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XXXV CYCLE

EFFECT OF COOKING AND *IN VITRO* GASTROINTESTINAL DIGESTION ON THE STABILITY, BIOACCESSIBILITY, AND BIOACTIVITIES OF PHENOLIC COMPOUNDS IN DARK PURPLE EGGPLANT AND RED-SKINNED ONION

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Abstract

Different cooking methods can positively and negatively influence the phenolic content of vegetable matrices. Indeed, cooking can increase the extractability of phenolic compounds due to a matrix softening effect but also cause their degradation or loss.

The first objective of this work was to evaluate the effect of four different types of cooking (boiling, baking, frying, grilling) on the phenolic content of two cornerstones of the Mediterranean diet: red-skinned onion and dark purple eggplant. At first, raw and cooked samples were characterized from a phenolic point of view using high-resolution mass spectrometry and were subjected to *in vitro* gastrointestinal digestion to assess their actual stability during cooking and bioaccessibility during the digestive process. As with the extracts, the digested samples were characterized from a phenolic point of view. Then, the antioxidant activity of samples was assessed by ABTS and FRAP assays. Hydroxycinnamic acid-derivatives, especially caffeoylquinic acids, dicaffeoylquinic acids and hydroxycinnamic acid-spermidine-derivatives were the major phenolic compounds, which made up eggplant phenolic profile. Instead, the phenolic profile of onion was dominated by flavonols such as quercetin, quercetin-4'-*O*-glucoside and quercetin-3-*O*-glucoside-4'-O-glucoside. The results indicate that, except for frying, cooking processes led to the degradation of individual phenolic compounds but were also crucial for their release from food matrices during *in vitro* gastrointestinal digestion. In this context, cooking processes such as frying, grilling, and baking meant an increase in the bioaccessibility of the phenolic compounds in dark purple eggplant and red-skinned onion, promoting their release from the food matrices.

Subsequently, the digested fractions of the two raw and cooked vegetables were subjected to colonic fermentation using the MICODE (multi-unit *in vitro* colon gut model) to investigate the impact of the different phenolic content of the samples on the colonic microbiota and vice versa. During the process, analyses of phenolic compound profiles and microbiomics were carried out. Gut bacteria were able to metabolize dark purple eggplant and red-skinned onion phenolic compounds promoting the formation of low-molecular weight phenolic acids that accumulated over the time. In particular, the most prominent metabolite identified after colon metabolism of dark purple eggplant hydroxycinnamic acids was 3-(3'-hydroxyphenyl)propanoic acid whereas fermentation of red-skinned onion flavonols resulted in the production of 3-(3'-hydroxyphenyl)propanoic acid, 3-(3'-hydroxyphenyl)acetic acid and 3-(3',4'-dihydroxyphenyl)acetic acid. Differences among the raw and cooked vegetables in the metabolite profiles were mainly quantitative, with the highest recorded amount of metabolites identified after frying and grilling for dark purple eggplant and red-skinned onion, respectively.

Furthermore, the results show that in dark purple eggplant, frying is better than grilling in terms of promoting the growth of beneficial bacterial taxa and limiting that of opportunistic taxa, whereas raw red-skinned onion

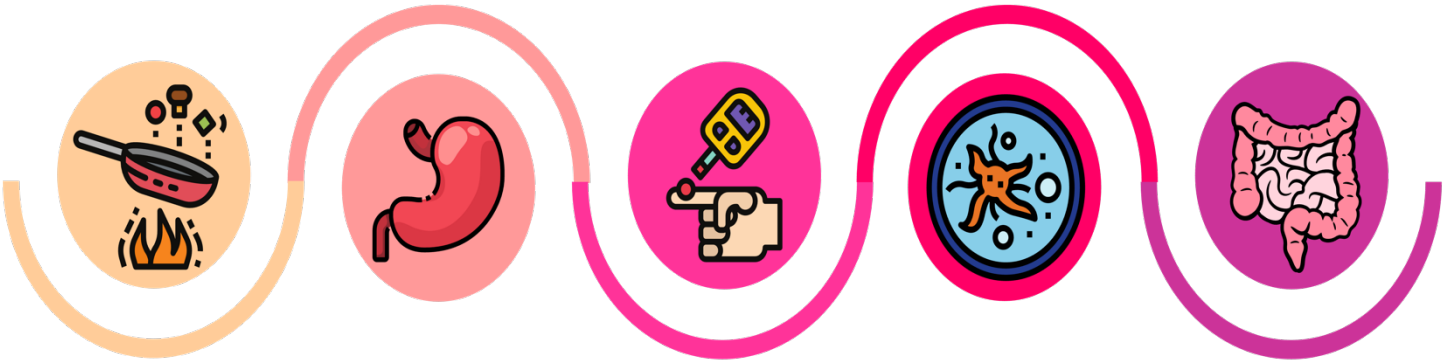
brought about a substantial increase in beneficial taxa, larger respect to the heat-treated onions, particularly on Lactobacillales and beneficial clostridia.

In the next step, the anti-diabetic activity of the digested fractions of the two vegetables, raw and subjected to the four cooking processes, was investigated by investigating their inhibitory activity against three key metabolic enzymes, DPP-IV, α-glucosidase, and α-amylase. The results show that all onion samples exhibit some inhibitory activity against the three key enzymes involved in the pathogenesis of diabetes, but to varying degrees depending on the cooking method and phenolic profile. For example, the class of flavonols was the most active against the enzymes α-glucosidase and DPP-IV. Furthermore, statistical analysis confirmed a significant correlation between the number of quercetin mono-hexosides and the IC50 of α-glucosidase and the number of quercetin di-hexosides and the IC50 of DPP-IV. Finally, the antiproliferative activity of the pure compounds present in the highest quantities in red-skinned onion and dark purple eggplant and their principal colonic metabolites against two model of human adenocarcinoma cell lines (Caco-2 and SW480) was investigated at physiologically relevant concentrations. The hydroxycinnamic acids 3-*O*-caffeoylquinic acid, 3,5-*O*-dicaffeoylquinic acid and the microbial metabolite 3-(3',4'-dihydroxyphenyl)acetic acid) were the most active against Caco-2 cell line whereas quercetin, glycosylated quercetin-derivatives and the metabolite 3-(3'-hydroxyphenyl)acetic acid were the most active against SW480. This last cell line metabolizes the tested parent phenolic compounds more rapidly and extensively than Caco-2, whereas colon metabolites were generally more stable during incubation with both the cell lines.

In conclusion, vegetable cooking had an important impact on the phenolic compounds stability and their following release during digestion. Despite the majority of cooking procedure caused a loss pf phenolic compound, thermal treatment, with the exception of boiling, increased the bioaccessibility of phenolic compounds promoting their release from the food matrix. Phenolic compounds released after *in vitro* digestion were extensively metabolized by gut bacteria releasing small phenolic acids. Indeed, dark purple eggplant and raw red-skinned onion exerted a prebiotic effect increasing the amount of beneficial bacteria taxa. Finally, the different cooking methods had an impact on the biological activities associated with the presence of phenolic compounds and metabolites modulating their anti-diabetic and anti-proliferative activities.

1

INTRODUCTION



1. Overview

1.1 Cooking treatments

At the time of the first hominids, the discovery of fire and thus of food cooking represented a turning point in human history. Cooking made food nutrients more accessible, and this helped the development of the brain size of humans and contributed to the process of mankind's evolution[1]. The discovery of cooking can be compared to other events that contributed to the development of humans such as the increase in meat consumption, agriculture, and the breeding of domestic animals [2]. As a result of cooking, the food was softer and easier to chew, as well as much more digestible and with a reduced amount of toxins [3,4]. At the end of the 19th century, people began to apply cooking in industry, focusing on microbial safety and nutritional quality issues [5]. In addition to increased food safety and quality, heat treatments also contribute to a longer shelf-life of food, reduced risk of contamination by pathogens, increased digestibility of macromolecules and the formation of flavouring compounds and aromas [6–9]. On the other hand, cooking can also adversely affect the quality of a food by causing certain undesirable effects such as the loss of certain nutrients, both essential and non-essentials (e.g., vitamin C and polyphenols), the formation of undesirable or toxic compounds (e.g., acrylamide), the formation of compounds that alter the perception of aroma, and the loss of texture and colour alteration [5,10]. In the domestic context, the most used cooking methods are baking, boiling, grilling, and frying and the foods that are best suited to all these types of cooking are vegetables. Vegetables constitute essential components of human diet. In fact, they represent, together with fruits, one of the most important sources of nutraceuticals, i.e., substances beneficial to human health, like antioxidants, colouring agents, minerals, and vitamins [11]. Among these substances, phenolic compounds, which are plant-derived secondary metabolites ubiquitously widespread in different botanical species, are of paramount importance for human health. In fact, these compounds have various beneficial effects on human health, they are powerful natural antioxidants, they are active against microbial proliferation and have anti-allergenic activities; from the cardiovascular point of view, they exert cardioprotective, antithrombotic, anti-atherogenic and vasodilatory activities and they also have anticarcinogenic effects [12]. To exert their beneficial effect, polyphenols must be bioavailable. The bioavailability of a compound can be affected by multiple factors, intrinsic to the food, such as stability during gastro-intestinal digestion and release from the food matrix, but also extrinsic, such as the cooking methods [13].

1.2 Polyphenols

1.2.1 Polyphenols and heat treatments

Heat treatments, whether industrial or home-based, can affect and modulate the phenolic content and the phenolic profile of foods[6,7]. Depending on the type of food matrix (type of vegetable) and the chemical structure of phenolic compounds, as well as certain technical parameters used during cooking (mode, time and temperature) the changes that occur are different [6,14]. Certain methods of cooking, such as boiling, cause greater loss of phenolic compounds, this is because certain hydrophilic compounds are lost in the cooking water[14,15]. Perhsson et al.[15] for example, comparing three cooking modes (boiling, steaming and microwaving) found that boiling was the technique that caused the greatest loss of flavonoids in Broccoli while the one that allowed the best preservation was microwaving. In contrast to boiling, frying seems to be the cooking method that better preserves the phenolic content in vegetables. Several studies have found an increase in onion flavonols after frying [9,16], and the same results were also observed for green pepper and cardoon[17]. Some classes of compounds then, such as anthocyanins and procyanidins, are often degraded during cooking. These classes of compounds, are in effect very unstable to heat treatments, especially at high temperatures[14]. Surh et al., observed that a significant loss of anthocyanins occurred during the cooking of black rice: in this case roasting resulted in the greatest decrease (94%), followed by steaming (88%), pan-frying (86%) and boiling (77%)[18]. The same results were also reported in another study on raspberries and elderberries by Cordeiro et al.[19], where all cooking methods tested resulted in a significant loss of the original anthocyanin content while in recipes without heat treatment the loss of anthocyanins was minimal. However, in almost all studies, increasing the temperature and cooking time caused a decrease in the phenolic content of the tested samples[14,15]. On the other hand, heat treatments, by breaking down the structures of the vegetables, exerts a softening effect on the food matrix that allows the release of phenolic compounds trapped in the fibers[9]. To exert the best effect, making phenolic compounds more bioaccessible without degrading them, it is therefore necessary to play with the cooking mode, timing, temperature, and type of food matrix used.

1.2.2. Polyphenols and gastro-intestinal digestion

To exert their beneficial effect on health phenolic compounds should be absorbed during gastro-intestinal digestion and reach the bloodstream. The more bioaccessible the compounds are, i.e., released from the food matrix into the gastrointestinal tract, the more likely they are to be absorbed[20]. After salivary digestion, in which the release of compounds is very limited because of the short time, the first step for absorption of phenolic compounds occurs in the stomach[21]. Tagliazucchi et al.[22] have called this first tract of the digestive system a "bioextractor" capable of release most phytocompounds from the food matrix of origin. For certain compounds, the hypothesis of absorption during the gastric phase is highly credited because their post-

prandial appearance in plasma occurs very rapidly[22]. However, as shown in Figure 1, most phenolic compounds are absorbed in the intestine[23]. Generally, aglycones and some glucosides are absorbed in the small intestine while the more complex forms such as esters, other glycosides, or polymers cannot be absorbed in the native form[23,24]. Specific absorption of aglycones and their glycosylated forms in the small intestine can occur in two ways: passive diffusion or active transport via the sodium glucose-bound transporter of enterocytes[25,26]. Compounds that cannot be absorbed in their native form (esters, glycosides, or polymers) undergo modifications carried out by intestinal β-glucosidases, lactase-phlorizin hydrolase and/or colonic microflora[23]. After these steps, even the more complex compounds can be absorbed. Before passage into the bloodstream, free aglycones are transported through the portal vein to the liver where they are conjugated by methylation, sulfation and glucuronidation. As a result of these transformations, metabolites with different bioactivities than the original compounds are found in the bloodstream[27]. The last step in the metabolism of phenolic compounds is excretion, which can occur through two routes: the biliary and urinary pathways. Urinary excretion is the most frequent, especially for the more polar compounds while for the more apolar one the main route of excretion is the biliary pathway[21].

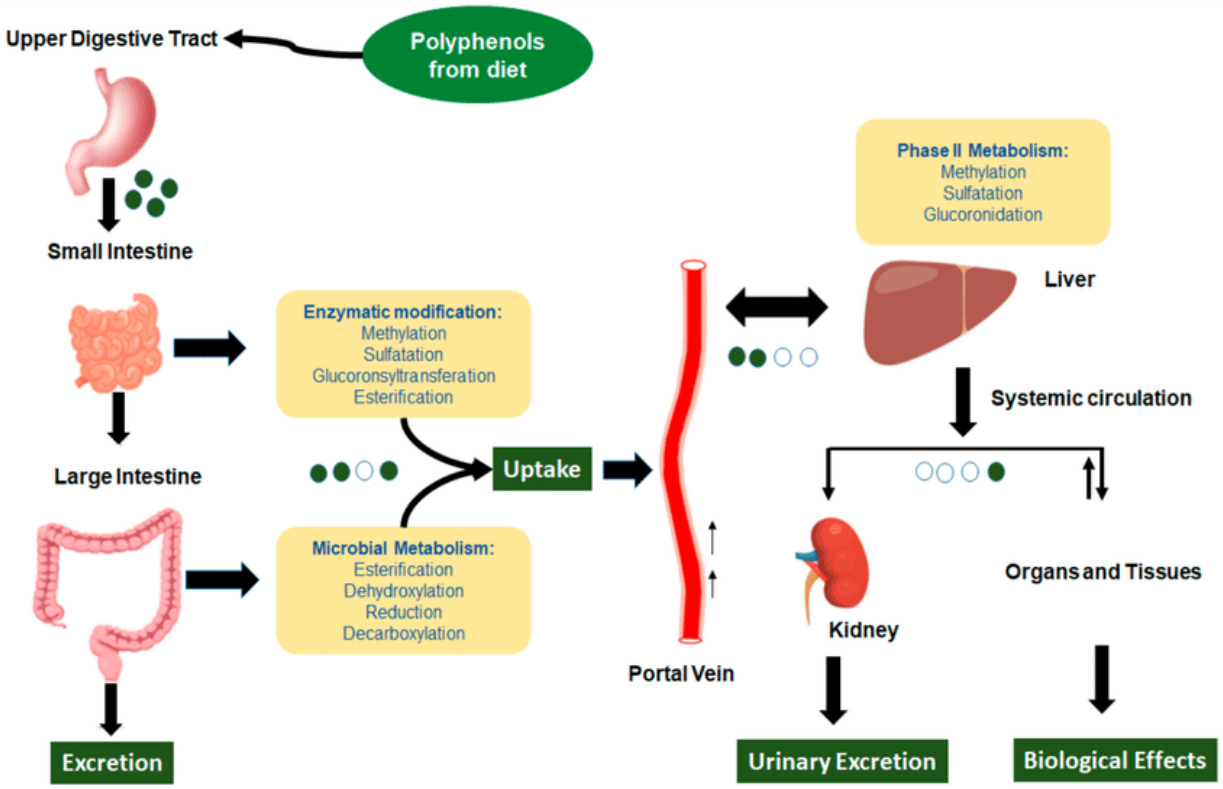


Figure 1. Schematic representation of the absorption and metabolism of dietary polyphenols. Green dots represent aglycon polyphenols, and white dots represent their metabolites. Adapted from Cipolletti et al.[23]

1.2.3. Polyphenols and colon microbiota

In recent years, many studies have focused on the investigation of the complex interaction between the colonic microbiota and phenolic compounds[28–31]. Indeed, increasing evidence suggests that the supposed health effects of phenolic compounds are probably related to the interaction with gut microbiota. About the 90-95% of phenolic compounds introduced with the diet are absorbed in the colon after being metabolized. Here, colonic bacteria are responsible for the changes in phenolic compounds that are transformed into their respective metabolites[32]. The microorganisms found in the distal regions of the colon constitute the highest concentration of microorganisms present in the human body and they are essential for the maintenance of intestinal homeostasis and the general health of the host[29,30]. The nature of the gut microbiota is influenced both by genetics but also by the environment in which it coexists[29]. While not much can be done genetically to improve the health of our gut, a diet rich in phenolic compounds can influence the environment of the gut microbiota by increasing the relative abundance of beneficial bacteria. The health properties associated with increased numbers of these bacteria range from protection against gastro-intestinal disorders and pathogens, strengthening the intestinal epithelial cell junctions, increasing mucus secretion, and even modulating the intestinal immune response[32]. As reported in Koudoufio et al.[31] consumption of red wine, for example, led to an increased abundance of *Bifidobacterium* and *Lactobacillus*, two taxa of beneficial bacteria active in protecting the intestinal barrier and *Faecalibacterium prausnitzii* known for its anti-inflammatory properties. It was also noted that as these beneficial taxa increased, opportunistic taxa such as *Escherichia coli* and *Enterobacter cloacae* decreased. The increase in beneficial taxa (*Bifidobacterium* and *Lactobacillus*) was also noted after consumption of green tea rich in phenolic compounds, blueberry juice and cocoa-derived flavanol drink[33], whereas after consumption of foods rich in betacyanin, ellagitannins and proanthocyanidins, increased growth of *Akkermansia muciniphila*, a bacterium active in regulating metabolic activity, was observed[34]. On the other hand, a diet high in fat and sugar negatively influences the composition and metabolic activity of the human gut microbiota, creating a situation of dysbiosis that can lead to intestinal, metabolic, and immune diseases[35]. For example, several studies reported that patients suffering from colorectal cancer and inflammatory Bowel diseases (which includes Chron's disease and ulcerative colitis) presented reduced relative abundance of beneficial taxa such as *Roseburia*, *Bifidobacterium adolescent*, and *Faecalibacterium prausnitzii* and increased opportunistic taxa such as *Streptococcus* and *Bacteroides*[33].

If phenolic compounds help the development of beneficial taxa in our intestines by acting as prebiotics, similarly these beneficial gut bacteria can modify the structure of phenolic compounds generating metabolites that are more easily absorbed than the parent compounds and presented important biological activities. For example, *Eubacterium ramulus* and *oxidoreducens* and *Clostridium orbiscidens* can metabolize flavanols such as glycosylated kaempferol, quercetin, and myricetin-derivatives by *O*-deglycosylation and C-ring fission into small metabolites and short-chain fatty acids while *Enterococcus casseliflavus* is able to

hydrolyse the sugar moiety from quercetin-3-*O*-glucoside releasing aglycone and producing lactic, formic, acetic, and ethanol acids[31].

1.3. Metabolic diseases and pathologies

Globally, in 2017, dietary factors were responsible for 11 million deaths, which represent the 22% of all deaths among adults. Ranking first as a cause of diet-related deaths are cardiovascular diseases, followed by type-2 diabetes and cancer. These pathologies, which have become popular in recent years, are also known as "civilization diseases"[36]. Therefore, in recent years, the study of the relationship between diet, lifestyle and noncommunicable diseases is becoming increasingly crucial[37–39]. The study of this relationship is entrusted to nutritional epidemiology, a discipline that in 2013 was defined by Bohn et al. as “a subdiscipline of epidemiology and provides specific knowledge to nutritional science”. Its objectives is to provide information on the diet-disease relationship so that appropriate prevention techniques can be developed.

The specific contributions of nutritional epidemiology include dietary assessment, description of nutritional exposure and statistical modelling of the diet-disease relationship[37]. Diet such as Western diet characterized by the high consumption of foods rich in calories, saturated and *trans*-unsaturated fatty acids, sugary drinks and red or processed meats undoubtedly lead to overweight and obesity[40]. While diets such as the Mediterranean diet, characterized by frequent consumption of fruits and vegetables, are known to exert beneficial effects on health[41].

Obesity is defined as “a chronic, progressive, relapsing, and treatable multi-factorial neurobehavioral disease, wherein an increase in body fat promotes adipose tissue dysfunction and abnormal fat mass physical forces, resulting in adverse metabolic, biomechanical, and psychosocial health consequences”[42,43]. In the diet of obese patients there is an imbalance between the amount of energy intake, which is too high, and the low, or absent, amount of energy consumed through physical activity. Based on the body mass index classification (MBI), an individual is considered overweight when his or her BMI exceeds 25 kg/m^2 and obese when it exceeds 30 kg/m^2 [44]. In addition to excessive adiposity, this condition may be accompanied by structural and functional abnormalities, increased risk of premature mortality, and comorbidities such as cardiovascular diseases, cerebrovascular diseases, diabetes, asthma, psychiatric disease, and depression[45]. For example, the link between obesity and type-2 diabetes is given by the fact that a condition of insulin resistance develops in obese patients with consequent hyperinsulinemia and impaired glucose homeostasis[46]. In addition, parameters leading to the activation of oncogenic pathways have been detected in the blood of obese patients[47]. Promotion and progression of cancer cells is linked to numerous factors related to the condition of obesity, such as the presence of certain hormones, modulation of energy balance, and various inflammatory processes[48]. Cancer types with which obesity is associated include colorectal, postmenopausal breast, kidney, oesophageal, pancreatic, gallbladder, liver, and hematologic malignancies[49].

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by high blood sugar, insulin resistance in target organs, and relative lack of insulin caused by β-cell dysfunction[50–53]. This condition is divided into type one (or insulin-dependent) diabetes of autoimmune origin, type two (or insulin-non-dependent) diabetes, and gestational diabetes. Type 2 diabetes mellitus (T2DM) is one of the leading causes of death worldwide and is also one of the fastest increasing diseases [53,54]. According to the 10th edition of the IDF diabetes Atlas, 10.5% of all adults worldwide have type 2 diabetes, and it is also estimated that if the world population grows by 20% by 2030, the number of people with diabetes will increase by 46%[55]. Diabetes often leads to the development of new diseases such as heart and kidney diseases, stroke, infection, and it can also cause retinal damage and blindness in adult age. Obesity associated with metabolic abnormalities, a sedentary lifestyle, and unhealthy diet are strong risk factors for T2DM[52]. Therefore, improvement in patients' dietary knowledge and practices associated with regular physical activity can help prevent and moderate metabolic syndrome and consequently diabetes[56].

Cardiovascular diseases (CVDs) are a group of disorders striking the heart and blood vessels. They include coronary heart diseases, cerebrovascular diseases, peripheral arterial diseases, rheumatic heart diseases, congenital heart diseases and deep vein thrombosis and pulmonary embolism[57,58]. According to the world health organization (WHO) in 2021, 32% of deaths globally were caused by cardiovascular disease (about 17.9 million people worldwide). Of these deaths, 85% occurred from heart attack and stroke[57]. These data make CVDs the leading cause of deaths globally. The main factors of the onset of this type of diseases are smoking, hypertension, obesity, and diabetes[59]. Given these risk factors, dietary habits are essential for prevention. for example, a diet such as the Mediterranean diet, rich in foods such as fruits and vegetables, whole grains, legumes, nuts, and fish, exerts beneficial effects on the risk of developing these diseases[59].

Cancer: the term cancer, or malignant tumour or neoplasm, encompasses a group of diseases that can potentially affect any part of the body. This disease consists of the development of abnormal cells that reproduce without restraint and can invade adjacent parts of the body and spread to other organs, thus creating metastasis. This pathology is a leading cause of death worldwide, accounting for nearly 10 million deaths in 2020[60]. The main risk factors for cancer are smoking, obesity, alcohol consumption, low intake of fruits and vegetables, and lack of physical activity[60]. Colorectal cancer is one of the most frequent forms of cancer (third place globally). In 2020, about 2 million cases were diagnosed worldwide and nearly 1 million deaths due to this disease[60–62]. To prevent the development of this disease, diet and eating habits are crucial[63]. Red meat consumption and unbalanced diets low in fiber and vegetables, for example, increase the risk of developing colorectal cancer while a diet such as the Mediterranean diet, rich in fiber, fruits and vegetables, and dairy products, can help prevent this disease[63,64].

1.4. Polyphenols and pathologies

A plethora of studies confirmed the beneficial effect of a diet rich in phenolic compounds on metabolic syndrome and diabetes, cardiovascular diseases, neurodegenerative diseases, and cancer[65–68]. For example, phenolic compounds can reduce carbohydrate absorption by inhibiting the amylolytic enzymes α-amylase and α-glucosidase and they also prevent the hydrolysis of incretins through inhibition of the enzyme dipeptidyl-peptidase IV[69–71]. In addition, they also improve β-cell function, stimulate insulin secretion, and help induce satiety[72,73]. Foods such as whole grains rich in phenolic compounds help prevent the development of this condition because they cause a lower glycaemic response. The same glucose-lowering effect has also been observed after consumption of fruits such as pomegranate and berries. Onions, on the other hand, are known for their inhibitory effects toward amylolytic enzymes while broccoli is known to improve insulin resistance[74].

Phenolic compounds also help decrease the risk of cardiovascular diseases. The ways in which they act are varied: from vasodilator, antithrombotic, and antiatherogenic and anti-inflammatory activity. Red wine polyphenols, for example, are known to reduce plasma lipid concentrations while those in chocolate help prevent inflammation in patients suffering from cardiovascular disease[66].

Finally, numerous studies investigated the anticarcinogenic properties of phenolic compounds[75–79]. Indeed, they are known to disrupt and reverse the process of carcinogenesis by acting on intracellular signals involved in tumour initiation or promotion. They also exert a dual cytoprotective effect on normal cells and cytotoxic effect on neoplastic cells. For example, foods such as onions rich in quercetins are known to reduce the proliferation of Caco-2 cancer cells by inducing apoptosis[75] while foods rich in phenolic acids, on the other hand, can reduce the continued proliferation of cancer cells by arresting the cell cycle in S phase, while curcumin, anthocyanins, and quercetin are reported to arrest the cell cycle in colon cancer cells in G2 and M phases[80].

2. References

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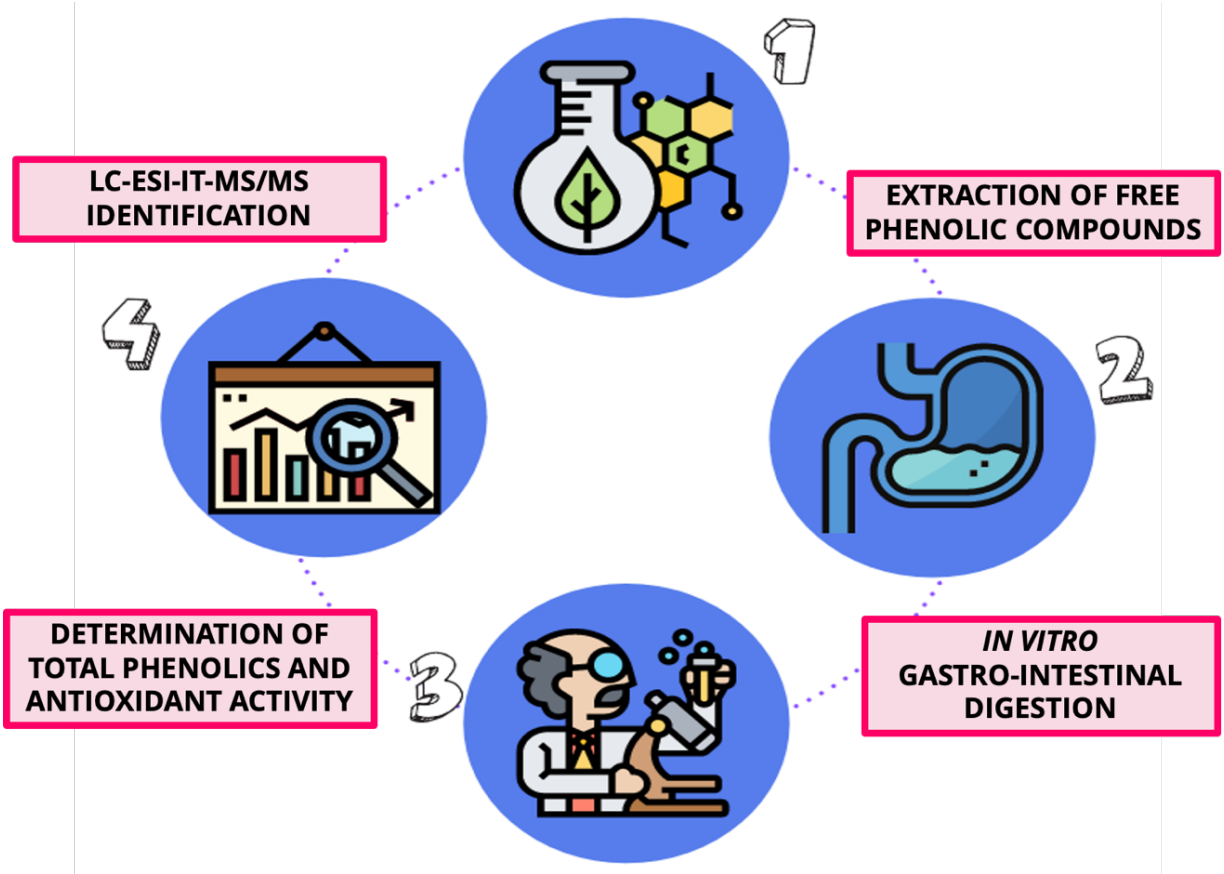
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S. Martini, A. Cattivelli, A. Conte, D. Tagliazucchi. Domestic cooking methods affects the stability and bioaccessibility of dark purple eggplant (Solanum melongena) phenolic compounds. *Food Chemistry*, **2021**, *341*, 128298. doi: 10.1016/j.foodchem.2020.128298

DOMESTIC COOKING METHODS AFFECT THE STABILITY AND BIOACCESSIBILITY OF DARK PURPLE EGGPLANT (*SOLANUM MELONGENA*) PHENOLIC COMPOUNDS

ABSTRACT

Eggplant is an important component of the Mediterranean Diet, which becomes edible after cooking. This study determined the fate of dark purple eggplant phenolic compounds after baking, boiling, frying, grilling and digestion. Thirty-seven phenolic compounds were identified and quantified in raw eggplant. Frying determined a 74% increase in total hydroxycinnamic acids whereas a decrease was observed after boiling (27%), grilling (51%), and baking (60%). After digestion, 45%, 33% and 22% of total phenolic compounds resulted bioaccessible in baked, grilled and fried dark purple eggplant. Fried eggplant displayed the highest amount of phenolic compounds (751.46 mg/100g) after digestion. The cooking methods differently affected the release of individual phenolic compounds. Baking and grilling resulted in higher amount of bioaccessible caffeoylquinic acids whereas frying in di-caffeoylquinic acids and hydroxycinnamic acid-amides. A careful design of the cooking method may be pivotal to modulate the release of specific phenolic compounds.

Keywords: mass spectrometry, food processing, bioaccessibility, polyphenols, gastro-intestinal digestion, thermal treatments

1. Introduction

The introduction of cooking processes represented an important breakthrough in human history. From an evolutionary point of view, food cooking was crucial for the increase of brain size in early hominids (Carmody, Weintraub, & Wrangham, 2011). Food processing increases the amount of calories accessible from raw foods and decreases the energy need for food digestion. Heat-treatment has also some important benefits involving the increase of food safety and quality, the reduction of contamination risk, the elongation of shelf-life, the enhancement of macromolecules digestibility and the formation of flavouring compounds and aromas (Van Boeckel et al., 2010). In contrast, thermal processes may result in undesired chemical reactions leading to the generation of toxic compounds and the loss of some nutrients (Koszucka, & Nowak, 2019). Undoubtedly, cooking has an influence on the total phenolic content and the phenolic profile of vegetable food as well as their bioaccessibility and bioavailability (Moyo, Serem, Bester, Mavumengwana, & Kayitesi, 2020; Palermo, Pellegrini, & Fogliano, 2014; Zhao et al., 2019). Changes in phenolic compound levels upon cooking could be related to several factors such as the cooking technique, the type of vegetable source as well as the chemical structure of the specific phenolic compound (Murador, Braga, Da Cunha, & De Rosso, 2018; Palermo et al., 2014; Zhao et al., 2019). For example, cooking processes involving water such as boiling normally resulted in a decrease of the phenolic content (Giusti, Capuano, Sagrantini, & Pellegrini, 2019; Miglio, Chiavaro, Visconti, Fogliano, Pellegrini, 2008). The cooking time also influenced the stability of phenolic compounds with a greater loss the longer the cooking time (Murador et al., 2018). However, the food matrix and the chemical nature of the compounds also influenced the release and stability of phenolic compounds during vegetable cooking. For example, Pellegrini et al. (2010) found that boiling but not steaming increased the amount of extractable phenolic compounds in broccoli, Brussel sprouts and cauliflower. In another study, Murador, Mercadante, and De Rosso (2016) found that steaming techniques increased the total phenolic content of kale whereas they observed a reduction in red cabbage phenolic content. Some classes of phenolic compounds such as procyanidins and anthocyanins appeared to be particularly unstable to the heat treatments with the majority of published papers recording a reduction in their content after cooking (Palermo et al., 2014; Zhao et al., 2019). In other cases, the amount of a specific class of phenolic compounds can be reduced or increased depending on the food matrix. For example, the flavonols amount was found to increase after frying and grilling of onion, green pepper and cardoon whereas domestic cooking of watercress resulted in a decrease in flavonols concentration (Juániz et al., 2016a; Giallourou, Oruna-Concha, & Harbourne, 2016). The loss of phenolic compounds after heat treatments has been attributed to several factors including the thermal degradation of the phenolic structures and their release in the cooking medium, especially during boiling (Palermo et al., 2014; Zhao et al., 2019). On the other hand, the sometimes observed increased phenolic content upon cooking has been attributed to a matrix softening effect. The latter condition was a consequence of the breakdown of vegetables structure as well as to the disruption of cell walls and the release of fiber-bound phenolic compounds (Palermo et al.,

2014; Zhao et al., 2019). All these phenomena may result in an increased and easier extractability of phenolic compounds.

Eggplant is an important and typical component of the Mediterranean cuisine being part of several traditional recipes and an important source of bioactive compounds (Gürbüz, Uluişik, Frary, Frary, & Doǵanlar, 2018). The most abundant phenolic compounds identified in eggplant were hydroxycinnamic acid-derivatives (mainly conjugated with quinic acids or amides) and anthocyanins (mainly delphinidin-derivatives) (Gürbüz et al., 2018; Niño-Medina, Urías-Orona, Muy-Rangel, Heredia, 2017). Eggplant has been suggested to have plenty of health benefits, playing a significant role in the prevention of chronic diseases (Gürbüz et al., 2018). Several beneficial effects have been ascribed to eggplant phenolic compounds on human health, including antioxidant, anti-carcinogenic, anti-diabetic, anti-inflammatory and cardio-protective activities (Gürbüz et al., 2018).

Despite its importance in the Mediterranean Diet and the fact that eggplant must be consumed exclusively after cooking, only few studies have been published on the effect of cooking methods on eggplant phenolic compounds. These studies mainly focused on the behaviour of chlorogenic acid suggesting that some cooking methods such as frying and grilling increased its concentration whereas boiling had an insignificant or slightly negative effect (Lo Scalzo et al., 2016; Ramírez-Anaya, Samaniego-Sánchez, Castañeda-Saucedo, Villalón-Mir, & López-García de la Serrana, 2015). Lo Scalzo et al. (2016) also found that both grilling and boiling caused a marked decrease in total anthocyanins content. However, a comprehensive picture of the behaviour of individual phenolic compounds during eggplant cooking is still missing in literature.

In addition, to be absorbed and reach the bloodstream, phenolic compounds have to be released from the solid food matrix during gastro-intestinal digestion (Del Rio, Rodriguez-Mateos, Spencer, Tognolini, Borges, & Crozier, 2013). Because the cooking methods may differently affect the softening and breaking of the cellular components, the cooking procedures could also have an impact on the subsequent release of phenolic compounds during gastro-intestinal digestion (Miglio e al., 2008).

We hypothesized that cooking technologies may differentially affect the stability and release of phenolic compounds from dark purple eggplant. In addition, due to the different effect of cooking on the food matrix, the subsequent gastro-intestinal digestion of dark purple eggplant subjected to the different treatments could result in a different profile of phenolic compounds potentially available for intestinal absorption. Therefore, the aim of the present study was to monitor the stability and release of eggplant phenolic compounds during different cooking procedures as well as the bioaccessibility during *in vitro* gastro-intestinal digestion to understand the crucial link between cooking method, the release and the bioaccessibility of eggplant phenolic compounds.

2. Materials and methods

2.1. Materials

Analytical reagents for total phenolic determination, antioxidant activity analysis and enzymes for the digestion procedure (α-amylase from porcine pancreas, pepsin from porcine gastric mucosa and pancreatin from porcine pancreas) were purchased from Sigma-Aldrich (Milan, Italy). The solvents for phenolic extraction and mass spectrometry were obtained from BioRad (Hercules, CA, USA). Dark purple eggplant samples (*Solanum melongena*) were purchased in a local supermarket (Reggio Emilia, Italy).

2.2. Cooking treatments

The four most common thermal treatments used to cook eggplants (boiling, grilling, frying and baking) were used. Each cooking experiment was carried out in three different trials. In each cooking trial, a single eggplant sample was weighed, washed, dried with paper towels and cut in slices of 1.0 ± 0.1 cm (approximatively 20 slices). After that, one portion (corresponding to about half the slices) was retained as the raw sample and immediately frozen at -80°C. The other portion was immediately cooked, as described below. All the cooking parameters are summarized in **Table 1**. For the boiling procedure, eggplant slices were added to boiling tap water in a covered stainless-steel pot (1:5 eggplant/water ratio) and cooked for 20 min at 100°C. At the end of the cooking, samples were drained off for 30 sec. Grilling treatment was carried out on a grilling plate where the eggplant slices were cooked at 120°C for 10 min. In the baking treatment, eggplant slices were placed in a steel tray and cooked for 30 min at 180°C in an electric oven. Frying was carried out by adding eggplant slices to sunflower oil in a domestic deep fryer at 170°C for 10 min. At the end of the frying procedure, eggplant slices were drained off and dabbed with paper towels to absorb the oil in excess. After all the thermal treatment, the cooked eggplant slices were weighed to monitor the loss of water and stored at -80°C until analysis.

Table 1. Cooking parameters and weight loss during eggplant cooking

	Baking	**Boiling**	**Frying**	**Grilling**
Slice thickness (cm)	1.0	1.0	1.0	1.0
Cooking temperature (°C)	180	100	170	120
Cooking time (min)	30	20	10	10
Initial weight (g)	260	230	272	233
Final weight (g)	106	222	213	125
Weight loss (%)	59.2	3.5	21.7	46.4

2.3. Extraction of phenolic compounds from raw and cooked dark purple eggplant samples

The extraction of free phenolic compounds from raw and cooked eggplant samples was carried out following the procedure reported in Martini, Conte, and Tagliazucchi (2017). For each cooking trial, three raw and three cooked slices were randomly selected and homogenized in a water/methanol/formic acid (28:70:2, v/v/v) solution with an Ultra-Turrax homogenizer for 2 min in a ratio 1:2 eggplant/extraction solution. The mixtures were then incubated for 30 min at 37°C, centrifuged (6000g, 20 min, 4 C) and the collected supernatant filtered on paper.

2.4. In vitro digestion of raw and cooked dark purple eggplant samples

For the *in vitro* digestion experiments, the method developed within the COST Action INFOGEST was applied (Minekus et al., 2014). All the gastro-intestinal fluids were prepared according to the INFOGEST protocol. The digestion procedure consisted of three consecutive phases. For each cooking trial, three raw and three cooked slices were randomly collected form the pool at –80°C and homogenized with a blender while still frozen. After that, 5 g of homogenized samples were added to 5 mL of simulated salivary fluid (containing 150 U/mL of porcine α-amylase) to create the oral step of the digestion. The samples were then incubated for 5 min at 37°C in a rotating wheel (10 rpm) before adding 10 mL of simulated gastric fluid (containing 2000 U/mL of pepsin) reproducing the gastric step of the digestion. In the gastric phase, the bolus was incubated for 120 min at 37°C in a rotating wheel (10 rpm). The last step of the digestion procedure, mimicking the intestinal phase, was carried out by adding 10 mL of pancreatic fluid (containing pancreatin, 200 U/mL based on trypsin activity) and 5 mL of bile fluid and incubating the chyme for further 120 min at 37°C in a rotating wheel (10 rpm). At the end of the digestion, an aliquot of the samples was collected for further analytical determinations.

All the digestion were carried out in triplicate.

Bioaccessibility index was calculated as reported in Martini, Conte, and Tagliazucchi (2019):

$$\text{Bioaccessibility} = \frac{\text{C}d}{\text{C}e} * 100$$

C*d* is the concentration of phenolic compounds after *in vitro* digestion whereas C*e* is the concentration in the methanolic extract.

2.5. Determination of total phenolic compounds and antioxidant activity in raw and cooked dark purple eggplant methanolic extracts and digested samples

Total phenolic content was quantified using the Folin-Ciocalteau assay (Singleton, Orthofer, & Lamuela-Raventos, 1999). Data were expressed as mg of gallic acid equivalent per 100 g of eggplant. The total antioxidant activity was determined by using the ABTS assay as reported in Re, Pellegrini, Proteggente, Pannala, Yang, and Rice-Evans (1999). The reducing capacity was quantified through the ferric reducing/antioxidant power (FRAP) assay (Benzie, & Strain, 1999). Results were expressed as mg of ascorbic acid equivalent per 100 g of eggplant.

2.6. Identification of phenolic compounds in raw and cooked dark purple eggplant methanolic extracts and digested samples by liquid chromatography electrospray ionization ion trap mass spectrometer (LC-ESI-IT-MS)

Identification of individual phenolic compounds was carried out on a HPLC Agilent 1200 Series (Agilent Technologies, Santa Clara, CA, USA) system provided with a C18 column (HxSil C18 Reversed phase, 250×4.6 mm, 5 µm particle size, Hamilton company, Reno, Nevada, USA) coupled with an Agilent 6300 ion trap mass spectrometer. The mobile phases and the elution gradient were described in detail in Martini, Cavalchi, Conte, Tagliazucchi (2018). Negative and positive ESI-MS parameters were the same as reported in Martini et al. (2017). Identification of phenolic compounds in the samples was carried out using full scan, data-dependent MS^2 scanning from *m/z* 100 to 1500.

Phenolic compounds were quantified by using representative standards for each identified phenolic class as reported in Martini et al. (2017). Hydroxycinnamic acids were quantified as ferulic, caffeic or coumaric acid equivalents. Flavonols were quantified as quercetin-3-*O*-rutinoside equivalents and anthocyanins were quantified as delphinidin-3-*O*-glucoside or cyanidin-3-*O*-glucoside equivalents.

2.7. Statistics

All data are presented as mean ± SD for three replicates for each prepared sample. Univariate analysis of variance (ANOVA) with Tukey's post-hoc test was applied using Graph Pad prism 6.0 (GraphPad Software, San Diego, CA, U.S.A.) when multiple comparisons were performed. The differences were considered significant with $P < 0.05$.

3. Result and discussion

3.1. Total phenolic content and antioxidant properties of raw and cooked dark purple eggplant methanolic extracts

Total phenolic compounds were extracted from raw and cooked eggplant samples and analysed by three different methodologies (Folin-Ciocalteau, ABTS and FRAP assays) for assessing the total phenolic content, the radical scavenging activity and the ferric reducing ability. In this section, the results are reported and discussed as mg of standard equivalents/100 g of fresh weight both for raw and cooked eggplant samples (**Figure 1**).

