with oxidative stress, and facilitate cell–cell signaling to promote cell proliferation [38].

Conclusions

This study provides valuable insights into the effects of various iminosugars on cellular energy metabolism, nutrient distribution, and tissue architecture, representing a significant advancement in this field. A thorough review of the available literature did not identify previous studies specifically addressing this issue, highlighting the originality and relevance of the present work. By filling this gap, our results offer a novel contribution to the field, opening new perspectives for future research aimed at understanding how iminosugars, both natural and synthetically derived, influence cellular bioenergetics. In particular, this study demonstrates that iminosugars alter cell growth in colon cancer Caco-2 and HCT-116 cells in a sugar- and environment-specific way, reducing their basal respiration and spare mitochondrial capacity while increasing glycolysis and lactate production. Moreover, long-term treatment induces changes in the substrate preferences for energy metabolism and alters lipid and glycogen deposits in intestinal monolayers. These changes in nutrient deposits are accompanied by alterations in tissue organization. Despite these promising results, our study has some limitations. *In vitro* models, although

representative, cannot fully replicate the complexity of the biological environment *in vivo*. Therefore, future studies should focus on clinical trials to further investigate the systemic effects of iminosugars in humans. In conclusion, this study demonstrates the potential of iminosugars in modulating cellular metabolism, opening new perspectives for cancer treatment. Future research should focus on dose optimization, safety assessment, and the integration of these compounds into innovative therapeutic approaches.

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CHAPTER 4

Conclusion

The research path developed in this PhD project focused on the study of the bioaccessibility of two fundamental classes of macronutrients, namely lipids and carbohydrates, whose unbalanced introduction into the diet represents one of the main determinants of numerous chronic-degenerative diseases [1] [2]. My objective was to propose a more functional nutritional perspective, centered not on the mere chemical content of foods, but rather on the fraction of nutrients that is effectively accessible after the digestive process. This orientation highlights the limitations of traditional compositional models and the importance of considering bioaccessibility, namely the fraction of a nutrient released during digestion and potentially absorbable [3], as a key parameter. Throughout the course of this work, the idea has clearly emerged that the food matrix, internal structure, chemical form of nutrients, and formulation strategies are not secondary aspects, but rather determinants of real nutritional efficacy. In agreement with recent reviews, processing significantly influences bioaccessibility by modifying the characteristics of the matrix and the release of molecules [4]. An important contribution from recent literature concerns the design of formulations as a lever to improve nutrient bioavailability. Strategies such as micro- and nanoemulsification, self-emulsification, chewable gels, and microencapsulation have been explored as means to overcome intrinsic digestive barriers [5]. These approaches represent possible ways to “bypass” the obstacles imposed by the food matrix, thereby improving the fraction of nutrients made accessible to the organism. Such considerations do not concern lipids alone, but also find a clear application in the field of carbohydrates, whose quality and mode of digestion are directly reflected in glycemic responses and the risk of developing metabolic disorders. Several studies have demonstrated that foods with identical starch content may elicit very different glycemic responses, depending on the degree of gelatinization, the presence of dietary fiber, and interactions with other nutrients. The inclusion of fiber, for example, has proven to be an effective strategy to modulate glucose release kinetics, favoring more gradual absorption and contributing to the prevention of postprandial glycemic peaks [6]. From this perspective, carbohydrates should not be evaluated solely as an energy source, but as elements that, depending on their bioaccessibility, can substantially modulate metabolic processes in both the short and long

term. Here again, a simple compositional determination proves reductive: what truly matters is the dynamics by which carbohydrates become available to the organism, and how these dynamics are modulated by the matrix and processing conditions.

Beyond its implications for health and nutritional innovation, the modulation of bioaccessibility also has a positive potential impact on the sustainability of food systems. Improving the fraction of nutrients that are effectively accessible and usable by the organism makes it possible to reduce nutritional waste, since foods with higher bioavailability provide a more efficient nutrient supply and require smaller quantities to meet physiological needs. This aspect is particularly relevant in a global context where malnutrition in all its forms, from micronutrient deficiencies to caloric excess, coexists with the need to reduce the environmental impact of food production. Promoting foods and formulations that can improve bioaccessibility therefore means optimizing the use of raw materials, enhancing local agricultural resources, and reducing the demand for resource-intensive inputs such as water, energy, and land. From this perspective bioaccessibility can be viewed not only as a nutritional parameter but also as an indicator of ecological efficiency. A food system that favors highly bioavailable foods, capable of delivering more nutrients per unit of raw material, helps make diets more sustainable from an environmental, economic, and social standpoint. Integrating the concepts of functional nutritional quality and sustainability represents one of the most important challenges for the future of food research and policy, as it allows the reconciliation of human health and planetary health through an approach based on the biological efficiency of foods.