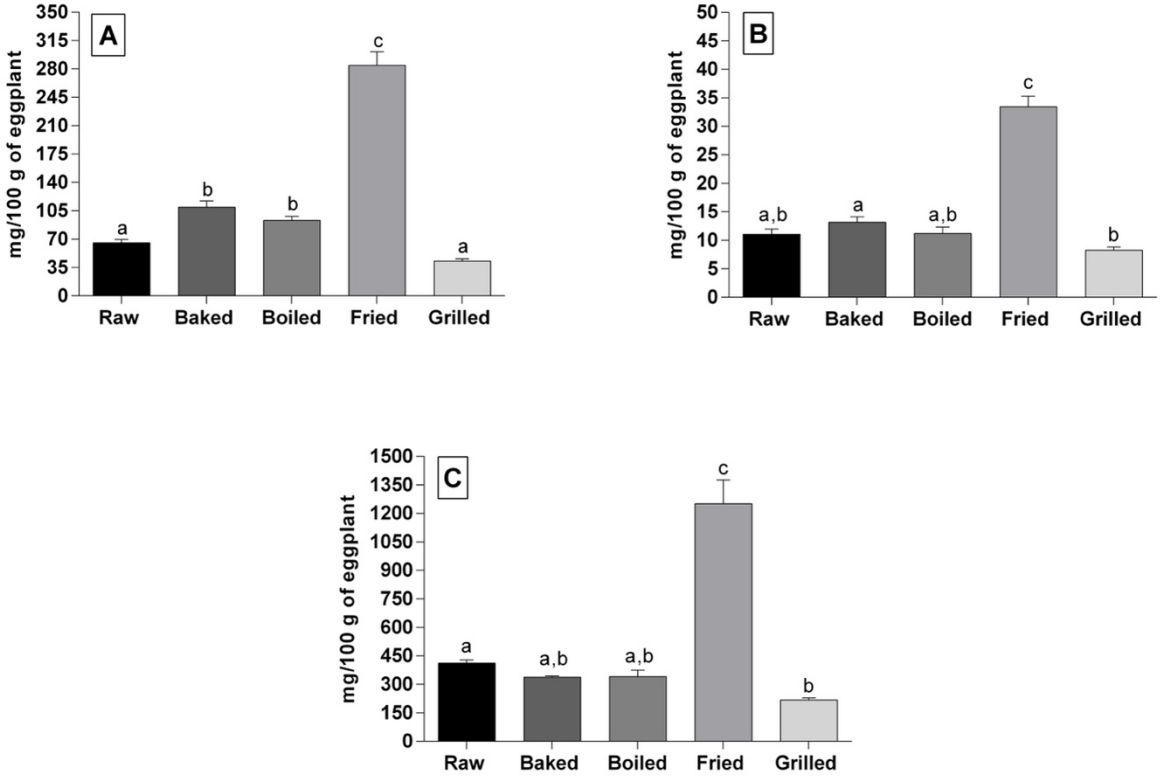


Figure 1. Effect of different cooking methods on the total phenolic compounds and antioxidant properties of dark purple eggplant. (A) Total phenolic compounds measured with the Folin-Ciocalteau assay. Results are expressed as mg of gallic acid equivalent/100 g of raw eggplant. (B) Radical scavenging activity measured with the ABTS assay. Results are expressed as mg of ascorbic acid equivalent/100 g of raw eggplant. (C) Reducing ability measured with the FRAP assay. Results are expressed as mg of ascorbic acid equivalent/100 g of raw eggplant. Different letters indicate that the values are significantly different ($P < 0.05$).

This means that the values were corrected for the weight loss due to the cooking procedure (**Table 1**) in order to get information on the effects of the cooking methods on the degradation/release of total phenolic and antioxidant compounds.

The total amount of phenolic compounds in raw eggplant phenolic-rich fraction was 65.46 ± 4.31 mg gallic acid/100 g of eggplant. Grilling resulted in a significant decrease (34.5%; $P<0.05$) compared to raw eggplant. By contrast, all of the other cooking treatments determined a significant increase ($P<0.05$) of total extractable phenolic compounds (**Figure 1A**). The increase was more pronounced after frying (>300%), followed by

baking (67%) and boiling (42%). The apparent increase in total phenolic compounds may be a consequence of the matrix softening effect of thermal treatment, which disrupted the cellular structure facilitating the release of phenolic compounds (Palermo et al., 2014; Zhao et al., 2019). Indeed, the release of free phenolic compounds from bound structures may partially account for the observed increment in extractable phenolic compounds (Rocchetti et al., 2017).

Several previous studies reported a reduction in the amount of total phenolic compounds after boiling, baking and frying (Giallourou et al., 2016; Murador et al., 2016; Murador et al., 2018; Palermo et al., 2014). Whereas, some other authors reported an increase in total phenolic content after the same thermal treatments (Moyo et al., 2020; Palermo et al., 2014; Pellegrini et al., 2010). The different cooking parameters (such as cooking temperature, cooking time and surface area of the vegetable exposed to the air or water) and/or the distinct food matrices may account for the large variability in the reported results (Palermo et al., 2014; Murador et al., 2018). According to the results reported in the present study, boiling and, especially, frying increased the recovery of total phenolic compounds in eggplants (Ramírez-Anaya et al., 2015). Therefore, it seemed that the cooking effect on total phenolic compounds was strictly related to the tested food matrix. Less attention has been paid to the grilling procedure. In contrast to the data reported in this study, Lo Scalzo et al. (2016) found an increase in the total phenolic content after eggplant grilling. **Figures 1B** and **1C** show the effect of cooking on the antioxidant activity of eggplant phenolic compounds tested with the ABTS and FRAP assays. Frying resulted in significant higher ABTS and FRAP values respect to the raw eggplants ($P<0.05$), whereas grilling induced a significant decrease in the antioxidant activity values with both the assays compared to the raw samples ($P<0.05$). No significant differences were found between baked, boiled and raw eggplant samples with both the assays ($P>0.05$).

3.2. Total phenolic content and antioxidant properties of in vitro digested samples

The effect of the *in vitro* digestion on the total phenolic compounds and antioxidant activity of raw and cooked eggplant samples is shown in **Figure 2**. No significant differences were found on the amount of total phenolic compounds after digestion of raw, boiled and baked eggplant (**Figure 2A**). For these samples, the bioaccessibility of total phenolic compounds was 112.5%, 93.4% and 101.8%, suggesting that all the phenolic compounds were released after *in vitro* digestion. Fried eggplant samples showed the highest amount of released total phenolic compounds after digestion but the lowest bioaccessibility (66.2%). On the contrary, grilled eggplant samples showed the highest total phenolic compounds bioaccessibility (217.4%). The data obtained with the ABTS and FRAP assays were consistent with those recorded with the Folin-Ciocalteau assay (**Figure 2B** and **C**). The only exceptions were for digested boiled sample in the ABTS assay and digested raw sample in the FRAP assay. In the first case, the radical scavenging activity measured after digestion was significantly higher than that detected in the methanolic extract (bioaccessibility of 336.4%). This could be due to the release of ABTS-reactive (but not Folin-Ciocalteau- or FRAP-reactive) compounds during the

digestion of boiled eggplant, which were not extracted with the solvents. Similarly, the lower FRAP value measured after digestion of raw eggplant respect to the value measured in the methanolic extract (24% of bioaccessibility) may be caused by the failure to extract the reducing compounds during digestion. The bioaccessibility values greater than 100% measured in all the assays for grilled sample may be due to the formation during cooking of water-soluble Maillard reaction products (Nicoli, Anese, & Parpinel, 1999). These compounds were easily released in the aqueous media of the digestive fluids but poorly soluble in the solvents used for the chemical extraction of phenolic compounds.

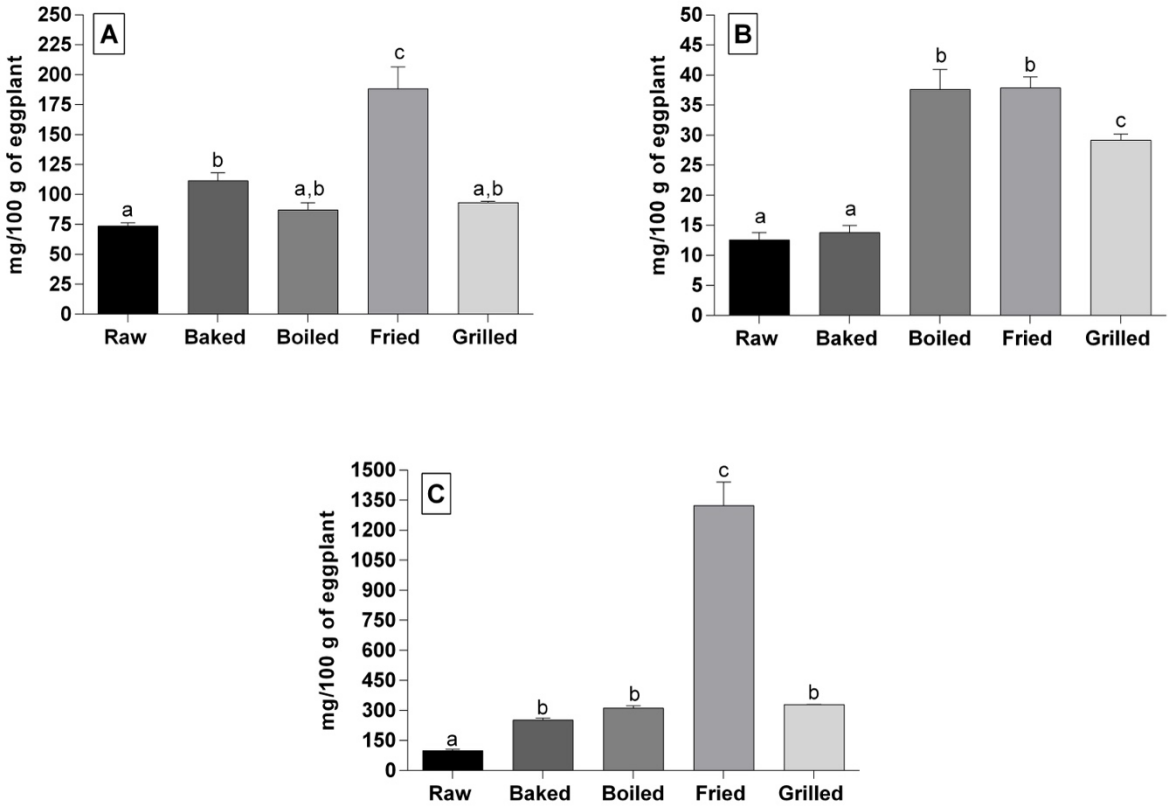


Figure 2. Effect of different cooking methods on the total phenolic compounds and antioxidant properties of dark purple eggplant after in vitro digestion. (A) Total phenolic compounds measured with the Folin-Ciocalteau assay. Results are expressed as mg of gallic equivalent/100 g of raw eggplant. (B) Radical scavenging activity measured with the ABTS assay. Results are expressed as mg of ascorbic acid equivalent/100 g of raw eggplant. (C) Reducing ability measured with the FRAP assay. Results are expressed as mg of ascorbic acid equivalent/100 g of raw eggplant. Different letters indicate that the values are significantly different ($P < 0.05$).

3.3. Phenolic profile of raw dark purple eggplant

The analysis of the methanolic extract of raw eggplant revealed the presence of 31 phenolic compounds (mainly hydroxycinnamic acid-derivatives and flavonols) and 6 anthocyanins identified in negative and positive ionization mode, respectively. The mass spectral data of the identified compounds are reported in supplementary **Tables S1** and **S2**, whereas the identified compounds, together with the quantitative data, are listed in **Table 2**. Most of the identified compounds have been already detected in eggplant samples (Gürbüz et al., 2018; Niño-Medina et al., 2017).

Table 2. Amount of phenolic compounds in raw and cooked eggplant. Results are expressed in mg of phenolic compound/100 of raw eggplant. Bioaccessibility index (BI) is the percentage ratio between the concentration after in vitro gastro-intestinal digestion and the concentration in the methanolic extract.

	Eggplant									
Compound	***Raw***		***Baked***		***Grilled***		***Fried***		***Boiled***	
	Before digestion	BI (%)	Before digestion	BI (%)	Before digestion	BI (%)	Before digestion	BI (%)	Before digestion	BI (%)
Hydroxycinnamic acids										
3-*O*-Caffeoylquinic acid	7.62 ± 2.52 [a]	8.46	18.26 ± 3.02 [b]	172.69	6.60 ± 1.08 [a]	672.49	42.24 ± 3.25 [c]	70.23	87.65 ± 7.28 [d]	29.24
5-*O*-Caffeoylquinic acid *trans*	307.06 ± 19.41 [a]	n.d.	146.35 ± 34.20 [b]	43.22	175.95 ± 9.99 [b]	48.31	321.02 ± 54.90 [c]	28.39	293.57 ± 71.71 [c]	30.20
5-*O*-Caffeoylquinic acid *cis*	140.54 ± 33.04 [a]	0.19	43.77 ± 1.89 [b]	10.78	68.23 ± 9.44 [c]	49.18	121.94 ± 10.53 [a]	7.41	117.02 ± 12.52 [a]	7.15
3-*O*-Feruloylquinic acid *cis*	2.85 ± 2.15 [ab]	n.d.	2.10 ± 0.37 [a]	n.d.	0.14 ± 0.10 [b]	n.d.	1.87 ± 0.40 [a]	1338.55	2.15 ± 0.64 [a]	n.d.
3-*O*-Feruloylquinic acid *trans*	24.21 ± 3.10 [a]	n.d.	14.55 ± 0.76 [b]	61.10	11.61 ± 7.60 [b]	15.64	23.36 ± 2.19 [a]	n.d.	20.88 ± 12.26 [ab]	n.d.
4-*O*-Feruloylquinic acid *cis*	53.03 ± 28.35 [a]	n.d.	20.50 ± 0.68 [b]	13.88	16.98 ± 4.26 [bc]	n.d.	36.90 ± 2.31 [a]	8.62	10.14 ± 3.64 [c]	n.d.
5-*O*-Feruloylquinic acid *trans*	18.96 ± 5.78 [a]	n.d.	4.88 ± 1.36 [b]	n.d.	5.51 ± 2.36 [b]	n.d.	11.10 ± 0.87 [c]	n.d.	11.84 ± 4.72 [ac]	n.d.
5-*O*-Feruloylquinic acid *cis*	3.47 ± 0.53 [a]	n.d.	0.56 ± 0.33 [b]	n.d.	0.63 ± 0.15 [b]	n.d.	2.91 ± 1.11 [a]	n.d.	0.28 ± 0.39 [b]	n.d.
3,4-Di-*O*-caffeoylquinic acid	7.89 ± 2.30 [a]	21.76	1.94 ± 2.75 [b]	615.65	3.51 ± 0.17 [b]	150.11	63.69 ± 3.67 [c]	145.75	24.22 ± 7.03 [d]	n.d.
4,5-Di-*O*-caffeoylquinic acid	160.44 ± 11.07 [a]	4.48	31.14 ± 9.82 [b]	23.88	28.52 ± 21.41 [bd]	7.37	318.28 ± 25.70 [c]	37.86	47.80 ± 3.16 [d]	n.d.
3,5-Di-*O*-caffeoylquinic acid	76.68 ± 45.86 [a]	18.26	2.97 ± 4.20 [b]	502.21	7.67 ± 6.81 [b]	117.21	129.99 ± 1.66 [c]	71.44	37.92 ± 1.65 [a]	n.d.
Caffeoylquinic acid dehydrodimer isomer	29.95 ± 2.64 [a]	381.14	77.25 ± 2.51 [b]	80.70	146.09 ± 10.41 [c]	17.43	36.27 ± 0.53 [d]	28.15	161.42 ± 42.69 [c]	5.95
Caffeoylquinic acid dehydrodimer isomer	10.95 ± 0.50 [a]	283.20	18.55 ± 0.78 [b]	112.61	45.28 ± 11.59 [c]	14.13	n.d.	n.d.	33.78 ± 3.77 [c]	33.54
Caffeoylquinic acid dehydrodimer isomer	6.63 ± 0.20 [a]	453.70	28.61 ± 0.90 [b]	79.90	57.01 ± 16.64 [c]	5.91	n.d.	n.d.	37.89 ± 6.50 [c]	8.92
N^1,N^5-Di-caffeoyl-spermidine	26.60 ± 5.39 [a]	n.d.	6.29 ± 0.84 [b]	21.53	6.23 ± 0.22 [b]	39.74	31.35 ± 8.27 [a]	4.48	17.32 ± 5.96 [a]	n.d.
N^1,N^{10}-Di-caffeoyl-spermidine	128.26 ± 50.50 [a]	n.d.	48.75 ± 9.91 [b]	1.25	29.22 ± 6.82 [c]	6.21	273.90 ± 1.39 [d]	0.06	86.65 ± 5.13 [a]	3.84
N^1,N^5-Dihydrocaffeoyl-caffeoyl-spermidine	182.23 ± 28.30 [a]	11.44	76.06 ± 37.01 [b]	10.60	77.19 ± 42.50 [b]	8.36	668.72 ± 73.18 [c]	14.44	44.82 ± 6.55 [b]	47.28
N^1,N^5-Di-dihydrocaffeoyl-spermidine	57.81 ± 3.96 [a]	0.34	5.65 ± 0.25 [b]	28.42	5.63 ± 0.90 [b]	27.65	55.59 ± 3.49 [a]	n.d.	12.75 ± 1.23 [c]	2.99
N^1,N^{10}-Dihydrocaffeoyl-caffeoyl-spermidine	226.95 ± 16.65 [a]	0.39	56.06 ± 16.98 [b]	0.62	43.71 ± 10.17 [b]	26.38	482.47 ± 16.96 [c]	n.d.	55.17 ± 2.69 [b]	1.40
Caffeoyl-hexose	1.55 ± 0.84 [a]	138.91	0.93 ± 1.01 [ab]	22.56	n.d.	n.d.	0.17 ± 0.24 [b]	268.68	0.46 ± 0.65 [ab]	n.d.
Caffeoylshikimic acid	10.73 ± 8.99 [a]	n.d.	1.97 ± 1.15 [ab]	n.d.	1.49 ± 1.23 [ab]	12.98	1.19 ± 0.91 [ab]	n.d.	0.35 ± 0.13 [b]	120.43
Caffeoyl-tryptophan-hexoside isomer	14.93 ± 1.0 [a]	20.79	n.d.	n.d.	0.82 ± 1.17 [b]	259.73	5.40 ± 0.78 [c]	29.57	1.31 ± 0.21 [b]	125.94
Caffeoyl-tryptophan-hexoside isomer	20.55 ± 5.03 [a]	23.67	n.d.	n.d.	2.50 ± 1.97 [b]	58.29	32.03 ± 3.89 [c]	15.64	3.78 ± 0.82 [b]	39.73
5-*O*-Coumaroylquinic acid *trans*	7.12 ± 3.24 [a]	n.d.	4.00 ± 0.62 [a]	43.13	3.19 ± 0.76 [ab]	31.55	2.54 ± 0.76 [b]	n.d.	4.76 ± 0.31 [a]	32.25
5-*O*-Coumaroylquinic acid *cis*	1.92 ± 0.08 [a]	n.d.	0.09 ± 0.13 [b]	711.37	0.76 ± 0.97 [bc]	20.85	0.55 ± 0.44 [bc]	7.11	0.86 ± 0.16 [c]	14.95
Total hydroxycinnamic acids	**1528.94**	**15.14**	**611.26**	**44.51**	**744.48**	**33.93**	**2664.04**	**22.03**	**1114.78**	**15.96**

Flavonols										
Quercetin-3-*O*-rutinoside	0.07 ± 0.00 [a]	42.00	0.03 ± 0.00 [b]	6462.80	0.02 ± 0.02 [b]	222.64	0.04 ± 0.01 [b]	229.88	0.06 ± 0.00 [a]	n.d.
Quercetin-di-*O*-hexoside	0.09 ± 0.01 [a]	n.d.	0.03 ± 0.01 [b]	n.d.	n.d.	n.d.	n.d.	n.d.	0.16 ± 0.08 [a]	n.d.
Quercetin-3-*O*-hexoside	0.04 ± 0.01 [a]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.02 ± 0.01 [b]	n.d.
Kaempferol-3-*O*-rutinoside	0.17 ± 0.00 [a]	59.55	0.05 ± 0.03 [b]	6143.01	0.05 ± 0.01 [b]	28.47	0.07 ± 0.01 [b]	73.86	0.05 ± 0.03 [b]	n.d.
Kaempferol-3-*O*-hexoside	0.05 ± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Myricetin-3-*O*-rutinoside	0.46 ± 0.24 [a]	n.d.	0.25 ± 0.02 [a]	498.16	0.17 ± 0.01 [b]	13.42	1.04 ± 0.08 [c]	3.95	0.44 ± 0.08 [a]	n.d.
Total flavonols	**0.88**	**14.75**	**0.37**	**1784.21**	**0.25**	**35.63**	**1.15**	**16.61**	**0.74**	**0.00**
Anthocyanins										
Delphinidin-3-*O*-hexoside	1.42 ± 0.21 [a]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.61 ± 0.3 [b]	n.d.
Delphinidin-3-*O*-rutinoside	57.77 ± 4.60 [a]	n.d.	3.58 ± 1.54 [b]	n.d.	8.18 ± 6.80 [b]	n.d.	23.82 ± 1.23 [c]	n.d.	34.28 ± 16.60 [c]	n.d.
Delphinidin-3-*O*-rutinoside-5-*O*-hexoside isomer	6.82 ± 1.02 [a]	n.d.	1.95 ± 0.48 [b]	n.d.	2.38 ± 1.27 [bc]	n.d.	3.69 ± 0.02 [c]	n.d.	3.24 ± 0.73 [c]	n.d.
Delphinidin-3-*O*-rutinoside-5-*O*-hexoside isomer	1.54 ± 0.06 [a]	n.d.	0.36 ± 0.15 [b]	n.d.	0.02 ± 0.03 [c]	n.d.	n.d.	n.d.	1.00 ± 0.54 [ab]	n.d.
Cyanidin-3-*O*-rutinoside isomer	0.26 ± 0.37 [a]	n.d.	n.d.	n.d.	n.d.	n.d.	0.05 ± 0.01 [b]	n.d.	n.d.	n.d.
Cyanidin-3-*O*-rutinoside isomer	1.67 ± 0.35 [a]	n.d.	0.24 ± 0.27 [b]	n.d.	0.27 ± 0.14 [b]	n.d.	n.d.	n.d.	n.d.	n.d.
Total anthocyanins	**69.49**	**0.00**	**6.14**	**0.00**	**10.85**	**0.00**	**27.56**	**0.00**	**39.12**	**0.00**
Total	**1599.31**	**14.48**	**617.77**	**45.11**	**755.57**	**33.44**	**2692.75**	**21.80**	**1154.64**	**15.41**

Different letters within the same row mean significant different (*P<0.05*) values.
n.d. means that the compound was not detected in the sample

However, to the best of our knowledge, three hydroxycinnamic acid-derivatives and one anthocyanin have been identified for the first time in eggplant in this study. Compound eluting at 10.7 min, showed a negative molecular ion $[M-H]^-$ at *m/z* 341 and MS^2 ion fragments at *m/z* 179 and 135. In comparison with previously published data, this compound was tentatively identified as caffeoyl-hexose (Martini et al., 2017). Two signals at *m/z* 337, eluting at 12.8 and 13.4 min, presented the same fragmentation pattern (MS^2 fragments at *m/z* 191, 173 and 163) and were identified as the *trans* and *cis* isomers of 5-*O*-coumaroylquinic acid (Martini et al., 2017). Two signals at *m/z* 527 eluted at 8.1 and 8.5 min. They showed the same MS^2 fragments at *m/z* 365 (loss of 162 amu, corresponding to a hexosyl moiety) and 161 (loss of 204 amu from the fragment at *m/z* 365 corresponding to a tryptophan molecules). In comparison with previous data, these compounds were tentatively identified as caffeoyl-tryptophan-hexoside isomers (Barros, Dueñas, Dias, Sousa, Santos-Buelga, & Ferreira, 2012). Finally, two signals with positive ions $[M]^+$ at *m/z* 595 eluted at 10 and 14 min. Their fragmentation spectra MS^2 were characterized for the presence of an intense signal at *m/*z 287 (corresponding to the cyanidin aglycone generated from the loss of a rutinosyl moiety; 308 amu) and a less intense signal at *m/z* 449 (from the loss of a rhamnose unit; 146 amu). These compounds were therefore tentatively identified as cyanidin-3-*O*-rutinoside isomers (Martini et al., 2017).

From a quantitative point of view, hydroxycinnamic acid-derivatives were the major phenolic compounds, which made up eggplant phenolic profile (**Table 2**). They account for the 95.60% of total identified phenolic compounds in eggplant. Anthocyanins represented the 4.34% of total phenolic compounds, whereas flavonols only the 0.06%. Among the hydroxycinnamic acids, 5-*O*-caffeoyquinic acid *trans* was found to be present at the highest concentration (20.08% of total hydroxycinnamic acids and 19.20% of total phenolic compounds) followed by N^1N^{10}-dihydrocaffeoyl-caffeoyl-spermidine (14.84% of total hydroxycinnamic acids and 14.19% of total phenolic compounds) and N^1N^5-di-dihydrocaffeoyl-spermidine (11.92% of total hydroxycinnamic acids and 11.39% of total phenolic compounds). All together, these three compounds accounted for about the 45% of total phenolic compounds in eggplant. Di-caffeoylquinic acids were also found in appreciable amount with the isomer 4,5-di-caffeoylquinic acid being the most representative (10.49% of total hydroxycinnamic acids and 10.03% of total phenolic compounds). The most important anthocyanin was delphinidin-3-*O*-rutinoside, which accounted for the 83.13% of total anthocyanins but only for the 3.61% of total phenolic compounds.

Interestingly, several isomers of caffeoylquinic acid dehydrodimer were also identified in raw eggplant sample. These compounds are well-known oxidation products of caffeoylquinic acids produced by the action of polyphenol oxidase (Castillo-Fraire, Poupard, Guilois-Dubois, Salas, & Guyot, 2019).

The total phenolic content, calculated as the sum of the amounts of the individual compounds quantified by mass spectrometry, in raw eggplant was 1599.31 mg/100 g fresh weight.

Large variations in total and individual phenolic content have been detected in eggplant samples depending on the variety, genotype and agro-environmental conditions (Whitaker, & Stommel, 2003; Plazas et al., 2013; Lo Scalzo et al., 2016).

3.4. Effect of cooking methods on dark purple eggplant phenolic profile

Table 2 also shows the effects of the different cooking methods on the phenolic profile of eggplant. As already stated above, also in this section the results are reported and discussed as mg of compound/100 g of fresh weight either for raw or cooked eggplant samples.

Considering the total amount of phenolic compounds (sum of the concentrations of the individual compounds identified by mass spectrometry), frying was the only cooking method that caused a significant increase ($P<0.05$) in extractable phenolic compounds (+68.4%). By contrast, the highest loss of phenolic compounds was observed after baking (61.4% loss), followed by grilling (52.8% loss) and boiling (27.8% loss). In this regard, the results attained by mass spectrometry experiments were quite different with respect to that obtained with the Folin-Ciocalteau assay. This discrepancy may be due to the formation of Maillard reaction products during cooking which react with the Folin-Ciocalteau's phenol reagent.

In the present study, a significant increase ($P<0.05$) in total hydroxycinnamic acids was observed only after the frying treatment (74.2% of increase compared to raw eggplant). Instead, a decrease of 27.1%, 51.3%, and

60.0% in total hydroxycinnamic acids was observed for boiling, grilling, and baking treatments, respectively, compared to raw eggplant. Previous research showed that frying and boiling the artichoke and cardoon promoted the release of caffeoylquinic and di-caffeoylquinic acids (Ferracane et al., 2008; Juániz et al., 2016a). In contrast, boiling but not baking or frying sweet potatoes caused a decrease in hydroxycinnamic acids (Sun, Mu, Xi, & Song, 2014). Similarly, boiling and grilling broccolini resulted in a significant reduction in the hydroxycinnamic acids content (Llorente-Martínez, Ortega-Vidal, Ruiz-Riaguas, Ortega-Barraless, & Fernández-de Córdova, 2020). As already suggested by Rothwell et al. (2015), hydroxycinnamic acids stability and release during cooking seemed to be more related to the food rather than to the process itself, with exception of frying, which resulted in an increase in hydroxycinnamic acids independently on the food type. The increase in total hydroxycinnamic acids after frying was almost completely due to the increase in N^1N^{10}-dihydrocaffeoyl-caffeoyl-spermidine, N^1N^5-di-dihydrocaffeoyl-spermidine, 4,5-di-*O*-caffeoylquinic acid and N^1N^5-di-caffeoyl-spermidine. Previous studies found an increase in di-caffeoylquinic acids after several cooking treatments in artichoke, cardoon and boiled potatoes (Ferracane et al., 2008; Juániz et al., 2016a).

As can be observed in **Table 2**, changes in concentrations of hydroxycinnamic acids after frying treatment may be the consequence of both the isomerization and hydrolysis reactions. This is particularly evident for di-caffeoylquinic acids and hydroxycinnamic acid-amides whose concentrations sharply increased after frying. Both di-caffeoylquinic acids and hydroxycinnamic acid-amides can be present in free form in vegetables but also covalently or ionically linked to other macromolecules such as polysaccharides or proteins (Monente, Ludwig, Irigoyen, De Peña, & Cid, 2015a; Moreau, Nuñez, & Singh, 2001). It can be supposed that frying may affect the cell-wall structure of eggplant, leading to depolymerization of polysaccharides and promoting the release of bound phenolic compounds.

Massive trans-esterification event occurred in the case of caffeoylquinic acids not only after frying but also after boiling and baking. High temperature promoted intramolecular trans-esterification of 5-*O*-caffeoylquinic acid to 3-*O*-caffeoylquinic acid as already observed during artichoke thermal treatments (Ferracane et al., 2008). During grilling and baking, a 45-55% loss of caffeoylquinic acids was observed, with the concurrent formation of the oxidation products caffeoylquinic acid dehydrodimers. In the context of baking and grilling, caffeoylquinic acids may also have taken part in the Maillard reaction, contributing to the observed reduction of caffeoylquinic acids levels.

Caffeoylquinic acid dehydrodimers were also generated during boiling; however, their formation was not associated to a decreasing content of caffeoylquinic acids. The hydrolysis of di-caffeoylquinic acids or the release of bound caffeoylquinic acids may explain this effect (Juániz et al., 2016a; Monente et al., 2015a).

Anthocyanins were quite sensitive to any heath treatments since their stability is inversely related to the temperature (Cavalcanti, Santos, & Meireles, 2011). The highest loss found after baking (91.2% loss) followed by grilling (84.4% loss), frying (60.3% loss) and boiling (43.7% loss) (**Table 3**). Noteworthy, the highest temperature was applied during baking whereas the lowest during boiling (**Table 1**). Interestingly, frying

induced a lower loss than the grilling treatment, even if higher temperature have been applied (170°C). This may be a consequence of the different rheological parameters and reaction mechanisms of the treatments themselves.

Table 3. Amount of phenolic compounds in cooked eggplant after in vitro gastro-intestinal digestion.
Results are expressed in mg of phenolic compound/100 of cooked eggplant.

	Eggplant			
Compound	***Baked***	***Grilled***	***Fried***	***Boiled***
		Hydroxycinnamic acids		
3-*O*-Caffeoylquinic acid	77.27 ± 0.24 [a]	82.97 ± 4.94 [a]	37.98 ± 1.26 [b]	26.66 ± 0.67 [c]
4-*O*-Caffeoylquinic acid	13.87 ± 0.06 [a]	13.96 ± 0.70 [a]	8.95 ± 0.34 [b]	n.d.
5-*O*-Caffeoylquinic acid *trans*	154.96 ± 0.10 [a]	158.96 ± 1.52 [b]	116.66 ± 0.98 [c]	92.22 ± 1.80 [d]
5-*O*-Caffeoylquinic acid *cis*	11.56 ± 0.28 [a]	62.75 ± 2.37 [b]	11.57 ± 0.13 [a]	8.70 ± 0.32 [c]
3-*O*-Feruloylquinic acid *cis*	n.d.	n.d.	32.02 ± 15.63	n.d.
3-*O*-Feruloylquinic acid *trans*	21.78 ± 12.86 [a]	3.39 ± 4.30 [b]	n.d.	n.d.
4-*O*-Feruloylquinic acid *cis*	n.d.	n.d.	n.d.	n.d.
5-*O*-Feruloylquinic acid *trans*	6.97 ± 5.87 [a]	n.d.	4.07 ± 0.11 [a]	n.d.
5-*O*-Feruloylquinic acid *cis*	n.d.	n.d.	n.d.	n.d.
3,4-Di-*O*-caffeoylquinic acid	29.30 ± 3.78 [a]	9.86 ± 0.25 [b]	118.83 ± 2.91 [c]	n.d.
4,5-Di-*O*-caffeoylquinic acid	18.22 ± 1.57 [a]	3.93 ± 0.15 [b]	154.51 ± 22.38 [c]	n.d.
3,5-Di-*O*-caffeoylquinic acid	36.58 ± 0.54 [a]	16.80 ± 2.30 [b]	118.87 ± 18.66 [c]	n.d.
Caffeoylquinic acid dehydrodimer isomer	152.74 ± 6.74 [a]	47.62 ± 0.16 [b]	13.07 ± 2.07 [c]	9.98 ± 0.37 [c]
Caffeoylquinic acid dehydrodimer isomer	51.18 ± 3.74 [a]	11.96 ± 0.88 [b]	n.d.	11.78 ± 0.19 [b]
Caffeoylquinic acid dehydrodimer isomer	56.01 ± 1.73 [a]	6.31 ± 0.25 [b]	n.d.	3.51 ± 0.36 [c]
N^1,N^5-Di-caffeoyl-spermidine	3.32 ± 0.09 [a]	4.63 ± 0.46 [b]	1.80 ± 0.06 [c]	n.d.
N^1,N^{10}-Di-caffeoyl-spermidine	1.49 ± 0.00 [a]	3.39 ± 0.12 [b]	0.20 ± 0.01 [c]	3.46 ± 0.20 [b]
N^1,N^5-Dihydrocaffeoyl-caffeoyl-spermidine	19.74 ± 2.49 [a]	12.06 ± 0.75 [b]	123.60 ± 44.03 [c]	22.04 ± 1.25 [a]
N^1,N^5-Di-dihydrocaffeoyl-spermidine	3.94 ± 0.28 [a]	2.91 ± 0.18 [b]	n.d.	0.40 ± 0.03 [c]

N^1,N^{10}-Dihydrocaffeoyl-caffeoyl-spermidine	0.85 ± 0.01 [a]	21.56 ± 0.58 [b]	n.d.	0.81 ± 0.01 [a]
Caffeoyl-hexose	0.51 ± 0.04 [a]	n.d.	0.59 ± 0.03 [b]	n.d.
Caffeoylshikimic acid	n.d.	0.36 ± 0.04 [a]	n.d.	0.44 ± 0.02 [b]
Caffeoyl-tryptophan-hexoside isomer	0.46 ± 0.11 [a]	4.01 ± 0.10 [b]	2.04 ± 0.69 [c]	1.72 ± 0.32 [c]
Caffeoyl-tryptophan-hexoside isomer	n.d.	2.73 ± 0.25 [a]	6.41 ± 0.41 [b]	1.56 ± 0.23 [c]
5-*O*-Coumaroylquinic acid *trans*	4.22 ± 0.00 [a]	1.88 ± 0.17 [b]	n.d.	1.60 ± 0.02 [b]
5-*O*-Coumaroylquinic acid *cis*	1.65 ± 0.20 [a]	0.30 ± 0.02 [b]	0.05 ± 0.00 [c]	0.13 ± 0.00 [d]
Total hydroxycinnamic acids	**666.64**	**472.34**	**751.21**	**185.00**
		Flavonols		
Quercetin-3-*O*-rutinoside	5.11 ± 0.84 [a]	0.10 ± 0.00 [b]	0.13 ± 0.00 [b]	n.d.
Quercetin-di-*O*-hexoside	n.d.	n.d.	n.d.	n.d.
Quercetin-3-*O*-hexoside	n.d.	n.d.	n.d.	n.d.
Kaempferol-3-*O*-rutinoside	7.92 ± 0.00 [a]	0.03 ± 0.00 [b]	0.07 ± 0.00 [c]	n.d.
Kaempferol-3-*O*-hexoside	n.d.	n.d.	n.d.	n.d.
Myricetin-3-*O*-rutinoside	3.10 ± 0.09 [a]	0.04 ± 0.00 [b]	0.05 ± 0.01 [c]	n.d.
Total flavonols	**16.13**	**0.17**	**0.25**	**n.d.**
Total	**682.77**	**472.51**	**751.46**	**185.00**

Different letters within the same row mean significant different (*P<0.05*) values.
n.d. means that the compound was not detected in the sample

In this study, eggplant slices were completely soaked in the frying medium (sunflower oil) whereas eggplant slices were in contact with air and grid during grilling.

As suggested by Liao et al. (2019) the same treatment but different conditions may affect the anthocyanin content in vegetable. Specifically, they stated that air-fried and fried cooking induced the losses in total anthocyanin content of 45% and 32%, respectively in purple-fleshed sweet potatoes.

Flavonols were found to be quite unstable especially during baking and grilling. Quercetin-3-*O*-glucoside was not detected after baking, frying and grilling. Previously, it has been reported that quercetin-3-*O*-glucoside can be de-glycosylated to quercetin after thermal treatment (Rohn, Buchner, Driemel, Rauser, & Kroh, 2007). Moreover, the flavonol-3-*O*-rutinoside derivatives appeared to be more stable than the monoglucosides derivatives after each cooking treatment.

3.5. Effect of cooking methods on the bioaccessibility of dark purple eggplant phenolic compounds

Because cooking methods may induce structural changes in plant material, the bioaccessibility of individual eggplant phenolic compounds after the different cooking treatments was assessed thorough *in vitro* digestion and mass spectrometry experiments (**Table 2**). Despite the importance of the topic, few studies have been aimed to characterize the interplay between the applied cooking methods and the release and stability of phenolic compounds after *in vitro* digestion. To the best of our knowledge, no studies about eggplant *in vitro* digestion and bioaccessibility are present in literature.

In all the tested samples, a significant reduction in the concentration of phenolic compounds was ascertained after *in vitro* digestion, although with substantial differences (**Table 2**). In raw eggplant, the bioaccessibility was the lowest and only the 14.5% of the total amount of phenolic compounds was released during *in vitro* digestion. The low bioaccessibility index of raw eggplant phenolic compounds was mainly due to the poor release of caffeoylquinic and di-caffeoylquinic acids, which dominated the phenolic profile. Indeed, the about three-fold increase in the amount of caffeoylquinic acid dehydrodimer may suggest that polyphenol oxidase was still, at least partially, active during digestion.

Baked eggplant phenolic compounds showed the highest bioaccessibility index (45.1%) followed by grilling (33.4%) and frying (21.8%). The highest bioaccessibility of caffeoylquinic acids was found in grilled eggplant sample (68.0%) whereas di-caffeoylquinic acids bioaccessibility was maximum after baking (95.2%). Generally, hydroxycinnamic acid-amides were characterized for a low bioaccessibility independently of the thermal treatment.

In cooked cardoon, thermal treatments resulted in a higher bioaccessibility of phenolic compounds (mainly caffeoylquinic and di-caffeoylquinic acids) respect to the raw sample (Juániz et al., 2017).

Similarly, cooking of green pepper enhanced the bioaccessibility of caffeoylquinic acids and flavonoids respect to the raw sample (Juániz et al., 2016b). Therefore, it can be stated that cooking is of paramount importance for the release of phenolic compounds, especially caffeoylquinic and di-caffeoylquinic acids during digestion. Compared to raw food, cooking may promote the release of phenolic compounds through a matrix softening effect, enhancing the release or the hydrolysis of phenolic compounds from the food macromolecules (Palermo et al., 2014; Zhao et al., 2019).

As reported in **Table 2**, the only exception was found after boiling where the bioaccessibility index was similar to that found after digestion of raw eggplant. However, there were qualitative differences between the two samples. In fact, caffeoylquinic acids, representing about the 69% of total phenolic compounds, dominated the phenolic profile of digested boiled eggplant. As already reported above, during raw eggplant digestion, most of the released caffeoylquinic acids were oxidized to caffeoylquinic acid dehydrodimer by the action of polyphenol oxidase. In boiled eggplant sample, the heath treatment resulted in the inactivation of the enzyme, avoiding the oxidation of caffeoylquinic acids.

Trans-esterification reactions also occurred during *in vitro* digestion. After digestion of baked and grilled eggplant, caffeoylquinic acids were subjected to isomerization from the 5-acyl to 4- and 3-acyl structures. Instead, in fried sample the migration of the acyl group was observed only from 5- to 4-*O*-caffeoylquinic acid. It is important to note that the 4-acyl isomer was absent both in raw sample and in all cooked eggplant samples. Previous works found acyl migration phenomena during digestion from 5-caffeoylquinic acid to the 4- and 3-isomers (Monente, Ludwig, Stalmach, De Pena, Cid, & Crozier, 2015b). However, Martini et al. (2019) observed trans-esterification reactions during *in vitro* digestion of cherry from 3-*O*-caffeoylquinic and 5-*O*-caffeoylquinic acids to the 4-acyl isomer. Considering that acyl migration proceeds via an 1,2-*ortho*-ester intermediate formation until reaching an equilibrium between 5-*O*-caffeoylquinic acid ⇋ 4-*O*-caffeoylquinic acid ⇋ 3-*O*-caffeoylquinic acid, the initial concentration of the isomers seems to be of paramount importance in determining the direction of the acyl migration reaction (Deshpande, Jaiswal, Matei, & Kuhnert, 2014). Acyl migration was also observed for di-caffeoylquinic acids in baked and grilled eggplant samples after digestion with the 4,5-di-*O*-caffeoylquinic acid isomer transformed mainly into the 3,4- and 3,5-di-*O*-caffeoylquinic acid isomers. Previously, Deshpande et al. (2014) demonstrated acyl migration under alkaline conditions from 4,5-di-*O*-caffeoylquinic acid to 3,4-di-*O*-caffeoylquinic acid and from the latter to 3,5-di-*O*-caffeoylquinic acid. Moreover, they also found that both the 4,5- and the 3,4-acyl isomers were subjected to hydrolysis generating the mono-acyl isomers. The degradation of di-caffeoylquinic acids may have contributed to the increase in concentration in 3- and 4-*O*-caffeoylquinic acid isomers, especially in digested grilled eggplant.

Finally, no anthocyanins were detected in all the samples after *in vitro* digestion suggesting that cooking and digestion strongly degraded the anthocyanin structure.

3.6. Effect of cooking methods on the amount of dark purple eggplant phenolic compounds after digestion expressed as serving size

The expression of the results referred to 100 g of raw eggplant allowed to obtain information on the release and stability of the phenolic compounds during cooking and digestion. However, from a nutritional point of view, it is pivotal to express the results as a function of the serving size considering the real amount of phenolic compounds in the intestinal fluids. Hence, the link to the usual intake should give an idea of the compounds potentially available for the absorption or able to exert their action in the gastro-intestinal tract. This approach provides nutritional recommendations concerning what type of cooking method may be used for maximizing the intake of phenolic compounds from eggplant. **Table 3** shows the amount of individual phenolic compounds after *in vitro* digestion of eggplant, subjected to the different cooking procedures, referring to a standard serving size (100 g of cooked eggplant).

Considering the total amount of phenolic compounds determined by mass spectrometry, frying was the cooking process that gave the highest amount of phenolic compounds at the end of the *in vitro* digestion, followed by baking, grilling and boiling.

Looking more in detail at the data shown in **Table 3**, it is possible to see that the different cooking methods had a different impact on the release of individual phenolic compounds. Baking and, especially, grilling resulted in a significantly higher amount of caffeoylquinic acids respect to the fried and boiled samples ($P<0.05$). On the contrary, frying determined the highest release in di-caffeoylquinic acids and hydroxycinnamic acid-amides (**Figure 3**).

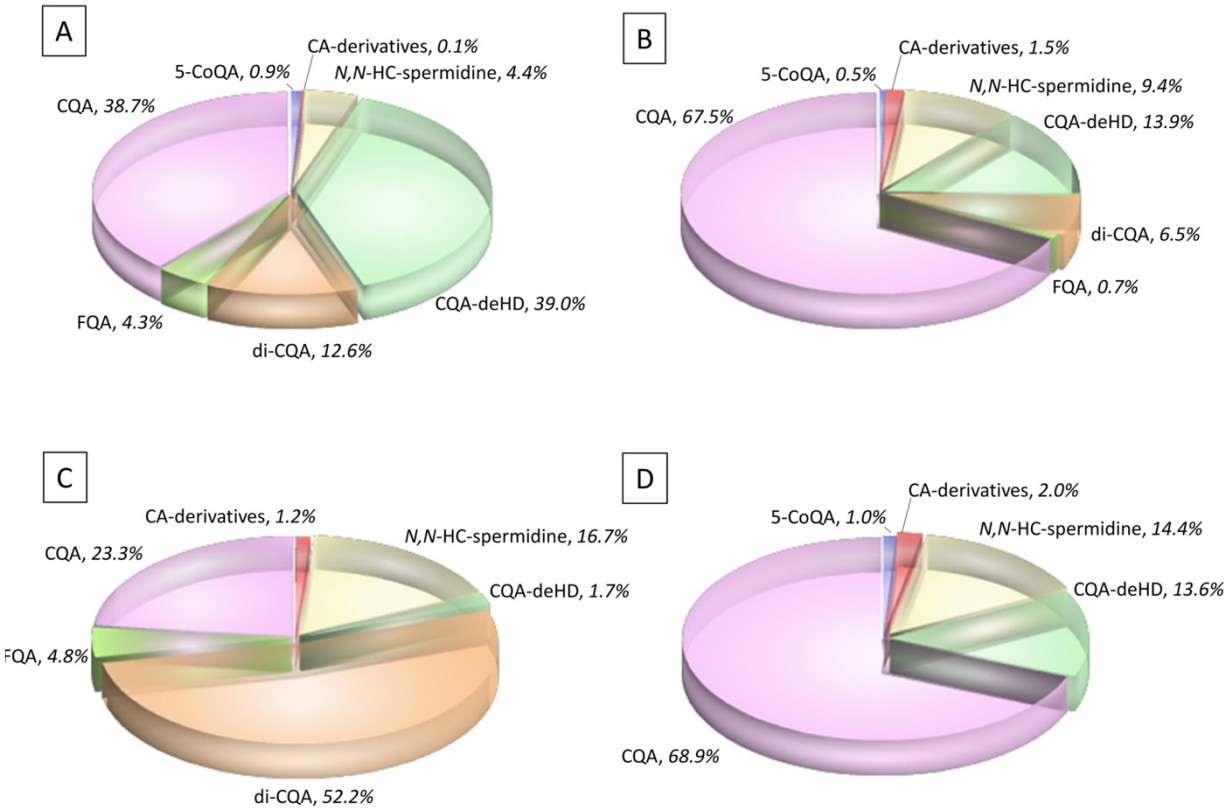


Figure 3. Percentage of individual hydroxycinnamic acids after in vitro digestion of cooked dark purple eggplant samples. The percentage values are referred to the total amount of hydroxycinnamic acids released after in vitro digestion expressed as mg of compound/100 g of cooked eggplant. The pie graphs underline the impact of digestion on the release of individual hydroxycinnamic acids. The cooking procedures were baking (A), grilling (B), frying (C) and boiling (D). CQA: caffeoylquinic acids; CoQA: coumaroylquinic acids; FQA: feruloylquinic acids; di-CQA: di-caffeoylquinic acids; N,N-HC-spermidine: hydroxycinnamic acid-spermidine derivatives; CQA-deHD: caffeoylquinic acid dehydrodimers; CA-derivatives: other caffeic acid derivatives.

4. Conclusions

This study provides evidence that cooking treatments have an important impact on the phenolic compounds stability and their following release during digestion. With the exception of frying, cooking processes resulted in the degradation of individual phenolic compounds. Compounds more susceptible to thermal degradation were hydroxycinnamic acid-amides and di-caffeoylquinic acids. On the contrary, monomeric caffeoylquinic acids were more stable during cooking. However, heat treatments were of paramount importance in the release of phenolic compounds from food matrices during digestion. In this context, heating processes (especially frying, grilling and baking) significantly increased the bioaccessibility of dark purple eggplant phenolic compounds promoting their release from the food matrix. This represent a pivotal result, since dark purple eggplant must be consumed only after cooking due to the presence of its typical toxic glycoalkaloids. Moreover, either cooking or digestion of dark purple eggplant induced isomerization reactions of caffeoylquinic and di-caffeoylquinic acids, which determined changes among the several stereoisomeric forms. Nevertheless, the different cooking procedures variedly affected the release of individual phenolic compounds. Whereas frying strongly fostered the release of di-caffeoylquinic acids and hydroxycinnamic acid-amides, grilling and baking were more effective in the release of caffeoylquinic acids. It is important to note that different eggplant cultivars and genotypes may behave differently during thermal treatments and digestion. These results indicated that distinct cooking methods might result in a different biological effect after dark purple eggplant consumption. Therefore, an accurate design of the cooking method may be useful to suggest the most appropriate process to maximize the amount of bioaccessible phenolic compounds and to modulate their release, making them available for the intestinal absorption or to exert their bioactivity in the gastro-intestinal tract.

5. Supplementary material

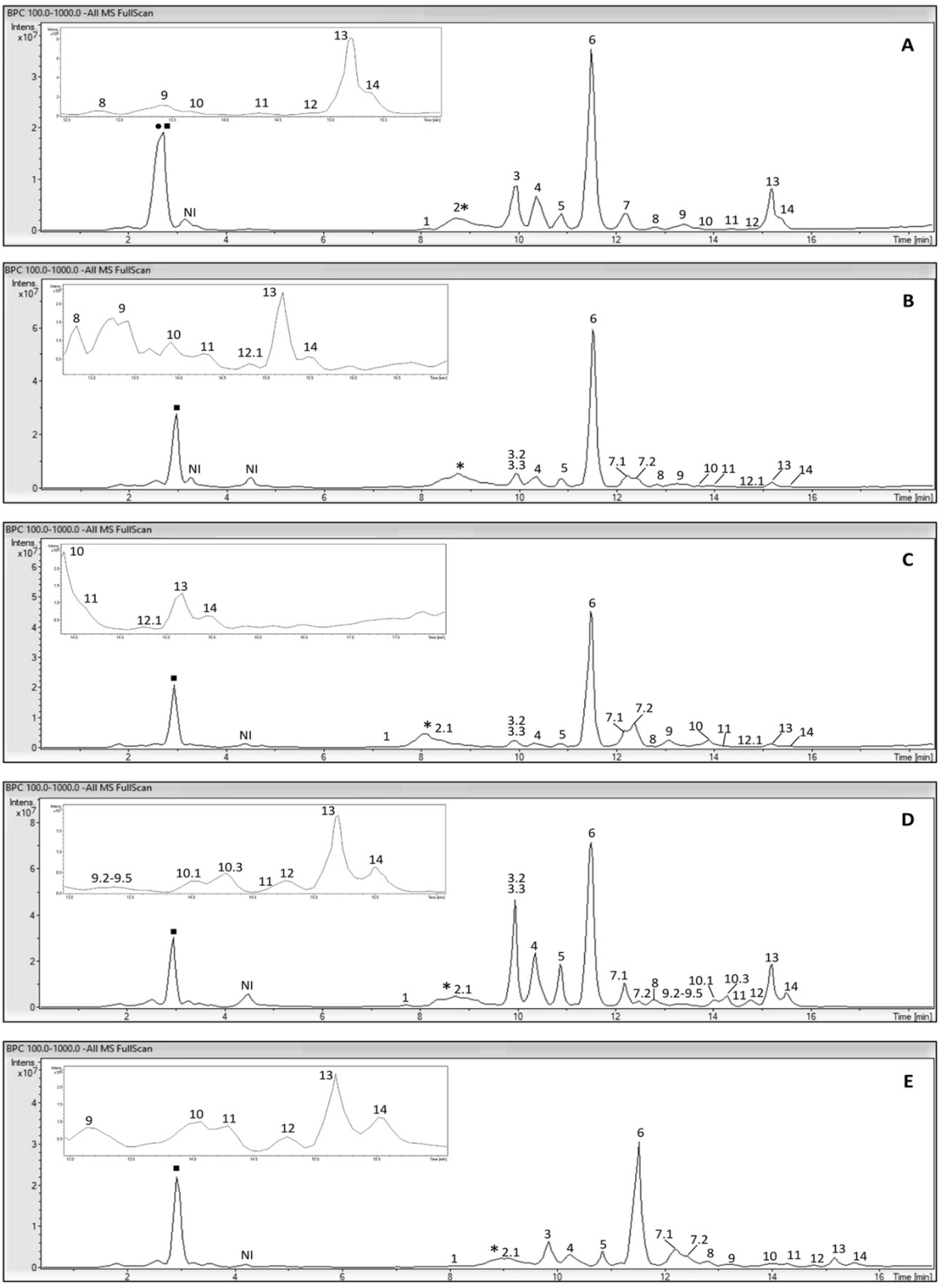


Figure S1. Representative negative ion mode base peak chromatograms (BPCs) of raw and cooked dark purple eggplant. The shown BPCs are representative of three independent experiments and represent the profile of the methanol extracts**.** (A) Raw dark purple eggplant methanol extract. (B) Baked dark purple eggplant methanol extract. (C) Grilled dark purple eggplant methanol extract. (D) Fried dark purple eggplant methanol extract. (E) Boiled dark purple eggplant methanol extract. The code number for the different peaks is reported in Table S1. ●Disaccharides; ■Quinic acid; *UDP-hexoside; n.i. means not identified compound in the peak.

Table S1. Mass spectral data of phenolic compounds identified in eggplant sample in negative ionization mode.

Peak N		Rt	Compound	$[M-H]^-$ *(m/z)*	MS^2 ion fragments *(m/z)*
1		8.1	Caffeoyl-tryptophan-hexoside isomer	527	161 (100%), 365 (70%), 421 (12%), 235 (13%), 483 (12%)
2	2.1	8.5	Caffeoyl-tryptophan-hexoside isomer	527	161 (100%), 365 (85%), 421 (16%), 235 (5%), 483 (14%)
	2.2	9.0	Quercetin-di-*O*-hexoside	625	463 (100%), 301 (25%)
	3.1	10.0	Quercetin-3-*O*-hexoside	463	300 (100%), 301 (85%)
3	3.2	10.0	N^1,N^5-Dihydrocaffeoyl-caffeoyl-spermidine	470	308 (100%), 334 (99%)
	3.3	10.0	N^1,N^5-Di-dihydrocaffeoyl-spermidine	472	308 (100%), 350 (25%), 332 (8%)
4	4.1	10.2	3-*O*-Caffeoylquinic acid	353	191 (100%), 179 (44%), 135 (17%)
	4.2	10.4	N^1,N^{10}-Dihydrocaffeoyl-caffeoyl-spermidine	470	334 (100%), 291 (6%)
	4.3	10.5	N^1,N^5-Di-caffeoyl-spermidine	468	332 (100%), 289 (18%)
5	5.1	10.7	Caffeoyl-hexose	341	233 (100%), 179 (90%), 281 (45%)
	5.2	10.9	N^1,N^{10}-Di-caffeoyl-spermidine	468	332 (100%), 289 (18%), 135 (11%), 306 (4%)
6		11.5	5-*O*-Caffeoylquinic acid *trans*	353	191 (100%), 173 (11%)
7	7.1	12.2	5-*O*-Caffeoylquinic acid *cis*	353	191 (100%)
	7.2	12.4	Caffeoylquinic acid dehydrodimer isomer	705	513 (100%), 339 (5%), 295 (1%)
8	8.1	12.8	Myricetin-3-*O*-rutinoside	625	316 (100%), 271 (6%), 179 (8%), 431 (9%), 358 (4%), 179 (89%)
	8.2	12.8	Caffeoylshikimic acid	335	179 (89%), 135 (30%)
	8.3	12.8	5-*O*-Coumaroylquinic acid *trans*	337	191 (100%), 179 (63%), 135 (15%)
	8.4	12.8	3-*O*-Feruloylquinic acid *cis*	367	193 (100%), 134 (26%)
	9.1	13.1	Caffeoylquinic acid dehydrodimer isomer	705	513 (100%), 339 (6%)
9	9.2	13.3	3-*O*-Feruloylquinic acid *trans*	367	193 (100%), 134 (14%)
	9.3	13.4	5-*O*-Coumaroylquinic acid *cis*	337	191 (100%), 179 (75%), 299 (11%), 163 (5%)
	9.4	13.5	5-*O*-Feruloylquinic acid *trans*	367	161 (100%), 135 (43%), 191 (21%), 161 (18%)
	9.5	13.5	Quercetin-3-*O*-rutinoside	609	301 (100%), 271 (7%)
10	10.1	13.7	4-*O*-Feruloylquinic acid	367	173 (100%)
	10.2	13.8	Caffeoylquinic acid dehydrodimer isomer	705	513 (100%), 351 (3%), 339 (2%)
	10.3	13.9	5-*O*-Feruloylquinic acid *cis*	367	179 (100%), 135 (45%), 191 (34%), 161 (7%)
11		14.3	Kaempferol-3-*O*-rutinoside	593	285 (100%), 257 (56%)
12	12.1	15.0	3,4-Di-*O*-caffeoylquinic acid	515	353 (100%), 173 (13%), 191 (8%), 335 (7%)
	12.2	15.0	Kaempferol-3-*O*-hexoside	447	285 (100%), 255 (23%)
13		15.2	4,5-Di-*O*-caffeoylquinic acid	515	353 (100%), 179 (4%), 191 (2%)
14		15.4	3,5-Di-*O*-caffeoylquinic acid	515	353 (100%), 191 (22%), 179 (11%)

Table S2. Mass spectral data of phenolic compounds identified in eggplant sample in positive ionization mode.

Rt	Compound	$[M]^+$ *(m/z)*	MS^2 ion fragments *(m/z)*
8.30	Delphinidin-3-*O*-rutinoside-5-*O*-hexoside isomer	773	303 (100%), 465 (32%)
8.90	Delphinidin-3-*O*-rutinoside-5-*O*-hexoside isomer	773	465 (100%), 303 (62%), 611 (33%)
9.40	Delphinidin-3-*O*-hexoside	465	303 (100%)
9.60	Delphinidin-3-*O*-rutinoside	611	303 (100%), 465 (26%)
10.00	Cyanidin-3-*O*-rutinoside isomer	595	287 (100%), 449 (20%)
14.00	Cyanidin-3-*O*-rutinoside isomer	595	287 (100%), 449 (84%)

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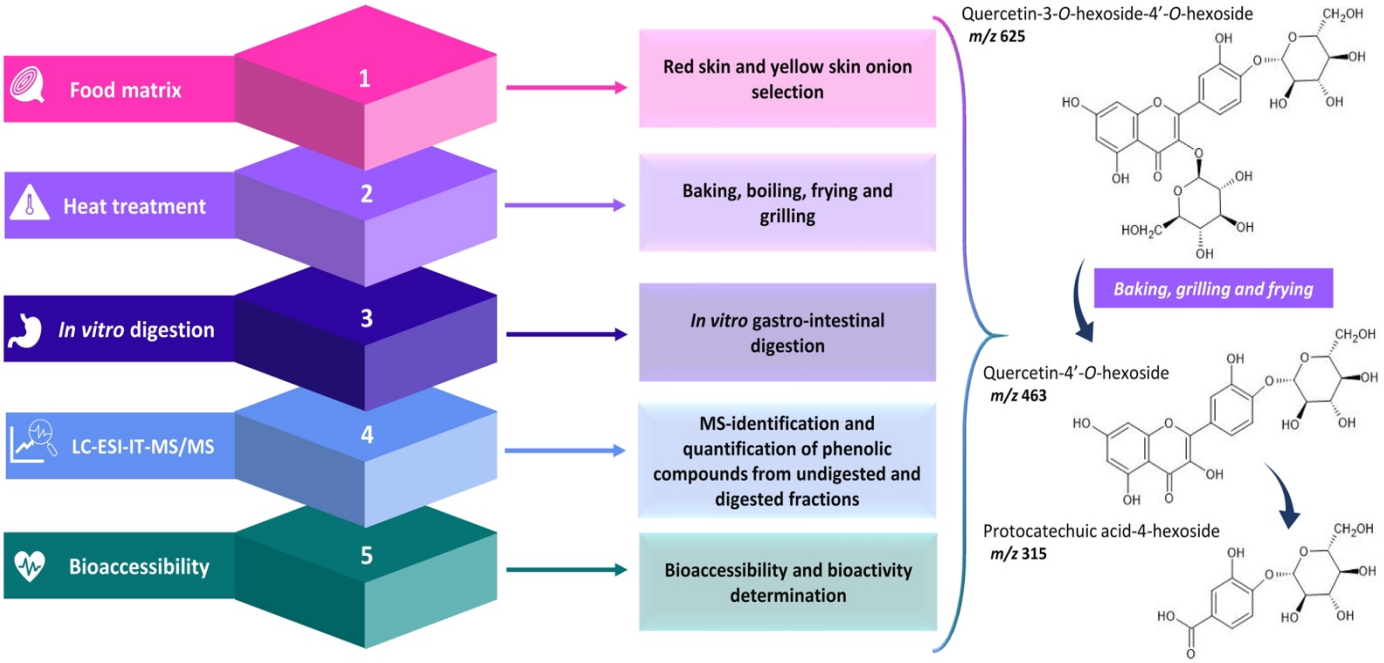
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A. Cattivelli, A. Conte, S. Martini, D. Tagliazucchi. Influence of cooking methods on onion phenolic compounds bioaccessibility. *Foods*, **2021**, *10*, 1023. doi: 10.3390/foods10051023

INFLUENCE OF COOKING METHODS ON ONION PHENOLIC COMPOUNDS BIOACCESSIBILITY

ABSTRACT

The impact of domestic cooking (baking, boiling, frying and grilling) and in vitro digestion on the stability and release of phenolic compounds from yellow-skinned (YSO) and red-skinned onions (RSO) have been evaluated. The mass spectrometry identification pointed out flavonols as the most representative phenolic class, led by quercetin-derivatives. RSO contained almost the double amount of phenolic compounds respect to YSO (50.12 and 27.42 mg/100 g, respectively). Baking, grilling and primarily frying resulted in an increased amount of total phenolic compounds, especially quercetin-derivatives, in both the onion varieties. Some treatments promoted the degradation of quercetin-3-*O*-hexoside-4'-*O*-hexoside, the main compound present in both the onion varieties, leading to the occurrence of quercetin-4'-*O*-hexoside and protocatechuic acid-4-*O*-hexoside. After in vitro digestion, the bioaccessibility index for total phenolic compounds ranged between 42.6% and 65.5% in grilled and baked YSO, respectively, and between 39.8% and 80.2% in boiled and baked RSO, respectively. Baking contributed to the highest amount of bioaccessible phenolic compounds for both the onion varieties after in vitro digestion. An in-depth design of the cooking process may be of paramount importance in modulating the gastro-intestinal release of onion phenolic compounds.

Keywords: mass spectrometry; food processing; metabolomics; polyphenols; in vitro digestion; thermal treatments

1. Introduction

Epidemiological data and human intervention trials have highlighted the protective effect of a polyphenol-rich food diet against the onset of chronic and non-communicable diseases including cardiovascular diseases and metabolic disorders [1,2]. Among fresh vegetables, onion was the second most cultivated, accounting for 8.1% of the European Union total fresh vegetables [3]. In recent years, several in vivo studies have described the beneficial effects of allium vegetables (including onion) consumption on cardiovascular diseases, hypertension and cancer [4,5].

The most important bioactive components found in onion are organosulfur and phenolic compounds, especially flavonols and, in the red varieties, anthocyanins [6,7].

Quercetin-derivatives are the major onion flavonols, with quercetin 3,4'-diglucoside and quercetin 4'-glucoside representing about the 90% of the total flavonols content [8]. Minor components such as kaempferol-and isorhamnetin-derivatives have also been detected in onion [6,7]. The onion flavonols content strongly depends on the variety. Red and yellow onion varieties may contain up to 1 g/kg of fresh weight of flavonols whereas the white onion variety displays a lower amount of flavonols (7 mg/kg) [6,8]. Red onion variety was also characterized by the presence of anthocyanins, mainly cyanidin-derivatives [7,9]. Flavonols have been reported to have a wide range of biological effects including antioxidant activity, protective effects against the onset of cardiovascular diseases, anti-diabetic and also anti-proliferative activities [6,10].

Onion can usually be consumed raw or following different cooking procedures such as boiling, frying, grilling and baking. Cooking processes may induce substantial changes on the physical structure of foods, which in turn result in a matrix softening effect due to a heat-induced breakdown of vegetables structure [11,12]. As a result, the extractability of phenolic compounds may increase after cooking due to the cell walls lysis and the release of fiber-bound phenolic compounds [11,12]. On the other hand, sometimes cooking may negatively affect the stability of phenolic compounds. Some phenolic structures may be thermolabile and are likely to experience thermal degradation during cooking [13]. Otherwise, hydrophilic phenolic compounds may be lost during cooking, especially during boiling, and released into cooking medium [11]. Cooking may also promote interactions between phenolic compounds and additional components in the food matrix such as proteins, fibers and lipids [14,15]. During high-temperature processes (such as grilling and baking) phenolic compounds may react with fibers and proteins in the Maillard reaction becoming part of the melanoidins structure and decreasing their content in the food [14,15].

The effect of cooking on phenolic compounds stability is dependent on a fine balance between their degradation or loss, their reaction with food matrix components and the increased extractability due to the matrix softening effect. This equilibrium strongly relies upon the cooking conditions (such as time, temperature and cooking medium), the food matrix and the phenolic structure [11,12].

Particular attention has been paid to the effect of cooking on onion phenolic compounds stability and extractability [16–21]. In all the studies, boiling was the thermal treatment that caused the highest loss of flavonols [17–21]. Conflicting results have been obtained when frying procedure was used. For example, Rodrigues et al. [17] did not find any effect of frying on flavonols content whereas Juaniz et al. [16] detected an increased extractability of flavonols after frying. By contrast, other authors found a small decrease in the onion flavonols content after frying [19,20]. Regarding the other cooking procedures, baking was found to have no or a slight negative effect on onion flavonols whereas grilling caused an increase in extractable flavonols concentration [16,17,21]. In red onion varieties, anthocyanins were always degraded independently of the thermal treatment but with different intensities [17].

To exert their positive health effects, phenolic compounds should be bioavailable, i.e., absorbed in the gastro-intestinal tract and reach the blood stream. The bioavailability of phenolic compounds strongly depends on their bioaccessibility, which can be defined as the fraction of phenolic compounds released from the food matrix in the gastro-intestinal tract [22]. Bioaccessibility quantification of phenolic compounds after digestion is of paramount importance for an appropriate evaluation of their intake, which is likely to be overestimated by considering only the mere phenolic content in the food [23]. Since the bioaccessibility is highly dependent on the phenolic compounds extractability, the different cooking procedures may have a pivotal impact on their bioaccessibility. For example, the application of distinct cooking methods resulted in a differential release of individual phenolic compounds in eggplant. Baked and grilled eggplant sample displayed the highest amount of bioaccessible caffeoylquinic acids whereas fried eggplant samples were rich in di-caffeoylquinic acids [13]. In this context, information on the effect of cooking on the bioaccessibility of onion phenolic compounds is still lacking.

Therefore, the objective of the present research was to assess the effect of the four different and most common cooking methods on the stability, bioaccessibility and antioxidant activity of phenolic compounds from two onion varieties (yellow-skinned and red-skinned onion) after cooking and in vitro gastro-intestinal digestion.

2. Materials and Methods

2.1. Materials

The reagents for total phenolic and antioxidant activity analysis as well as the materials and enzymes for the in vitro digestion were purchased from Sigma-Aldrich (Milan, Italy). Methanol, formic acid and acetonitrile for phenolic extraction and mass spectrometry analysis were obtained from BioRad (Hercules, CA, USA). Ferulic acid (catalogue number 52229), caffeic acid (catalogue number 51868), protocatechuic acid (catalogue number 03930590), quercetin-3-*O*-glucoside (catalogue number 16654), epicatechin (catalogue number 68097) and cyanidin-3-*O*-glucoside (catalogue number PHL89616) were supplied by Sigma-Aldrich (Milan, Italy). Yellow-skinned (YSO) and red-skinned onion (RSO) samples (*Allium cepa* L.) were purchased in a local supermarket (Reggio Emilia, Italy).

2.2. Onion Cooking

Four different procedures (i.e., baking, boiling, frying and grilling) were applied to cook onion. Each cooking experiment was carried out in three different trials as reported in Martini et al. [13]. Before cooking experiments, onions were peeled and cut longitudinally into thin slices of approximatively 0.3 cm. Briefly, for the baking procedure, onion was placed in a steel tray and cooked for 30 min at 180 °C in an electric oven. In the boiling treatment, onion was added to boiling tap water in a covered stainless-steel pot (1:5 onion/water ratio) and cooked for 30 min. After cooking, samples were drained for 30 sec. Frying treatment was performed by adding onion to sunflower oil in a domestic deep fryer at 140 °C for 8 min. Grilling was carried out on a two-sided electric grill and the onion was cooked at 110 °C for 15 min. Before and after all the thermal treatments, the peeled and chopped onion was weighed to monitor the water loss caused by the cooking and samples stored at −80 °C until analysis.

All the cooking parameters are summarized in **Table 1**.

Table 1. Cooking parameters and weight loss during cooking of yellow-skinned (YSO) and red-skinned onion (RSO).

	YSO				RSO			
	Baking	**Boiling**	**Frying**	**Grilling**	**Baking**	**Boiling**	**Frying**	**Grilling**
Cooking temperature (°C)	180	100	140	110	180	100	140	110
Cooking time (min)	30	30	8	15	30	30	8	15
Initial weight (g)	173	129	200	170	133	129	158	155
Final weight (g)	57	49	126	80	48	79	90	88
Weight loss (%)	67	62	37	53	64	38	43	43
Initial/final weight ratio	3.04	2.63	1.59	2.13	2.77	1.63	1.76	1.76

2.3. Preparation of Phenolic Compounds Methanol Extracts from Raw and Cooked YSO and RSO

The phenolic compounds extraction procedure was carried out following the method described by Martini, Conte, and Tagliazucchi [24]. Briefly, 15 g of raw and cooked onion samples were homogenized in 30 mL of a water/methanol/formic acid (28:70:2, *v*/*v*/*v*) solution. After 30 min of incubation at 37 °C, the homogenates were centrifuged (6000*g*, 20 min, 4 °C) and the collected supernatant stored at −20 °C until further analysis.

2.4. In Vitro Gastro-Intestinal Digestion of Raw and Cooked Onion Samples

The in vitro digestion procedure was carried out applying the COST Action INFOGEST protocol [25]. The in vitro gastro-intestinal digestion comprised three sequential steps. The oral step was carried out by mixing 5 g of chopped raw or cooked onion with 5 mL of simulated salivary fluid added of 150 U/mL of salivary α-amylase. After homogenization, the oral bolus was incubated for 5 min at 37 °C in a rotating wheel (10 rpm). The gastric phase was performed by adding 10 mL of simulated gastric fluid (containing 2000 U/mL of pepsin) to the oral bolus and incubated for 2 h at 37 °C in a rotating wheel (10 rpm). Then, 15 mL of the intestinal fluid (containing pancreatin, 200 U/mL based on trypsin activity) were added to the gastric bolus, mimicking the final intestinal phase. After 2 h of incubation at 37 °C in a rotating wheel (10 rpm), an aliquot of the samples was withdrawn. After centrifugation (10,000 g; 20 min; 4 °C), the supernatants were collected and stored at −20 °C until further analysis.

The digestion experiments were performed in triplicate.

Bioaccessibility index (BI) was determined as follow:

$$\text{Bioaccessibility } (\%) = \frac{\text{Cd}}{\text{Ce}} * 100 \qquad (1)$$

Cd is the concentration of phenolic compounds in the supernatants collected at the end of the in vitro gastro-intestinal digestion whereas Ce is the concentration in the methanol/formic acid extract.

2.5. Determination of Total Phenolic Compounds and Antioxidant Activity in Raw and Cooked Onion Samples

Total phenolic compounds were quantified in the methanol extracts and in vitro digested samples of raw and cooked onion by using the Folin-Ciocalteu assay [26]. Data were reported as mg of gallic acid equivalent per 100 g of onion fresh weight. The antioxidant activity was measured as total radical scavenging activity and ferric reducing/antioxidant power by using the ABTS and FRAP assays, respectively [27,28]. Results were expressed as mg of ascorbic acid equivalent per 100 g of onion fresh weight.

2.6. Liquid Chromatography Electrospray Ionization Ion Trap Mass Spectrometry (LC-ESI-IT-MS) Profiling of Phenolic Compounds in Raw and Cooked Onion Samples

Phenolic compounds profiling of methanol/formic acid extracts and in vitro digested samples of raw and cooked onion was carried out by using a liquid chromatography system (HPLC Agilent 1200 Series, Agilent Technologies, Santa Clara, CA, USA) coupled with an Agilent 6300 ion trap mass spectrometer. Separation was performed with a C18 column (HxSil C18 Reversed phase, 250 × 4.6 mm, 5 µm particle size, Hamilton Company, Reno, Nevada, USA) by using a binary gradient of water/formic acid and acetonitrile. The mobile phase composition, the flow rate, the elution gradient, the negative and positive ESI-MS parameters as well as the quantification method are fully described in Martini et al. [24,29] and Mekam et al. [30].

Ferulic acid-, caffeic acid- and protocatechuic acid-derivatives were quantified by using ferulic acid, caffeic acid and protocatechuic acid as standard references, respectively; flavonols and di-hydro-flavonols were quantified in quercetin-3-*O*-glucoside equivalents; flavan-3-ols were quantified by using epicatechin as standard compound; cyanidin-3-*O*-glucoside was used as reference compound for anthocyanins quantification.

2.7. Statistics

Each analysis was carried out in triplicates and data are shown as mean ± SD. One-way ANOVA with Tukey's post-hoc test and Pearson correlation were performed by using Graph Pad prism 6.0 (GraphPad Software, San Diego, CA, U.S.A.) The differences were considered significant with $p < 0.05$.

3. Results and Discussion

3.1. Total Phenolic Content and Phenolic Profiles of YSO and RSO

A total of 13 and 28 phenolic compounds were identified and quantified in raw YSO and RSO samples, respectively. In particular, in YSO, 9 flavonols and 4 hydroxycinnamic acids were identified whereas in RSO, flavonols was the major representative class (14 individual compounds), followed by anthocyanins (8 individual compounds), di-hydro-flavonols (4 individual compounds) and flavan-3-ols (2 individual compounds). The compounds mass spectral data are reported in supplementary **Tables S1** and **S2**, whereas the identified compounds, together with the quantitative data, are listed in **Tables 2** and **3**.

From a quantitative point of view, flavonols dominated the phenolic profiles of both the onion varieties, representing 94.1% and 74.2% of total phenolic compounds (**Tables 2** and **3**).

Anthocyanins represented the 25.1% of total phenolic compounds in RSO whereas, in YSO, hydroxycinnamic acids accounted for the 5.9% of total phenolic compounds.

The most abundant compounds in both the onion varieties were quercetin-3-*O*-hexoside-4'-*O*-hexoside and quercetin-4'-*O*-hexoside, which together accounted for approximately 93.1% and 62.5% of total phenolic content in YSO and RSO, respectively.

These results complied with other authors' studies [16,21,31] which reported percentages of quercetin mono- and di-hexoside between 80% and 93%, depending on the onion varieties.

Total phenolic content of YSO was roughly half the content of RSO, 27.42 ± 1.70 and 50.12 ± 0.65 mg/100 g of fresh weight, respectively (Tables 2 and 3) as stated by previous studies [6,19]. The amount of onion phenolic compounds has been found to be strongly dependent on the onion variety, harvest year and meteorological conditions [17].

Similarly, the total flavonols content was 1.4 times higher in raw RSO compared to YSO (**Tables 2** and **3**). Looking at the two most important compounds, the amount of quercetin-4'-*O*-hexoside was almost the double in RSO respect to YSO, whereas there were no differences in the amount of quercetin-3-*O*-hexoside-4'-*O*-hexoside between the two varieties (**Tables 2** and **3**).

The total amount of phenolic compounds measured with the Folin-Ciocalteu's assay was 43.21 ± 1.57 and 65.44 ± 1.88 mg/100 g of fresh weight in YSO and RSO, respectively (Tables 2 and 3). The total amount determined through mass spectrometry experiments was 64% and 77% of the Folin-Ciocalteu values in YSO and RSO, respectively.

Table 2. Amount of phenolic compounds in raw and cooked yellow-skinned onion (YSO). Results are expressed in mg of phenolic compound/100 g of raw or cooked onion.

	YSO				
Compound	**Raw**	**Baked**	**Boiled**	**Fried**	**Grilled**
			Hydroxycinnamic acids		
Caffeic acid-*O*-hexoside	0.41 ± 0.01 [a]	n.d.	n.d.	n.d.	0.03 ± 0.00 [b]
Ferulic acid-*O*-hexoside	0.43 ± 0.02 [a]	n.d.	n.d.	n.d.	0.10 ± 0.01 [b]
Sinapic acid-*O*-hexoside isomer	0.11 ± 0.00 [c]	n.d.	0.08 ± 0.00 [d]	0.25 ± 0.00 [b]	0.46 ± 0.00 [a]
Sinapic acid-*O*-hexoside isomer	0.66 ± 0.01 [c]	0.99 ± 0.01 [b]	0.30 ± 0.00 [e]	0.58 ± 0.01 [d]	1.39 ± 0.03 [a]
Total hydroxycinnamic acids	1.61 ± 0.03 [b]	0.99 ± 0.01 [c]	0.38 ± 0.01 [e]	0.83 ± 0.01 [d]	1.98 ± 0.03 [a]
			Flavonols		
Quercetin	0.03 ± 0.00 [a]	n.d.	n.d.	0.01 ± 0.00 [b]	0.03 ± 0.00 [a]
Quercetin-3-*O*-hexoside	0.07 ± 0.00 [b]	0.06 ± 0.00 [b]	0.03 ± 0.00 [c]	0.13 ± 0.00 [a]	n.d.
Quercetin-4′-*O*-hexoside	4.99 ± 0.29 [c]	13.76 ± 0.06 [a,b]	1.00 ± 0.05 [d]	15.01 ± 0.48 [a]	12.07 ± 0.77 [b]
Quercetin-3-*O*-glucoside	n.d.	0.19 ± 0.01 [a]	0.04 ± 0.00 [c]	n.d.	0.12 ± 0.00 [b]
Quercetin-3-*O*-hexoside-7-*O*-hexoside	0.02 ± 0.00	n.d.	n.d.	n.d.	n.d.
Quercetin-7-*O*-hexoside-4′-*O*-hexoside	0.04 ± 0.00 [b]	0.13 ± 0.00 [a]	n.d.	n.d.	0.05 ± 0.00 [b]
Quercetin-3-*O*-hexoside-4′-*O*-hexoside	20.56 ± 1.68 [c]	27.67 ± 0.21 [a]	15.58 ± 0.98 [d]	28.05 ± 0.66 [a]	24.26 ± 0.27 [b]
Quercetin-tri-*O*-hexoside	0.02 ± 0.00 [b]	0.06 ± 0.00 [a]	0.02 ± 0.00 [b]	0.03 ± 0.00 [b]	n.d.
Isorhamnetin-4′-*O*-hexoside	0.06 ± 0.00 [d]	0.45 ± 0.00 [a]	0.05 ± 0.00 [d]	0.19 ± 0.00 [b]	0.15 ± 0.00 [c]
Isorhamnetin-3-*O*-hexoside-4′-*O*-hexoside	0.02 ± 0.00 [d]	0.21 ± 0.01 [a]	0.05 ± 0.00 [c]	0.06 ± 0.00 [b,c]	0.07 ± 0.00 [b]
Total flavonols	25.81 ± 1.70 [c]	42.53 ± 0.22 [a]	16.77 ± 0.98 [d]	43.48 ± 0.81 [a]	36.75 ± 0.82 [b]
Total phenolic by MS *	27.42 ± 1.70 [c]	43.52 ± 0.22 [a]	17.15 ± 0.98 [d]	44.31 ± 0.81 [a]	38.73 ± 0.82 [b]
Total phenolic by FC **	43.21 ± 1.57 [d]	168.75 ± 1.03 [a]	20.85 ± 0.85 [e]	51.71 ± 1.13 [c]	79.11 ± 0.31 [b]

Different letters within the same row mean significant different ($p < 0.05$) values. n.d. means that the compound was not detected in the sample. * Total phenolic compounds by mass spectrometry. Sum of the amount of individual phenolic compounds. ** Total phenolic compounds by Folin-Ciocalteu's assay. Data are expressed as mg of gallic acid equivalent per 100 g of raw or cooked onion.

Table 3. Amount of phenolic compounds in raw and cooked red-skinned onion (RSO). Results are expressed in mg of phenolic compound/100 g of raw or cooked onion.

	RSO				
Compound	**Raw**	**Baked**	**Boiled**	**Fried**	**Grilled**
			Hydroxybenzoic acids		
Protocatechuic acid-*O*-hexoside	n.d.	2.99 ± 0.03 ^{b}	n.d.	2.20 ± 0.06 ^{c}	4.56 ± 0.03 ^{a}
Total hydroxybenzoic acids	n.d.	2.99 ± 0.03 ^{b}	n.d.	2.20 ± 0.06 ^{c}	4.56 ± 0.03 ^{a}
			Flavan-3-ols		
(Epi)catechin-3-*O*-hexoside isomer	0.04 ± 0.00 ^{d}	0.72 ± 0.01 ^{b}	0.02 ± 0.00 ^{e}	0.20 ± 0.00 ^{c}	0.82 ± 0.02 ^{a}
(Epi)catechin-3-*O*-hexoside isomer	0.01 ± 0.00	n.d.	n.d.	n.d.	n.d.
Total flavan-3-ols	0.05 ± 0.00 ^{d}	0.72 ± 0.01 ^{b}	0.02 ± 0.00 ^{e}	0.20 ± 0.00 ^{c}	0.82 ± 0.02 ^{a}
			Di-hydro-flavonols		
Taxifolin-*O*-hexoside isomer	0.06 ± 0.00 ^{b}	n.d.	0.04 ± 0.00 ^{b}	0.06 ± 0.00 ^{b}	0.10 ± 0.00 ^{a}
Taxifolin-*O*-hexoside isomer	0.06 ± 0.00 ^{d}	n.d.	0.20 ± 0.00 ^{b}	0.29 ± 0.00 ^{a}	0.16 ± 0.00 ^{c}
Taxifolin-*O*-hexoside isomer	0.05 ± 0.00 ^{b}	0.09 ± 0.00a	n.d.	0.04 ± 0.00 ^{b}	0.02 ± 0.00 ^{c}
Taxifolin-*O*-hexoside isomer	0.12 ± 0.00 ^{a}	0.06 ± 0.00 ^{c}	0.03 ± 0.00 ^{d}	0.09 ± 0.00 ^{b}	0.05 ± 0.00 ^{c}
Taxifolin-*O*-hexoside isomer	n.d.	n.d.	n.d.	0.07 ± 0.00	n.d.
Total di-hydro-flavonols	0.29 ± 0.00 ^{c}	0.15 ± 0.00 ^{d}	0.27 ± 0.00 ^{c}	0.55 ± 0.01 ^{a}	0.33 ± 0.00 ^{b}
			Flavonols		
Quercetin	0.89 ± 0.05 ^{a}	0.43 ± 0.00 ^{b}	0.06 ± 0.01 ^{c}	0.03 ± 0.01 ^{c}	0.73 ± 0.01 ^{a}
Quercetin-3-*O*-hexoside	0.36 ± 0.01 ^{d}	1.01 ± 0.01 ^{a}	0.29 ± 0.00 ^{d}	0.71 ± 0.01 ^{b}	0.58 ± 0.00 ^{c}
Quercetin-4′-*O*-hexoside	10.46 ± 0.01 ^{d}	12.08 ± 0.05 ^{b,c}	11.47 ± 0.31 ^{c}	12.94 ± 0.46 ^{a,b}	13.49 ± 0.06 ^{a}
Quercetin-3-*O*-hexoside-7-*O*-hexoside	n.d.	n.d.	n.d.	n.d.	0.15 ± 0.00
Quercetin-7-*O*-hexoside-4′-*O*-hexoside	0.37 ± 0.00 ^{c}	0.65 ± 0.00 ^{a}	0.31 ± 0.00 ^{d}	0.55 ± 0.00 ^{b}	0.38 ± 0.00 ^{c}
Quercetin-3-*O*-hexoside-4′-*O*-hexoside	20.87 ± 0.14 ^{c}	32.55 ± 0.30 ^{a}	23.02 ± 0.34 ^{c}	36.29 ± 0.37 ^{a}	27.20 ± 0.58 ^{b}
Quercetin-tri-*O*-hexoside isomer	0.15 ± 0.00 ^{a}	0.14 ± 0.00 ^{a}	0.11 ± 0.00 ^{b}	n.d.	0.14 ± 0.00 ^{a}
Quercetin-tri-*O*-hexoside isomer	n.d.	0.03 ± 0.00 ^{a}	n.d.	0.02 ± 0.00 ^{a}	0.02 ± 0.00 ^{a}
Kaempferol-7-*O*-hexoside isomer	0.26 ± 0.01 ^{b}	0.05 ± 0.00 ^{d}	0.20 ± 0.00 ^{c}	0.20 ± 0.00 ^{c}	0.33 ± 0.00 ^{a}
Kaempferol-7-*O*-hexoside isomer	n.d.	0.07 ± 0.00 ^{a}	0.02 ± 0.00 ^{c}	0.02 ± 0.00 ^{c}	0.04 ± 0.00 ^{b}
Kaempferol-3-*O*-hexoside isomer	n.d.	0.09 ± 0.00 ^{a}	n.d.	0.02 ± 0.00 ^{b}	0.01 ± 0.00 ^{b}
Kaempferol-3-*O*-hexoside-7-*O*-hexoside isómer	0.55 ± 0.00 ^{a}	0.19 ± 0.00 ^{b}	0.04 ± 0.00 ^{d}	0.32 ± 0.00 ^{c}	n.d.
Kaempferol-3-*O*-hexoside-7-*O*-hexoside isómer	0.07 ± 0.00 ^{b}	0.16 ± 0.00 ^{a}	0.05 ± 0.00 ^{b}	0.08 ± 0.00 ^{b}	n.d.
Kaempferol-hexoside-rhamnoside-rhamnoside	0.05 ± 0.00 ^{a}	0.04 ± 0.00 ^{a}	n.d.	n.d.	n.d.
Isorhamnetin-3-*O*-hexoside isomer	0.28 ± 0.00 ^{c}	0.98 ± 0.00 ^{b}	n.d.	1.23 ± 0.02 ^{a}	0.81 ± 0.01 ^{b}
Isorhamnetin-3-*O*-hexoside isomer	n.d.	0.21 ± 0.00 ^{a}	n.d.	0.12 ± 0.01 ^{b}	0.13 ± 0.01 ^{b}
Isorhamnetin-4′-*O*-hexoside	2.25 ± 0.04 ^{d}	2.82 ± 0.04 ^{b}	n.d.	3.55 ± 0.07 ^{a}	2.47 ± 0.01 ^{c}
Isorhamnetin-3-*O*-hexoside-4′-*O*-hexoside	0.52 ± 0.00 ^{e}	2.38 ± 0.03 ^{a}	1.05 ± 0.05 ^{d}	2.20 ± 0.01 ^{b}	1.40 ± 0.01 ^{c}
Isorhamnetin-*O*-hexoside-*O*-pentoside	0.11 ± 0.00 ^{a}	n.d.	n.d.	0.04 ± 0.00 ^{b}	n.d.
Myricetin-*O*-hexoside-*O*-hexoside isómer	n.d.	0.11 ± 0.00 ^{a}	0.04 ± 0.00 ^{c}	0.04 ± 0.00 ^{c}	0.07 ± 0.00 ^{b}
Myricetin-*O*-hexoside-*O*-hexoside isómer	n.d.	n.d.	n.d.	0.04 ± 0.00	n.d.
Total flavonols	37.19 ± 0.53 ^{c}	53.98 ± 0.78 ^{a}	36.66 ± 0.88 ^{c}	58.40 ± 0.99 ^{a}	47.95 ± 1.08 ^{b}
			Anthocyanins		
Cyanidin-3-*O*-hexoside	1.00 ± 0.02 ^{a}	0.36 ± 0.01 ^{c}	0.74 ± 0.01 ^{b}	n.d.	n.d.
Cyanidin-*O*-hexoside-*O*-hexoside isómer	0.09 ± 0.00 ^{b}	n.d.	0.07 ± 0.00 ^{b}	0.07 ± 0.00 ^{b}	0.31 ± 0.02 ^{a}
Cyanidin-*O*-hexoside-*O*-hexoside isómer	0.31 ± 0.01 ^{b}	0.10 ± 0.00 ^{d}	0.14 ± 0.00 ^{c}	0.07 ± 0.01 ^{d}	2.20 ± 0.02 ^{a}
Cyanidin-*O*-malonyl-hexoside isomer	5.97 ± 0.37 ^{a}	n.d.	3.25 ± 0.03 ^{b}	2.48 ± 0.01 ^{c}	4.93 ± 0.08 ^{a}
Cyanidin-*O*-malonyl-hexoside isomer	n.d.	n.d.	n.d.	0.50 ± 0.01	n.d.
Cyanidin-*O*-hexoside-*O*-malonyl-hexoside isómer	4.60 ± 0.01 ^{a}	1.29 ± 0.07 ^{d}	1.67 ± 0.03 ^{c}	2.82 ± 0.06 ^{b}	3.96 ± 0.08 ^{a}
Peonidin-3-*O*-hexoside	0.21 ± 0.00 ^{a}	n.d.	0.13 ± 0.00 ^{b}	n.d.	0.16 ± 0.00 ^{b}
Peonidin-*O*-malonyl-hexoside	0.37 ± 0.01 ^{a}	0.17 ± 0.00 ^{b}	0.19 ± 0.00 ^{b}	0.39 ± 0.00 ^{a}	n.d.
Malvidin-*O*-hexoside-acetaldehyde	0.02 ± 0.00 ^{a}	n.d.	0.02 ± 0.00 ^{a}	n.d.	n.d.
Total anthocyanins	12.57 ± 0.38 ^{a}	1.92 ± 0.07 ^{c}	6.22 ± 0.04 ^{b}	6.33 ± 0.06 ^{b}	11.57 ± 0.12 ^{a}
Total phenolic by MS *	50.12 ± 0.65 ^{c}	59.76 ± 0.78 ^{b}	43.15 ± 0.89 ^{d}	67.70 ± 0.99 ^{a}	65.22 ± 1.09 ^{a}
Total phenolic by FC **	65.44 ± 1.88 ^{d}	137.85 ± 1.23 ^{a}	36.79 ± 0.31 ^{e}	81.85 ± 0.54 ^{c}	111.79 ± 1.44 ^{b}

Different letters within the same row mean significant different ($p < 0.05$) values. n.d. means that the compound was not detected in the sample. * Total phenolic compounds by mass spectrometry. Sum of the amount of individual phenolic compounds. ** Total phenolic compounds by Folin-Ciocalteu's assay. Data are expressed as mg of gallic acid equivalent per 100 g of raw or cooked onion.

3.2. Effect of Cooking Methods on Total and Individual Phenolic Content in YSO and RSO

Tables 2 and **3** also show the effects of the different cooking treatments on the total and individual phenolic compounds in YSO and RSO. Considering the total amount of phenolic compounds (sum of the concentrations of the individual compounds identified by mass spectrometry), boiling was the only thermal treatment that caused a decrease in total phenolic content in both the onion varieties (37.5% and 13.9% decrease in YSO and RSO, respectively). On the contrary, frying was the cooking method that triggered the highest increase in total phenolic content determined by mass spectrometry (61.6% and 35.1% increase in YSO and RSO, respectively) followed by baking for YSO (58.7% increase) and grilling for the RSO (30.1% increase). These results were fairly different from those achieved with the Folin-Ciocalteu's assay, where baking and grilling caused the highest increase in total phenolic content (**Tables 2** and **3**). This discrepancy may be a consequence of the Maillard reaction, which occurs during cooking, generating compounds able to directly interact with the Folin-Ciocalteu's reagent [32]. Supporting this, previous studies already reported that grilling and baking processes determined the formation of higher amounts of Maillard reaction products respect to frying and boiling in onion [33].