In summary, the message that emerges from this PhD work is that the value of a food or supplement does not lie in what it contains “on paper,” but in what it is able to actually make available to the organism. This principle, applied to both lipids and carbohydrates, allows for a better understanding of the relationship between diet and health, and enables the design of healthier foods that, when part of a balanced diet, could contribute to the prevention of chronic diseases. Embracing the paradigm of bioaccessibility thus entails a shift in perspective: from nutrition based on quantity to nutrition based on function. Such an evolution has the potential to improve the quality of research, guide industrial innovation, support stronger nutritional policies, and ultimately contribute to a future in which food and health are more closely and consciously interconnected.

The present work is not without inherent limitations, which should be acknowledged in light of the methodological choices adopted. The use of static in vitro digestion models, while ensuring high reproducibility and robust comparability across experimental

conditions, cannot fully capture the multifaceted complexity of human digestion, including dynamic intestinal absorption processes, endocrine regulation, and microbiota–host interactions.

In this context, glucose release following simulated digestion should be interpreted as an index of potential bioaccessibility rather than as a direct surrogate of postprandial glycemic responses *in vivo*. Likewise, lipid bioaccessibility reflects the fraction theoretically available for absorption, without providing information on actual bioavailability, tissue distribution, or metabolic fate.

These limitations do not detract from the value of the approach adopted; rather, they delineate the conceptual boundaries within which the results should be interpreted. Accordingly, future research should aim to integrate the bioaccessibility data generated in this thesis with more advanced experimental platforms, including dynamic digestion systems, intestinal cell-based models, and *in vivo* postprandial studies, in order to more effectively bridge the gap between food digestion, nutrient absorption, and systemic metabolic outcomes.

From an applied and translational perspective, the methodological framework and analytical strategies developed herein represent a valuable tool to support the rational design and reformulation of foods with improved nutritional quality. In this sense, the work contributes to the broader effort of informing public health strategies aimed at mitigating the metabolic burden associated with highly processed dietary patterns.

In parallel, this thesis investigated the biological activity of food-derived iminosugars (DNJ, FGM) and selected synthetic derivatives in colon cancer cell models. The results provide evidence that iminosugars are capable of inhibiting cell proliferation and reprogramming cellular energy metabolism in a highly context-dependent manner, influenced by cell line characteristics, glucose availability, exposure duration, and cellular architecture (monolayer versus spheroid models). This metabolic reprogramming was characterized by a reduction in mitochondrial respiratory capacity, accompanied by a compensatory increase in glycolytic flux and lactate production. Prolonged exposure further impacted intracellular nutrient storage, including lipid and glycogen deposition, as well as epithelial and tissue-like organization. The principal limitations of this experimental section stem from the *in vitro* nature of the models employed and their intrinsically limited physiological complexity. Future investigations should therefore seek to validate and extend these findings using more physiologically relevant systems, such as advanced co-culture models, three-dimensional organoids, and *in vivo* approaches, in order to better

capture tissue-level organization and systemic interactions. Moreover, further research is warranted to elucidate the molecular mechanisms linking glycosidase inhibition to the observed metabolic adaptations. In particular, comprehensive analyses of gene expression and signaling pathways will be essential to identify the molecular networks activated or suppressed in response to iminosugar treatment. Finally, careful evaluation of dose–response relationships, safety profiles, and translational relevance will be crucial to assess the potential of iminosugars both as dietary prevention tools and as adjunct strategies in metabolic or oncological contexts.

While acknowledging the limitations discussed above, the work presented in this thesis offers an integrated and original contribution to the understanding of how food structure, processing, and food-derived bioactive compounds shape nutrient bioaccessibility and cellular metabolic responses. By bridging food science, nutritional biochemistry, and cellular metabolism through complementary *in vitro* approaches, this research delineates a conceptual framework that can inform future translational and systems-level investigations. In a broader perspective, the thesis contributes to the evolving paradigm of precision and preventive nutrition, supporting the development of food strategies and bioactive-based interventions aimed at promoting metabolic health and reducing disease risk in increasingly complex dietary environments.

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