Similar results were obtained when considering the amount of total flavonols determined by mass spectrometry as a function of the cooking procedure (**Tables 2** and **3**). The highest decrease in total flavonols was found for boiled (35.0% decrease) YSO whereas a marginal 1.4% decrease was observed in the boiled RSO. Once again, frying was the cooking method with the highest increase in total flavonols for both the varieties (68.5% and 57.1% increase in YSO and RSO, respectively).

In accordance, previous studies confirmed this trend of flavonols content dependent on different cooking processes. Specifically, flavonols concentration increased after grilling, baking and frying but decreased after boiling, compared to the raw samples [16,18,21,34]. Nevertheless, other studies found no effect or a small decrease in flavonols after frying. The variability in the reported data may be due to the different cooking parameters applied. It has been suggested that phenolic compounds stability and extractability after thermal treatments are strongly dependent on the cooking time and temperature as well as on the chopping procedure [11,35].

A significant increase of both quercetin-3-*O*-hexoside-4'-*O*-hexoside and quercetin-4'-*O*-hexoside was observed in the baked, fried and grilled YSO and RSO, compared to the raw samples (**Tables 2** and **3**). In YSO, the percentage incidence of quercetin-di-hexosides on total phenolic compounds determined by mass spectrometry decreased from 75% in raw onion to 64%, 63% and 63% after baking, frying and grilling, respectively. In parallel, an increase in the quercetin-mono-hexosides percentage incidence was observed after baking, frying and grilling where they represented the 32%, 34% and 31% of total phenolic compounds, respectively, compared to the raw onion (percentage incidence of 18%) (**Table 2**). Vice versa, in boiled YSO, the percentage incidence of quercetin-di-hexosides increased, compared to the raw onion, reaching more than 90% of total phenolic compounds determined by mass spectrometry (**Table 2**). In RSO, the percentage

incidence of quercetin-mono-hexosides was steady whatever the thermal treatment, whereas an increased incidence of quercetin-di-hexosides was observed after boiling and frying, compared to the raw onion (**Table 3**).

In YSO, the amount of total hydroxycinnamic acids determined by mass spectrometry decreased for all treatments with the exception of grilling where a 22.4% increase was obtained (**Table 2**). Boiling was responsible for the highest loss of total hydroxycinnamic acids (76.4% decrease). In RSO, grilling was the only treatment where the total amount of anthocyanins determined by mass spectrometry was similar to that of the raw sample (**Table 3**). In this case, baking caused the highest loss of total anthocyanins (84.7% decrease).

3.3. Effect of Cooking Methods on Total and Individual Phenolic Content in YSO and RSO as a Function of the Initial Fresh Weight

The phenolic increase observed after baking, frying and grilling could be a consequence of water loss during cooking, which resulted in a surge of onion phenolic compounds, or to an easier extractability due to a matrix softening effect [11,12]. Therefore, to gain more information on the stability/release of phenolic compounds, the values were corrected for the weight loss due to the cooking procedure (cooking ratio as reported in **Table 1**).

As reported in **Figure 1A**, all the treatments caused a significant reduction in extractable total phenolic compounds (sum of the amounts of individual phenolic compounds identified by mass spectrometry). The only exception was noted for fried YSO, where the amount of extractable total phenolic compounds was not significantly different ($p > 0.05$) compared to the raw sample. The highest loss of total phenolic compounds was recorded after boiling (76.2% loss) in YSO and after baking (56.9% loss) in RSO. In this context, the data acquired by mass spectrometry were fairly different compared to those observed with the Folin-Ciocalteu's assay (**Figure 1B**). This incongruity may be due to the formation of Folin-Ciocalteu-reactive Maillard reaction products during cooking and/or to the thermally-induced degradation of Folin-Ciocalteu-reactive compounds (i.e., ascorbic acid or sulphur-containing compounds) [36–38].The behaviour of quercetin-di-hexosides was similar between the two varieties (**Figure 1C**). Frying was the thermal treatment that caused the lowest decrease of quercetin-di-hexosides, whereas the highest losses were recorded after boiling and grilling. Considering quercetin-mono-hexosides, decreases of 56.4%, 32.3%, 27.9% and 19.7% were observed for baking, boiling, frying and grilling treatments in RSO, respectively, compared to the raw sample (**Figure 1D**). Contrariwise, grilling and, especially, frying boosted quercetin-mono-hexosides concentration in YSO (**Figure 1D**).

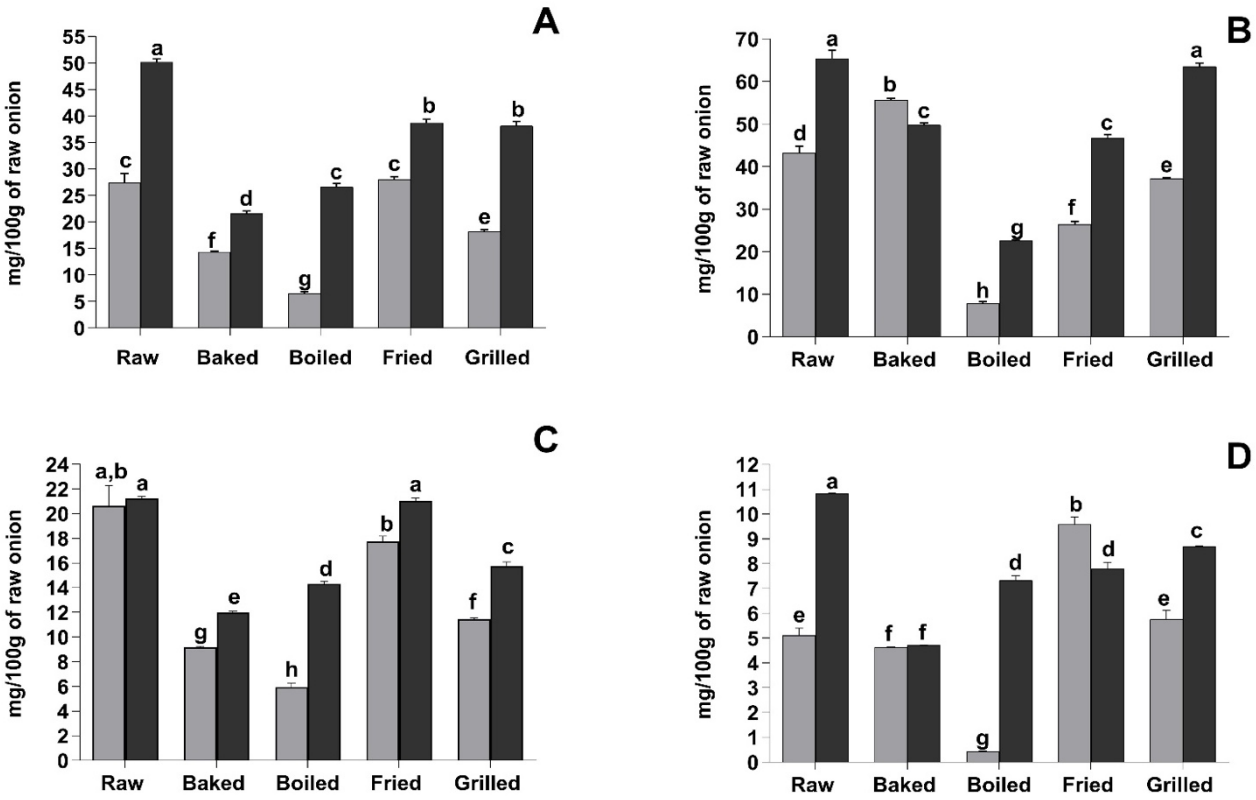


Figure 1. Effect of different cooking methods on YSO and RSO total phenolic compounds and quercetin-derivatives referred to the original fresh weight. Light grey bars indicated the YSO whereas black bars the RSO. (A) Total phenolic compounds measured by mass spectrometry. Results are expressed as mg/100 g of fresh weight. (B) Total phenolic compounds with the Folin-Ciocalteu's assay. Results are expressed as mg of gallic acid equivalent/100 g of fresh weight. (C) Total quercetin-di-hexosides measured by mass spectrometry. Results are expressed as mg/100 g of fresh weight. (D) Total quercetin-mono-hexosides measured by mass spectrometry. Results are expressed as mg/100 g of fresh weight. Different letters mean significant difference ($p < 0.05$).

Total hydroxycinnamic in the YSO and total anthocyanins in the RSO followed a similar trend resulting in a decrease for all of the thermal treatments (**Figure 2A, B**).

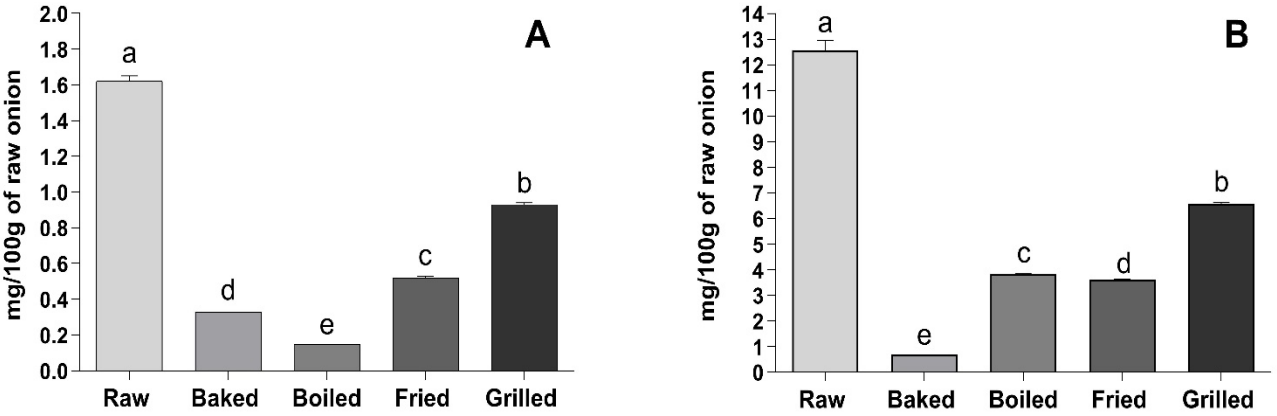


Figure 2. Effect of different cooking methods on YSO hydroxycinnamic acids and RSO anthocyanins referred to the original fresh weight. (**A**) Total hydroxycinnamic acids measured by mass spectrometry in YSO. Results are expressed as mg/100 g of fresh weight. (**B**) Total anthocyanins measured by mass spectrometry in RSO. Results are expressed as mg/100 g of fresh weight. Different letters indicate that the values are significantly different ($p < 0.05$).

In YSO, the molar ratio between quercetin-di-hexosides and quercetin-mono-hexosides decreased after baking, grilling and frying. The raw YSO had a quercetin-di-hexosides/quercetin-mono-hexosides molar ratio of 4.03 whereas baked, grilled and fried samples showed ratios of 1.98, 1.99 and 1.85, respectively. These results suggested that quercetin-di-hexosides were hydrolysed in the corresponding mono-hexosides during thermal treatments as already reported in other studies for YSO varieties [39]. This is particularly evident in fried YSO where the sum of the molar concentrations of the quercetin-derivatives was not significantly different respect to the raw sample (**Figure 3A**). During frying, quercetin-derivatives were quite stable and only a net molar conversion of quercetin-di-hexosides (≈ 8 µmol/100 g decrease respect to the raw sample) in quercetin-mono-hexosides (≈ 9 µmol/100 g increase respect to the raw sample) was observed. In particular, quercetin-3-*O*-hexoside-4'-*O*-hexoside was de-glycosylated to quercetin-4'-*O*-hexoside without any formation of the 3-*O*-hexoside (**Table 2**). As stated by Rohn et al. [39], the hexoside in 4'-*O*-position displayed a higher stability versus de-glycosylation than the hexoside in 3-*O*-position. However, contrary to the above-mentioned study, no increase in quercetin aglycone was found after frying. Similarly, also grilling and baking resulted in a decrease in the quercetin-di-hexosides/quercetin-mono-hexosides molar ratio. This aspect highlighted that the conversion of quercetin-3-*O*-hexoside-4'-*O*-hexoside in quercetin-4'-*O*-hexoside took place during these thermal treatments, without any formation of quercetin aglycone (**Figure 3A and Table 2**). On molar basis, boiling was the thermal treatment that triggered the highest loss of quercetin-derivatives, respect to the raw sample (75% loss) as a consequence of the leaching of these compounds in the cooking water as previously suggested [18,19,21].

In RSO, all the treatments caused a decrease in the sum of the molar concentrations of quercetin-derivatives, respect to the raw sample (**Figure 3B**). The highest decrease was observed after baking (49%), whereas the lowest one was detected after frying (13%). Furthermore, despite a decrease in the quercetin-di-hexosides amount (**Table 3**), the quercetin-di-hexosides/quercetin-mono-hexosides molar ratio after the thermal treatments was similar or higher than the raw sample. Altogether, these data suggest that both the quercetin-glycosides (mono- and di-hexosides) degraded in baked, grilled and fried samples without the occurrence of quercetin aglycone. Interestingly, as reported in Table 3, protocatechuic acid-hexoside, which was not present in the raw RSO, was first detected after baking, grilling and frying. Protocatechuic acid is a well-known quercetin degradation product, resulting from the oxidative decarboxylation by means of oxygen nucleophilic attack, followed by the cleavage of the C-ring [40,41]. We hypothesize that the same mechanism may occur in the case of quercetin-4'-*O*-hexoside resulting in the release of protocatechuic acid-hexoside in the food matrix. When the molar concentration of protocatechuic acid-hexoside was added to the sum of molar concentrations of quercetin-derivatives, a 100% recovery was observed after frying and grilling respect to the raw onion (**Figure 3B**).

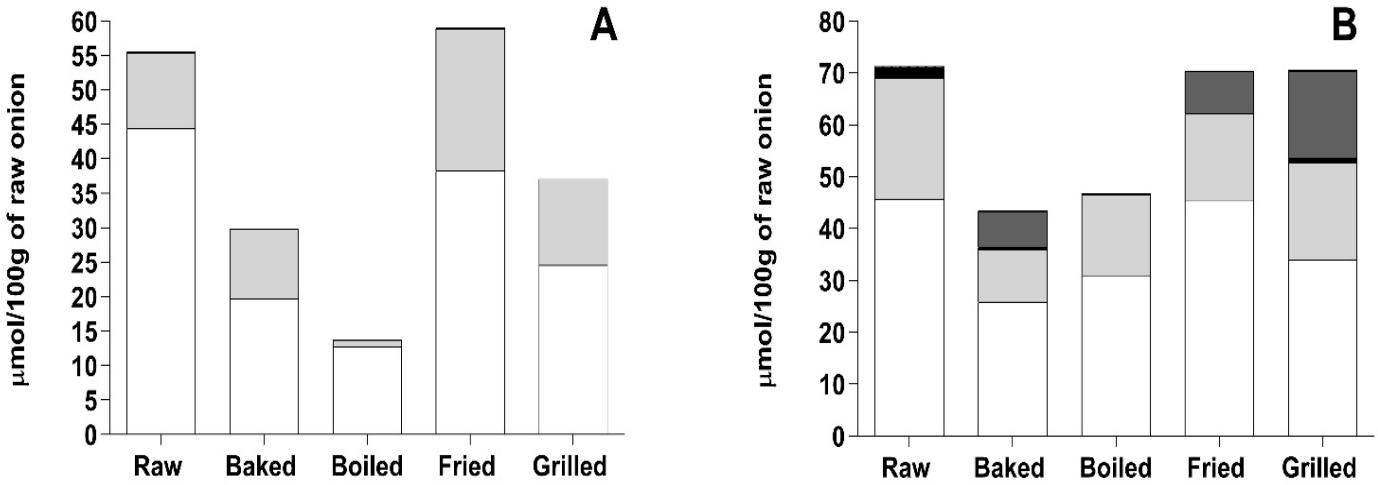


Figure 3. Sum of the molar concentrations of quercetin-derivatives and the degradation product protocatechuic acid-hexoside in YSO and RSO as affected by the cooking method. The data are referred to the original fresh weight and expressed as µmol of compound/100 g of fresh weight. (**A**) YSO; (**B**) RSO. Reported compounds are total quercetin-di-hexosides (white), total quercetin-mono-hexosides (light grey), quercetin (black) and protocatechuic acid-hexoside (dark grey).

Furthermore, the molar ratio calculated by including the molar concentration of protocatechuic acid-hexoside at the denominator (i.e., quercetin-di-hexosides/(quercetin-mono-hexosides + protocatechuic acid-hexoside) molar ratio) gave values for frying (1.82 molar ratio) and grilling (0.96 molar ratio) significantly lower respect to the raw onion (1.95 molar ratio). In light of the degradation pathway proposed in **Figure 4**, these results suggest a net conversion of quercetin-3-*O*-hexoside-4'-*O*-hexoside in quercetin-4'-*O*-hexoside, which was further degraded in protocatechuic acid-hexoside.

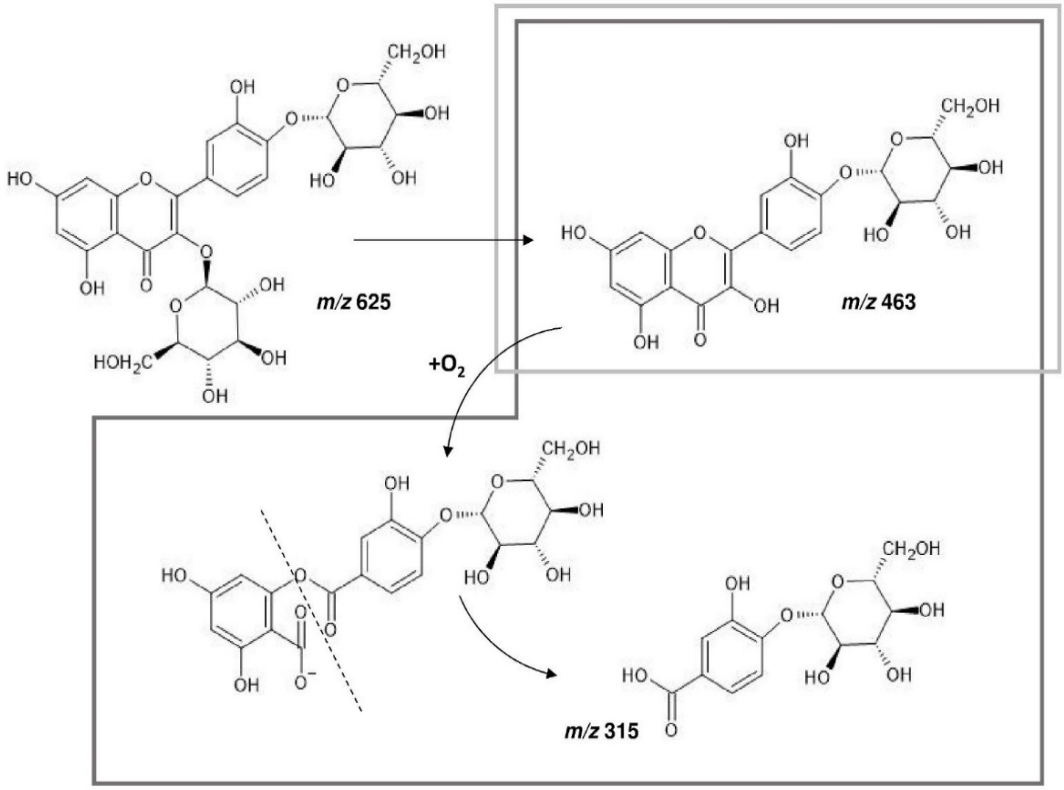


Figure 4. Proposed degradation pathway for quercetin-3-*O*-hexoside-4'-*O*-hexoside during cooking of YSO and RSO. Quercetin-3-*O*-hexoside-4'-*O*-hexoside (*m/z* = 625) was firstly de-glycosylated at the C3 level, releasing quercetin-4'-*O*-hexoside (*m/z* = 463), under heating treatment and in presence of oxygen. Quercetin-4'-*O*-hexoside may face the nucleophilic attack by oxygen and the cleavage of the C-ring, resulting in the release of protocatechuic acid-hexoside (*m/z* = 315). Light grey box records the hypothesized degradation pathway detected in YSO. Dark grey box records the hypothesized degradation pathway detected in RSO.

Also during baking, the same hypothesized degradation pathway may occur, as suggested by the presence of protocatechuic acid-hexoside and the calculation of a molar ratio, which included the molar concentration of protocatechuic acid-hexoside at the denominator, lower than the raw sample (1.50 vs 1.95 molar ratio). However, the sum of the molar concentrations of the quercetin-derivatives and protocatechuic acid-hexoside accounted for only the 61% of the total amount of quercetin-derivatives in the raw onion. Similarly to YSO, boiled RSO confirmed lower quercetin-derivatives concentration than the raw sample, due to the leaching of these compounds in cooking water.

3.4. Effect of Cooking Methods on the Release and Bioaccessibility of YSO and RSO Phenolic Compounds

Recently, several works focused on the influence of domestic cooking processes on the release and the bioaccessibility of phenolic compounds [13,42–46]. Notwithstanding, the very high incidence of phenolic compounds in onion, no studies paid attention to characterize the possible correlation between the cooking procedures and the bioaccessibility of onion phenolic compounds after in vitro gastro-intestinal digestion.

Data reported in Tables 4 and 5 show that the bioaccessibility index (BI) was below 100% for either raw or cooked samples in both the onion varieties.

In YSO, the BI for total phenolic compounds determined by mass spectrometry was between 42.6% and 65.5% in grilled and baked samples, respectively (**Table 4**).

Boiled YSO showed the lowest quantity of bioaccessible phenolic compounds (8.76 mg/100 g of cooked sample), since only quercetin-3-*O*-hexoside-4'-*O*-hexoside was released after the in vitro gastro-intestinal digestion. Baked YSO had the largest amount of total phenolic compounds after in vitro digestion (28.53 mg/100 g of cooked sample), followed by fried (22.12 mg/100 g of cooked sample) and grilled (16.50 mg/100 g of cooked sample) samples (**Table 4**). Raw YSO showed about half the quantity of bioaccessible total phenolic compounds of the baked sample. Total flavonols accounted for the 100% of bioaccessible phenolic compounds in all of the samples, with the exception of baked YSO, where only a mere 3% of hydroxycinnamic acids were detected.

Quercetin-3-*O*-hexoside-4'-*O*-hexoside was the most abundant phenolic compound in all of the yellow-skinned samples even though its BI was between 50.9% and 64.4% in grilled and baked onion, respectively. The second most abundant compound in all of the samples was quercetin-4'-*O*-hexoside, except for the boiled sample where it was not detected after in vitro digestion.

The highest amount of quercetin-3-*O*-hexoside-4'-*O*-hexoside and quercetin-4'-*O*-hexoside was found after in vitro digestion of baked YSO (**Table 4**).

In RSO, the BI for total phenolic compounds after in vitro gastro-intestinal digestion was between 39.8% and 80.2% in boiled and baked samples, respectively (**Table 5**). The boiled RSO sample also showed the lowest amount of total phenolic compounds after digestion (17.22 mg/100 g of cooked sample) whereas baked onion had the highest amount of bioaccessible phenolic compounds (45.19 mg/100 g of cooked sample). Quercetin-3-*O*-hexoside-4'-*O*-hexoside and quercetin-4'-*O*-hexoside were the most abundant bioaccessible phenolic compounds in all the RSO samples. The highest amount of quercetin-3-*O*-hexoside-4'-*O*-hexoside was detected after in vitro digestion of baked RSO, whereas grilled RSO showed the highest amount of quercetin-4'-*O*-hexoside (**Table 5**). Total flavonols accounted for more than 95% of total bioaccessible phenolic compounds in all the RSO samples. Anthocyanins showed low bioaccessibility (between 3.8% and 17.6%) and were not detected after digestion of the baked sample. The highest amount of bioaccessible

anthocyanins was found in fried RSO, but they accounted only for the 3.2% of total bioaccessible phenolic compounds (**Table 5**).

Generally, the BI calculated for the total phenolic compounds through the Folin-Ciocalteu's assay was higher than that calculated by mass spectrometry for both the onion varieties (**Tables 4** and **5**). In most cases, the BI was greater than 100% suggesting the presence of gastro-intestinal interfering products, which were actually absent during the extraction with the methanol/formic acid solution.

Table 4. Amount of phenolic compounds in raw and cooked yellow-skinned onion (YSO) after in vitro gastro-intestinal digestion. Results are expressed in mg of phenolic compound/100 of raw or cooked onion. Bioaccessibility index (BI) is the percentage ratio between the concentration after in vitro gastro-intestinal digestion and the concentration before digestion.

	YSO									
Compound	**Raw**		**Baked**		**Boiled**		**Fried**		**Grilled**	
	After Digestion	**BI (%)**	**After Digestion**	**BI (%)**	**After Digestion**	**BI (%)**	**After Digestion**	**BI (%)**	**After Digestion**	**BI (%)**
Hydroxycinnamic acids										
Caffeic acid-*O*-hexoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ferulic acid-*O*-hexoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Sinapic acid-*O*-hexoside isómer	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Sinapic acid-*O*-hexoside isómer	n.d.	n.d.	0.76 ± 0.01	77.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total hydroxycinnamic acids	n.d.	n.d.	0.76 ± 0.01	77.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Flavonols										
Quercetin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Quercetin-3-*O*-hexoside	0.02 ± 0.00 [c]	29.3	0.19 ± 0.00 [a]	264.1	n.d.	n.d.	0.10 ± 0.00 [b]	80.3	0.09 ± 0.00 [b]	n.d.
Quercetin-4′-*O*-hexoside	3.20 ± 0.00 [d]	64.0	9.17 ± 0.16 [a]	66.6	n.d.	n.d.	5.67 ± 0.18 [b]	37.8	4.01 ± 0.02 [c]	33.2
Quercetin-3-*O*-glucoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Quercetin-3-*O*-hexoside-7-*O*-hexoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Quercetin-7-*O*-hexoside-4′-*O*-hexoside	n.d.	n.d.	0.14 ± 0.00	112.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Quercetin-3-*O*-hexoside-4′-*O*-hexoside	11.54 ± 0.13 [b]	56.1	17.83 ± 1.09 [a]	64.4	8.76 ± 0.12 [c]	56.2	16.24 ± 0.18 [a]	57.9	12.35 ± 0.49 [b]	50.9
Quercetin-tri-*O*-hexoside	n.d.	n.d.	0.06 ± 0.00	86.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Isorhamnetin-4′-*O*-hexoside	0.02 ± 0.00 [c]	38.8	0.19 ± 0.00 [a]	43.3	n.d.	n.d.	0.06 ± 0.00 [b]	30.4	n.d.	n.d.
Isorhamnetin-3-*O*-hexoside-4′-*O*-hexoside	n.d.	n.d.	0.19 ± 0.00 [a]	92.0	n.d.	n.d.	0.04 ± 0.00 [b]	74.2	0.05 ± 0.00 [b]	65.9
Total flavonols	14.78 ± 0.13 [d]	57.2	27.77 ± 1.11 [a]	65.3	8.76 ± 0.12 [e]	52.2	22.12 ± 0.26 [b]	50.9	16.50 ± 0.49 [c]	44.9
Total phenolic by MS *	14.78 ± 0.13 [d]	53.8	28.53 ± 1.11 [a]	65.5	8.76 ± 0.12 [e]	51.0	22.12 ± 0.26 [b]	49.9	16.50 ± 0.49 [c]	42.6
Total phenolic by FC **	63.38 ± 3.13 [c]	146.7	207.66 ± 7.08 [a]	123.1	57.24 ± 2.19 [d]	117.8	74.76 ± 4.14 [c]	179.2	111.23 ± 0.43 [b]	140.6

Different letters within the same row mean significant different ($p < 0.05$) values. n.d. means that the compound was not detected in the sample. * Total phenolic compounds by mass spectrometry. Sum of the amount of individual phenolic compounds. ** Total phenolic compounds by Folin-Ciocalteu's assay. Data are expressed as mg of gallic acid equivalent per 100 g of raw or cooked onion.

Table 5. Amount of phenolic compounds in raw and cooked red-skinned onion (RSO) after in vitro gastro-intestinal digestion. Results are expressed in mg of phenolic compound/100 of raw or cooked onion. Bioaccessibility index (BI) is the percentage ratio between the concentration after in vitro gastro-intestinal digestion and the concentration before digestion.

	RSO									
Compound	**Raw**		**Baked**		**Boiled**		**Fried**		**Grilled**	
	After Digestion	**BI (%)**	**After Digestion**	**BI (%)**	**After Digestion**	**BI (%)**	**After Digestion**	**BI (%)**	**After Digestion**	**BI (%)**
Hydroxybenzoic acids										
Protocatechuic acid-*O*-hexoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total hydroxybenzoic acids	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Flavan-3-ols										
(Epi)catechin-3-*O*-hexoside isomer	n.d.	n.d.	0.51 ± 0.00 ^{b}	70.0	n.d.	n.d.	0.27 ± 0.01 ^{c}	133.4	0.71 ± 0.10 ^{a}	86.3
(Epi)catechin-3-*O*-hexoside isomer	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total flavan-3-ols	n.d.	n.d.	0.51 ± 0.00 ^{b}	70.0	n.d.	n.d.	0.27 ± 0.01 ^{c}	133.4	0.71 ± 0.10 ^{a}	86.3
Di-hydro-flavonols										
Taxifolin-*O*-hexoside isomer	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Taxifolin-*O*-hexoside isomer	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Taxifolin-*O*-hexoside isomer	0.03 ± 0.00 ^{b}	56.4	n.d.	n.d.	n.d.	n.d.	0.08 ± 0.00 ^{a}	217.5	n.d.	n.d.
Taxifolin-*O*-hexoside isomer	0.03 ± 0.00	24.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Taxifolin-*O*-hexoside isomer	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total di-hydro-flavonols	0.06 ± 0.00 ^{b}	19.8	n.d.	n.d.	n.d.	n.d.	0.08 ± 0.00 ^{a}	217.5	n.d.	n.d.
Flavonols										
Quercetin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Quercetin-3-*O*-hexoside	n.d.	n.d.	1.28 ± 0.01 ^{a}	126.9	n.d.	n.d.	0.89 ± 0.09 ^{b}	126.6	0.34 ± 0.01 ^{c}	19.1
Quercetin-4'-*O*-hexoside	4.27 ± 0.01 ^{a}	40.8	5.59 ± 0.06 ^{b}	46.3	1.89 ± 0.03 ^{c}	16.5	4.99 ± 0.05 ^{d}	38.5	7.07 ± 0.06 ^{e}	52.4
Quercetin-3-*O*-hexoside-7-*O*-hexoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Quercetin-7-*O*-hexoside-4'-*O*-hexoside	0.22 ± 0.01 ^{b}	59.7	0.47 ± 0.01 ^{a}	72.6	n.d.	n.d.	0.20 ± 0.02 ^{b}	36.6	0.26 ± 0.01 ^{b}	68.0
Quercetin-3-*O*-hexoside-4'-*O*-hexoside	17.73 ± 0.17 ^{d}	84.9	35.35 ± 0.36 ^{a}	108.6	14.70 ± 0.73 ^{e}	63.8	25.08 ± 0.22 ^{c}	69.1	31.73 ± 0.52 ^{b}	116.7
Quercetin-tri-*O*-hexoside isómer	0.11 ± 0.00 ^{c}	73.0	0.23 ± 0.00 ^{a}	165.6	0.11 ± 0.00 ^{c}	102.3	0.14 ± 0.00 ^{b}	n.d.	0.23 ± 0.00 ^{a}	204.6
Quercetin-tri-*O*-hexoside isómer	n.d.	n.d.	0.09 ± 0.00	279.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Kaempferol-7-*O*-hexoside isomer	0.05 ± 0.00	18.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Kaempferol-7-*O*-hexoside isomer	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Kaempferol-3-*O*-hexoside isomer	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Kaempferol-3-*O*-hexoside-7-*O*-hexoside isomer	0.13 ± 0.00	23.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Kaempferol-3-*O*-hexoside-7-*O*-hexoside isomer	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Kaempferol-hexoside-rhamnoside-rhamnoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Isorhamnetin-3-*O*-hexoside isomer	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.55 ± 0.01 ^{b}	44.9	0.60 ± 0.00 ^{a}	73.8
Isorhamnetin-3-*O*-hexoside isomer	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Isorhamnetin-4′-*O*-hexoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Isorhamnetin-3-*O*-hexoside-4′-*O*-hexoside	0.92 ± 0.01 ^{b}	174.8	1.68 ± 0.02 ^{a}	70.5	0.29 ± 0.00 ^{d}	27.4	0.85 ± 0.02 ^{c}	38.7	0.73 ± 0.08 ^{c}	52.4
Isorhamnetin-*O*-hexoside-*O*-pentoside	0.08 ± 0.00 ^{b}	71.6	n.d.	n.d.	n.d.	n.d.	0.14 ± 0.00 ^{a}	344.8	n.d.	n.d.
Myricetin-di-*O*-hexoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Myricetin-di-*O*-hexoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total flavonols	23.50 ± 0.17 ^{d}	64.7	44.68 ± 0.37 ^{a}	83.4	16.98 ± 0.73 ^{e}	46.2	32.85 ± 0.25 ^{c}	56.3	41.02 ± 0.53 ^{b}	83.6
Anthocyanins										
Cyanidin-3-*O*-hexoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.11 ± 0.00	n.d.
Cyanidin-*O*-hexoside-*O*-hexoside isómer	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Cyanidin-*O*-hexoside-*O*-hexoside isómer	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.24 ± 0.00	328.2	n.d.	n.d.
Cyanidin-*O*-malonyl-hexoside isomer	0.52 ± 0.00 ^{b}	8.7	n.d.	n.d.	0.14 ± 0.00 ^{d}	4.2	0.60 ± 0.00 ^{a}	24.0	0.45 ± 0.00 ^{c}	9.2
Cyanidin-*O*-malonyl-hexoside isomer	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Cyanidin-*O*-hexoside-*O*-malonyl-hexoside isomer	0.31 ± 0.00 ^{a}	6.7	n.d.	n.d.	0.10 ± 0.00 ^{c}	5.8	0.28 ± 0.00 ^{b}	9.9	0.34 ± 0.01 ^{a}	8.4
Peonidin-3-*O*-hexoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Peonidin-*O*-malonyl-hexoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Malvidin-*O*-hexoside-acetaldehyde	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total anthocyanins	0.83 ± 0.00 ^{c}	6.6	n.d.	n.d.	0.24 ± 0.00 ^{d}	3.8	1.11 ± 0.00 ^{a}	17.6	0.90 ± 0.00 ^{b}	8.6
Total phenolic by MS *	24.39 ± 0.17 ^{c}	49.5	45.19 ± 0.37 ^{a}	80.2	17.22 ± 0.73 ^{d}	39.8	34.32 ± 0.25 ^{b}	52.1	42.63 ± 0.53 ^{a}	65.3
Total phenolic by FC **	65.19 ± 3.26 ^{d}	99.6	187.61 ± 4.81 ^{a}	136.1	43.34 ± 2.26 ^{e}	117.8	102.92 ± 1.74 ^{c}	125.7	127.66 ± 3.92 ^{b}	114.2

Different letters within the same row mean significant different ($p < 0.05$) values. n.d. means that the compound was not detected in the sample. * Total phenolic compounds by mass spectrometry. Sum of the amount of individual phenolic compounds. ** Total phenolic compounds by Folin-Ciocalteu's assay. Data are expressed as mg of gallic acid equivalent per 100 g of raw or cooked onion.

3.5. Effect of Cooking and In Vitro Gastro-Intestinal Digestion on the Antioxidant Activity of YSO and RSO

Figure 5 depicts the impact of cooking methods and in vitro digestion on the antioxidant activity of YSO and RSO phenolic compounds, tested with the ABTS and FRAP assays. Generally, the ABTS and FRAP data showed a significant correlation with the total phenolic compounds determined with the Folin-Ciocalteu assay (Pearson coefficient $r = 0.968$ $p = 4.3 \times 10^{-6}$ and Pearson coefficient $r = 0.752$ $p = 0.012$, for ABTS and FRAP assay, respectively). On the contrary, no significant correlations were found between the antioxidant activities data and the total amount of phenolic compounds determined by mass spectrometry.

The highest ABTS and FRAP values were found for baked samples, whereas the lowest values were found for boiled samples in both the onion varieties (**Figure 5**). In the ABTS assay, the raw YSO and RSO exhibited higher antioxidant activity than the boiled samples but lower than the baked, grilled and fried ones. In the FRAP assay, baking and grilling resulted in an increased antioxidant activity respect to the raw samples whereas boiling determined a decrease in the FRAP values in both the onion varieties. In YSO, frying also caused a decrease in the FRAP value respect to the raw sample, whereas in the RSO no significant differences were found between fried and raw onion.

Conflicting results are present in literature about the variation in the antioxidant activity after onion cooking. According to the present study, Pellegrini et al. [47] found an increase in the antioxidant activity determined with the ABTS assay after frying respect to the raw onion. However, other authors found no loss or decrease in the ABTS values after grilling and frying [16,48].

After in vitro digestion, baked samples still showed the highest ABTS and FRAP values in both the onion varieties (**Figure 5**). In the ABTS assay, raw and cooked YSO and RSO showed higher antioxidant activity after digestion compared to the methanol/formic acid extract, suggesting that the digestion procedure released antioxidant compounds, which were not extracted with the methanol/formic acid solution. On the contrary, when antioxidant activity was assayed with the FRAP procedure, only frying, for both the onion varieties, and boiling for YSO showed higher data after in vitro digestion respect to the methanol/formic acid extracts.

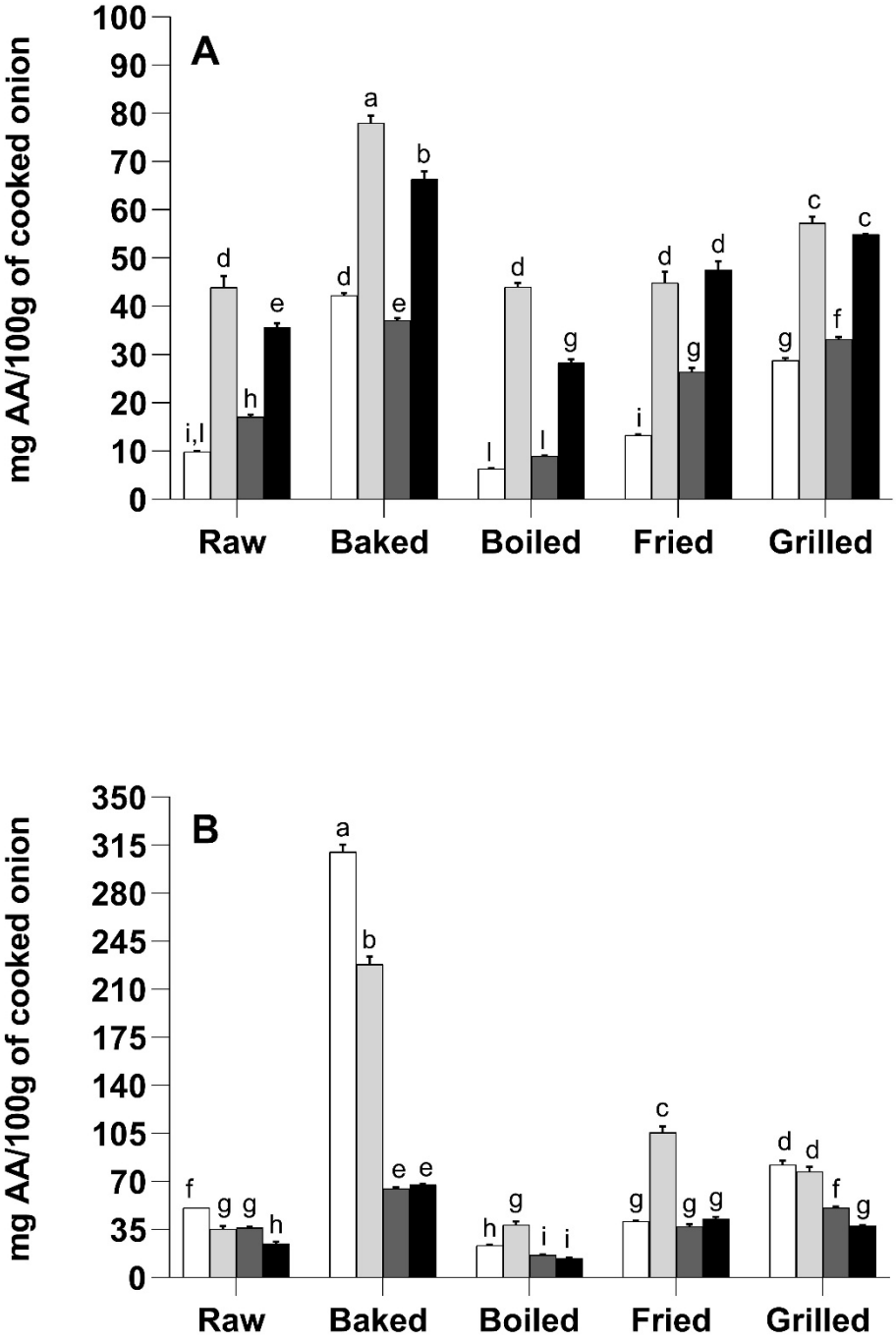


Figure 5. Effect of different cooking methods and in vitro digestion on yellow-skinned (YSO) and red-skinned (RSO) onions antioxidant activity. (**A**) YSO antioxidant activity as determined by the ABTS assay in the methanolic/formic acid extract (white) and after in vitro digestion (light grey bars). RSO antioxidant activity as determined by the ABTS assay in the methanolic/formic acid extract (dark grey bars) and after in vitro digestion (black bars). (**B**) YSO antioxidant activity as determined by the FRAP assay in the methanolic/formic acid extract (light grey bars) and after in vitro digestion (grey bars). RSO antioxidant activity as determined by the FRAP assay in the methanolic/formic acid extract (dark grey bars) and after in vitro digestion (black bars). Results are expressed as mg of ascorbic acid equivalent/100 g of raw or cooked onion. Different letters indicate that the values are significantly different ($p < 0.05$).

4. Conclusions

This study demonstrates that cooking treatments may modulate the release and bioaccessibility of onion phenolic compounds. Based on the initial fresh weight, all the treatments tended to decrease the phenolic compounds content respect to the raw samples as determined by mass spectrometry. The only exception was found for fried YSO. However, when the results were expressed referring to 100 g of cooked samples, baking, frying and grilling resulted in an increased amount of phenolic compounds in comparison with the raw onion samples. After in vitro digestion, baking for both the onion varieties and grilling for RSO significantly increased the bioaccessibility of onion phenolic compounds, fostering their delivery from the onion matrices. For both the onion varieties, baking was the thermal treatment that provided the highest amount of phenolic compounds after in vitro digestion. In particular, baking and grilling resulted in an increased gastro-intestinal availability of quercetin-3-*O*-hexoside-4'-*O*-hexoside, underlining the highest bioaccessibility respect to its aglycone or mono-hexosides. Considering its reported anti-proliferative activity against colon cancer cell lines, it is a potential front-runner in the prevention strategies designing [49,50]. Additionally, quercetin-3-*O*-hexoside-4'-*O*-hexoside can also be absorbed due to its considerable hydrophilic character and undergo further de-glycosylation at intestinal level. The corresponding aglycone may actually be absorbed and reach the systemic level where it can carry out its biological actions [50].

These results suggest how baking and grilling are the recommended cooking methods, not only for the healthy lack of use of cooking oils or fats (i.e., sunflower oil in frying), but also for the evidence of the proved bioaccessibility characteristics of onion health-promoting phenolic compounds (e.g., flavonols).

5. Supplementary materials

Table S1. Mass spectral data of phenolic compounds identified in onion samples in negative ionization mode.

Rt	Compound	$[M-H]^-$ *(m/z)*	MS^2 ion fragments *(m/z)*
7.7	Protocatechuic acid-hexoside	315	153 (100%), 109 (4%)
9.5	Quercetin-tri-*O*-hexoside isomer	786	625 (100%), 463 (10%), 301 (2%)
9.9	Kaempferol-7-*O*-hexoside isomer	447	285 (100%), 284 (18%), 257 (4%)
9.9	Taxifolin-*O*-hexoside isomer	465	285 (100%), 241 (8%), 303 (5%)
9.9	Kaempferol-hexoside-rhamnoside-rhamnoside	739	447 (100%), 284 (4%), 285 (3%)
10.0	Kaempferol-3-*O*-hexoside-7-*O*-hexoside isomer	609	447 (100%), 285 (4%)
10.1	(Epi)catechin-3-*O*-hexoside isomer	451	289 (100%), 245 (10%), 205 (5%)
10.2	Isorhamnetin-*O*-hexoside-*O*-pentoside	609	315 (100%), 447 (11%)
10.3	Caffeic acid-*O*-hexoside	341	179 (100%)
10.5	Taxifolin-*O*-hexoside isomer	465	303 (100%), 285 (43%), 241 (3%)
10.6	(Epi)catechin-3-*O*-hexoside isomer	451	289 (100%), 245 (16%), 205 (6%)
10.6	Quercetin-3-*O*-hexoside-7-*O*-hexoside	625	463 (100%), 301 (14%)
10.7	Myricetin-*O*-hexoside-*O*-hexoside isomer	641	479 (100%), 317 (11%)
10.8	Taxifolin-*O*-hexoside isomer	465	303 (100%), 285 (45%), 241 (5%)
10.8	Sinapic acid-*O*-hexoside isomer	385	223 (100%), 208 (5%)
11.4	Taxifolin-*O*-hexoside isomer	465	303 (100%), 285 (4%), 241 (4%)
11.5	Ferulic acid-*O*-hexoside	355	193 (100%), 178 (65%)
11.5	Sinapic acid-*O*-hexoside isomer	385	223 (100%), 208 (29%)
11.6	Myricetin-*O*-hexoside-*O*-hexoside isomer	641	479 (100%), 317 (12%)
11.8	Quercetin-7-*O*-hexoside-4'-*O*-hexoside	625	463 (100%), 301 (5%)
12.2	Quercetin-tri-*O*-hexoside isomer	786	625 (100%), 301 (7%), 463 (2%)
12.3	Quercetin-3-*O*-hexoside-4'-*O*-hexoside	625	463 (100%), 301 (9%). 179 (2%)
12.4	Kaempferol-3-*O*-hexoside-7-*O*-hexoside isomer	609	447 (100%), 285 (66%)
12.5	Isorhamnetin-3-*O*-hexoside isomer	477	315 (100%)
12.6	Isorhamnetin-3-*O*-hexoside-4'-*O*-hexoside	639	477 (100%), 315 (4%)
12.8	Taxifolin-*O*-hexoside isomer	465	303 (100%), 285 (93%), 241 (2%)
14.0	Quercetin-3-*O*-hexoside isomer	463	301 (100%), 179 (3%)
14.8	Isorhamnetin-3-*O*-hexoside isomer	477	315 (100%)
15.1	Quercetin-4'-*O*-hexoside	463	301 (100%), 179 (4%), 151 (3%)
15.4	Kaempferol-7-*O*-hexoside isomer	447	285 (100%), 284 (6%), 257 (4%), 151 (3%)
15.5	Isorhamnetin-4'-*O*-hexoside isomer	477	315 (100%)

16.1	Quercetin-3-*O*-glucoside	463	301 (100%), 151 (13%), 179 (4%)
16.1	Kaempferol-3-*O*-hexoside isomer	447	284 (100%), 285 (80%), 255 (5%)
18.1	Quercetin	301	179 (100%), 151 (65%)

Table S2. Mass spectral data of phenolic compounds identified in onion sample in positive ionization mode.

re	Compound	$[M]^+$ *(m/z)*	MS^2 ion fragments *(m/z)*
9.0	Cyanidin-*O*-hexoside-*O*-hexoside isomer	611	287 (100%), 449 (90%)
9.9	Cyanidin-3-*O*-hexoside	449	287 (100%)
10.0	Cyanidin-*O*-hexoside-*O*-hexoside isomer	611	287 (100%), 449 (20%)
10.5	Peonidin-3-*O*-hexoside	463	301 (100%)
10.7	Cyanidin-*O*-malonyl-hexoside isomer	535	287 (100%)
11.0	Cyanidin-*O*-malonyl-hexoside isomer	535	287 (100%), 449 (5%)
11.1	Cyanidin-*O*-hexoside-*O*-malonyl-hexoside	697	287 (100%)
11.4	Malvidin-*O*-hexoside-acetaldehyde	517	355 (100%), 339 (32%)
11.7	Peonidin-*O*-malonyl-hexoside isomer	549	301 (100%), 463 (3%)

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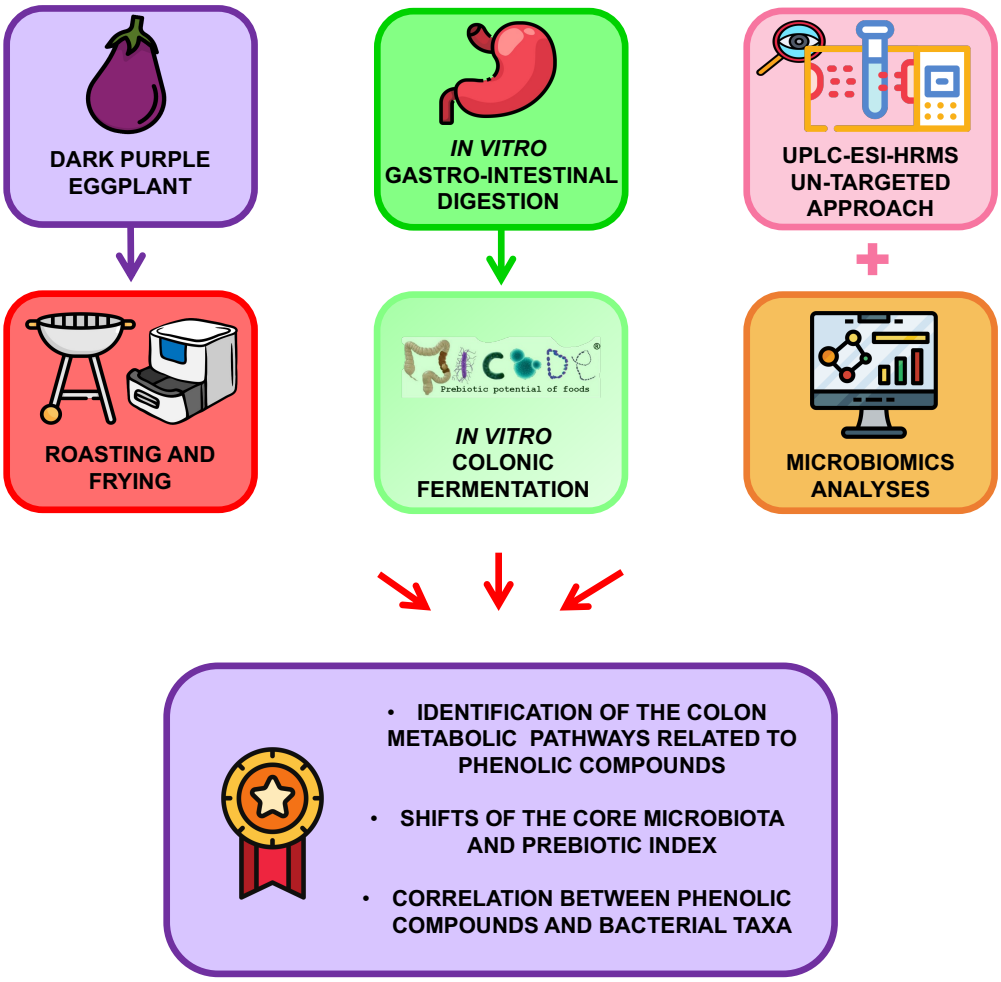
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L. Nissen[#], A. Cattivelli[#], F. Casciano, A. Gianotti, D. Tagliazucchi. Roasting and frying modulate the phenolic profile of dark purple eggplant and differently change the colon microbiota and phenolic metabolites after *in vitro* digestion and fermentation in a gut model. *Food Research International*, **2022**, *160*, 111702. doi: 10.1016/j.foodres.2022.111702

ROASTING AND FRYING MODULATE THE PHENOLIC PROFILE OF DARK PURPLE EGGPLANT AND DIFFERENTLY CHANGE THE COLON MICROBIOTA AND METABOLITES AFTER IN VITRO DIGESTION AND FERMENTATION IN A GUT MODEL

ABSTRACT

The way of cooking vegetables could differently affect the phenolic profiles of foods and their impact on human colon microbiota. In this work, we investigated the stability and bioaccessibility as well as the impact and fate of dark purple eggplant (DPE) phenolic compounds in the gut microbiota after grilling or frying in comparison to the raw one. After cooking, DPE underwent a gastro-intestinal digestion along with a proximal colon fermentation using the short-term batch model MICODE (multi-unit *in vitro* colon gut model). During the process, the phenolic compounds profiles (through high-resolution mass spectrometry) and microbiomics (qPCR of 14 core taxa) analyses were performed. Results showed that thermal treatments increased the amount of extractable phenolic compounds as well as their bioaccessibility. The highest gastro-intestinal release was observed in fried DPE (2468.46 ± 13.64 µmol/100g), followed by grilled DPE (1007. 96 ± 12.84 µmol/100g) and raw DPE (900.93 ± 10.56 µmol/100g). Mass spectrometry analysis confirmed that colonic bacteria were able to metabolize DPE phenolic compounds mainly to 3-(3'-hydroxyphenyl)propanoic acid. Furthermore, results indicated that frying was better than grilling in terms of fostering more the growth of beneficial bacterial taxa and limiting that of opportunistic taxa. For example, fried DPE determined an increase in abundance of *Bifidobacteriaceae Lactobacillales* of 2.66 and 3.80 times. This work is one of the first exploring how cooking methods can affect the phenolic composition of DPE and differently impact on the colon microbiota tuning and modifying the food functionalities.

Keywords: thermal treatments, hydroxycinnamic acids, prebiotic index, food processing, mass spectrometry, microbiomics

1. Introduction

Eggplant is a typical component of the Mediterranean Diet, which is considered a notable source of phenolic compounds, especially hydroxycinnamic acid-derivatives mainly conjugates with quinic acid or amides (Martini, Conte, Cattivelli, & Tagliazucchi, 2021). The worldwide eggplant production in 2019 was more than 50 million of tons per year making this vegetable one of the most important crop of the solanaceous family together with tomato, potato and pepper. In the USA the consumption per capita of eggplant was estimated about 0.5 kg in 2020 (Karimi, Kazemi, Samani, & Simal-Gandara, 2021). Several health benefits have been linked to eggplant intake, especially related to the prevention of chronic pathologies such as cardiovascular diseases, cancer and inflammatory conditions (Gürbüz et al., 2018; Nishimura et al., 2019). These supposed beneficial effects of eggplant consumption have been correlated to the presence of phenolic compounds and in particular of hydroxycinnamic acids (Gürbüz et al., 2018). Various *in vivo* and *in vitro* studies demonstrated the potential cardio-protective effects as well as the anti-diabetic, anti-carcinogenic and anti-inflammatory properties of hydroxycinnamic acids and derived metabolites (Clifford, 2000; Sova, & Saso, 2020).

Besides composition, technological treatments may alter the food composition including the quali/quantitative profile of beneficial compounds and their possibility to be bioaccessible and thereafter bioavailable. Indeed, differently from many Mediterranean Diet vegetable foods, eggplant is consumed exclusively after cooking, largely after frying and grilling that are the two most common thermal treatments to which eggplant are subjected. Various studies pointed out that cooking procedure may modify the phenolic profile of vegetables foods also resulting in a differential release of individual phenolic compounds after *in vitro* gastro-intestinal digestion (Valli, et al., 2016; Saa, Di Silvestro, Dinelli, & Gianotti, 2017; Cattivelli, Conte, Martini, & Tagliazucchi, 2021; Martini et al., 2021). Furthermore, variations in the phenolic composition after cooking and *in vitro* digestion resulted in a different pattern of colonic metabolites after *in vitro* fermentation of raw and cooked cardoon, green pepper and cactus cladodes (De Santiago, Gill, Carafa, Tuohy, De Peña, & Cid, 2019; Juániz et al., 2016; Juániz et al., 2017). In addition, it has been shown that both food composition and thermal treatments altered the composition of the gut microbiota (Pérez-Burillo et al., 2018).

Specifically, increasing evidence suggests that the supposed health effects of phenolic compounds are probably related to the interaction with gut microbiota including the bio-activation of phenolic compounds by gut bacterial metabolism and the preservation of a healthy microbial population (Marhuenda-Muñoz et al., 2019). For example, phenolic compounds may enhance the relative abundance of *Lactobacillus* and *Bifidobacterium* species that are involved in the protection of the intestinal epithelium, as well as of *Faecalibacterium prausnitzii* and *Akkermansia muciniphila*, which exhibited anti-inflammatory effects (Corrêa et al., 2019; Kasprzak-Drozd, Oniszczuk, Stasiak, & Oniszczuk, 2021; Mena, & Bresciani, 2020). On the other hand, gut bacteria can modify the structure of phenolic compounds generating metabolites that are

more easily absorbed than the parent compounds and presented important biological activities (Koudoufio et al., 2020; Marhuenda-Muñoz et al., 2019).

In order to shed light on the network involving food, processes, bioaccessibility, and bioavailability for humans, more recently *in vitro* gut models were used to study some mechanisms involved in gut microbiota interactions with food components including plant phytochemicals. *In vitro* gut models are considered a proper solution because they can explain the impact of prebiotics on human gut microbiota, focusing on the shift in the core microbial groups and that of selected species and their metabolites, assaying composition and abundance in the community over time (Nissen, Casciano & Gianotti, 2020a). In particular, we performed this study employing the gut model MICODE (Multi-Unit *in vitro* Colon Model), previously used with success to study food matrices rich in phenolic compounds (Nissen et al., 2021a).

Therefore, the objective of this study was to assess the effect of the cooking methods (grilling and frying) on the dark purple eggplant phenolic compounds, their release during *in vitro* gastro-intestinal digestion and the main metabolites produced after their *in vitro* colonic fermentation. Moreover, the shift of fecal microbiota groups associated to those changes were also evaluated.

2. Materials and methods

2.2. Dark purple eggplant (DPE) cooking and in vitro gastro-intestinal digestion

DPE (Black Beauty variety) samples underwent to two different cooking treatments, i.e. frying and grilling, as reported in Martini et al. (2021). Briefly, for each treatment, a whole DPE fruit was weighed, washed with distilled water, dried with paper towels and cut in slices of approximatively 1.0 cm. One portion (corresponding to about half of the eggplant weight) was immediately frozen at −80 °C and used as raw sample, whereas the other portion was subjected to the cooking treatment. For the grilling procedure, the DPE slices were cooked at 120 °C for 10 min on a grilling plate. For the frying procedure, the DPE slices were fried at 170 °C for 10 min in sunflower oil by using a domestic deep fryer. After frying, DPE slices were drained of and dabbed with paper towels to absorb the oil in excess, and stored at −80 °C until analysis. The *in vitro* gastro-intestinal digestion was carried out on raw and cooked DPE samples by applying the INFOGEST protocol (Minekus et al., 2014). The full description of the digestion protocol is reported in Martini et al (2021). At the end of the intestinal phase, an aliquot of sample (1 mL) was withdrawn, centrifuged (10000*g*, 10 min, 4 °C) and the supernatant stored at -80 °C for mass spectrometry analysis. Another portion of 1 g of the sample was subjected to the *in vitro* colonic fermentation trials as described below. The remaining material (about 30 mL of digested sample) was stored at -80 °C. Cooking treatments and digestions were carried out in triplicate.

2.3. Colonic fermentation

2.3.1 Fecal donors

The fecal samples used for this study were obtained from three lean healthy individuals that respected the inclusion criteria as previously reported (Connoly et al, 2012; Koutsos et al., 2017; Oba et al., 2020; Nissen et al., 2021a; Nissen et al., 2021b; Arnal et al., 2021). Briefly, the three healthy subjects, two females and one male aged between 30 and 45 y did not undergo antibiotic treatment for at least 3 months prior to stool collection, did not intentionally consume pre- or probiotic supplements before the experiment, and had no history of bowel disorders. Additionally, the donors were normal weight, not smokers, not chronically consuming any drug, and not alcoholic drink consumers. Fecal samples were donated two times (with an interval of seven days) for the two biological replicas. Fecal samples were collected, and processed as previously described (Nissen et al., 2021a; Nissen et al., 2021b). Briefly, to collect fecal samples, donors were provided and instructed to use a collection kit, which includes a stool collector (Sarstedt AG & Co. KG, Nümbrecht, Germany) and an anaerobic jar with a O_2 catalyst (Oxoid, Thermo Fisher Scientific, Waltham, MA, USA). Fecal samples were then maintained at 4 °C and processed within 2 h. The fecal slurry was prepared by homogenizing 6 g of feces (2 g of each donation) in 54 mL of O_2 reduced phosphate-buffered saline (PBS). The three healthy donors were told of the study's aims and procedures and gave their verbal consent for

their fecal matter to be used for the experiments, in agreement with the ethics procedures required at the University of Bologna.

2.3.2 Fecal batch-culture fermentation and sampling

Colonic fermentations were conducted for 24 hours in independent vessels using an *in vitro* gut model, MICODE (Nissen et al., 2021a; Nissen et al., 2021b), obtained by the assembly of Minibio Reactors (Applikon Biotechnology BV, Delft, NL) and controlled by Lucullus PIMS software (Applikon Biotechnology BV, NL). The preparation of the experiments was made according to published procedures (Connoly et al., 2012; Koutsos et al., 2017; Wang et al., 2020) and described in detail in Nissen et al. (2021a). Briefly, fermentation vessels were filled aseptically with 90 mL of basal medium (Connoly et al., 2012; Koutsos et al., 2017). Once the proximal colon ecological condition was reached, a first vessel was aseptically loaded with 10 mL of a mixtures including fecal slurry (FS) (10% w/v of human feces in O_2 reduced PBS) and 1 g of the solid portion of *in vitro* digested fried DPE (FDPE), a second vessel was loaded with 10 mL of FS including 1 g of the solid portion of *in vitro* digested grilled DPE (GDPE), a third vessel was loaded with 10 mL of FS including 1 g of the solid portion of *in vitro* digested raw DPE (RDPE), and a fourth vessel was set as the blank control (BC) and was loaded with 10 mL of FS including 1 mL of digestive enzymes. Batch cultures were run under controlled conditions for a period of 26.55 h including the baseline (BL) (2.55 ± 0.11 h) as described in Nissen et al. (2021b). Sampling was performed as reported in Nissen et al. (2021b). Samples of the different time points were used for UHPL/HR-MS and qPCR. Technical replicas of analyses were conducted in triplicates from two independent biological experiments.

2.4. Identification and quantification of the phenolic compounds by ultra-high-performance liquid chromatography/high resolution mass spectrometry (UHPLC/HR-MS)

2.4.1. Phenolic compounds and faecal metabolites extraction

The extraction of phenolic compounds from raw and cooked non-digested DPE was performed according to Martini et al. (2021). Raw and cooked samples were mixed with a water/methanol/formic acid (28:70:2, v/v/v) solution in a ratio of 1:2 (w/v). After homogenization, the samples were incubated at 37 °C for 30 min, centrifuged (6000*g*, 20 min, 4 °C) and the collected supernatant analysed by UHPLC/HR-MS.

Samples collected at the end of the *in vitro* gastro-intestinal digestion were analysed by UHPLC/HR-MS without any further extraction procedure.

Faecal metabolites were extracted following the protocol of Juániz et al. (2017). Briefly, fermented samples were mixed with the extraction solution (water/methanol/formic acid; 19.9:80:0.1, v/v/v) in a ratio of 1:1

(v/v). Then the mixtures were vortexed for 1 min and centrifuged at 12000*g* for 10 min at 4 °C. The supernatants were then collected and analysed by UHPLC/HR-MS.

2.4.2. Phenolic profile of raw and cooked non-digested, in vitro digested and in vitro fermented dark purple eggplant

Qualitative and quantitative profiles of phenolic compounds were performed using an UHPLC Ultimate 3000 separation module equipped with a C18 column (Acquity UPLC HSS C18 Reversed phase, 2.1 × 100 mm, 1.8 µm particle size, Waters, Milan, Italy) and coupled with a Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). Data were acquired by both negative and positive ionization modes. The composition of the mobile phases, the elution gradient and the mass spectrometry parameters are fully described in Martini, Tagliazucchi, Minelli, & Lo Fiego (2020).

Quantification was carried out by building external calibration curves with the available standard compounds as depicted in **Table S1**.

The name of the phenolic metabolites generated during colonic bacterial fermentation is reported by following the recommendation previously reported (Kay et al., 2020).

2.5 Microbiota analyses

2.5.1 Enumeration of bacterial groups and qPCR Prebiotic Index (qPI)

Bacterial DNA was extracted just after sampling (Purelink Microbiome DNA Purification Kit by Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA) and quantified (Biodrop, Biochrom Ltd., Cambridge, UK). Enumeration of bacterial groups was made by qPCR to evidence changes in the microbiota after fermentation (Tanner et al., 2014; Westfall, Lomis & Prakash, 2018; Tsitko et al., 2019; Tamargo et al., 2022) following previous protocols (Modesto et al., 2011; Nissen et al., 2020b; Nissen et al., 2021b). 14 different bacterial taxa (*Eubacteria*, *Firmicutes*, *Bacteroidetes*, *Lactobacillales*, *Bifidobacteriaceae, Enterobacteriaceae*, *Clostridium* group I, *Clostridium* group IV, *Bacteroides-Prevotella-Porphyromonas* (BPP) group, *Atopobium-Collinsella-Eggerthella* (ATOP) group, *Bifidobacterium longum*, *Escherichia coli*, *Faecalibacterium prausnitzii*, and *Akkermansia muciniphila*) (**Table S2**) were assessed by qPCR on a QuantStudio 5 System (Applied Biosystem, Thermo Fisher, USA). The qPCR Prebiotic Index (qPI) was calculated as reported in Nissen et al. (2021b).

2.6. Data processing and statistical analysis

The shifts in abundance of bacterial groups were calculated as $Log_2(F/C)$ on raw data (Love, Huber & Anders, 2014) obtained from sextuplicates. Also, from the raw data, the qPI was obtained after normalization with the mean centering method. ANOVA followed by a Tukey post-hoc test was applied using Statistica v 8.0 (Tibco, Palo Alto, CA, USA). The differences were considered significant with $p < 0.05$. To address specific correlations among bacteria and metabolites, two independent datasets were merged and computed by Spearman Rank analysis and visualized with a two-way joining heatmap, including Pearson dendrograms with complete linkage. The dataset of correlations was performed with Statistica v. 8.0 (Tibco, USA) and the heatmap was produced with the Expression tool on www.heatmapper.ca (Babicki et al., 2016).

3. Results and discussion

3.1. Phenolic compound profiles of raw and cooked DPE

The phenolic compound profiles of raw (RDPE) fried (FDPE) and grilled (GDPE) eggplant was investigated through high-resolution mass spectrometry. The mass spectral data of the identified phenolic compounds are reported in supplementary **Table S3** whereas the quantitative data are displayed in **Table 1**. A total of 48 phenolic compounds were detected and quantified in DPE samples, being the *trans* isomer of 5-*O*-caffeolyquinic acid the principal compound in both raw and cooked DPE samples, where it accounted for more than 65% of the total amount of phenolic compounds. In general, caffeoylquinic acids was the most representative class of phenolic compound accounting for the 78.0%, 73.0% and 79.8% of total phenolic compounds in RDPE, FDPE and GDPE, respectively. In addition, feruloylquinic acids made up the 15.9%, 14.3% and 17.3% of total phenolic compounds in RDPE, FDPE and GDPE. Small amounts of flavonols and anthocyanins were also identified in eggplant samples, especially in RDPE (**Table 1**). These data are in agreement with previous studies showing that hydroxycinnamic acids and, in particular, caffeoylquinic acids dominated the phenolic profiles of eggplant (Gürbüz, Uluişik, Frary, Frary, & Doǵanlar, 2018; Martini et al., 2021; Niño-Medina, Urías-Orona, Muy-Rangel, & Heredia, 2017).

RDPE displayed a total amount of 900.9 ± 14.8 µmol/100 g of eggplant of phenolic compounds (**Table 1**). After submitting eggplant to frying and grilling, total phenolic compounds increased by 174% and 12%, respectively. After grilling, a small increase in the amount of caffeoylquinic and feruloylquinic acids of 14.5% and 21.8%, respectively, was detected. At the same time, the amount of dicaffeoylquinic acids and hydroxycinnamic acids-spermidine derivatives decreased by about 29% and 64%. Grilling also caused strong degradation of flavonols (~63%) and especially of anthocyanins (~81%). On the contrary, frying treatment brought about an increase in all of the classes of hydroxycinnamic acids. The highest increase in terms of concentration was found for caffeoylquinic acids and feruloylquinic acids. However, the utmost percentage increase was detected for hydroxycinnamic acids-spermidine derivatives (~932%) and dicaffeoylquinic acids (~272%).

3.2. Phenolic compound profiles of raw and cooked DPE after in vitro gastro-intestinal digestion

As reported in **Table 1**, the bioaccessibility of phenolic compounds in DPE following *in vitro* gastro-intestinal digestion was greatly influenced by the applied cooking method. In RDPE, only trace amounts of some hydroxycinnamic acids were detected after the digestion procedure. On the contrary, the two thermal treatments caused an important increase in the amount of phenolic compounds released after *in vitro* digestion, with a bioaccessibility index of 47.9% and 24.7% in FDPE and GDPE, respectively. In absolute terms, frying was the thermal treatment that caused the highest release of phenolic compounds after digestion

(1182.2 ± 24.4 µmol/100 g of eggplant) whereas after grilling the amount of released phenolic compounds was 4.8 times lower (248.7 ± 6.9 µmol/100 g of eggplant).

Table 1. Concentration of individual phenolic compounds in raw and cooked dark purple eggplant. Results are expressed in µmol of phenolic compound/100 g of eggplant. Bioaccessibility index (BI) is defined as the ratio between the amount of a specific phenolic compound after *in vitro* digestion and the amount in the solvent extract expressed as percentage value.

Compound	*RDPE*			*FDPE*			*GDPE*		
	Before digestion	After digestion	BI (%)	Before digestion	After digestion	BI (%)	Before digestion	After digestion	BI (%)
Hydroxycinnamic acids									
3-*O*-Caffeoylquinic acid *cis*	1.54 ± 0.03	n.d.	0	31.42 ± 1.09	76.56 ± 0.73	243.7	2.36 ± 0.01	80.9 ± 0.54	3427
4-*O*-Caffeoylquinic acid *cis*	1.15 ± 0.02	n.d.	0	4.04 ± 0.02	2.37 ± 0.01	58.7	2.34 ± 0.01	1.51 ± 0.01	64.5
5-*O*-Caffeoylquinic acid *trans*	609.33 ± 8.73	0.11 ± 0.01	0.02	1618.40 ± 9.27	792.92 ± 17.18	49.0	697.16 ± 11.41	118.95 ± 5.70	17.1
4-*O*-Caffeoylquinic acid *trans*	20.02 ± 0.12	n.d.	0	140.26 ± 0.50	175.67 ± 4.26	125.2	30.28 ± 0.20	13.19 ± 0.20	43.6
5-*O*-Caffeoylquinic acid *cis*	70.66 ± 0.84	n.d.	0	8.29 ± 0.10	25.61 ± 0.06	308.9	72.12 ± 0.98	17.78 ± 0.25	24.7
Di-hydro-caffeoylquinic acid	0.03 ± 0.00	n.d.	0	0.41 ± 0.01	0.10 ± 0.01	24.4	0.04 ± 0.00	n.d.	0
3-*O*-Feruloylquinic acid *cis*	0.31 ± 0.01	0.34 ± 0.01	109.7	1.81 ± 0.03	0.63 ± 0.01	34.8	n.d.	n.d.	n.d.
4-*O*-Feruloylquinic acid *cis*	0.13 ± 0.01	n.d.	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
5-*O*-Feruloylquinic acid *trans*	24.85 ± 0.26	0.46 ± 0.01	1.9	44.30 ± 0.33	34.80 ± 0.95	78.6	27.73 ± 0.96	11.06 ± 0.14	39.9
O-Feruloylquinic acid	2.13 ± 0.04	n.d.	0	14.10 ± 0.23	n.d.	0	3.88 ± 0.01	n.d.	0
4-*O*-Feruloylquinic acid *trans*	97.50 ± 2.95	n.d.	0	274.44 ± 5.77	0.59 ± 0.01	0.2	120.77 ± 1.11	n.d.	0
O-Feruloylquinic acid	9.13 ± 0.63	n.d.	0	8.56 ± 0.58	5.16 ± 0.03	60.3	13.55 ± 0.33	1.07 ± 0.01	7.9
5-*O*-Feruloylquinic acid *cis*	9.20 ± 0.15	n.d.	0	9.91 ± 0.07	n.d.	0	8.60 ± 0.07	n.d.	0
1,4-Di-*O*-caffeoylquinic acid	0.60 ± 0.03	n.d.	0	9.15 ± 0.12	14.85 ± 0.53	162.3	0.48 ± 0.02	0.36 ± 0.01	75.0
1,5-Di-*O*-caffeoylquinic acid	19.45 ± 0.07	n.d.	0	55.77 ± 0.37	15.83 ± 0.21	28.4	6.31 ± 0.04	0.13 ± 0.01	2.1
3,5-Di-*O*-caffeoylquinic acid	3.06 ± 0.10	n.d.	0	6.47 ± 0.07	n.d.	0	1.05 ± 0.02	n.d.	0
4,5-Di-*O*-caffeoylquinic acid	n.d.	n.d.	n.d.	14.23 ± 0.53	13.69 ± 0.10	96.2	0.50 ± 0.01	0. 46 ± 0.02	92.0
3,4-Di-*O*-caffeoylquinic acid	n.d.	n.d.	n.d.	0.36 ± 0.01	0.20 ± 0.00	55.6	n.d.	n.d.	n.d.
N^1,N^5-Caffeoyl-caffeoyl-spermidine	0.67 ± 0.01	n.d.	0	2.94 ± 0.08	0.38 ± 0.01	12.9	0.49 ± 0.01	0.14 ± 0.01	28.6
N^1,N^{10}-caffeoyl-caffeoyl-spermidine	3.65 ± 0.03	n.d.	0	33.99 ± 0.39	1.77 ± 0.01	5.2	2.61 ± 0.05	n.d.	0
N^1,N^5-Di-hydro-caffeoyl-caffeoyl-spermidine	6.88 ± 0.15	n.d.	0	54.18 ± 0.60	2.30 ± 0.01	4.2	3.54 ± 0.09	n.d.	0
N^1,N^{10}- Di-hydro-caffeoyl-caffeoyl-spermidine	1.63 ± 0.01	n.d.	0	14.34 ± 0.17	0.63 ± 0.01	4.4	0.90 ± 0.01	n.d.	0
N^1,N^5-Di-hydro-caffeoyl-di-hydro-caffeoyl-spermidine	7.92 ± 0.52	n.d.	0	108.79 ± 1.43	4.30 ± 0.02	4.0	7.11 ± 0.08	n.d.	0
Hydroxycinnamic acid	n.d.	n.d.	n.d.	0.31 ± 0.01	0.14 ± 0.01	45.2	0.14 ± 0.01	n.d.	0
Coumaric acid	n.d.	0.52 ± 0.01	n.f.	0.53 ± 0.01	0.52 ± 0.01	98.1	n.d.	0.40 ± 0.01	n.f.
Di-hydro-coumaric acid isomer	n.d.	n.d.	n.d.	n.d.	0.31 ± 0.01	n.f.	0.17 ± 0.01	0.28 ± 0.01	164.7

Di-hydro-coumaric acid isomer	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.17 ± 0.01	0.24 ± 0.01	141.2
Caffeic acid	0.16 ± 0.01	n.d.	0	1.02 ± 0.02	9.68 ± 0.16	949.0	0.25 ± 0.01	0.16 ± 0.01	64.0
Di-hydroxycinnamic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d	0.02 ± 0.01	0.03 ± 0.00	150.0
Di-hydro-caffeic acid	n.d.	n.d.	n.d.	n.d.	0.03 ± 0.00	n.f.	n.d.	n.d.	n.d.
Caffeoylshikimic acid isomer	0.42 ± 0.01	n.d.	0	0.05 ± 0.01	0.04 ± 0.00	80.0	0.14 ± 0.01	n.d.	0
Caffeoylshikimic acid isomer	n.d.	n.d.	n.d.	n.d.	0.06 ± 0.00	n.f.	n.d.	n.d.	n.d.
Caffeoyl-tryptophan-hexoside isomer	0.52 ± 0.01	n.d.	0	0.42 ± 0.01	n.d.	0	0.07 ± 0.01	n.d.	0
Caffeoyl-tryptophan-hexoside isomer	0.76 ± 0.01	n.d.	0	3.50 ± 0.10	n.d.	0	0.18 ± 0.01	n.d.	0
Caffeoyl-hexoside isomer	0.09 ± 0.01	n.d.	0	0.01 ± 0.00	n.d.	0	0.05 ± 0.01	n.d.	0
Caffeoyl-hexoside isomer	0.04 ± 0.01	n.d.	0	0.44 ± 0.01	0.11 ± 0.00	25.0	0.08 ± 0.01	0.03 ± 0.00	37.5
Caffeoyl-hexoside isomer	0.01 ± 0.00	0.02 ± 0.00	200.0	0.08 ± 0.00	0.05 ± 0.00	62.5	0.04 ± 0.01	0.04 ± 0.00	100.0
Caffeoyl-hexoside isomer	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.01 ± 0.01	n.d.	0
3-*O*-Coumaroylquinic acid	n.d.	0.42 ± 0.01	n.f.	n.d.	n.d.	n.d.	n.d.	1.25 ± 0.01	n.f.
5-*O*-Coumaroylquinic acid *trans*	2.52 ± 0.04	0.15 ± 0.01	6.0	2.63 ± 0.04	1.90 ± 0.04	72.2	2.63 ± 0.02	0.58 ± 0.01	22.1
4-*O*-Coumaroylquinic acid	n.d.	0.17 ± 0.01	n.f.	n.d.	0.37 ± 0.01	n.f.	n.d.	0.28 ± 0.01	n.f.
5-*O*-Coumaroylquinic acid *cis*	0.83 ± 0.02	0.12 ± 0.01	14.5	0.29 ± 0.01	0.28 ± 0.01	96.6	0.63 ± 0.01	0.32 ± 0.01	50.8
Total hydroxycinnamic acids	**895.22 ± 10.56**	**2.31 ± 0.03**	**0.3**	**2465.44 ± 13.34**	**1181.88 ± 17.75**	**47.9**	**1006.43 ± 12.84**	**248. 44 ± 5.74**	**24.7**
Hydroxybenzoic acids									
Hydroxybenzoic acid isomer	0.03 ± 0.00	n.d.	0	0.14 ± 0.00	0.06 ± 0.00	42.9	0.06 ± 0.00	0.06 ± 0.00	100.0
Hydroxybenzoic acid isomer	0.04 ± 0.00	0.06 ± 0.00	150.0	0.07 ± 0.00	0.08 ± 0.00	114.3	0.07 ± 0.00	0.09 ± 0.00	112.5
Protocatechuic acid	0.01 ± 0.00	n.d.	0	0.07 ± 0.00	0.10 ± 0.00	14.3	0.08 ± 0.00	0.08 ± 0.00	100.0
Total hydroxybenzoic acids	**0.08 ± 0.00**	**0.06 ± 0.00**	**75.0**	**0.28 ± 0.00**	**0.24 ± 0.00**	**85.7**	**0.21 ± 0.00**	**0.23 ± 0.00**	**109.5**
Flavonols									
Quercetin-3-*O*-rutinoside	1.10 ± 0.01	n.d.	0	0.58 ± 0.01	n.d.	0	0.26 ± 0.01	n.d.	0
Quercetin-3-*O*-hexoside-rhamnoside	0.03 ± 0.00	n.d.	0	0.02 ± 0.00	0.01 ± 0.00	50.0	0.02 ± 0.00	n.d.	0
Kaempferol-3-*O*-hexoside	0.02 ± 0.00	n.d.	0	n.d.	n.d.	n.d.	0.01 ± 0.00	n.d.	0
Kaempferol-3-*O*-rutinoside	0.09 ± 0.00	n.d.	0	0.03 ± 0.00	0.01 ± 0.00	33.3	0.07 ± 0.00	0.01 ± 0.00	14.3
Kaempferol-3-*O*-hexoside-rhamnoside	0.01 ± 0.00	n.d.	0	0.01 ± 0.00	n.d.	0	0.01 ± 0.00	n.d.	0
Kaempferol-3-*O*-hexoside-hexoside	0.02 ± 0.00	n.d.	0	0.02 ± 0.00	0.01 ± 0.00	50.0	0.02 ± 0.00	n.d.	0
Myricetin-3-*O*-rutinoside	0.12 ± 0.01	n.d.	0	0.80 ± 0.01	0.01 ± 0.00	1.25	0.14 ± 0.01	n.d.	0
Total flavonols	**1.39 ± 0.01**	**n.d.**	**0**	**1.46 ± 0.01**	**0.04 ± 0.00**	**2.7**	**0.52 ± 0.01**	**0.01 ± 0.00**	**1.9**

Anthocyanins									
Delphinidin-3-*O*-rutinoside isomer	3.83 ± 0.03	n.d.	0	1.12 ± 0.02	n.d.	0	0.64 ± 0.02	n.d.	0
Delphinidin-3-*O*-rutinoside isomer	0.05 ± 0.00	n.d.	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Delphinidin-3-*O*-rutinoside-5-*O*-hexoside isomer	0.23 ± 0.01	n.d.	0	0.15 ± 0.00	n.d.	n.d.	0.13 ± 0.00	n.d.	0
Delphinidin-3-*O*-rutinoside-5-*O*-hexoside isomer	0.06 ± 0.01	n.d.	0	n.d.	n.d.	0	n.d.	n.d.	n.d.
Cyanidin-3-*O*-rutinoside isomer	0.09 ± 0.00	n.d.	0	0.01 ± 0.00	n.d.	n.d.	0.04 ± 0.00	n.d.	0
Total anthocyanins	**4.25 ± 0.03**	**n.d.**	**0**	**1.28 ± 0.02**	**n.d.**	**0**	**0.81 ± 0.02**	**n.d.**	**0**
Total	**900.93 ± 10.56**	**2.37 ± 0.03**	**0.3**	**2468.46 ± 13.64**	**1182. 16 ± 17.75**	**47.9**	**1007. 96 ± 12.84**	**248. 67 ± 5.74**	**24.7**

Different letters within the same row mean significant different ($P<0.05$) values.
n.d. means that the compound was not detected in the sample
n.f. means newly formed compound
Abbreviations are: RDPE, raw dark purple eggplant; FDPE, fried dark purple eggplant; GDPE, grilled dark purple eggplant

A similar cooking effect was already described for other food matrices such as cardoon, green pepper and onion as well as for eggplant (Cattivelli et al., 2021; Juániz et al., 2016; Juániz et al., 2017; Martini et al., 2021). In all of these samples, frying and grilling resulted in a higher bioaccessibility of phenolic compounds respect to the raw food. These results may be a consequence of the matrix softening effect due to the thermal treatments, which enhance the release of phenolic compounds from the food matrices (Palermo, Pellegrini, & Fogliano, 2014).

The classes of compounds with the lowest bioaccessibility in both FDPE and GDPE were feruloylquinic acids and hydroxycinnamic acids-spermidine derivatives. Caffeoylquinic and dicaffeoylquinic acids showed higher bioaccessibility than the other hydroxycinnamic acids in both the cooked samples (**Table 1**). Some compounds in both FDPE and GDPE showed a bioaccessibility index higher than 100%. The increase in concentration of these compounds may be the consequence of both isomerization and hydrolysis reactions. Acyl migration from 5-*O*-caffeoylquinic acid to the 4- and 3-*O*-isomers was observed after *in vitro* digestion of both GDPE and FDPE. Former works revealed isomerization phenomena during *in vitro* digestion of several food matrices from 5-caffoylquinic acid to the 4- and 3-isomers (Juániz et al., 2017; Martini et al., 2019; Martini et al., 2021; Monente, Ludwig, Stalmach, De Pena, Cid, & Crozier, 2015). Similarly, the increase in 1,4-di-*O*-caffeoylquinic acid concentration observed in digested FDPE may be due to the isomerization of other di-caffeoylquinic acids isomers as already observed (Juániz et al., 2017; Martini et al., 2021). Likewise, isomerization of 5-*O*-coumaroylquinic acid to the 4- and 3-acyl structure may account for the changes in the concentration of coumaroylquinic acid isomers observed after digestion (Kahle et al., 2011; Martini et al., 2019). Furthermore, the increase in the concentration of 5-*O*-caffeoylquinic acid and caffeic acid may result from the hydrolysis of di-caffeoylquinic acids and caffeoylquinic acids, respectively. Previous studies pointed out the formation of hydrolysis products of di- and mono-caffeoylquinic acids after incubation under alkaline

conditions (as found in the intestinal fluid) or with pancreatic juice (Da Encarnação, Farrel, Ryder, Kraut, & Williamson, 2015; Deshpande, Jaiswal, Matei, & Kuhnert, 2014; Kahle et al., 2011).

3.3. Identification of phenolic compound metabolites after in vitro faecal fermentation

Previous studies involving healthy humans and ileostomists demonstrated that, after consumption of coffee, only about the 30% of hydroxycinnamic acids were absorbed in the small intestine and appeared in plasma as parent compounds and/or sulphate and glucuronide conjugates (Stalmach et al., 2009; Stalmach, Steiling, Williamson, & Crozier, 2010). On the contrary, the 70% of ingested hydroxycinnamic acids reached the colon where they underwent to the action of the colonic microbiota (Stalmach et al., 2009; Stalmach et al., 2010). Similar results were obtained after consumption of artichoke or yerba mate where more than 80% of absorbed hydroxycinnamic acids appeared in the plasma or urine as colonic metabolites (Domínguez-Fernández, Young Tie Yang, Ludwig, Clifford, Cid, & Rodríguez-Mateos, 2022; Gómez-Juaristi, Martínez-López, Sarria, Bravo, & Mateos, 2018). Therefore, following *in vitro* gastro-intestinal digestion, raw and cooked DPE were subjected to faecal fermentation and the phenolic compounds and metabolites were analysed at different time points (6, 18 and 24 h) by high-resolution mass spectrometry. As reported in **Tables 2-4**, most of the native phenolic compounds were degraded after 6 hours of *in vitro* fermentation. Independently on the treatment, isomers of caffeoylquinic acids, dicaffeoylquinic acids, coumaroylquinic acids and caffeic acid-hexosides were totally degraded after 6 hours of incubation. Contemporaneously, an increase of 3',4'-dihydroxycinnamic acid (caffeic acid) concentration as well as the appearance of several isomers of two related metabolites, namely di-hydro-caffeoylquinic acid and 3-(3',4'-dihydroxyphenyl)propanoic acid (di-hydro-caffeic acid), was observed. Otherwise, some residual amounts of two feruloylquinic acid isomers were recorded together with the appearance of 4'-hydroxy-3'-methoxycinnamic acid (ferulic acid) and 3-(4'-hydroxy-3'-methoxyphenyl)propanoic acid (di-hydro-ferulic acid). Additional newly formed metabolites detected after 6 h of fermentation were 3-(3'-hydroxyphenyl)propanoic acid (di-hydro-coumaric acid), 3-(4'-hydroxyphenyl)propanoic acid (phloretic acid), 3,4-dihydroxybenzoic acid (protocatechuic acid), 4-hydroxy-3-methoxybenzoic acid (vanillic acid) and 4'-hydroxy-3'-methoxyphenylacetic acid (homovanillic acid) (**Tables 2-4**). After 18 hours of fermentation, the majority of the previously identified phenolic compounds and metabolites disappeared in all the DPE samples. The metabolite present at the highest concentration after 18 and 24 h in each sample was 3-(3'-hydroxyphenyl)propanoic acid although some other metabolites such as 3-(3',4'-dihydroxyphenyl)propanoic acid, 3-(4'-hydroxyphenyl)propanoic acid, 3,4-dihydroxybenzoic acid, 4-hydroxy-3-methoxybenzoic acid and 4'-hydroxy-3'-methoxyphenylacetic acid were still present.

Table 2. Amount of phenolic metabolites produced during *in vitro* fecal fermentation of raw dark purple eggplant. Results are expressed in µmol of phenolic compound/100 of eggplant.

Compound	Synonim	6 h	18 h	24 h
Di-hydro-caffeoylquinic acid isomer 1	/	n.d.	n.d.	n.d.
Di-hydro-caffeoylquinic acid isomer 2	/	32.72 ± 1.63	8.00 ± 0.11	n.d.
Di-hydro-caffeoylquinic acid isomer 3	/	1.60 ± 0.02	0.38 ± 0.01	n.d.
Di-hydro-caffeoylquinic acid isomer 4	/	2.55 ± 0.05	0.54 ± 0.01	n.d.
5-*O*-Feruloylquinic acid *trans*	/	15.11 ± 0.64	2.31 ± 0.02	n.d.
4-*O*-Feruloylquinic acid	/	14.96 ± 0.21	2.96 ± 0.04	n.d.
3',4'-Dihydroxycinnamic acid	Caffeic acid	0.97 ± 0.03	0.64 ± 0.03	n.d.
4'-Hydroxy-3'-methoxycinnamic acid	Ferulic acid	26.11 ± 0.59	n.d.	n.d.
3-(3'-Hydroxyphenyl)propanoic acid	Di-hydro-coumaric acid	20.61 ± 0.69	37.67 ± 0.47	27.18 ± 0.22
3-(4'-Hydroxyphenyl)propanoic acid	Phloretic acid	2.48 ± 0.02	3.14 ± 0.05	2.68 ± 0.04
3-(3',4'-Dihydroxyphenyl)propanoic acid	Di-hydro-caffeic acid	0.69 ± 0.02	0.52 ± 0.01	0.47 ± 0.01
3-(4'-Hydroxy-3'-methoxyphenyl)propanoic acid	Di-hydro-ferulic acid	n.d.	n.d.	n.d.
Hydroxybenzoic acid	/	n.d.	n.d.	n.d.
3,4-Dihydroxybenzoic acid	Protocatechuic acid	4.09 ± 0.02	0.15 ± 0.06	0.08 ± 0.02
4-Hydroxy-3-methoxybenzoic acid	Vanillic acid	2.11 ± 0.03	0.02 ± 0.00	0.20 ± 0.01
4'-Hydroxy-3'-methoxyphenylacetic acid	Homovanillic acid	0.16 ± 0.00	0.03 ± 0.00	0.02 ± 0.00
N^1,N^5-Di-hydro-caffeoyl-di-hydro-caffeoyl-spermidine	/	n.d.	n.d.	n.d.
Total		**124.16 ± 1.99**	**56.34 ± 0.57**	**30.63 ± 0.23**

Different letters within the same row mean significant different (*P<0.05*) values.
n.d. means that the compound was not detected in the sample

Table 3. Amount of phenolic metabolites produced during *in vitro* fecal fermentation of fried dark purple eggplant. Results are expressed in µmol of phenolic compound/100 of eggplant.

Compound	Synonim	6 h	18 h	24 h
Di-hydro-caffeoylquinic acid isomer 1	/	n.d.	n.d.	n.d.
Di-hydro-caffeoylquinic acid isomer 2	/	27.36 ± 0.02	7.63 ± 0.04	7.51 ± 0.02
Di-hydro-caffeoylquinic acid isomer 3	/	1.60 ± 0.01	n.d.	n.d.
Di-hydro-caffeoylquinic acid isomer 4	/	2.30 ± 0.02	n.d.	n.d.
5-*O*-Feruloylquinic acid *trans*	/	8.68 ± 0.13	n.d.	n.d.
4-*O*-Feruloylquinic acid	/	11.28 ± 0.26	n.d.	n.d.
3',4'-Dihydroxycinnamic acid	Caffeic acid	0.76 ± 0.01	n.d.	n.d.
4'-Hydroxy-3'-methoxycinnamic acid	Ferulic acid	21.19 ± 0.85	2.90 ± 0.11	2.06 ± 0.16
3-(3'-Hydroxyphenyl)propanoic acid	Di-hydro-coumaric acid	92.03 ± 3.09	215.00 ± 3.50	216.01 ± 4.76

3-(4’-Hydroxyphenyl)propanoic acid	Phloretic acid	3.63 ± 0.22	5.38 ± 0.09	4.81 ± 0.14
3-(3’,4’-Dihydroxyphenyl)propanoic acid	Di-hydro-caffeic acid	0.28 ± 0.01	0.32 ± 0.01	0.31 ± 0.01
3-(4’-Hydroxy-3’-methoxyphenyl)propanoic acid	Di-hydro-ferulic acid	4.21 ± 0.02	n.d.	n.d.
Hydroxybenzoic acid	/	n.d.	0.16 ± 0.01	0.36 ± 0.01
3,4-Dihydroxybenzoic acid	Protocatechuic acid	5.58 ± 0.02	0.61 ± 0.07	0.54 ± 0.02
4-Hydroxy-3-methoxybenzoic acid	Vanillic acid	0.98 ± 0.01	0.01 ± 0.00	0.17 ± 0.01
4’-Hydroxy-3’-methoxyphenylacetic acid	Homovanillic acid	0.16 ± 0.00	0.14 ± 0.01	0.06 ± 0.00
N^1,N^5-Di-hydro-caffeoyl-di-hydro-caffeoyl-spermidine	/	0.09 ± 0.00	0.48 ± 0.02	0.36 ± 0.01
Total		**180.34 ± 3.23**	**232.64 ± 3.50**	**232.20 ± 4.76**

Different letters within the same row mean significant different (*P<0.05*) values.
n.d. means that the compound was not detected in the sample

Table 4. Amount of phenolic metabolites produced during *in vitro* fecal fermentation of grilled dark purple eggplant.
Results are expressed in µmol of phenolic compound/100 of eggplant.

Compound	Synonim	6 h	18 h	24 h
Di-hydro-caffeoylquinic acid isomer 1	/	9.47 ± 0.02	n.d.	n.d.
Di-hydro-caffeoylquinic acid isomer 2	/	36.21 ± 0.28	n.d.	n.d.
Di-hydro-caffeoylquinic acid isomer 3	/	1.80 ± 0.08	n.d.	n.d.
Di-hydro-caffeoylquinic acid isomer 4	/	2.95 ± 0.13	n.d.	n.d.
5-*O*-Feruloylquinic acid *trans*	/	10.23 ± 0.31	n.d.	n.d.
4-*O*-Feruloylquinic acid	/	17.05 ± 0.24	n.d.	n.d.
3’,4’-Dihydroxycinnamic acid	Caffeic acid	1.20 ± 0.06	n.d.	n.d.
4’-Hydroxy-3’-methoxycinnamic acid	Ferulic acid	31.68 ± 0.19	0.14 ± 0.01	n.d.
3-(3’-Hydroxyphenyl)propanoic acid	Di-hydro-coumaric acid	26.33 ± 0.14	41.28 ± 0.79	49.13 ± 1.26
3-(4’-Hydroxyphenyl)propanoic acid	Phloretic acid	3.58 ± 0.09	2.86 ± 0.07	2.10 ± 0.05
3-(3’,4’-Dihydroxyphenyl)propanoic acid	Di-hydro-caffeic acid	0.42 ± 0.01	0.32 ± 0.01	0.42 ± 0.02
3-(4’-Hydroxy-3’-methoxyphenyl)propanoic acid	Di-hydro-ferulic acid	2.70 ± 0.26	n.d.	n.d.
Hydroxybenzoic acid	/	n.d.	n.d.	n.d.
3,4-Dihydroxybenzoic acid	Protocatechuic acid	4.80 ± 0.03	1.46 ± 0.01	0.01 ± 0.00
4-Hydroxy-3-methoxybenzoic acid	Vanillic acid	2.27 ± 0.05	n.d.	n.d.
4’-Hydroxy-3’-methoxyphenylacetic acid	Homovanillic acid	0.18 ± 0.00	0.07 ± 0.01	n.d.
N^1,N^5-Di-hydro-caffeoyl-di-hydro-caffeoyl-spermidine	/	n.d.	n.d.	n.d.
Total		**124.16 ± 0.63**	**46.12 ± 0.79**	**51.66 ± 1.26**

Different letters within the same row mean significant different (*P<0.05*) values.
n.d. means that the compound was not detected in the sample

Metabolic pathways of eggplant hydroxycinnamic acids degradation by colonic microbiota are described in **Figure 1**. The first step in the metabolic pathway of caffeoylquinic acids (**Figure 1**) was the reduction of the C2-C3 double bond leading to the appearance of several isomers of di-hydro-caffeoylquinic acid (Juániz et al., 2017; Tomas-Barberan et al., 2014). Caffeoylquinic acids may also be a substrate of bacterial esterase, which are able to cleave the bond between quinic acid and 3',4'-dihydroxycinnamic acid releasing this last compound (Andreasen, Kroon, Williamson, & Garcia-Conesa, 2001; Juániz et al., 2017; Ludwig, De Peña, Cid, & Crozier, 2013). Several cinnamoyl esterase-producing bacteria have been isolated from human microbiota belonging to species of *Lactobacillus gasseri*, *Bifidobacterium lactis* and *Escherichia coli* (Couteau, McCartney, Gibson, Williamson, & Faulds, 2001). However, as already suggested, caffeoylquinic acids reduction proceeded faster than ester hydrolysis being di-hydro-caffeoylquinic acids the major detected metabolic products after 6 h of fermentation (Juániz et al., 2017; Tomas-Barberan et al., 2014). The next metabolic step was represented by the hydrolysis of the ester bond of di-hydro-caffeoylquinic acid and the reduction of 3',4'-dihydroxycinnamic acid, which converged on the formation of 3-(3',4'-dihydroxyphenyl)propanoic acid (Juániz et al., 2017; Ludwig et al., 2013; Tomás-Barberán et al., 2014). This last intermediate may have different fates as reported in **Figure 1**.

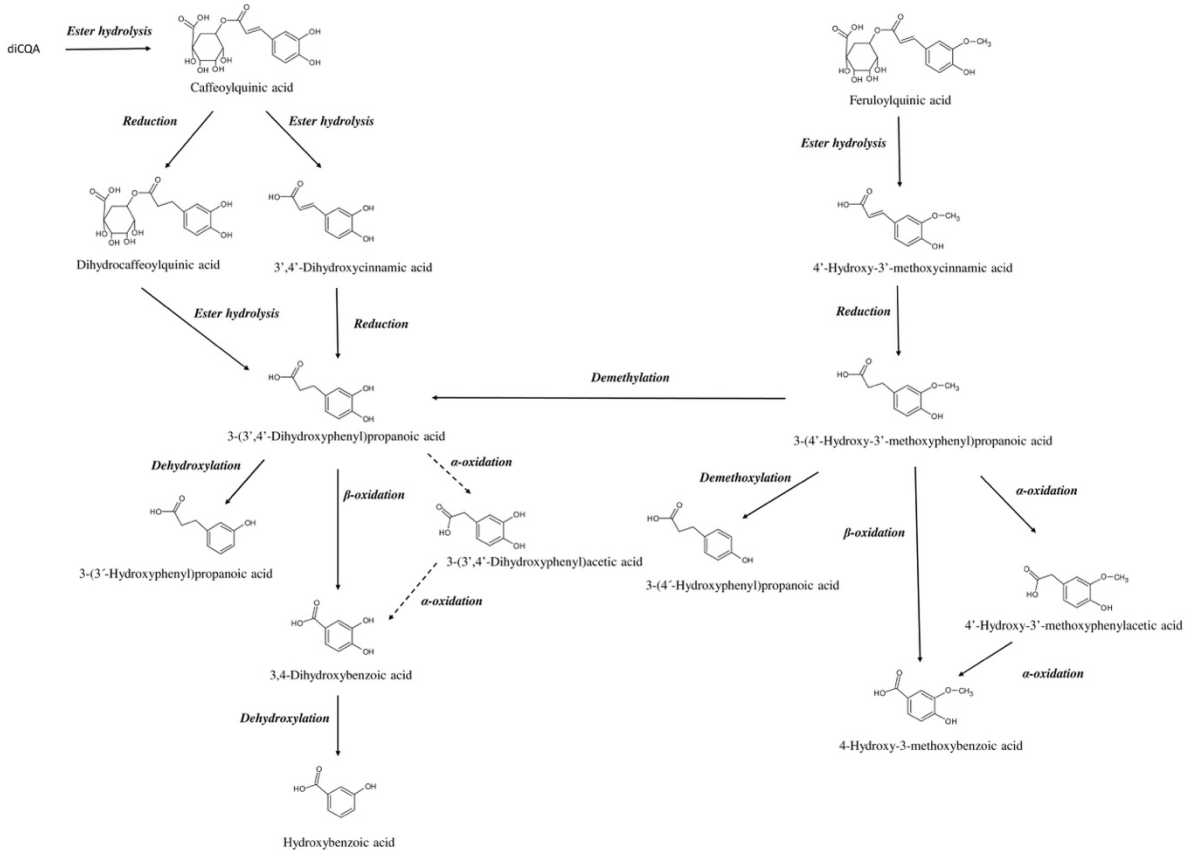


Figure 1. Proposed metabolic pathways for phenolic compounds microbial metabolism after in vitro gastro-intestinal digestion and faecal fermentation of dark purple eggplant.

The propionic side chain of 3-(3',4'-dihydroxyphenyl)propanoic acid may underwent to β-oxidation producing 3,4-dihydroxybenzoic acid, which can be further dehydroxylated to hydroxybenzoic acid (Cortés-Martín, Selma, Tomás-Barberán, González-Sarrías, & Espín, 2020; Williamson, & Clifford, 2017). Alternatively, 3,4-dihydroxybenzoic acid may be formed from 3-(3',4'-dihydroxyphenyl)propanoic acid by two consecutive α-oxidation steps that proceeded through the formation of 3',4'-dihydroxyphenylacetic acid (homoprotocatechuic acid) (Juániz et al., 2017). However, this last intermediate was not detected at any time during fermentation, suggesting that this pathway could be of minor importance respect to direct β-oxidation. However, the major catabolic pathway for 3-(3',4'-dihydroxyphenyl)propanoic acid degradation was the dehydroxylation to 3-(3'-hydroxyphenyl)propanoic acid.

Another important class of hydroxycinnamic acids found in *in vitro* digested eggplant samples was represented by the different isomers of feruloylquinic acids, whose metabolic pathway during fermentation is depicted in **Figure 1**. Feruloylquinic acids were metabolized slowly respect to caffeoylquinic acids by gut microbiota since some residual amounts of these compounds were detected after 6 h of fermentation. Furthermore, the most important degradative pathway for feruloylquinic acids was the ester hydrolysis that resulted in the fast appearance of 4'hydroxy-3'-methoxycinnamic acid that derived from the cleavage of the quinic acid moiety from the parent compound (Ludwig et al., 2013). Indeed, 4'hydroxy-3'-methoxycinnamic acid was reduced to the 3-(4'-hydroxy-3'-methoxyphenyl)propanoic acid intermediate that was further degraded to 4-hydroxy-3-methoxybenzoic acid (directly through β-oxidation or through two steps of α-oxidation via the intermediate 4'-hydroxy-3'-methoxyphenylacetic acid) and 3-(4'-hydroxyphenyl)propanoic acid (**Figure 1**). The low measured amounts of these last compounds in the fermented eggplant samples may suggest the presence of alternative metabolic pathways. Previous studies reported that the most important metabolites identified after colonic fermentation of ferulic acid (4'hydroxy-3'-methoxycinnamic acid) were 3-(4'-hydroxy-3'-methoxyphenyl)propanoic acid, 3-(3',4'-dihydroxyphenyl)propanoic acid and 3-(3'-hydroxyphenyl)propanoic acid (Duncan et al., 2016; Russel et al., 2008). In this case, the metabolic pathway involved the reduction of 4'-hydroxy-3'-methoxycinnamic acid to 3-(4'-hydroxy-3'-methoxyphenyl)propanoic acid followed by demethylation of the methoxy group at C3' and further dehydroxylation at C4' (Duncan et al., 2016; Russel et al., 2008). It is possible to speculate that this catabolic pathway also occurred during *in vitro* fermentation of raw and cooked DPE such that the metabolism of feruloylquinic acids converged to the formation of 3-(3'-hydroxyphenyl)propanoic acid.

Concerning the effect of cooking, DPE heat treatment appeared to influence only the amount of produced metabolites, without any effect on the catabolic pathway. These results are in accordance with previous studies that found that cooking of cardoon, green pepper and artichoke had only a quantitative impact on the metabolites produced during *in vitro* colonic fermentation (Domínguez-Fernández, Ludwig, De Peña, & Cid, 2021; Juániz et al., 2016; Juániz et al., 2017). FDPE, which showed the highest amount of phenolic compounds after *in vitro* digestion, also displayed the highest amount of metabolites after 24 h of *in vitro*

fermentation (232.2 ± 5.1 µmol/100 g of eggplant) followed by GDPE (51.7 ± 1.3 µmol/100 g of eggplant) and RDPE (30.6 ± 0.3 µmol/100 g of eggplant) eggplant. In all the samples, 3-(3'-hydroxyphenyl)propanoic acid was the major produced metabolite accounting for the 89%, 93% and 95% of total phenolic compounds after 24 h of *in vitro* fermentation in RDPE, FDPE and GDPE, respectively. *In vitro* studies showed that 3-(3'-hydroxyphenyl)propanoic acid exhibited anti-inflammatory properties by reducing prostanoid production in human fibroblast stimulated with cytokines and anti-diabetic activity by promoting survival of pancreatic β-cells and insulin secretion (Fernández-Millán et al., 2014; Larrosa et al., 2009; Russell et al., 2008). Moreover, 3-(3'-hydroxyphenyl)propanoic acid was able to relax *in vitro* vascular smooth cells and to decrease *in vivo* blood pressure in healthy and spontaneously hypertensive rats (Najmanová et al., 2016).

3.4 Shift of total bacteria and microbiota eubiosis

qPCR absolute quantifications were targeted to 14 different bacterial taxa (**Table S2**) related to the core microbiota, including total Eubacteria and the two main phyla of the human healthy colon, i.e., *Bacteroidetes* and *Firmicutes*. Considering the total Eubacteria, in respect to the abundances at the baseline (BL) and apart from the values of the blank control (BC), just GDPE at 24 h scored a significant reduction in abundance of 1.66 folds less ($p < 0.05$) (**Table 5**). The two other samples, independently from the time points, had slight not significant increases ($p > 0.05$). It is noteworthy that at the 24 h the value of GDPE was 2.91 and 2.72 folds significantly lower than RDPE and FDPE, respectively ($p < 0.05$). These evident results indicated that GDPE, alike the BC, limited the growth of total colon Eubacteria.

Quantifications of *Bacteroidetes* phylum showed significant changes principally at the 24 h, where all values were significant in respect to the BL ($p < 0.05$) (**Table 5**). In particular, RDPE had the top increase of 1.75 folds and GDPE had the top reduction of 1.56 folds, in comparison to the BL ($p < 0.05$). Additionally, RDPE was the sole that significantly increased *Bacteroidetes* content at 18 h ($p < 0.05$). The higher loads of *Bacteroidetes* after fermentation of RDPE could indicate a preferable substrate described by longer fiber chains, and thus more specific sites for *Bacteroidetes*, which is rich in fiber degrading enzymes.

Considering *Firmicutes* (**Table 5**), significant changes were seen for each sample at the 24 h and for GDPE at 18 h ($p < 0.05$). At 24 h, increases were found for each eggplant sample, with no difference among each other ($p > 0.05$). These outputs could indicate opposite scenarios depending on which group of *Firmicutes* was the most fostered; a positive outlook could be due to increase of *Lactobacillales*, while a negative could be due to increase of *Clostridiaceae*.

As a general parameter for microbiota eubiosis, we chose the *Firmicutes/Bacteroidetes* (F/B) ratio (**Table 6**). Within this ratio, a value over two is usually linked to microbiota dysbiosis (Blaut et al., 2002; Koliada et al., 2017; Zhou et al., 2017; Martinez et al., 2021). The fecal samples at the baseline had a F/B of 0.81 and this eubiosis condition was maintained by RDPE (0.51) and by FDPE (0.88) ($p > 0.05$), but not by GDPE (2.64) ($p < 0.05$). These results indicate that during the 24 h of fermentation RDPE and FDPE did not perturb the colon

core microbiota of healthy donors and were able to provide a substrate nutritionally adapted. Oppositely, fermentations on GDPE triggered a dysbiosis condition that mirrored the higher *Firmicutes* content. A potential explanation could be related to the occurrence of the Maillard reaction between DPE fiber and amino compounds during grilling, making DPE fiber no longer available for gut microbiota fermentation (Pérez-Burillo et al., 2018). Previous studies suggested that during grilling the degree of the Maillard reaction was stronger than during frying (Pérez-Burillo, Rufián-Henares, & Pastoriza, 2019).

Table 5. Shift in bacterial abundances of selected targets by enumeration with qPCR.

qPCR Target	Cells/mL	Log_2(F/C)		ANOVA*
Eubacteria	**Baseline**	**18 h**	**24 h**	
RDPE	9.55E+08 ± 2.27E+07	0.02^{A}	0.57^{A}	*0.139711*
GDPE	9.55E+08 ± 2.27E+07^{a}	-0.06^{aA}	-1.66^{bB}	*0.001852*
FDPE	9.55E+08 ± 2.27E+07	0.04^{A}	0.61^{A}	*0.063910*
BC	9.55E+08 ± 2.27E+07^{a}	-1.88^{bB}	-3.34^{cC}	*0.000001*
		0.002320	*0.000001*	**P value**
Bacteroidetes	**Baseline**	**18 h**	**24 h**	
RDPE	2.73E+08 ± 3.24E+07^{b}	1.84^{aA}	1.75^{aA}	*0.015355*
GDPE	2.73E+08 ± 3.24E+07^{a}	0.88^{aA}	-1.56^{bB}	*0.006992*
FDPE	2.73E+08 ± 3.24E+07^{b}	-0.08^{bB}	1.30^{aA}	*0.002790*
BC	2.73E+08 ± 3.24E+07^{a}	-0.79^{aB}	-4.15^{bC}	*0.025874*
		0.015736	*0.000542*	**P value**
Firmicutes	**Baseline**	**18 h**	**24 h**	
RDPE	2.20E+08 ± 6.43E+07	0.22^{bAB}	1.09^{A}	*0.087025*
GDPE	2.20E+08 ± 6.43E+07^{b}	1.04^{abA}	1.35^{aA}	*0.035807*
FDPE	2.20E+08 ± 6.43E+07^{b}	-0.16^{bB}	1.43^{aA}	*0.000006*
BC	2.20E+08 ± 6.43E+07^{a}	0.07^{abAB}	-1.51^{bB}	*0.000262*
		0.000091	*0.010083*	**P value**
Lactobacillales	**Baseline**	**18 h**	**24 h**	
RDPE	5.99E+06 ± 7.00E+05^{b}	-0.03^{bA}	3.82^{aA}	*0.000974*
GDPE	5.99E+06 ± 7.00E+05^{b}	0.51^{bA}	3.07^{aA}	*0.000023*
FDPE	5.99E+06 ± 7.00E+05^{b}	1.23^{bA}	3.80^{aA}	*0.000001*
BC	5.99E+06 ± 7.00E+05^{a}	-1.24^{bB}	-0.86^{bB}	*0.001798*
		0.006568	*0.000322*	**P value**
Bifidobacteriaceae	**Baseline**	**18 h**	**24 h**	
RDPE	9.67E+07 ± 9.60E+06^{b}	2.13^{aA}	-4.02^{cBC}	*0.045509*
GDPE	9.67E+07 ± 9.60E+06^{b}	1.31^{aA}	-2.32^{cB}	*0.012910*
FDPE	9.67E+07 ± 9.60E+06^{b}	3.13^{aA}	2.66^{aA}	*0.014113*
BC	9.67E+07 ± 9.60E+06^{a}	-1.66^{bB}	-6.41^{cC}	*0.004711*
		0.007112	*0.002270*	**P value**
Enterobacteriaceae	**Baseline**	**18 h**	**24 h**	
RDPE	5.94E+06 ± 1.17E+06^{b}	0.53^{bAB}	2.31^{aA}	*0.040556*
GDPE	5.94E+06 ± 1.17E+06^{b}	-0.09^{bB}	1.57^{aB}	*0.000202*
FDPE	5.94E+06 ± 1.17E+06	0.30^{AB}	0.56^{C}	*0.054133*
BC	5.94E+06 ± 1.17E+06^{c}	0.86^{bA}	2.75^{aA}	*0.000006*
		0.000001	*0.000171*	**P value**
BPP group	**Baseline**	**18 h**	**24 h**	
RDPE	1.38E+07 ± 1.99E+06^{b}	1.52^{aA}	2.42^{aAB}	*0.040071*
GDPE	1.38E+07 ± 1.99E+06^{b}	0.55^{bAB}	1.30^{aB}	*0.000353*
FDPE	1.38E+07 ± 1.99E+06^{b}	-0.59^{bB}	2.83^{aA}	*0.017136*
BC	1.38E+07 ± 1.99E+06	-0.97^{B}	-0.77^{C}	*0.391845*
		0.049358	*0.025514*	**P value**

ATOP group	**Baseline**	**18 h**	**24 h**	
RDPE	3.10E+04 ± 1.37E+03	0.27^{B}	0.87^{B}	*0.328140*
GDPE	3.10E+04 ± 1.37E+03^{b}	3.76^{aA}	5.32^{aA}	*0.011461*
FDPE	3.10E+04 ± 1.37E+03^{b}	0.62^{abB}	1.26^{aB}	*0.014034*
BC	3.10E+04 ± 1.37E+03^{b}	0.62^{bB}	8.54^{aA}	*0.001073*
		0.004022	*0.000656*	**P value**
Clostridium Group I	**Baseline**	**18 h**	**24 h**	
RDPE	1.34E+03 ± 2.58E+02	0.88^{B}	0.72^{C}	*0.040768*
GDPE	1.34E+03 ± 2.58E+02^{b}	3.60^{aA}	2.58^{aB}	*0.000002*
FDPE	1.34E+03 ± 2.58E+02	0.90^{B}	0.64^{C}	*0.059008*
BC	1.34E+03 ± 2.58E+02^{c}	4.78^{bA}	8.74^{aA}	*0.007743*
		0.000002	*0.000044*	**P value**
Clostridium Group IV	**Baseline**	**18 h**	**24 h**	
RDPE	1.28E+07 ± 1.40E+06^{a}	-0.94^{bB}	-1.74^{bB}	*0.002343*
GDPE	1.28E+07 ± 1.40E+06^{a}	-0.88^{bB}	-1.40^{bB}	*0.001558*
FDPE	1.28E+07 ± 1.40E+06^{b}	1.52^{aA}	1.97^{aA}	*0.002567*
BC	1.28E+07 ± 1.40E+06^{a}	-3.36^{bC}	-4.17^{bC}	*0.043406*
		0.000001	*0.001605*	**P value**
Faecalibacterium prausnitzii	**Baseline**	**18 h**	**24 h**	
RDPE	1.46E+03 ± 5.20E+02^{b}	4.58^{a}	8.56^{a}	*0.001407*
GDPE	1.46E+03 ± 5.20E+02^{b}	3.59^{a}	5.69^{a}	*0.001564*
FDPE	1.46E+03 ± 5.20E+02^{b}	3.78^{a}	8.81^{a}	*0.001359*
BC	1.46E+03 ± 5.20E+02^{a}	-0.88^{ab}	-3.62^{b}	*0.001090*
		0.010691	*0.023811*	**P value**
Bifidobacterium longum	**Baseline**	**18 h**	**24 h**	
RDPE	6.58E+07 ± 1.35E+06	-0.28	-0.57^{AB}	*0.053207*
GDPE	6.58E+07 ± 1.35E+06	-0.37	0.41^{AB}	*0.060214*
FDPE	6.58E+07 ± 1.35E+06^{b}	-0.12^{b}	1.86^{aA}	*0.000033*
BC	6.58E+07 ± 1.35E+06^{a}	-1.09^{b}	-3.40^{cB}	*0.003734*
		0.051701	*0.006037*	**P value**
Akkermansia muciniphila	**Baseline**	**18 h**	**24 h**	
RDPE	4.24E+03 ± 6.04E+02^{b}	3.05^{aA}	3.30^{aAB}	*0.000001*
GDPE	4.24E+03 ± 6.04E+02^{b}	2.05^{aA}	1.88^{aB}	*0.000002*
FDPE	4.24E+03 ± 6.04E+02^{b}	2.24^{aA}	3.70^{aA}	*0.000024*
BC	4.24E+03 ± 6.04E+02^{a}	-2.42^{bB}	-5.16^{bC}	*0.000303*
		0.000012	*0.000096*	**P value**
Escherichia coli	**Baseline**	**18 h**	**24 h**	
RDPE	9.10E+02 ± 2.28E+02	0.40^{B}	0.82^{B}	*0.131025*
GDPE	9.10E+02 ± 2.28E+02^{b}	0.12^{bB}	3.43^{aB}	*0.033926*
FDPE	9.10E+02 ± 2.28E+02^{b}	0.06^{bB}	2.42^{aB}	*0.000017*
BC	9.10E+02 ± 2.28E+02^{c}	4.54^{bA}	9.50^{aA}	*0.000019*
		0.000109	*0.043282*	**P value**

*ANOVA and post hoc Tukey ($P < 0.05$). For each bacterial target, values with different letters are significantly different among a sample and among a time point, with lower- or upper-case letter, respectively. RDPE = raw dark purple eggplant; GDPE = grilled dark purple eggplant; FDPE = fried dark purple eggplant; BC = blank control.

Table 6. Shift in *Firmicutes* to *Bacteroidetes* ratio.

Sample	*Firmicutes*/*Bacteroidetes*			ANOVA*
	Baseline	**18 h**	**24 h**	
RDPE	0.81 ± 0.50^{a}	0.26 ± 0.16^{cB}	0.51 ± 0.20^{bC}	*0.001769*
GDPE	0.81 ± 0.50^{b}	0.90 ± 0.40^{bA}	2.64 ± 0.48^{aB}	*0.000161*
FDPE	0.81 ± 0.50^{b}	1.24 ± 0.54^{aA}	0.88 ± 0.55^{bC}	*0.000558*
BC	0.81 ± 0.50^{b}	1.46 ± 0.67^{bA}	5.04 ± 0.67^{aA}	*0.002569*
		0.000353	*0.000545*	**P value**

*ANOVA and post hoc Tukey ($P < 0.05$). For each bacterial target, values with different letters are significantly different among a sample and among a time point, with lower- or upper-case letter, respectively. RDPE = raw dark purple eggplant; GDPE = grilled dark purple eggplant; FDPE = fried dark purple eggplant; BC = blank control.

3.6. Shift in selected bacterial taxa

To give evidence to the shifts of colon core microbiota, we quantify the abundance of some bacterial taxa selected as renown to be active responders to fiber substrate fermentation and modulators of host wellbeing (**Table 5**). A first set was related to beneficial microbes, while a second set was related to opportunistic and dysbiontic microbes.

Lactobacillales and *Bifidobacteriaceae* with the exceptions of few species are related to the host's wellbeing and eubiosis of the colon microbiota (Sanders et al., 2019). *Lactobacillales* includes also *Lactobacillaceae* and *Enterococcaceae*. These three families are implicated in the biotransformation of phenolic compounds; for example *Lactobacillaceae* are able to increase epicatechin as well as gallic, caffeic, and ferulic acids content (Liu et al., 2021); *Enterococcaceae* are able to promote *O*-deglycosylation and *C*-ring fission of several flavonols (Corrêa et al., 2019), whereas *Bifidobacteriaceae* are able to metabolize homovanillic acids (Boto-Ordonez et al., 2014) and hydrolyze chlorogenic acids (Iqbal et al., 2020). In our experiments, *Lactobacillales* and *Bifidobacteriaceae* had a different trend during fermentation (**Table 5**). The first taxon at 18 h scored a significant increase in abundance just for FDPE. At the 24 h, all DPE substrates were able to increase the abundance of *Lactobacillales*. Similarly, on a long-term fermentation of phenolic compounds on gut models, other authors found that *Enterococcaceae* was the most fostered family (Kemperman et al., 2013; Tamargo et al., 2022).

From our results at 18 h, *Bifidobacteriaceae* taxa were significantly fostered ($p < 0.05$) by any DPE substrates, while at 24 h a significant increase was seen just for FDPE ($p < 0.05$) (**Table 5**). A similar fashion was observed even at the species level (**Table 5**). In fact, targeting *B. longum*, a significant increase was seen just after FDPE fermentation ($p < 0.05$). This feature could mean that *Bifidobacteriaceae* are more specific in metabolizing phenolic compounds than *Lactobacillales*, as well as are more sensible to some detrimental compounds generated by pyrolysis of grilled foods. It is reported that *Bifidobacteriaceae* are somehow inhibited by a certain class of polyphenol, *e.g.* that of black tea (Kemperman et al., 2013) rather than other, *e.g.* that of olive (Nissen et al., 2021a). Additionally, it is known that *Lactobacillales* can resist to high content of phenolic compound (Khubber et al., 2022), because owns an enzymatic arsenal with a wide target range, able to de-

esterification, hydrolysis, or conversion of phenolics to individual acids (gallic, quinic, caffeic, *p*-coumaric, ferulic, dihydrocaffeic, dihydroferulic acid, vinylcatechol, vinylguaiacol) (Filannino, Di Cagno & Gobbetti, 2018; Khubber et al., 2022).

Among other beneficial taxa, *Clostridium* group IV and *Faecalibacterium prausnitzii* are reported to be involved in biotransformation of phenolic compounds (Braune, & Blaut, 2016). From our results (**Table 5**), *Clostridium* group IV increased significantly at the 24 h just after FDPE fermentation that had even the highest significant increase in *F. prausnitzii* too. *F. prausnitzii* belongs to *Clostridium* group IV related to important SCFA producers and its presence is associated to a healthy gut. A decrease in the number of these bacteria is observed in patients with inflammatory bowel disease (Zhang et al., 2019). Like our results, these taxa are reported to increase after dietary clinical trial with phenolic compounds from red wine (Moreno-Indias et al., 2016; Wiciński et al., 2020). Lastly, *Akkermansia muciniphila* has been reported to be exalted from phenolic compounds presence, alike that some phenolic compounds are acting as prebiotics (Rodríguez-Daza et al., 2021). From our results (**Table 5**) this taxon was significantly fostered both at 18 h and at 24 h by any eggplant substrate, but was not by the BC ($p < 0.05$). In particular, RDPE and FDPE behaved similarly, and their mean increment, 3.5 ± 0.28 Log_2(F/C), was 1.8-fold higher than that after GDPE fermentation.

Another commensal group that is involved mainly in fiber degradation is the *Bacteroides-Prevotella* group, covered by the BPP (*Bacteroides–Prevotella–Porphyromonas*) target. Generally, these bacteria are sensible to both fibers (Patrascu et al., 2017) and phenolic compounds. For example, Tamargo et al., (2022) found a significant increase of this group after phenolic compounds fermentation on a gut model. From our results, the BPP group increased significantly at 18 h ($p < 0.05$) just after RDPE fermentation, while at 24 h for all the DPE ($p < 0.05$) (**Table 5**).

Considering the opportunistic bacterial taxa, we have targeted the *Enterobacteriaceae*, *Clostridium* group I, the *Atopobium–Collinsella-Eggerthella* (ATOP) group, and *Escherichia coli* (**Table 5**).

Enterobacteriaceae at 24 h increased after fermentation of all sample, including the BC. Although this increment was not significant for FDPE ($p < 0.05$). In particular, the increment scored by FDPE was 2.8, 4.1, and 4.9-folds lower than RDPE, GDPE and BC, respectively.

Likely was the fashion of fermentation for *E. coli* that at 24 h increased in any fermentation condition, including the BC, and significantly in three out of four samples. Even in this case, the increase scored by the BC was the top one, more than four time higher than the mean increment observed by the eggplant samples, 2.2 ± 1.32 Log_2(F/C). In comparison to each other, this time was GDPE that made *E. coli* grow more; 1.4 and 4.2-folds more than FDPE and RDPE, respectively.

Clostridium group I surged in any case at any time point, but significantly just for GDPE and BC ($p < 0.05$). In particular, at 24 h, RDPE and FDPE had a similar increase that was 3.8 and 12.9-folds lower than GDPE and BC, respectively.

Lastly, considering the ATOP group, increases were observed in any substrate at any time. In detail, ATOP raised at 24 h significantly for GDPE, FDPE and BC ($p < 0.05$), but GDPE and BC had respectively 4.2 and 6.7-fold more abundance in comparison to FDPE. This group engages in the biotransformation of phenolic compounds, because *Atopobium parvulum* for example possess tannases (Jimenez, et al., 2014), while *Atopobium* spp. is correlated to protocatechuic acid production after colonic fermentation of berry phenolic compounds (Williamson & Clifford, 2017). In similar works of fermentation, the abundance of *Eggerthella* was enhanced by berry phenolic compounds (Rodríguez-Daza et al., 2021). From this scenario, seems that GDPE was the substrates that made the opportunistic group grow mostly, whereas FDPE brought about a considerable increase in many beneficial taxa Previous *in vitro* and *in vivo* studies showed that vegetable cooking reshapes the gut microbiota with effects attributable to an altered physical access to dietary fibers, to changes in the amount and/or type of plant-derived compounds or to the generation of new compounds, such as Maillard reaction products, after thermal treatments (Carmody et al., 2019; Pérez-Burillo et al., 2018).

3.7. qPCR Prebiotic Index

Considering the results (**Table 7**), we found out that the fermented substrate with the best prebiotic activity was FDPE after 24 h, followed by RDPE and GDPE after 24 h. Thus, GDPE had the lowest prebiotic index that was 2.48 folds lower than that of FDPE at 24 h. In comparison to FDPE, RDPE scored 1.35-fold lower values at 24 h. The blank control scored for any time points lower values than any FDPE, GDPE and RDPE cases (all significant, but one) and reached the lowest value of the dataset at the endpoint (7.47-fold lower than FDPE 24 h).

Anyhow, at 18 h, results showed that the qPI values were similar among the three eggplant samples, but FDPE and RDPE were still higher than GDPE ($p > 0.05$). Lastly, the BC sample scored significant lower values than each eggplants sample ($p < 0.05$). Thus, the different prebiotic index of FDPE, RDPE and GDPE could be mostly due to a different structural composition of the eggplant fibers with different portion of soluble fibers generated after the different cooking conditions, as well as by different transformation of eggplant phenolics with prebiotic capacity. Similarly to most know prebiotics (*e.g*. fructooligosaccharides), it is recognized that soluble fibers are excellent substrates for production of postbiotics (*e.g.* short chain fatty acids) in the large intestine (Oba et al, 2020). Additionally, in line with the latest definition of prebiotics (Gibson et al., 2017), there is evidence that some phenolics could foster the growth of beneficial bacteria, thus owning a prebiotic nature (Rodríguez-Daza et al., 2021).

Table 7. Scores of qPCR Prebiotic Index (qPI).

Sample	qPCR Prebiotic Index			ANOVA*
	Baseline	**18 h**	**24 h**	
RDPE	0.30 ± 0.05^{c}	0.51 ± 0.01^{bB}	0.72 ± 0.07^{aC}	*0.000032*
GDPE	0.30 ± 0.05^{c}	0.39 ± 0.01^{aA}	0.33 ± 0.01^{bB}	*0.000012*
FDPE	0.30 ± 0.05^{c}	0.97 ± 0.03^{aA}	0.53 ± 0.03^{bC}	*0.000001*
BC	0.30 ± 0.05^{b}	0.29 ± 0.01^{abA}	0.01 ± 0.01^{aA}	*0.000001*
		0.000022	*0.000001*	**P value**

*ANOVA and post hoc Tukey ($P < 0.05$). For each bacterial target, values with different letters are significantly different among a sample and among a time point, with lower- or upper-case letter, respectively. RDPE = raw dark purple eggplant; GDPE = grilled dark purple eggplant; FDPE = fried dark purple eggplant; BC = blank control.

3.8 Correlations among phenolics and microbial taxa

Spearman Rank Correlations ($p < 0.05$), two-joining-way Heatmaps, and Pearson cluster analysis were performed by comparison of two different normalized datasets, each derived from values of relative quantification (microbial taxa and phenolics) of the whole experimental dataset (**Figure 2**). The significance of correlations is reported in **Figure S1**. From the left-side dendrograms, two main clusters and a smaller one were identified. The two main clusters were then distributed in two different subgroups defined by the topside dendrograms related to phenolics variables. In cluster 1A the most significant positive correlations were held by taxa *Bifidobacteriaceae*, *B. longum* and *Clostridium* group IV towards 3,4-dihydroxybenzoic, 4'-hydroxy-3'-methoxyphenylacetic, hydroxybenzoic, 3-(3'-hydroxyphenyl)propanoic and 4'-hydroxy-3'-methoxycinnamic acids. In cluster 1B, the same bacterial variables were negatively correlated mainly to 3',4'-dihydroxycinnamic, di-hydrocaffeoylquinic isomer 3, 5-*O*-feruloylquinic and 3-(3',4'-dihydroxyphenyl)propanoic acids. The most significant negative correlations were seen for *B. longum* to each of the four compounds aforementioned. *Bifidobacteriaceae* are able of both *O*-deglycosylation and *C*-ring fission of kaempferol, quercetin, and myricetin releasing protocatechuic acid (Murota, Nakamura & Uehara, 2018). *Bifidobacteriaceae* and species from *Clostridium* group IV are able also to hydrolyze ester bonds, *C*-ring cleavage, and dihydroxylation of anthocyanins and proanthocyanidins releasing hydroxybenzoic acids (Braune, & Blaut 2016).

In cluster 3 the trend was opposite. The bacterial variables that resulted to have more significant correlations were *Enterobacteriaceae* and *Bacteroidetes* (and minorly *Bacteroides-Prevotella* of BPP target) principally towards 3',4'-dihydroxycinnamic acid, di-hydrocaffeoylquinic isomer 3, 5-*O*-feruloylquinic and 3-(3',4'-dihydroxyphenyl)propanoic acids (cluster 3B). While on cluster 3A the negative correlations were significant just for 3,4-dihydroxybenzoic acid and *Bacteroidetes*.

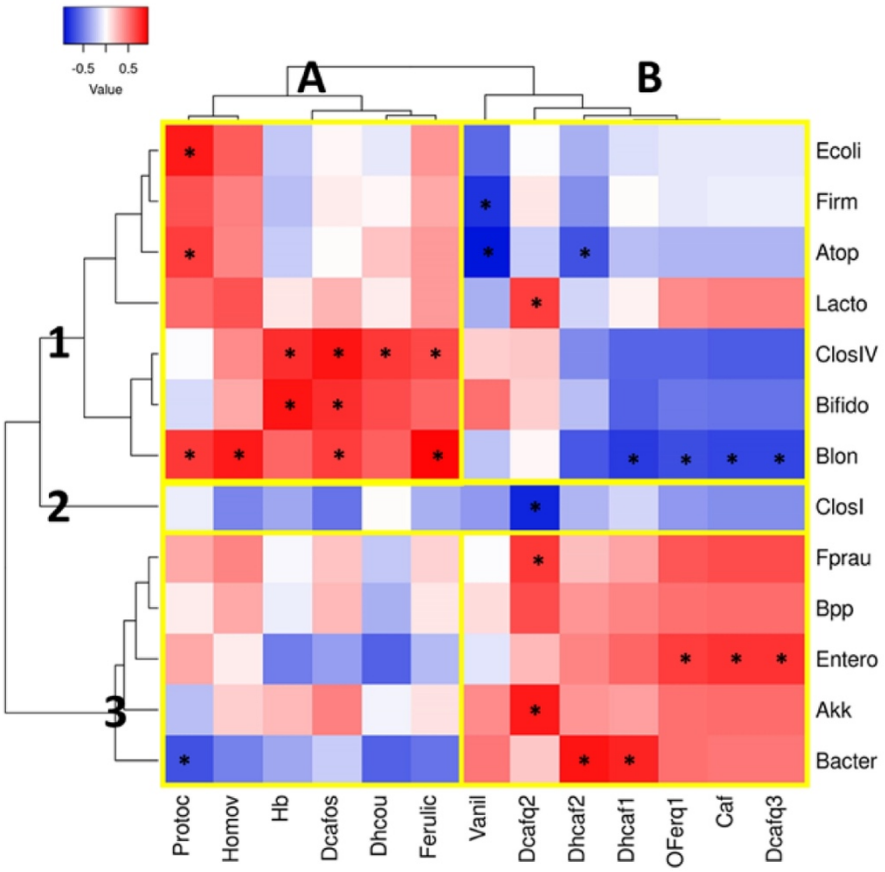


Figure 2. Interomics, Spearman Rank Correlations among the microbiota dataset and the phenolic compounds dataset. Left side dendrogram identifies by Pearson analysis three major clusters among bacterial groups. Top dendrogram identifies by Pearson analysis two major clusters among phenolic compounds. Significance of correlations are provided as supplementary material (**Figure S1**).

X axis labels: Protoc = protocatechuic acid (3,4-dihydroxybenzoic acid); Homov = homovanillic acid (4'-hydroxy-3'-methoxyphenylacetic acid); HB = hydroxybenzoic acid; Dcafos = N^1,N^5-di-dihydrocaffeoylsperimidine; Dhcou = Di-hydro-coumaric acid (3-(3'-hydroxyphenyl)propanoic acid); Ferulic = ferulic acid (4'-hydroxy-3'-methoxycinnamic acid); Vanil = vanillic acid (4-hydroxy-3-methoxybenzoic acid); Dcafq2 = di-hydro-caffeoylquinic acid isomer 2; Dhcaf2 = di-hydro-caffeic acid isomer 2 (3-(3',4'-dihydroxyphenyl)propanoic acid); Dhcaf1 = di-hydro-caffeic acid isomer 1 (3-(3',4'-dihydroxyphenyl)propanoic acid); Oferq1 = 5-O-feruloylquinic acid trans; Caf = caffeic acid (3',4'-dihydroxycinnamic acid); Dcafq3 = di-hydro-caffeoylquinic acid isomer 3.

Y axis labels: Ecoli = Escherichia coli; Firm = Firmicutes; Atop = Atopobium-Collinsella-Eggerthella group; Lscto = Lactobacillales; Clos IV = Clostridium group IV; Bifido = Bifidobacteriaceae; Blon = Bifidobacterium longum; Clos I = Clostridium group I; Fprau = Faecalibacterium prausnitzii; Bpp = Bacteroides-Prevotella-Porphyromonas group; Entero = Enterobacteriaceae; Akk = Akkermansia muciniphila; Bacter = Bacteroidetes.

4. Conclusion

Heat treatments increased the extractability of eggplant phenolic compounds and were essential to promote the release of DPE phenolic compounds during *in vitro* gastro-intestinal digestion. Fried DPE displayed the highest amount of phenolic compounds following *in vitro* gastro-intestinal digestion. Additionally, gut microbiota exhibited a considerable metabolic activity resulting in a thorough modification in the chemical structure of DPE phenolic compounds. The colon metabolic pathways of the different phenolic compounds in DPE converged towards the production of 3-(3'-hydroxyphenyl)propanoic acid, independently from the samples. Fried DPE displayed the highest amount of metabolites.

Colonic fermentation of fried and raw DPE resulted in a considerable increase in many beneficial taxa. A positive correlation among the most important phenolic metabolites and *Bifidobacteriaceae*, *B. longum* and *Clostridium group IV* suggesting their involvement in the colonic metabolism of hydroxycinnamic acids.

Therefore, eggplant frying can be considered as the most appropriate cooking treatment to achieve the highest amount of bioaccessible phenolic compounds and metabolites also favoring the growth of beneficial colonic bacteria resulting in a shift in the microbiota composition that more efficaciously metabolize phenolic compounds. Since the amount and type of phenolic metabolites and the colonic microbiota composition influence human health, presented results suggest that the application of different cooking methods may have distinctive effects on human health. Meanwhile the gut microbiota composition and its metabolic activity can be modulate not only by the cooking treatments but also by the type of food and phenolic compounds profile. Therefore, more studies are needed in order to unravel the effect of different foods in combination with diverse cooking techniques on gut microbiota diversity and phenolic compounds metabolism.

5. Supplementary materials

Table S1. Classes of identified phenolic compounds and metabolites and standard used for the quantification.

Class of phenolic compound and metabolites	*Standard used for quantification*
Hydroxybenzoic acid-derivatives	3,4-Dihydroxybenzoic acid (protocatechuic acid)
4-Hydroxy-3-methoxybenzoic acid	4-Hydroxy-3-methoxybenzoic acid (vanillic acid)
Caffeoylquinic acids	5-*O*-Caffeoylquinic acid
Dicaffeoylquinic acids	5-*O*-Caffeoylquinic acid
Dihydro-caffeoylquinic acids	5-*O*-Caffeoylquinic acid
3-(3',4'-Dihydroxyphenyl)propanoic acid (di-hydro-caffeic acid)	3-(3',4'-Dihydroxyphenyl)propanoic acid (di-hydro-caffeic acid)
3-(4'-Hydroxy-3'-methoxyphenyl)propanoic acid (di-hydro-ferulic acid)	3-(4'-Hydroxy-3'-methoxyphenyl)propanoic acid (di-hydro-ferulic acid)
3-(3'-Hydroxyphenyl)propanoic acid and 3-(4'-hydroxyphenyl)propanoic acid	3-(3',4'-Dihydroxyphenyl)propanoic acid (di-hydro-caffeic acid)
4'-Hydroxy-3'-methoxyphenylacetic acid (homovanillic acid)	4'-Hydroxy-3'-methoxyphenylacetic acid (homovanillic acid)
Caffeic acid-derivatives	Caffeic acid
Coumaric acid-derivatives	Coumaric acid
Ferulic acid-derivatives	Ferulic acid
Flavonols	Quercetin-3-*O*-glucoside
Anthocyanins	Cyanidin-3-*O*-glucoside

Table S2. Primers pairs employed for PCR and qPCR reactions and quantifications.

Bacterial taxa	Target	Sequence 3'-5'	Bp*	MT** (°C)	Reference
Eubacteria	*V3-V4*	Eub518-R: ATTACCGCGGCTGCTGG	147	57.6	Lane et al, 1991
	16S	Eub338-F: ACTCCTACGGGAGGCAG		63.5	
Firmicutes	*V3-V4*	Firm-F: GGAGYATGTGGTTTAATT	300	60.5	Guo et al, 2008
	16S	Firm-R: ACTCCTACGGGAGGCAG		63.5	
Bacteroidetes	*V3-V4*	Bact-F: GGARCATGTGGTTTAATT	250	58.9	Guo et al, 2008
	16S	Bact-R: AGCTGACGACAACCATG		59.4	
Lactobacillales	*V3-V4*	F-Lac: GCAGCAGTAGGGAATCT	340	59.8	Walter et al, 2001
	16S	R-Lac: GCATTYCACCGCTACACA		58.3	
Bifidobacteriaceae	*RecA*	RecA-F: CGTYTCBCAGCCGGAYA	220	60.3	Masco et al, 2006
		RecA-R: CCARVGCRCCGGTCATC		59.2	
Enterobacteriaceae	*V3-V4*	Enterb-F: TGCCGTAACTTCGGGAG	450	64.2	Bartosh et al, 2004
	16S	Enterb-R: TCAAGGACCAGTGTTCAG		60.3	
Clostridium group I	*V3-V4*	ClosI-F: TACCHRAGGAGGAAGCCAC	148	54.6	Bartosh et al, 2004
	16S	ClosI-R: GTTCTTCCTAATCTCTACGCAT		53.0	
Clostridium group IV	*V3-V4*	ClosIV-F: TTAACACAATAAGTWATC	400	58.1	Goldberg et al,
	16S	ClosIV-R: ACCTTCCTCCGTTTTGTC		57.9	2013
ATOP group	*V3-V4*	ATOP-F: GGGTTGAGAGACCGACC	190	57.6	Matsuki et al., 2004
	16S	ATOP-R: CGGRGCTTCTTCTGCAG		59.4	
BPP group	*V3-V4*	BPP-F: GAGAGGAAGGTCCCCCA	140	60.5	Pachikian et al., 2011
	16S	BPP-R: CGCKACTTGGCTGGTTCA		59.9	
Escherichia coli	*FtsZ*	EcFtsZ-F: GGTATCCTGACCGTTGCT	250	59.4	Zhou &
		EcFtsZ-R: ATACCTCGGCCCAGAACT		57.3	Helmstetter, 1994
Akkermansia	*V3-V4*	AkM1: CAGCACGTGAAGGTGGG	327	63.5	Guo et al, 2016
muciniphila	*16S*	AkM2: CCTTGCGGTTGGCTTCA		59.4	
Bifidobacterium	*V3-V4*	Blon-F: GATTCTGGCTCAGGATGA	220	62.1	Chen et al., 2007
longum	*16S*	Blon-R: CTGATAGGACGCGACCC		61.4	
Faecalibacterium	*V3-V4*	Fprau223-F: GATGGCCTCGCGTCCGA	199	63.7	Wang et al, 1996
prausnitzii	*16S*	Fprau420-R: CCGAAGACCTTCTTCCTC		58.8	

*Base pairs, **Melting Temperature.

Table S3. Mass spectral data of phenolic compounds and metabolites identified in the eggplant samples

	Formula	*z*	*m/z*	*Error (ppm)*	*MS^2 ion fragments (m/z)*
Hydroxybenzoic acid	$C_7H_6O_3$	-1	137.0233	-0.15	93.0332
3,4-Dihydroxybenzoic acid (protocatechuic acid)	$C_7H_6O_4$	-1	153.0183	0.19	109.0283
Hydroxycinnamic acid	$C_9H_8O_3$	-1	163.0392	1.41	119.0490
Coumaric acid	$C_9H_8O_3$	-1	163.0392	1.41	119.0490
3-(3'-Hydroxyphenyl)propanoic acid (di-hydro-coumaric acid)	$C_9H_{10}O_3$	-1	165.0548	0.87	121.0648
3-(4'-Hydroxyphenyl)propanoic acid (phloretic acid)	$C_9H_{10}O_3$	-1	165.0547	0.59	121.0648
4-Hydroxy-3-methoxybenzoic acid (Vanillic acid)	$C_8H_8O_4$	-1	167.0341	1.03	152.0103; 123.0439
Di-hydroxycinnamic acid	$C_9H_8O_4$	-1	179.0341	1.30	135.0441
3',4'-Dihydroxycinnamic acid (caffeic acid)	$C_9H_8O_4$	-1	179.0341	1.30	135.0441
3-(3',4'-Dihydroxyphenyl)propanoic acid (di-hydro-caffeic acid)	$C_9H_{10}O_4$	-1	181.0499	1.74	137.0595
4'-Hydroxy-3'-methoxyphenylacetic acid (homovanillic acid)	$C_9H_{10}O_4$	-1	181.0499	1.74	137.0597; 122.0361
4'-Hydroxy-3'-methoxycinnamic acid (ferulic acid)	$C_{10}H_{10}O_4$	-1	193.0500	2.34	178.0262; 149.0600
3-(4'-Hydroxy-3'-methoxyphenyl)propanoic acid (di-hydro-ferulic acid)	$C_{10}H_{12}O_4$	-1	195.0657	2.66	136.0518; 151.0755
Caffeoylshikimic acid	$C_{16}H_{16}O_8$	-1	335.0773	3.45	179.0342; 135.0440
3-*O*-Coumaroylquinic acid	$C_{16}H_{18}O_8$	-1	337.0931	3.99	163.0392; 119.0490; 191.0554
5-*O*-Coumaroylquinic acid	$C_{16}H_{18}O_8$	-1	337.0930	3.72	191.0554; 119.0490; 163.0392; 173.0447
4-*O*-Coumaroylquinic acid	$C_{16}H_{18}O_8$	-1	337.0931	3.99	173.0447; 163.0393; 119.0490
Caffeoyl-hexoside	$C_{15}H_{18}O_9$	-1	341.0880	3.64	179.0341; 135.0441
3-*O*-Caffeoylquinic acid	$C_{16}H_{18}O_9$	-1	353.0862	-1.32	191.0552; 135.0440; 179.0340
4-*O*-Caffeoylquinic acid	$C_{16}H_{18}O_9$	-1.	353.0879	3.43	173.0447; 179.0340; 135.0440; 191.0552
5-*O*-Caffeoylquinic acid	$C_{16}H_{18}O_9$	-1	353.0877	2.83	191.0552; 173.0447
Di-hydro-caffeoylquinic acid	$C_{16}H_{20}O_9$	-1	355.1033	2.66	181.0498; 137.0597
3-*O*-Feruloylquinic acid	$C_{17}H_{20}O_9$	-1	367.1035	3.15	161.0235; 173.0446; 193.0501
4-*O*-Feruloylquinic acid	$C_{17}H_{20}O_9$	-1	367.1035	3.15	191.0550; 135.0440; 179.0340; 173.0444
5-*O*-Feruloylquinic acid	$C_{17}H_{20}O_9$	-1	367.1034	2.90	161.0235: 135.0441; 191.0550; 173.0443
O-Feruloylquinic acid	$C_{17}H_{20}O_9$	-1	367.1035	3.15	161.0235
O-Feruloylquinic acid	$C_{17}H_{20}O_9$	-1	367.1035	3.15	191.0550; 135.0440; 193.0494

Kaempferol-3-*O*-hexoside	$C_{21}H_{20}O_{11}$	-1	447.0937	3.49	255.0301; 227.0346; 284.0327; 285.0406
N^1,N^5-Caffeoyl-caffeoyl-spermidine	$C_{25}H_{31}O_6N_3$	-1	468.2144	3.08	135.0441; 332.1619; 306.1826
N^1,N^{10}-Caffeoyl-caffeoyl-spermidine	$C_{25}H_{31}O_6N_3$	-1	468.2141	2.63	135.0441; 332.1619; 306.1826
N^1,N^5-Di-hydro-caffeoyl-caffeoyl-spermidine	$C_{25}H_{33}O_6N_3$	-1	470.2298	2.63	135.0440; 334.1775; 308.1938
N^1,N^{10}- Di-hydro-caffeoyl-caffeoyl-spermidine	$C_{25}H_{33}O_6N_3$	-1	470.2299	2.82	135.0440; 334.1775; 308.1981
N^1,N^5-Di-hydro-caffeoyl-di-hydro-caffeoyl-spermidine	$C_{25}H_{35}O_6N_3$	-1	472.2455	2.76	308.1984; 350.2089; 186.1605
1,4-Di-*O*-caffeoylquinic acid	$C_{25}H_{23}O_{12}$	-1	515.1198	2.74	135.0441; 173.0447; 179.0342; 191.0556; 353.0871; 93.0333
1,5-Di-*O*-caffeoylquinic acid	$C_{25}H_{23}O_{12}$	-1	515.1197	2.50	191.0554; 135.0441; 179.0342; 353.0880; 85.0281
3,5-Di-*O*-caffeoylquinic acid	$C_{25}H_{23}O_{12}$	-1	515.1197	2.85	191.0554; 135.0441; 179.0342; 353.0880; 173.0449; 85.0281
4,5-Di-*O*-caffeoylquinic acid	$C_{25}H_{23}O_{12}$	-1	515.1198	2.74	173.0447; 135.0441; 179.0342; 191.0554; 353.0871; 93.0333
3,4-Di-*O*-caffeoylquinic acid	$C_{25}H_{23}O_{12}$	-1	515.1198	2.74	135.0441; 173.0446; 179.0340; 191.0553; 353.0887; 93.0333
Caffeoyl-tryptophan-hexoside	$C_{33}H_{40}O_4N_2$	-1	527.1671	0.92	161.0235; 365.2546; 135.0440
Kaempferol-3-*O*-hexoside-rhamnoside	$C_{27}H_{30}O_{15}$	-1	593.1516	0.60	284.0320; 255.0304; 227.0345; 285.0413
Kaempferol-3-*O*-rutinoside	$C_{27}H_{30}O_{15}$	-1	593.1519	1.12	285.0407; 255.0300; 227.0348; 284.0330
Cyanidin-3-*O*-rutinoside	$C_{27}H_{30}O_{15}$	+1	595.1660	0.35	287.0546
Quercetin-3-*O*-rutinoside	$C_{27}H_{30}O_{16}$	-1	609.1468	-1.08	300.0276; 301.0353; 271.0254
Kaempferol-3-*O*-hexoside-hexoside	$C_{27}H_{30}O_{16}$	-1	609.1468	-1.08	284.0330; 255.0301: 227.0347; 285.0405
Quercetin-3-*O*-hexoside-rhamnoside	$C_{27}H_{30}O_{16}$	-1	609.1471	1.62	300.0279; 271.0246; 301.0351; 255.0302; 151.0030
Delphinidin-3-*O*-rutinoside	$C_{27}H_{30}O_{16}$	+1	611.1605	-0.23	303.0495
Myricetin-3-*O*-rutinoside	$C_{27}H_{30}O_{17}$	-1	625.1416	0.93	316.0225; 271.0251; 287.0199; 317.0304
Delphinidin-3-*O*-rutinoside-5-*O*-hexoside	$C_{33}H_{40}O_{21}$	+1	711.2137	0.26	303.0496; 465.1023; 611.1581

Spearman Rank Order Correlations (Spreadsheet2)
MD pairwise deleted
Marked correlations are significant at p <,05000

Variable	Hydroxybenzoic acid	Protocatechuic acid	Di-hydrocoumaric acid	Vanillic acid	Caffeic acid	Di-hydrocaffeic acid isomer 1	Di-hydrocaffeic acid isomer 2	Homovanillic acid	Ferulic acid	Di-hydrocaffeoylquinic acid isomer 2	Di-hydrocaffeoylquinic acid isomer 3	Di-hydrocaffeoylquinic acid isomer 4	O-Feruloylquinic acid isomer 1	O-Feruloylquinic acid isomer 2	N1,N5-Di-diidrocaffeoylspermidine
Firmicutes	0,386158	0,816667	0,333333	-0,237322	-0,103510	-0,483333	-0,435665	0,900000	0,731126	0,470010	-0,068465	-0,068465	-0,068465	-0,068465	0,504975
Bacteroidetes	-0,455468	-0,283333	-0,783333	0,559402	0,517549	0,500000	0,841625	-0,466667	-0,626680	-0,182782	0,502079	0,502079	0,502079	0,502079	-0,613891
Lactobacillales	-0,277241	0,566667	-0,133333	-0,576354	0,414039	0,216667	-0,158424	0,400000	0,121854	0,382971	0,456435	0,456435	0,456435	0,456435	-0,079212
Bifidobacteriaceae	-0,386158	-0,066667	-0,050000	-0,406838	-0,517549	-0,133333	-0,257438	-0,450000	-0,278524	-0,940019	-0,547723	-0,547723	-0,547723	-0,547723	-0,504975
Enterobacteriaceae	-0,207931	0,066667	-0,416667	0,067806	0,724569	0,516667	0,435665	0,200000	-0,078335	0,609272	0,730297	0,730297	0,730297	0,730297	0,029704
Bpp	-0,574286	0,400000	-0,666667	-0,152564	0,517549	0,416667	0,386158	0,066667	-0,252413	-0,008704	0,524901	0,524901	0,524901	0,524901	-0,495074
Atopobium	0,653497	0,800000	0,666667	-0,254274	-0,310530	-0,700000	-0,683202	0,983333	0,940019	0,478714	-0,296683	-0,296683	-0,296683	-0,296683	0,772315
Clostridium Group I	0,841625	-0,233333	0,716667	0,440741	-0,414039	-0,550000	-0,297044	0,233333	0,496121	0,287228	-0,433614	-0,433614	-0,433614	-0,433614	0,762414
Clostridium Group IV	-0,188128	0,300000	0,116667	-0,440741	-0,621059	-0,433333	-0,455468	-0,100000	0,017408	-0,844277	-0,639010	-0,639010	-0,639010	-0,639010	-0,346552
Fprau	-0,524778	0,400000	-0,516667	-0,339032	0,621059	0,466667	0,237635	0,150000	-0,182782	0,217597	0,639010	0,639010	0,639010	0,639010	-0,366355
B.longum	0,009901	0,716667	0,266667	-0,644160	-0,724569	-0,533333	-0,653497	0,500000	0,539641	-0,435194	-0,730297	-0,730297	-0,730297	-0,730297	0,168325
Akkermansia	-0,762414	0,083333	-0,700000	-0,220371	0,517549	0,500000	0,386158	-0,366667	-0,609272	-0,313340	0,524901	0,524901	0,524901	0,524901	-0,841625
Ecoli	0,217832	0,766667	0,166667	-0,288177	-0,103510	-0,266667	-0,306946	0,833333	0,670199	0,391675	-0,091287	-0,091287	-0,091287	-0,091287	0,495074

Figure S1. Significance of Spearman Rank correlations related to Figure 2.

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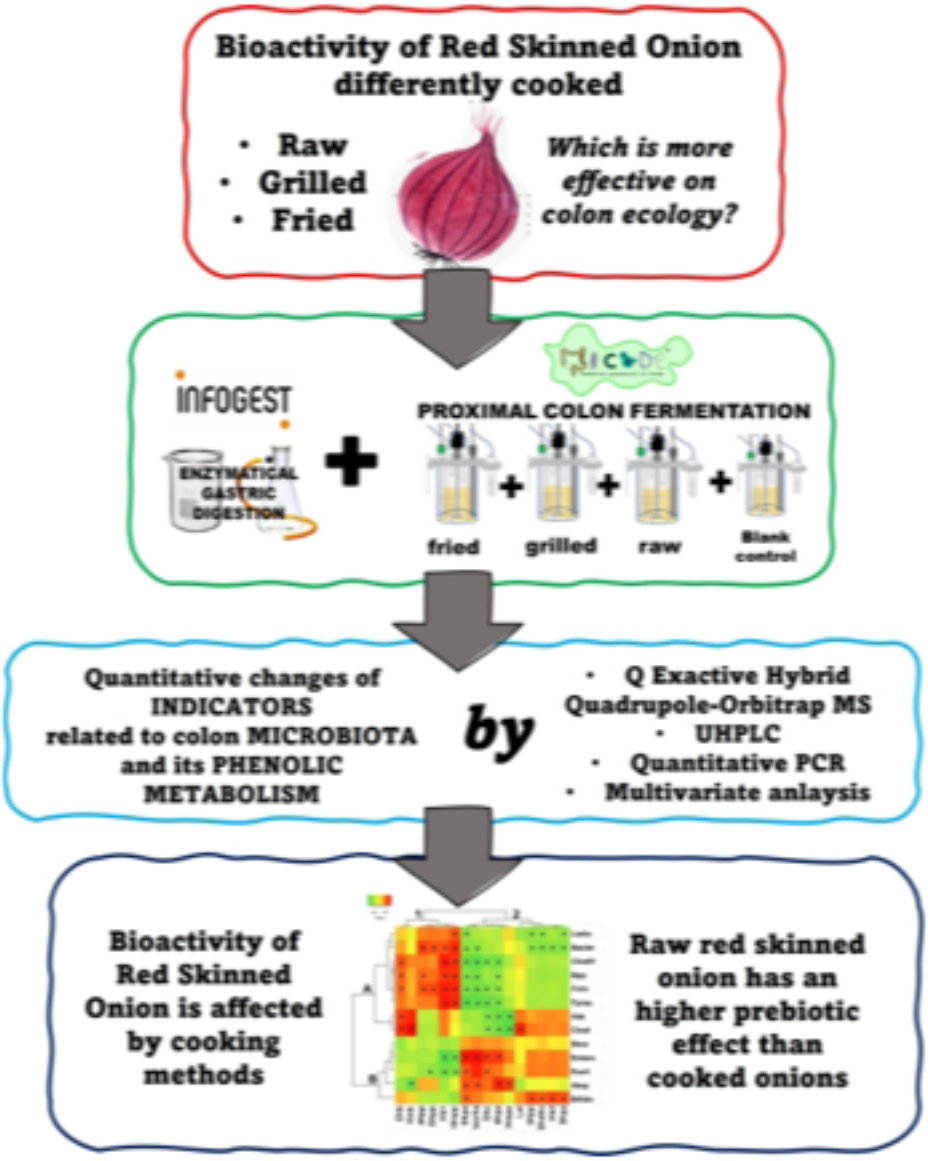
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A. Cattivelli[#], L. Nissen[#], F. Casciano, D. Tagliazucchi, A. Gianotti. Impact of cooking methods on *in vitro* colon metabolic transformation of phenolic compounds and gut microbiota changes. Submitted to *Food & Function*.

IMPACT OF COOKING METHODS OF RED-SKINNED ONION ON METABOLIC TRANSFORMATION OF PHENOLIC COMPOUNDS AND GUT MICROBIOTA CHANGES

ABSTRACT

The recipient work investigated on the stability and bioaccessibility of phenolics of red-skinned onion (RSO) differently cooked and in turn on its impact on the gut microbiota and phenolics metabolism. In fact, the different process used to cook vegetables can modify and re-arrange the molecular profiles of bioactives compounds, likely that of phenolics for phenolics-rich vegetables, such as RSO. Fried and grilled RSO were compared to raw RSO and to a blank control and were subject to oro-gastro-intestinal digestion and subsequent colonic fermentation. For upper gut digestion the INFOGEST protocol was used and for the lower gut fermentation a short-term batch model, namely MICODE (multi-unit in vitro colon gut model) was employed. During the process, the phenolic compounds profiles (through high-resolution mass spectrometry) and colon microbiomics (qPCR of 14 core taxa) analyses were performed. From the results obtained, the degradation driven by colon microbiota of RSO flavonols resulted in the accumulation of three main metabolites, 3-(3'-hydroxyphenyl)propanoic acid, 3-(3'-hydroxyphenyl)acetic acid and 3-(3',4'-dihydroxyphenyl)acetic acid. Also, colonic fermentation of raw onions resulted in a substantial increase in beneficial taxa, larger in respect to the heat-treated onions, particularly on Lactobacillales and beneficial clostridia. Also, a higher level of inhibition of opportunistic bacteria was seen for raw onion samples, namely Clostridium perfringens group and Escherichia coli. Thus, our results showed that RSO, and especially the raw one, is an excellent dietary source of flavonols that are strongly metabolized by gut bacteria and able to modulate positively the gut microbiota. Even if additional in vivo studies are necessary, this work is one of the first exploring how RSO processed with different cooking methods can differently impact on the phenolic metabolism and microbiota composition of human large intestine, tuning and fining the food antioxidant nature.

Keywords: thermal treatments, hydroxycinnamic acids, prebiotic index, food processing, mass spectrometry, microbiomics

1. Introduction

Onion (*Allium cepa L.*) is one of the most consumed vegetables worldwide and is also traditionally part of the Mediterranean Diet. Beyond the typical taste and flavour, onion is considered a healthy food due to the presence of several classes of phenolic compounds such as flavonols (mainly quercetin and its glycosylated derivatives), hydroxycinnamic acids and, in the red-skinned onion, anthocyanins[1-3]. Recently, onion consumption has been associated with a reduction in the onset of some chronic diseases such as cardiovascular diseases, type-2 diabetes, and gastro-intestinal tract cancers[4,5].

Generally, onion can be consumed, in the daily diet, either raw or cooked through different methods such as grilling and frying, which are the two most usual cooking procedures applied to onion. Several studies evidenced that heat treatments during cooking may modify the phenolic profile and structures of vegetables foods resulting in a different and sometimes higher bioactivity, and improved bioaccessibility after *in vitro* gastro-intestinal digestion[1,6,7]. Additionally, these modified compounds undergo an ulterior transformation when they enter the colon, where can feed the colon microbiota to foster beneficial bacteria and inhibit the opportunistic microbes[8]. Unfortunately, such improvement could also impact differently on the colon microbiota, resulting unfriendly to sensitive beneficial bacteria, such as bifidobacterial[9]. Moreover, it has been shown that both food composition and thermal treatments altered the composition of the gut microbiota[10]. In addition, previous studies highlighted that the different cooking procedures may affect the profile and concentration of phenolic metabolites deriving from the gut microbiota metabolic activity during *in vitro* fermentation of several vegetables[10-13].

The two-way interaction between gut microbiota and phenolic compounds is considered pivotal in determining the beneficial effect of phenolic compounds in human health[14]. In this sense, the colon bacteria can metabolize phenolic compounds in low molecular weight phenolic metabolites that are better absorbed and often more active than the parent compounds as well as phenolic compounds may modulate the gut microbiota population[15,16]. In particular, phenolic compounds may increase the relative abundance of beneficial bacteria involved in the protection of the intestinal epithelium (such as *Lactobacillus* and *Bifidobacterium* species) or that displayed anti-inflammatory effects (such as *Faecalibacterium prausnitzii* and *Akkermansia muciniphila*)[10,17-19]. In turn, colonic bacteria use to transform the structure of parent phenolic compounds in metabolites with improved biological activities and more bioavailable for the host[10,15,20].

In vitro gut models are considered the model of choice to study the complex relationships between the chemical constituents of food and the colon microbiota because they can clarify the effect on human gut microbiota of prebiotics such as phenolic compounds, through the analysis in the shift in the core microbial groups or of selected species, as well as assessing the modification in the produced

metabolites by individual bacterial specie[21]. In this study, we employed a gut model based on healthy adults, coupling the INFOGEST static protocol to simulate the oro-gastro-duodenal digestion[22] to the colon model MICODE (Multi-Unit *in vitro* Colon Model) to simulate proximal colonic fermentation, previously used with success to study food matrices rich in phenolic compounds[8-10]. Therefore, the aim of this study was to assess the bioaccessibility of phenolic compounds of both raw and cooked (grilled and fried) red-skinned onion as well as to elucidate the principal metabolic pathways and produced metabolites after *in vitro* colonic fermentation. Furthermore, the changes in fecal microbiota groups and selected bacterial taxa were also investigated.

2. Materials and Methods

2.1. Red-skinned onion (RSO) thermal treatments and in vitro gastro-intestinal digestion

Red-skinned onion (RSO) was subjected to two distinct thermal treatments to mimic the most common home cooking methods to which onion is subjected, i.e., grilling and frying. The cooking procedure and the cooking parameters were previously fully described[1]. Thereafter, raw (RRSO), grilled (GRSO) and fried (FRSO) red-skinned onion samples were subjected to *in vitro* gastro-intestinal digestion by using the INFOGEST protocol[22] as previously described for onion[1]. Finally, an aliquot of sample was collected at the end of the digestion procedure, centrifuged (10 min, 10000*g*, 4 °C) and the supernatant stored at -80 °C for further analysis whereas the remaining material (denser portion) was used for the *in vitro* colonic fermentation trials as described in paragraph 2.2. Cooking treatments and digestions were carried out in triplicate. Afterwards, for colonic in vitro fermentations, the triplicates of a given digestate were pooled together.

2.2. Colonic fermentation

2.2.1 Human colon microbiota

For this study, stool donations obtained from three lean healthy individuals that respected the inclusion criteria as previously reported[8-10,23-25] were used. Human colon microbiota (HCM) was extracted from stool samples, that were donated two times (with an interval of seven days) for the two biological replicas. Stool samples were collected with a collection kit, maintained at 37 °C under anaerobiosis and processed within two hours as previously described[8-10]. Briefly, to obtain the HCM a faecal slurry (FS) was prepared by homogenizing 6 g of stools (2 g of each donation) in 54 mL of O_2 reduced phosphate-buffered saline (PBS). The three healthy donors were told of the study's aims and procedures and gave their verbal consent for their faecal matter to be used for the experiments, in agreement with the ethics procedures required at the University of Bologna.

2.2.2 Colonic fermentation and sampling

Colonic fermentations were conducted for 24 hours in independent vessels using an *in vitro* gut model, MICODE [8-10,26,27], obtained by the assembly of Minibio Reactors (Applikon Biotechnology BV, Delft, NL) and controlled by Lucullus PIMS software (Applikon Biotechnology BV, NL). The preparation of the experiments was made according to published procedures [8-10,23,27,28] and recently described in details[9]. Briefly, four fermentation bioreactors were filled with 90 mL of basal medium[23] and then each one loaded as follows: i) with 10 mL of a mixtures of FS and 1 g of the solid portion of *in vitro* digested raw onion (RRSO); ii) with 10 mL of FS including 1 g of the solid portion of *in vitro* digested

grilled onion (GRSO); iii) with 10 mL of FS including 1 g of the solid portion of *in vitro* digested fried onion (FRSO), and iv) with 10 mL of FS including 1 mL of digestive enzymes, as the blank control (BC). Batch cultures were run under controlled conditions for a period of 27.48 h including the baseline (BL) (3.48 ± 0.22 h) as previously described[9]. The BL represents the time of adaptation of the HCM to *in vitro* conditions. BL was defined on the first pH changes detected by Lucullus® (1 read/10 s) via the pH sensors (Applisense, Applikon Biotechnology BV, NL) of MICODE. Sampling was performed at different time points, specifically BL; T1 = intermediate time point (18 h of fermentation); EP = endpoint (24 h of fermentation), as previously reported[9]. Samples of the different time points were used for UHPL/HR-MS and qPCR. Technical replicas of analyses were conducted in triplicates from two independent biological experiments.

2.3. High-resolution mass spectrometry analysis of phenolic compounds and fecal metabolites.

2.3.1 Extraction of phenolic compounds from undigested, digested and fermented red-skinned onion samples.

Phenolic compounds were extracted from raw, fried and grilled undigested RSO following a previously reported protocol[6]. RSO samples (15 g) were homogenised (6000 rpm for 2 min) with an Ultra-Turrax (Heidolph DIAX900, Sigma-Aldrich, Milan, Italy) after mixing with 30 mL a water/methanol/formic acid (28:70:2, v/v/v) solution. The mixtures were then incubated for 30 min at 37 °C and, after centrifugation (20 min, 6000*g*, 4 °C), the supernatants were collected and analysed by mass spectrometry for phenolic compounds identification and quantification.

Phenolic compounds in *in vitro* digested samples and faecal metabolites were extracted as previously described[12,13]. Briefly, 500 µL of extraction solution (water/methanol/formic acid; 19.9:80:0.1, v/v/v) were mixed with 500 µL of digested or fermented samples. After vortexing for 1 min the mixtures were centrifuged (10 min; 12000*g*; 4 °C). The supernatants were then used for mass spectrometry identification and quantification of phenolic compounds and metabolites.

2.3.2 Phenolic compounds profiles of undigested, digested and fermented red-skinned onion samples.

Phenolic compounds identification and quantification was performed by high resolution mass spectrometry analysis by using a Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). Chromatographic separation was carried out through an UHPLC Ultimate 3000 separation module equipped with a C18 column (Acquity UPLC HSS C18 Reversed phase, 2.1 × 100 mm, 1.8 µm particle size, Waters, Milan, Italy). The chromatographic and mass spectrometry parameters were previously fully detailed[29]. Quantification was carried out by building external calibration curves with the available standard phenolic compounds[2] and faecal metabolites[10] as previously reported. Mass spectral data of phenolic compounds[2] and faecal

metabolites[10] can be found in selected literature. For the phenolic metabolites the nomenclature rules previously suggested[30] was applied.

2.4. Microbiota analyses

2.4.1. Enumeration of bacterial groups and qPCR Prebiotic Index (qPI).

Bacterial DNA was extracted just after sampling (Purelink Microbiome DNA Purification Kit by Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA) and quantified (Biodrop, Biochrom Ltd., Cambridge, UK). Enumeration of bacterial groups was made by qPCR to evidence changes in the microbiota after fermentation[31-34] and expressed as cells/mL (obtained from values in gene copy numbers/mL multiplied by three for multicopy genes, as *V3-V4 16S*, and by one for monocopy genes, as *RecA*), following previous protocols[8-10,26,27,35]. 14 different bacterial taxa (*Eubacteria*, *Firmicutes*, *Bacteroidetes*, *Lactobacillales*, *Bifidobacteriaceae, Enterobacteriaceae*, *Clostridium* group I, *Clostridium* group IV, *Bacteroides-Prevotella-Porphyromonas* (BPP) group, *Atopobium-Collinsella-Eggerthella* (ATOP) group, *Bifidobacterium longum*, *Escherichia coli*, *Faecalibacterium prausnitzii*, and *Akkermansia muciniphila*) (**Table S1**) were assessed by qPCR on a QuantStudio 5 System (Applied Biosystem, Thermo Fisher, USA) and SYBR Green I chemistry. The qPCR Prebiotic Index (qPI) was calculated as previously reported[9,10]. Briefly, qPI is based on qPCR data of bacterial targets entered on this equation:

$$qPI = (Bifidobacteriaceae/Eubacteria) - (Enterobacteriaceae/Eubacteria) + (Lactobacillales/Eubacteria) - (Clostridium\ group\ I/Eubacteria).$$

2.5. Data processing and statistical analysis.

The shifts in abundance of bacterial groups were calculated as $Log_2(F/C)$ on raw data[36] obtained from sextuplicates. Also, from the raw data, the qPI was obtained after normalization with the mean centering method. Normality and Homeoscedasticity of the datasets were considered with Levene's and Shapiro-Wilks' test, respectively. To compare "time points" and "samples" among each other, the two factors used to categorize the MANOVAs were "time" and "food matrix", respectively. MANOVA for "time" and "food matrix" as categorical descriptors and followed by a Duncan's Multiple Range post-hoc test was applied using Statistica v 8.0 (Tibco, Palo Alto, CA, USA). The differences were considered significant with $p < 0.05$. To address specific correlations among bacteria and metabolites, two independent datasets were merged and computed by Spearman Rank analysis and visualized with a two-way joining heatmap, including Pearson dendrograms with complete linkage for both variables[8-10]. The dataset of correlations was performed with Statistica v. 8.0 (Tibco, USA) and the heatmap was produced with the Expression tool on www.heatmapper.ca[37] (last accessed 15/12/22).

3. Results and Discussion

3.1. Effect of cooking and in vitro digestion on red-skinned onion phenolic compounds bioaccessibility

A total of 54 phenolic compounds belonging to different classes were identified and quantified in the red-skinned onion (RSO) samples (**Table 1**). The most representative class of phenolic compounds in RSO were flavonols and, in particular, quercetin and quercetin-derivatives. In the raw sample (RRSO) flavonols represented the 85.3% of total phenolic compounds. Among flavonols, quercetin-derivatives accounted for the 74.2% of total phenolic compounds. The individual compound found at the highest concentration in RRSO was quercetin that represented the 50.1% of total phenolic compounds. Two glycosylated-derivatives of quercetin, namely quercetin-4'-*O*-glucoside and quercetin-3-*O*-glucoside-4'-*O*-glucoside were also present in appreciable amount in RRSO representing the 12.3% and 10.9% of total phenolic compounds, respectively. The second most important class of phenolic compounds in RRSO was represented by anthocyanins that accounted for the 11.2% of total phenolic compounds. A similar phenolic profile in RRSO have been already reported[2,38].

Both the applied thermal treatments induced an increase in the amount of extractable total phenolic compounds in RSO, mainly because of the matrix softening effect and to the loss of water that occurred during cooking, as previously suggested[1,2]. Specifically, grilling determined a 30.4% increase in total phenolic compounds whereas a recorded increase of 12.1% was observed in fried RSO. The increase in phenolic compounds in both the grilled (GRSO) and fried (FRSO) red-skinned onion was imputable to an augment in the concentration of quercetin-4'-*O*-glucoside and quercetin-3-*O*-glucoside-4'-*O*-glucoside. Vice versa, the concentration of quercetin decreased in both GRSO and FRSO in comparison with the raw sample. Similar results have been already reported[1,2,39,40]. Both grilling and frying also positively affected the release of onion phenolic compounds during *in vitro* gastro-intestinal digestion (**Table 1**). The highest amount of released phenolic compounds after in vitro digestion was found for the FRSO sample (92.05 ± 2.75 µmol/100 g) that also showed the highest bioaccessibility index (55.5%). RRSO and GRSO samples displayed similar bioaccessibility index for total phenolic compounds (30.6% and 32.7%, respectively) although in vitro digestion of GRSO resulted in a significantly higher amount of bioaccessible phenolic compounds respect to RRSO (63.21 ± 2.25 and 45.26 ± 1.52 µmol/100 g, respectively).The highest bioaccessibility index of FRSO was mainly due to the high bioaccessibility of quercetin (48.0%), which was present in trace or not detected in *in vitro* digested RRSO and GRSO. As previously suggested, the highest concentration of quercetin in *in vitro* digested FRSO respect to RRSO and GRSO may be due to the occurrence of oil that favoured quercetin solubility and stability during digestion[2,41]. A similar behaviour was also observed for the aglycone isorhamnetin that displayed a bioaccessibility index near to 100% in FRSO,

whereas was not detected in digested RRSO and GRSO. Looking at the behaviour of the other two most important flavonols, quercetin-3-*O*-glucoside-4'-*O*-glucoside was characterized by a high bioaccessibility index in all the samples (from 74.3% to 98.3% in FRSO and RRSO, respectively) whereas quercetin-4'-*O*-glucoside showed high bioaccessibility index only in RRSO (**Table 1**). In all the RSO digested samples the lowest bioaccessibility index was found for anthocyanins (from 2.5% in RRSO to 12.9% in FRSO) whereas the class of hydroxybenzoic acids was always characterized by a bioaccessibility index over than 100%. This is indicative of the occurrence of oxidative degradation of quercetin-mono- glucosides and quercetin during *in vitro* digestion, which resulted in the production of protocatechuic acid and di-hydroxybenzoic acid-hexosides[1,42,43].

Table 1. Amount of phenolic compounds in raw and cooked red-skinned onion determined by high-resolution mass spectrometry. Results are expressed in µmol of phenolic compound/100 g of onion. Bioaccessibility index (BI), expressed as percentage value, is the ratio between the concentration of each individual phenolic compound after *in vitro* digestion and the concentration in the extract.

Compound	*RRSO*			*FRSO*			*GRSO*		
	Before digestion	After digestion	BI (%)	Before digestion	After digestion	BI (%)	Before digestion	After digestion	BI (%)
Hydroxycinnamic acids									
Di-hydro-coumaric acid	n.d.	0.30 ± 0.02^{a}	n.f.	0.17 ± 0.01^{b}	0.35 ± 0.03^{a}	205.9	0.13 ± 0.01^{c}	0.29 ± 0.02^{a}	223.1
Caffeic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.03 ± 0.00	n.d.	0
Sinapic acid	0.08 ± 0.00^{a}	0.03 ± 0.00^{b}	37.5	0.02 ± 0.00^{b}	0.14 ± 0.01^{c}	700.0	0.04 ± 0.00^{b}	0.03 ± 0.00^{b}	67.7
Ferulic acid-4-*O*-hexoside	0.18 ± 0.01^{a}	0.18 ± 0.01^{a}	100.0	0.35 ± 0.03^{b}	0.11 ± 0.01^{c}	31.4	0.19 ± 0.01^{a}	0.14 ± 0.01^{d}	75.0
Sinapic acid-*O*-hexoside isomer 1	0.70 ± 0.03^{a}	0.40 ± 0.02^{b}	57.1	2.05 ± 0.15^{c}	1.86 ± 0.09^{d}	90.7	0.40 ± 0.02^{b}	0.35 ± 0.03^{b}	87.5
Sinapic acid-*O*-hexoside isomer 2	1.59 ± 0.08^{a}	0.12 ± 0.01^{b}	7.5	1.42 ± 0.08^{a}	0.73 ± 0.03^{c}	51.4	0.53 ± 0.03^{d}	0.15 ± 0.01^{b}	28.3
Sinapic acid-*O*-hexoside isomer 3	1.00 ± 0.10^{a}	0.15 ± 0.01^{b}	15.0	1.09 ± 0.09^{a}	0.54 ± 0.04^{c}	49.5	0.10 ± 0.00^{d}	n.d.	0
Sinapic acid-*O*-hexoside isomer 4	0.45 ± 0.04^{a}	0.08 ± 0.00^{b}	17.8	0.49 ± 0.03^{a}	0.54 ± 0.04^{a}	110.2	n.d.	n.d.	n.d.
Total hydroxycinnamic acids	**4.00 ± 0.14^{a}**	**1.26 ± 0.03^{b}**	**31.5**	**5.59 ± 0.20^{c}**	**4.27 ± 0.12^{a}**	**76.4**	**1.42 ± 0.04^{d}**	**0.96 ± 0.04**	**67.6**
Hydroxybenzoic acids									
Hydroxybenzoic acid isomer 1	0.08 ± 0.01^{a}	0.04 ± 0.00^{b}	50.0	0.17 ± 0.01^{c}	0.12 ± 0.01^{d}	70.6	0.14 ± 0.01^{d}	0.12 ± 0.01^{d}	85.7
Hydroxybenzoic acid isomer 2	0.04 ± 0.00^{a}	0.06 ± 0.00^{a}	150.0	0.05 ± 0.00^{a}	0.06 ± 0.00^{a}	120.0	0.04 ± 0.00^{a}	0.06 ± 0.00^{a}	150.0
Protocatechuic acid	0.18 ± 0.01^{a}	1.00 ± 0.09^{b}	555.6	0.22 ± 0.01^{c}	0.86 ± 0.05^{b}	390.9	0.24 ± 0.01^{c}	1.26 ± 0.07^{d}	525.0
Di-hydroxybenzoic acid	0.15 ± 0.01^{a}	0.04 ± 0.00^{b}	26.7	0.16 ± 0.01^{a}	0.05 ± 0.00^{b}	31.3	0.17 ± 0.01^{a}	0.03 ± 0.00^{b}	17.6
Vanillic acid	0.12 ± 0.01^{a}	0.01 ± 0.00^{b}	8.3	0.07 ± 0.00^{c}	n.d.	0	0.12 ± 0.01^{a}	n.d.	0
Di-hydroxybenzoic acid-hexoside isomer 1	0.14 ± 0.01^{a}	3.51 ± 0.25^{b}	2507.1	0.26 ± 0.01^{c}	1.45 ± 0.09^{d}	557.7	0.51 ± 0.04^{e}	2.35 ± 0.12^{f}	460.8
Di-hydroxybenzoic acid-hexoside isomer 2	0.03 ± 0.00^{a}	0.08 ± 0.00^{a}	266.7	0.05 ± 0.00^{a}	0.05 ± 0.00^{a}	100.0	0.07 ± 0.00^{a}	0.07 ± 0.00^{a}	100.0
Di-hydroxybenzoic acid-hexoside isomer 3	0.03 ± 0.00^{a}	n.d.	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total hydroxybenzoic acids	**0.77 ± 0.02^{a}**	**4.74 ± 0.27^{b}**	**615.6**	**0.98 ± 0.02^{c}**	**2.59 ± 0.10^{d}**	**264.3**	**1.29 ± 0.04^{e}**	**3.89 ± 0.14^{f}**	**308.7**
Flavonols									
Quercetin	74.14 ± 3.61^{a}	n.d.	0	50.01 ± 2.57^{b}	23.98 ± 1.77^{c}	48.0	56.59 ± 3.10^{b}	0.38 ± 0.01	0.67
Quercetin-3-*O*-hexoside	n.d.	n.d.	n.d.	0.04 ± 0.00^{a}	n.d.	0	0.04 ± 0.00^{a}	n.d.	0
Quercetin-3-*O*-glucoside	0.96 ± 0.05^{a}	0.61 ± 0.03^{b}	63.5	0.79 ± 0.01^{c}	1.90 ± 0.09^{d}	240.5	1.87 ± 0.11^{d}	1.76 ± 0.07	94.1
Quercetin-4'-*O*-glucoside	18.28 ± 1.03^{a}	17.50 ± 1.11^{a}	95.7	45.11 ± 1.80^{b}	18.88 ± 1.21^{a}	41.9	50.60 ± 3.24^{b}	10.60 ± 0.81^{c}	20.9
Quercetin-7-*O*-hexoside-4'-*O*-hexoside	0.03 ± 0.00^{a}	0.17 ± 0.01^{b}	566.7	0.14 ± 0.01^{b}	0.17 ± 0.01^{b}	121.4	0.22 ± 0.01^{c}	0.07 ± 0.00^{a}	31.8

Quercetin-3-*O*-glucoside-4'-*O*-glucoside	16.21 ± 0.99[a]	15.93 ± 0.99[a]	98.3	31.12 ± 1.51[b]	23.13 ± 1.56[c]	74.3	46.65 ± 2.39[d]	39.76 ± 2.09[e]	85.2
Quercetin-tri-*O*-hexoside	0.05 ± 0.00[a]	0.04 ± 0.00[a]	80.0	0.12 ± 0.00[b]	0.06 ± 0.00[a]	50.0	0.25 ± 0.02[c]	0.07 ± 0.00[a]	28.0
Quercetin-*O*-hexoside-*O*-malonyl-hexoside isomer 1	0.04 ± 0.00[a]	0.04 ± 0.00[a]	100.0	0.03 ± 0.00[a]	0.03 ± 0.00[a]	100.0	0.05 ± 0.00[a]	0.05 ± 0.00[a]	100.0
Quercetin-*O*-hexoside-*O*-malonyl-hexoside isomer 2	0.05 ± 0.01[a]	0.03 ± 0.00[a]	60.0	0.04 ± 0.00[a]	0.02 ± 0.00[a]	50.0	0.06 ± 0.00[a]	0.04 ± 0.00[a]	66.7
Quercetin-7-*O*-acetyl-hexoside	0.07 ± 0.00[a]	0.03 ± 0.00[a]	42.9	0.05 ± 0.00[a]	0.03 ± 0.00[a]	60.0	0.06 ± 0.00[a]	0.03 ± 0.00[a]	50.0
Quercetin-*O*-hexoside-*O*-acetyl-hexoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.01 ± 0.00	0.01 ± 0.00	100.0
Isorhamnetin	9.14 ± 0.52[a]	n.d.	0	8.66 ± 0.51[a]	8.30 ± 0.60[a]	95.8	9.78 ± 0.61[a]	n.d.	0
Isorhamnetin-3-*O*-hexoside	0.09 ± 0.00[a]	0.13 ± 0.01[b]	144.4	0.20 ± 0.01[c]	0.12 ± 0.01[b]	65.0	0.27 ± 0.01[d]	0.16 ± 0.01[b]	66.7
Isorhamnetin-4'-*O*-hexoside	5.63 ± 0.20[a]	3.49 ± 0.14[b]	62.0	9.73 ± 0.63[c]	5.48 ± 0.35[a]	56.3	5.84 ± 0.67[a]	2.68 ± 0.16[d]	45.9
Isorhamnetin-*O*-hexoside-*O*-pentoside	0.02 ± 0.00[a]	n.d.	0	0.03 ± 0.00[a]	0.02 ± 0.00[a]	66.7	0.02 ± 0.00[a]	0.02 ± 0.00[a]	100.0
Isorhamnetin-3-*O*-hexoside-4'-*O*-hexoside	0.40 ± 0.02[a]	0.73 ± 0.03[b]	182.5	1.43 ± 0.08[c]	1.38 ± 0.06[c]	96.5	1.40 ± 0.06[c]	1.04 ± 0.03[d]	74.3
Kaempferol-7-*O*-hexoside-isomer 1	0.35 ± 0.02[a]	n.d.	0	0.32 ± 0.02[a]	0.03 ± 0.00[b]	9.4	0.91 ± 0.07[c]	0.63 ± 0.04[d]	69.2
Kaempferol-7-*O*-hexoside-isomer 2	0.01 ± 0.00[a]	0.01 ± 0.00[a]	100.0	0.02 ± 0.00[a]	0.01 ± 0.00[a]	50.0	0.01 ± 0.00[a]	0.01 ± 0.00[a]	100.0
Kaempferol-3-*O*-hexoside	0.10 ± 0.00[a]	0.05 ± 0.00[b]	50.0	0.07 ± 0.00[b]	0.03 ± 0.00[a]	42.9	0.04 ± 0.00[a]	0.02 ± 0.00[a]	50.0
Kaempferol-*O*-hexoside-*O*-hexoside isomer 1	0.71 ± 0.05[a]	0.01 ± 0.00[b]	1.4	0.33 ± 0.02[c]	0.03 ± 0.00[b]	9.1	0.81 ± 0.06[a]	0.02 ± 0.00[b]	2.5
Kaempferol-*O*-hexoside-*O*-hexoside isomer 2	0.08 ± 0.00[a]	0.05 ± 0.00[b]	62.5	0.05 ± 0.00[b]	0.04 ± 0.00[b]	80.0	0.04 ± 0.00[b]	0.03 ± 0.00[b]	75.0
Total flavonols	**126.36 ± 3.92[a]**	**38.82 ± 1.50[b]**	**30.7**	**148.29 ± 3.58[c]**	**83.65 ± 2.74[d]**	**56.4**	**175.52 ± 5.16[e]**	**57.39 ± 2.25[f]**	**32.7**
Other flavonoids									
Epicatechin-*O*-hexoside isomer 1	0.02 ± 0.00[a]	n.d.	0	0.01 ± 0.00[a]	n.d.	0	0.01 ± 0.00[a]	n.d.	0
Epicatechin-*O*-hexoside isomer 2	0.01 ± 0.00[a]	n.d.	0	n.d.	n.d.	n.d.	0.01 ± 0.00[a]	n.d.	0
Epicatechin-*O*-hexoside isomer 3	0.01 ± 0.00[a]	n.d.	0	n.d.	n.d.	n.d.	0.01 ± 0.00[a]	n.d.	0
Taxifolin-*O*-hexoside isomer 1	0.06 ± 0.00[a]	0.01 ± 0.00[b]	16.7	0.09 ± 0.00[a]	0.05 ± 0.00[a]	55.6	0.02 ± 0.00[b]	0.01 ± 0.00[b]	50.0
Taxifolin-*O*-hexoside isomer 2	0.16 ± 0.01[a]	n.d.	0	0.20 ± 0.01[a]	0.08 ± 0.00[b]	40.0	0.11 ± 0.01[b]	n.d.	0
Taxifolin-*O*-hexoside isomer 3	0.11 ± 0.01[a]	n.d.	0	0.11 ± 0.01[a]	n.d.	0	0.15 ± 0.01[b]	n.d.	0.
Taxifolin-*O*-hexoside isomer 4	0.06 ± 0.00[a]	0.01 ± 0.00[b]	16.7	0.10 ± 0.01[c]	0.04 ± 0.00[a]	40.0	0.04 ± 0.00[a]	0.01 ± 0.00[b]	25.0
Total other flavonoids	**0.43 ± 0.01[a]**	**0.02 ± 0.00[b]**	**4.7**	**0.51 ± 0.02[c]**	**0.17 ± 0.00[d]**	**33.3**	**0.35 ± 0.01[e]**	**0.02 ± 0.00[b]**	**5.7**
Anthocyanins									
Cyanidin-3-*O*-hexoside isomer 1	1.86 ± 0.15[a]	0.02 ± 0.00[b]	1.1	1.40 ± 0.11[c]	0.11 ± 0.01[d]	7.9	2.11 ± 0.18[a]	0.09 ± 0.01[d]	4.3
Cyanidin-3-*O*-hexoside isomer 2	0.09 ± 0.01[a]	0.05 ± 0.00[b]	55.6	0.06 ± 0.00[b]	0.02 ± 0.00[c]	33.3	0.06 ± 0.00[b]	0.02 ± 0.00[c]	50.0

Peonidin-3-*O*-hexoside	0.13 ± 0.01[a]	n.d.	0	0.19 ± 0.02[b]	0.02 ± 0.00[c]	10.5	0.16 ± 0.01[b]	0.09 ± 0.00[d]	56.3
Cyanidin-*O*-malonyl-hexoside	8.99 ± 0.74[a]	0.19 ± 0.02[b]	2.1	6.15 ± 0.51[c]	0.86 ± 0.07[d]	14.0	7.51 ± 0.48[c]	0.43 ± 0.03[e]	5.7
Peonidin-*O*-malonyl-hexoside	0.16 ± 0.01[a]	0.01 ± 0.00[b]	6.3	0.14 ± 0.01[a]	0.03 ± 0.00[b]	21.4	0.14 ± 0.01[a]	0.01 ± 0.00[b]	7.1
Cyanidin-*O*-hexoside-*O*-hexoside isomer 1	0.10 ± 0.01[a]	0.01 ± 0.00[b]	10.0	1.01 ± 0.09[c]	n.d.	0	0.21 ± 0.02[d]	0.03 ± 0.00[b]	14.3
Cyanidin-*O*-hexoside-*O*-hexoside isomer 2	1.63 ± 0.12[a]	0.03 ± 0.00[b]	1.8	0.04 ± 0.00[b]	0.09 ± 0.01[c]	225.0	1.52 ± 0.11[a]	0.07 ± 0.00[c]	4.6
Cyanidin-*O*-hexoside-*O*-hexoside isomer 3	0.05 ± 0.00[a]	0.05 ± 0.00[a]	100.0	0.03 ± 0.00[a]	0.03 ± 0.00[a]	100.0	0.04 ± 0.00[a]	0.03 ± 0.00[a]	75.0
Cyanidin-*O*-hexoside-*O*-malonyl-hexoside isomer 1	n.d.	n.d.	n.d.	0.05 ± 0.00[a]	0.01 ± 0.00[b]	20.0	0.10 ± 0.01[c]	0.01 ± 0.00[b]	10.0
Cyanidin-*O*-hexoside-*O*-malonyl-hexoside isomer 2	3.49 ± 0.29[a]	0.06 ± 0.00[b]	1.7	1.55 ± 0.10[c]	0.20 ± 0.01[d]	12.9	2.58 ± 0.19[e]	0.16 ± 0.01[d]	6.2
Total anthocyanins	**16.50 ± 0.82[a]**	**0.42 ± 0.02[b]**	**2.5**	**10.62 ± 0.54[c]**	**1.37 ± 0.07[d]**	**12.9**	**14.43 ± 0.56[a]**	**0.94 ± 0.03[e]**	**6.5**
Total	**148.06 ± 4.01[a]**	**45.26 ± 1.52[b]**	**30.6**	**165.99 ± 3.62[c]**	**92.05 ± 2.75[d]**	**55.5**	**193.01 ± 5.19[e]**	**63.21 ± 2.25**	**32.7**

Different letters within the same row mean significant different (*P<0.05*) values.
n.d. means that the compound was not detected in the sample
n.f. means newly formed compound
Abbreviations are: RRSO, raw red-skinned onion; FRSO, fried red-skinned onion; GRSO, grilled red-skinned onion

3.2. Red-skinned onion phenolic compound metabolism during in vitro colonic fermentation.

Quercetin and glycosylated quercetin-derivatives absorption and bioavailability have been fairly studied in the past years[44,45]. In general, quercetin-mono-glucosides (such as quercetin-4'-O-glucoside and quercetin-3-O-glucoside) are quite well absorbed in the small intestine reaching the 50% of the ingested dose[46]. The absorption pathway involved the hydrolysis of the glucose moiety by brush-border glucosidases (such as lactase phloridzin hydrolase) and the absorption of the quercetin aglycone that is sufficiently hydrophobic to cross the membrane of enterocytes by passive diffusion[47]. Differently, the bioavailability of quercetin aglycone is much lower than that of the glucoside-derivatives (about 20% of the ingested doses) mainly due to its low bioaccessibility and solubility in the intestinal fluid[48]. Therefore, most of the quercetin and its derivatives present in onion are supposed to reach the colon where they are thoroughly metabolized by the colon microbiota. Fecal metabolism of quercetin (and its derivatives) involves deglycosylation reaction, followed by C-ring fission that led to the production of several low-molecular weight phenolic acids[49,50]. Although there are some in vitro studies aimed to elucidate colonic quercetin metabolism, there are no research aimed to investigate the effect of cooking on the colonic metabolism of onion quercetins and on the on changes in the microbiota. Therefore, the in vitro digested RSO samples were further subjected to in vitro faecal fermentation and the generated metabolites were identified and quantified by means of high-resolution mass spectrometry at BL, T1 and EP h of fermentation (**Tables 2-4**).

Table 4. Changes in phenolic compounds metabolites concentration during in vitro colonic fermentation of raw red-skinned onion (RRSO). Results are expressed in µmol of phenolic compound/100 g of onion.

Compound	Time points of colonic fermentation		
	BL	**T1**	**EP**
Hydroxybenzoic acid isomer 1	0.47 ± 0.02[a]	1.18 ± 0.09[b]	1.17 ± 0.11[b]
Hydroxybenzoic acid isomer 2	n.d.	0.71 ± 0.05[a]	0.70 ± 0.05[a]
3-(3'-Hydroxyphenyl)acetic acid	7.51 ± 0.61[a]	7.93 ± 0.59[a]	12.49 ± 1.00[b]
3-(4'-Hydroxyphenyl)acetic acid	n.d.	n.d.	n.d.
3,4-Dihydroxybenzoic acid	1.89 ± 0.11[a]	1.98 ± 0.15[a]	2.13 ± 0.11[a]
Dihydroxybenzoic acid isomer 1	0.31 ± 0.01[a]	n.d.	n.d.
Dihydroxybenzoic acid isomer 2	n.d.	n.d.	n.d.
3-(3'-Hydroxyphenyl)propanoic acid	183.31 ± 11.29[a]	32.66 ± 2.36[b]	39.50 ± 1.63[c]
3-(4'-Hydroxyphenyl)propanoic acid	0.20 ± 0.01[a]	2.69 ± 0.19[b]	6.12 ± 0.48[c]
4-Hydroxy-3-methoxybenzoic acid	0.21 ± 0.01[a]	0.27 ± 0.02[b]	0.29 ± 0.01[b]
3-(3',4'-Dihydroxyphenyl)acetic acid	0.68 ± 0.02[a]	1.79 ± 0.16[b]	3.05 ± 0.11[c]
3',4'-Dihydroxycinnamic acid	0.41 ± 0.02[a]	0.27 ± 0.02[b]	0.29 ± 0.02[b]
Dihydroxyphenyl)propanoic acid	0.22 ± 0.01[a]	n.d.	0.05 ± 0.00[b]
3-(3',4'-Dihydroxyphenyl)propanoic acid	0.14 ± 0.01[a]	1.25 ± 0.09[b]	0.19 ± 0.01[c]
3-(4'-Hydroxy-3'-methoxyphenyl)acetic acid	0.02 ± 0.00[a]	0.02 ± 0.00[a]	0.08 ± 0.00[b]
3-(4'-Hydroxy-3'-methoxyphenyl)propanoic acid	0.68 ± 0.04[a]	0.66 ± 0.05[a]	1.16 ± 0.09[b]
Luteolin	7.51 ± 0.51[a]	3.96 ± 0.28[b]	3.39 ± 0.21[b]
Quercetin	96.41 ± 5.66[a]	1.09 ± 0.10[b]	n.d.
Isorhamnetin	17.39 ± 1.08[a]	0.43 ± 0.03[b]	0.20 ± 0.02[c]
Total	317.36 ± 12.70[a]	56.89 ± 2.47[b]	70.81 ± 1.99[c]

[a,b,c]Different letters within the same row mean significant different (p < 0.05) values; n.d. = not detected compound; BL = baseline (3.48 h); T1 = intermediate time point (21.48 h); EP = endpoint (27.48 h).

Table 3. Changes in phenolic compounds metabolites concentration during *in vitro* colonic fermentation of fried red-skinned onion (FRSO). Results are expressed in µmol of phenolic compound/100 g of onion.

Compound	Time points of colonic fermentation		
	BL	**T1**	**EP**
Hydroxybenzoic acid isomer 1	0.44 ± 0.03[a]	1.24 ± 0.10[b]	1.08 ± 0.09[b]
Hydroxybenzoic acid isomer 2	0.11 ± 0.00[a]	1.01 ± 0.10[b]	1.09 ± 0.09[b]
3-(3'-Hydroxyphenyl)acetic acid	7.83 ± 0.58[a]	11.69 ± 0.99[b]	12.84 ± 1.13[b]
3-(4'-Hydroxyphenyl)acetic acid	n.d.	15.53 ± 0.81[a]	8.58 ± 0.72[b]
3,4-Dihydroxybenzoic acid	1.45 ± 0.11[a]	6.83 ± 0.49[b]	1.52 ± 0.11[a]
Dihydroxybenzoic acid isomer 1	0.24 ± 0.02[a]	n.d.	n.d.
Dihydroxybenzoic acid isomer 2	n.d.	n.d.	n.d.
3-(3'-Hydroxyphenyl)propanoic acid	170.43 ± 12.84[a]	49.70 ± 2.92[b]	44.20 ± 2.66[b]
3-(4'-Hydroxyphenyl)propanoic acid	0.17 ± 0.01[a]	4.13 ± 0.28[b]	4.13 ± 0.36[b]
4-Hydroxy-3-methoxybenzoic acid	0.19 ± 0.01[a]	0.24 ± 0.02[a]	0.19 ± 0.01[a]
3-(3',4'-Dihydroxyphenyl)acetic acid	1.16 ± 0.09[a]	10.68 ± 0.61[b]	14.40 ± 0.97[c]
3',4'-Dihydroxycinnamic acid	0.37 ± 0.01[a]	0.41 ± 0.03[a]	0.35 ± 0.03[a]
Dihydroxyphenyl)propanoic acid	0.29 ± 0.02[a]	2.01 ± 0.11[b]	0.61 ± 0.04[c]
3-(3',4'-Dihydroxyphenyl)propanoic acid	0.12 ± 0.00[a]	0.09 ± 0.00[b]	0.15 ± 0.01[c]
3-(4'-Hydroxy-3'-methoxyphenyl)acetic acid	0.01 ± 0.00[a]	0.11 ± 0.00[b]	0.12 ± 0.01[b]
3-(4'-Hydroxy-3'-methoxyphenyl)propanoic acid	0.13 ± 0.01[a]	0.63 ± 0.05[b]	n.d.
Luteolin	7.97 ± 0.60[a]	7.62 ± 0.45[a]	4.39 ± 0.31[b]
Quercetin	121.16 ± 8.22[a]	12.44 ± 1.02[b]	n.d.
Isorhamnetin	37.99 ± 2.18[a]	4.48 ± 0.36[b]	1.58 ± 0.09[c]
Total	350.06 ± 15.42[a]	128.84 ± 3.41[b]	95.23 ± 3.09[c]

[a,b,c]Different letters within the same row mean significant different ($p < 0.05$) values; n.d. = not detected compound; BL = baseline (3.48 h); T1 = intermediate time point (21.48 h); EP = endpoint (27.48 h).

Table 4. Changes in phenolic compounds metabolites concentration during in vitro colonic fermentation of grilled red-skinned onion (GRSO). Results are expressed in µmol of phenolic compound/100 g of onion.

Compound	Time points of colonic fermentation		
	BL	**T1**	**EP**
Hydroxybenzoic acid isomer 1	0.47 ± 0.03[a]	1.08 ± 0.08[b]	1.15 ± 0.10[b]
Hydroxybenzoic acid isomer 2	n.d.	1.14 ± 0.09[a]	2.21 ± 0.12[b]
3-(3'-Hydroxyphenyl)acetic acid	8.45 ± 0.78[a]	11.86 ± 1.28[b]	13.53 ± 0.97[b]
3-(4'-Hydroxyphenyl)acetic acid	n.d.	n.d.	n.d.
3,4-Dihydroxybenzoic acid	1.74 ± 0.14[a]	2.22 ± 0.11[b]	2.12 ± 0.09[b]
Dihydroxybenzoic acid isomer 1	0.99 ± 0.10[a]	n.d.	n.d.
Dihydroxybenzoic acid isomer 2	0.31 ± 0.03[a]	n.d.	n.d.
3-(3'-Hydroxyphenyl)propanoic acid	203.89 ± 11.64[a]	76.01 ± 4.58[b]	91.32 ± 6.64[c]
3-(4'-Hydroxyphenyl)propanoic acid	0.20 ± 0.01[a]	1.49 ± 0.09[b]	1.88 ± 0.12[c]
4-Hydroxy-3-methoxybenzoic acid	0.27 ± 0.02[a]	0.27 ± 0.02[a]	0.31 ± 0.02[a]
3-(3',4'-Dihydroxyphenyl)acetic acid	0.94 ± 0.08[a]	2.56 ± 0.19[b]	3.37 ± 0.19[c]
3',4'-Dihydroxycinnamic acid	0.40 ± 0.03[a]	0.32 ± 0.02[b]	0.29 ± 0.02[b]
Dihydroxyphenyl)propanoic acid	0.28 ± 0.02[a]	1.85 ± 0.11[b]	1.54 ± 0.10[c]
3-(3',4'-Dihydroxyphenyl)propanoic acid	0.15 ± 0.01[a]	0.03 ± 0.00[b]	0.02 ± 0.00[b]
3-(4'-Hydroxy-3'-methoxyphenyl)acetic acid	0.02 ± 0.00[a]	0.01 ± 0.00[a]	0.01 ± 0.00[a]
3-(4'-Hydroxy-3'-methoxyphenyl)propanoic acid	0.20 ± 0.01[a]	n.d.	n.d.
Luteolin	7.81 ± 0.57[a]	6.64 ± 0.46[b]	5.97 ± 0.32[b]
Quercetin	144.91 ± 7.31[a]	8.50 ± 0.71[b]	n.d.
Isorhamnetin	18.92 ± 1.04[a]	1.23 ± 0.09[b]	0.32 ± 0.03[c]
Total	389.95 ± 13.82[a]	115.21 ± 4.84[b]	124.04 ± 6.73[b]

[a,b,c]Different letters within the same row mean significant different ($p < 0.05$) values; n.d. = not detected compound; BL = baseline (3.48 h); T1 = intermediate time point (21.48 h); EP = endpoint (27.48 h).

In all the RSO samples, quercetin-glucosides and isorhamnetin-glucosides were rapidly hydrolysed to the corresponding aglycones since they almost completely disappeared already at the BL of fermentation. These results are in agreement with previous studies that showed a complete hydrolysis of O-glucosides of flavonols in the first hours of colon fermentation[39,51]. In addition to quercetin and isorhamnetin aglycones also luteolin aglycone appeared at the BL of fermentation in all the RSO samples. Since luteolin aglycone and luteolin-derivatives were not identified in RSO samples, we can speculate that luteolin aglycone derived from quercetin dehydroxylation at C3 position in the C-ring, as already suggested[39]. The concentration of quercetin and isorhamnetin aglycones strongly decreased at T1 of fermentation and quercetin was no more detectable at the EP of fermentation (**Tables 2-4**).

The metabolite found at the highest concentration in all the RSO samples at the BL of fermentation was 3-(3'-hydroxyphenyl)propanoic acid, which represented the 57.8%, 48.7% and 52.3% of total metabolites in RRSO, GRSO and FRSO. Additional metabolites identified in appreciable amount at the BL of fermentation were 3-(3'-hydroxyphenyl)acetic acid, 3,4-dihydroxybenzoic acid and 3-(3',4'-dihydroxyphenyl)acetic acid. At the BL of fermentation only quantitative differences in the amount of total metabolites were found among samples. The highest concentration of metabolites was found in GRSO followed by FRSO and RRSO. In all the RSO samples, the concentration of metabolites at the BL of fermentation strongly exceed the amount of parent phenolic compounds identified in onion, suggesting the presence of high amount of bound phenolic compounds in onion that can be released from fiber or other macromolecules by the action of the gut bacteria.

At the end of the fermentation process, a decline in the concentration of phenolic metabolites was observed and GRSO once again was the sample releasing the highest amount of phenolic metabolites. In all the sample 3-(3'-hydroxyphenyl)propanoic acid was still the metabolite present at the highest concentration although with a different incidence depending on the sample. In GRSO 3-(3'-hydroxyphenyl)propanoic acid accounted for the 73.6% of total metabolites whereas in FRSO and RRSO its incidence was lower (46.4% and 55.8%, respectively). In addition, also the other most important metabolites were found in different proportion depending on the thermal treatment at the end of the fermentation process. RRSO was the richest in term of percentage of incidence of 3-(3'-hydroxyphenyl)acetic acid (17.6% of total phenolic compounds) followed by FRSO (13.4% of total phenolic compounds) and GRSO (10.4% of total phenolic compounds). Moreover, in FRSO sample the metabolite 3-(3',4'-dihydroxyphenyl)acetic acid was found in high proportion, representing the 15.1% of total metabolites, whereas it represented only the 2.7% and 4.3% of total metabolites in GRSO and RRSO. Generally, the three metabolites 3-(3'-hydroxyphenyl)propanoic acid, 3-(3'-hydroxyphenyl)acetic acid and 3-(3',4'-dihydroxyphenyl)acetic acid tended to accumulate in high amount in fermented samples but with a different proportion depending on the sample. In this way, cooking may modulate the metabolite profile after fermentation both from a quantitative and qualitative point-of-view.

Figure 1 displays the colonic microbiota metabolic pathway of red-skinned onion flavonols. The first step was the de-glycosylation of quercetin-glucosides and isorhamnetin-glucosides into the corresponding aglycones quercetin and isorhamnetin[39,51]. The appearance of luteolin, which was not present as aglycone or any derivatives, suggested the possible de-hydroxylation of quercetin[39]. The next step in the flavonols metabolism was the ring fission leading 3-(3',4'-dihydroxyphenyl)propanoic acid in the case of quercetin and luteolin or 3-(4'-hydroxy-3'-methoxyphenyl)propanoic acid in the case of isorhamnetin[49]. Ring fission of quercetin may also result in the production of 3,4-dihydroxybenzoic acid[52].

The metabolic intermediate 3-(3',4'-dihydroxyphenyl)propanoic acid may have different fates as depicted in **Figure 1**.

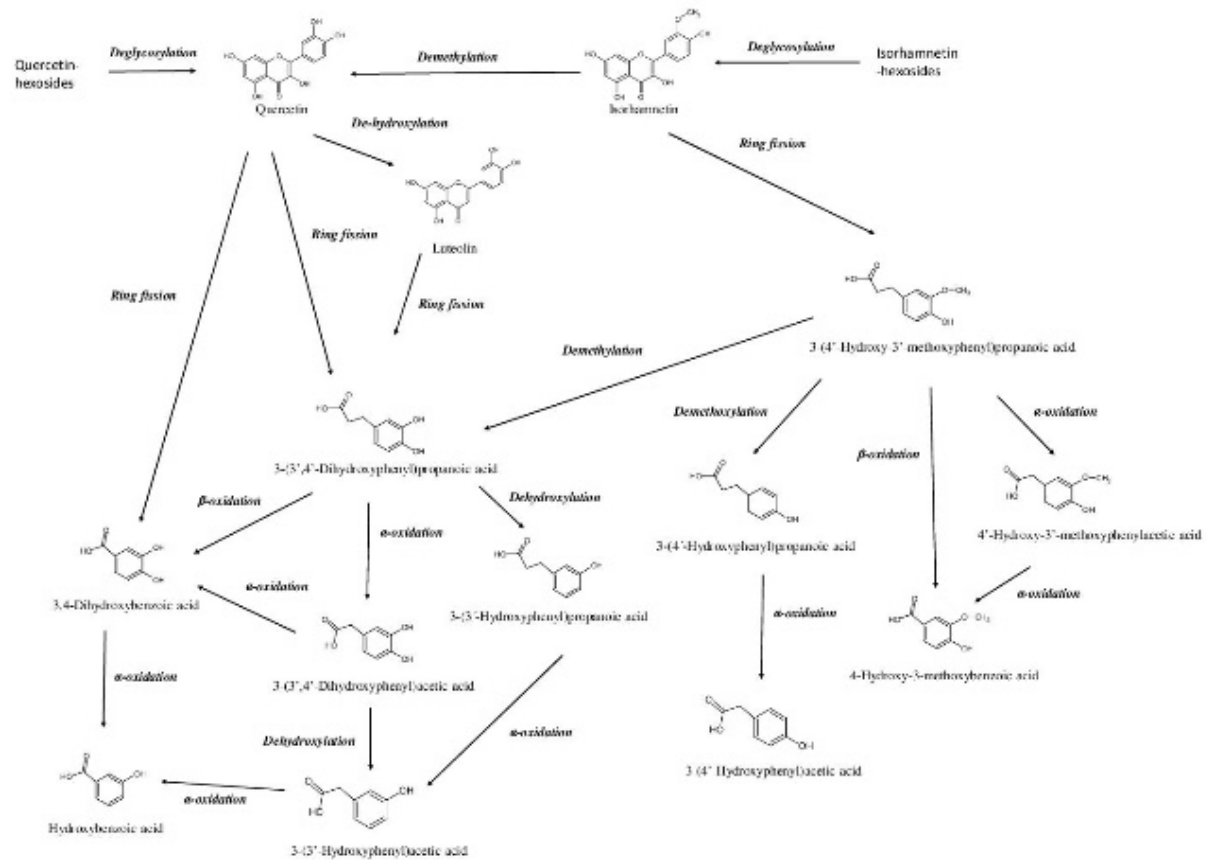


Figure 1. Proposed metabolic pathways for phenolic compounds microbial metabolism after *in vitro* gastro-intestinal digestion and colonic fermentation of red-skinned onion.

The major metabolic pathway for this intermediate was the dehydroxylation to 3-(3'-hydroxyphenyl)propanoic acid, which was the major metabolite found at any time in each RSO sample during fermentation[10]. Alternatively, 3-(3',4'-dihydroxyphenyl)propanoic acid may underwent to α-oxidation step producing 3-(3',4'-dihydroxyphenyl)acetic acid that can be further de-hydroxylated to 3-(3'-hydroxyphenyl)acetic acid or subjected to a second α-oxidation step releasing 3,4-dihydroxybenzoic acid[13]. Additional metabolic pathways involved direct β-oxidation of 3-(3',4'-dihydroxyphenyl)propanoic acid producing 3,4-dihydroxybenzoic acid[53]. All these metabolites were found in high amount in the fermented RSO samples suggesting that the described pathways were predominant during flavonols fermentation. Regarding isorhamnetin metabolism, the C-ring fission product 3-(4'-hydroxy-3'-methoxyphenyl)propanoic acid may underwent to β-oxidation or two steps of α-oxidation to produce 4-hydroxy-3-methoxybenzoic acid. Otherwise, 3-(4'-hydroxy-3'-methoxyphenyl)propanoic acid may be demethoxylated producing 3-(4'-hydroxy-phenyl)propanoic acid that can subsequently be subject to α-oxidation generating 3-(4'-hydroxyphenyl)acetic acid.

However, all these metabolites were found in low concentration in fermented samples suggesting a possible alternative metabolic pathway for 3-(4'-hydroxy-3'-methoxyphenyl)propanoic acid. Indeed, this last intermediate may be demethylated resulting in the formation of 3-(3',4'-dihydroxyphenyl)propanoic acid[10,54,55]. Phenolic metabolites generated by the action of gut microbiota are generally considered to be better absorbed and responsible for the beneficial effects following the consumption of phenolic compounds-rich food[15]. Several *in vitro* and *in vivo* studies demonstrated the biological potential of these metabolites. For example, 3-(3'-hydroxyphenyl)propanoic acid displayed *in vitro* anti-inflammatory and anti-diabetic activities[55-57]. Furthermore, 3-(3'-hydroxyphenyl)propanoic acid and 3-(3'-hydroxyphenyl)acetic acid exhibited blood pressure-reducing effect *ex vivo* and *in vivo*[58,59]. Moreover, 3-(3',4'-dihydroxyphenyl)acetic acid was reported to inhibit colon cancer cell proliferation more efficiently than 3-(3'-hydroxyphenyl)propanoic acid and 3-(3'-hydroxyphenyl)acetic acid[60]. Finally, all the three metabolites showed neuroprotective activity in vitro[61].

3.3. Shift of total bacteria and microbiota eubiosis

qPCR absolute quantifications were targeted to 14 different bacterial taxa (**Table S1**) related to the core microbiota, including total *Eubacteria* and the two main phyla of the human healthy colon, i.e., *Bacteroidetes* and *Firmicutes*. Considering the total Eubacteria, in respect to the abundances at the baseline (BL) and apart from the values of the blank control (BC), just RRSO either at T1 and at the EP was significantly overrepresented up to 1.43 folds more at the EP ($p < 0.05$) (**Table 5**). Oppositely, both the samples of cooked onions, independently from the time points of fermentation, were underrepresented, in particular, GRSO had slight not significant reduction ($p > 0.05$), but FRSO had a 1.68 folds significant reduction. These evident results indicated that the cooked onion samples, alike the BC, limited or were not able to support the growth of total colon Eubacteria. Quantifications of *Bacteroidetes* phylum showed significant changes at each time point for any samples tested, including the BC, in respect to the BL ($p < 0.05$) (**Table 5**). In particular, fermentation of RRSO generated the lowest underrepresentation of Bacteroidetes, up to 1.54-fold less at the EP, and FRSO generated the highest underrepresentation, up to 6.44-fold less at the EP. In details, FRSO brought a reduction of 4.18 times higher than that brought by RRSO. The relative low loads of Bacteroidetes after FRSO fermentation could indicate that this cooking process can particularly reduce the availability of fiber for fermentation, asmany genera of this phylum are fibrolytic[62]. Considering *Firmicutes* (**Table 5**), overrepresentations were seen for each sample at both time points of fermentation, although almost all not significantly in respect to the BL ($p > 0.05$). The sole time sample and time point that scored a slight increase, but significant in abundance was RRSO at the EP with just 1.00 Log_2(FC). This latter increase was significantly higher than that of GRSO and FRSO at

the EP (p > 0.05), but not than the BC (p > 0.05). These outputs could indicate opposite scenarios depending on which group of *Firmicutes* was the most fostered; a positive outlook could be due to increase of *Lactobacillales*, while a negative could be due to increase of *Clostridiaceae*.

Table 5. Absolute quantifications and changes in the abundances of bacterial taxa of the core-microbiota of colonic fermentation of red-skinned onion, by qPCR.

Target & Sample	Cells/mL		Log_2(F/C)		MANOVA*	
	BL raw values**	BL ± SD*§	T1	EP	p (Time)	-Log_{10}(p)
			Eubacteria			
RRSO	5.13E+08	7.42E+08 ± 2.08E+08^{b}	0.47^{abA}	1.43^{aA}	0.001469	2.833075
GRSO	6.30E+08	7.42E+08 ± 2.08E+08^{a}	-0.36^{abB}	-0.81^{bB}	0.000122	3.930721
FRSO	8.59E+08	7.42E+08 ± 2.08E+08^{a}	-0.55^{abB}	-1.68^{bB}	0.000970	3.013253
BC	9.67E+08	7.42E+08 ± 2.08E+08^{a}	-0.71^{aB}	-4.21^{bC}	0.002812	2.551055
p (Food matrix)	0.726777		0.002947	0.000004		
			Firmicutes			
RRSO	4.80E+07	5.25E+07 ± 6.86E+06^{b}	0.66^{abA}	1.00^{aA}	0.029213	1.534484
GRSO	6.20E+07	5.25E+07 ± 6.86E+06	0.27^{B}	0.28^{B}	0.272088	0.565290
FRSO	4.69E+07	5.25E+07 ± 6.86E+06^{b}	0.39^{aAB}	0.27^{aB}	0.000182	3.750942
BC	5.32E+07	5.25E+07 ± 6.86E+06^{b}	0.44^{abA}	0.76^{aAB}	0.001091	2.962952
p (Food matrix)	0.589376		0.000126	0.000002		
			Bacteroidetes			
RRSO	2.37E+08	1.74E+08 ± 6.44E+07^{a}	-1.36^{bA}	-1.54^{bA}	0.017556	1.755573
GRSO	2.21E+08	1.74E+08 ± 6.44E+07^{a}	-6.05^{bC}	-5.94^{bB}	0.002734	2.563179
FRSO	1.05E+08	1.74E+08 ± 6.44E+07^{a}	-6.30^{bC}	-6.44^{bB}	0.002727	2.564311
BC	1.35E+08	1.74E+08 ± 6.44E+07^{a}	-3.46^{bB}	-4.16^{bB}	0.003134	2.503885
p (Food matrix)	0.972251		0.000018	0.000003		

*Multivariate ANOVA model with category "time" on column and category "food matrix" on rows (p < 0.05). **BL raw values are the means of sextuplicates from each sample at the beginning of its colonic fermentations; *§BL ± SD is BL mean ± standard deviation and is obtained from BL raw values. ^{A,B,C}Upperscore letter are for statistical difference within "food matrix" and ^{a,b,c}lowerscore letter are for statistical difference within "time ", according to Duncan's Multiple Range post hoc test (p < 0.05); -Log10(p) = Significance of Log_2(F/C); BL = baseline (3.48 h); T1 = intermediate time point (21.48 h); EP = endpoint (27.48 h); RRSO = raw red skinned onion; GRSO = grilled red skinned onion; FRSO = fried red skinned onion; BC = blank control.

As a general parameter for microbiota eubiosis, we chose the *Firmicutes/Bacteroidetes* (F/B) ratio (**Table 6**). Within this ratio, a value over two is usually linked to microbiota dysbiosis[8,10,63-66]. The fecal samples at the baseline had a F/B of 0.30 and this eubiosis condition was strongly maintained after 24 h just by RRSO (0.88), while the cooked onion samples failed. GRSO had a rise in the ratio up to 1.90, and FRSO quite overcame significantly in respect to the BL the dysbiontic value of 2.00 (p < 0.05). These results indicate that during the 24 h of fermentation just the raw onion samples did not perturb the colon core microbiota of healthy donors and were able to provide a substrate nutritionally adapted. Oppositely, fermentations of FRSO triggered a dysbiosis condition that mirrored the lower Bacteroidetes content.

Table 6. *Firmicutes* to *Bacteroidetes* ratio (F/B) of colonic fermentation of red-skinned onion, by qPCR.

Target & Sample	*Firmicutes* to *Bacteroidetes* ratio (F/B)			MANOVA*
	BL ± SD**	T1	EP	*p* (Time)
RRSO	0.30 ± 0.11^{b}	0.51± 0.45^{ab}	0.88 ± 0.77^{aC}	0.002296
GRSO	0.30 ± 0.11	1.64 ± 0.25	1.90 ± 0.69^{B}	0.230897
FRSO	0.30 ± 0.11^{b}	1.96 ± 0.90^{a}	2.05 ± 2.20^{aB}	0.004958
BC	0.30 ± 0.11^{c}	1.46 ± 0.15^{b}	4.08 ± 0.45^{aA}	0.016104
p (Food matrix)	0.124932	0.328928	0.020129	

*Multivariate ANOVA model with category "time" on column and category "food matrix" on rows ($p < 0.05$); **BL ± SD is BL mean ± standard deviation and is obtained from BL raw values; ^{A,B,C}Upperscore letter are for statistical difference within "food matrix" and ^{a,b,c}lowerscore letter are for statistical difference within "time", according to Duncan's Multiple Range post hoc test ($p < 0.05$). BL = baseline (3.48 h); T1 = intermediate time point (21.48 h); EP = endpoint (27.48 h); RRSO = raw red skinned onion; GRSO = grilled red skinned onion; FRSO = fried red skinned onion; BC = blank control.

3.4. Shift in selected beneficial bacterial taxa.

To give evidence to the shifts of colon core microbiota, we quantify the abundance of some bacterial taxa selected as renown to be active responders to fiber substrate fermentation and modulators of host wellbeing (**Table 7**). A first set was related to beneficial microbes, while a second set was related to opportunistic and dysbiontic microbes. *Lactobacillales* and *Bifidobacteriaceae* with the exceptions of few species are related to the host's wellbeing and eubiosis of the colon microbiota[61]. *Lactobacillales* includes also *Lactobacillaceae* and *Enterococcaceae*. These three families are implicated in the biotransformation of phenolic compounds; for example, *Lactobacillaceae* are able to increase epicatechin as well as gallic, caffeic, and ferulic acids content[68]; *Enterococcaceae* are able to promote *O*-deglycosylation and *C*-ring fission of several flavonols[17], whereas *Bifidobacteriaceae* are able to metabolize homovanillic acids[69] and hydrolyze chlorogenic acids[70]. Although, in our experiments, just the class of *Lactobacillales* was fostered by any onions fermentations but targets such *Bifidobacteriaceae* and also specific *Bifidobacterium longum* underwent a large depletion (**Table 7**). In details, the *Lactobacillales* were overrepresented in any sample, and still the raw onions almost doubled in the abundance than both the cooked onions. Thus, all onion substrates once fermented by the colon microbiota were able to increase the abundance of *Lactobacillales* significantly or not in respect to the BL and the BC. Similarly, on a long-term fermentation of phenolic compounds on gut models, other authors found that *Enterococcaceae* was the most fostered family[34,71].

Although from our results, *Bifidobacteriaceae* and the *B. longum* taxa was not fostered by any onion substrates. In particular, the fact that the significant reduction in respect to the BL were achieved just on onion substrates and not in the BC could mean that the high flavonols content of the samples had somehow inhibited the sensitive taxon of *Bifidobacteriaceae* (**Table 7**). It is in fact reported, that quercetin is inhibitory in respect to bifidobacteria[72], but also other studies in similar *in vitro* environments had reported fosterage of this vital group[50]. This scenario on beneficial core microbiota could mean that *Lactobacillales* are more specific in metabolizing onion flavonols compounds than

Bifidobacteriaceae, as well as this latter group is more sensible to some detrimental compounds generated by pyrolysis of cooked foods. It is reported that *Bifidobacteriaceae* are somehow inhibited by a certain class of phenolic compounds, *e.g.,* that of black tea[71] rather than other, *e.g.* that of olive[8]. Additionally, it is known that *Lactobacillales* can resist to high content of phenolic compounds[73], because owns an enzymatic arsenal with a wide target range, able to de-esterification, hydrolysis, or conversion of phenolics to individual acids (gallic, quinic, caffeic, *p*-coumaric, ferulic, dihydrocaffeic, dihydroferulic acid, vinylcatechol, vinylguaiacol)[73-74]. Also, Date and colleagues[75] reported that onion is able to foster the growth of three probiotic lactobacilli after in vitro fermentation with fecal inoculum. Among other beneficial taxa, *Clostridium* group IV and *Faecalibacterium prausnitzii* are reported to be involved in biotransformation of phenolic compounds[76]. From our results (**Table 7**), *Clostridium* group IV increased significantly at the EP in any onions fermentations, but not in the BC. Although, its resident *F. prausnitzii* was fostered just by the raw onions and was limited by the cooked samples, even if less than twice than the BC.

The *Clostridium* group IV and its resident *F. prausnitzii* are important SCFA producers and its presence is associated to a healthy gut. A decrease in the number of these bacteria is observed in patients with inflammatory bowel disease[77]. Comparably to our results, these taxa are reported to increase after dietary clinical trial with phenolic compounds from red wine[78-79].

Lastly, *Akkermansia muciniphila* has been reported to be exalted from phenolic compounds presence, alike that some phenolic compounds are acting as prebiotics[16]. From our results (**Table 7**) this taxon was fostered both at T1 and at EP by RRSO and GRSO substrate but not significantly, while was limited at the EP by both FRSO and by BC, which in comparison a thrice strength by this latter. Another commensal group that is involved mainly in fiber degradation is the *Bacteroides-Prevotella* group, covered by the BPP (*Bacteroides–Prevotella–Porphyromonas*) target. Generally, these bacteria are sensible to both fibers[80] and phenolic compounds. For example, Tamargo and colleagues[34] found a significant increase of this group after phenolic compounds fermentation on a gut model. Although from our results, but in line with our findings about the superior phylum of *Bacteroidetes*, the BPP group was significantly underrepresented at the end of any fermentations, in a strength larger than the BC. Even in this case, we can argue that the low fiber content of onions had impacted on the growth of this group, which is highly fibrolytic[56] (**Table 7**). These results could be applied as indicators of health-related effects when RSO is consumed and could be translated to in vivo condition.

Table 7. Absolute quantifications and changes in the abundances of beneficial bacterial taxa of colonic fermentation of red-skinned onion, by qPCR.

Target & Sample	Cells/mL		Log_2(F/C)		MANOVA*	
	BL raw values **	BL ± SD*§	T1	EP	*p* (Time)	-Log_{10}(*p*)
Lactobacillales						
RRSO	7.70E+05	8.79E+05 ± 1.32E+05^{b}	1.05^{a}	1.90^{aA}	0.014742	1.831452
GRSO	1.05E+06	8.79E+05 ± 1.32E+05^{b}	0.46^{ab}	0.97^{aAB}	0.000859	3.065816
FRSO	7.81E+05	8.79E+05 ± 1.32E+05	0.38	1.03^{AB}	0.084676	1.072238
BC	9.16E+05	8.79E+05 ± 1.32E+05	-0.49	-0.65^{B}	0.924597	0.034047
p (Food matrix)	*0.709415*		0.369291	0.004476		
Bifidobacteriaceae						
RRSO	1.22E+05	5.08E+04 ± 5.24E+04^{a}	-5.17^{bC}	-5.06^{bC}	0.031567	1.500769
GRSO	5.77E+04	5.08E+04 ± 5.24E+04^{a}	-5.11^{bC}	-5.26^{bC}	0.012853	1.891000
FRSO	1.55E+04	5.08E+04 ± 5.24E+04^{a}	-2.40^{bB}	-3.27^{bB}	0.000041	4.388389
BC	7.85E+03	5.08E+04 ± 5.24E+04	0.19^{A}	0.28^{A}	0.777184	0.109475
p (Food matrix)	0.683959		0.000962	0.014112		
***Bacteroides-Prevotella-Porphyromonas* (BPP) group**						
RRSO	2.34E+07	2.09E+07 ± 3.41E+06^{a}	-0.34^{bA}	-0.98^{bA}	0.001932	2.714073
GRSO	2.39E+07	2.09E+07 ± 3.41E+06^{a}	-3.45^{bB}	-3.17^{bB}	0.000005	5.324022
FRSO	1.95E+07	2.09E+07 ± 3.41E+06^{a}	-2.58^{bB}	-3.69^{bB}	0.000008	5.081399
BC	1.67E+07	2.09E+07 ± 3.41E+06^{a}	-1.32^{bA}	-1.10^{bA}	0.015634	1.805925
p (Food matrix)	0.509081		0.000009	0.033771		
***Clostridium* IV (CLOS IV) group**						
RRSO	1.25E+07	1.79E+07 ± 6.42E+06	0.25^{B}	5.65^{A}	0.072745	1.138197
GRSO	2.72E+07	1.79E+07 ± 6.42E+06^{b}	1.76^{aA}	2.53^{aB}	0.012853	1.891000
FRSO	1.57E+07	1.79E+07 ± 6.42E+06^{b}	1.16^{bA}	2.99^{aB}	0.000511	3.291724
BC	1.61E+07	1.79E+07 ± 6.42E+06^{a}	-2.28^{bC}	-1.35^{bC}	0.021311	1.671394
p (Food matrix)	0.643268		0.008103	0.009921		
Bifidobacterium longum						
RRSO	1.76E+06	2.53E+06 ± 1.91E+06^{a}	-0.17^{Ab}	-0.69^{Bb}	0.014742	1.831452
GRSO	5.04E+06	2.53E+06 ± 1.91E+06^{b}	0.39^{abA}	0.71^{aA}	0.000859	3.065816
FRSO	5.25E+05	2.53E+06 ± 1.91E+06	0.04^{A}	0.39^{A}	0.084676	1.072238
BC	2.78E+06	2.53E+06 ± 1.91E+06	-2.59^{B}	-3.55^{C}	0.924597	0.034047
p (Food matrix)	0.036929		0.042285	0.004473		
Akkermansia muciniphila						
RRSO	6.04E+05	5.26E+05 ± 8.23E+04^{b}	0.51^{aA}	0.81^{aA}	0.000201	3.695973
GRSO	5.85E+05	5.26E+05 ± 8.23E+04^{b}	0.45^{abA}	0.95^{aA}	0.000181	3.743186
FRSO	4.80E+05	5.26E+05 ± 8.23E+04^{a}	0.06^{aA}	-0.53^{bAB}	0.018797	1.725910
BC	4.34E+05	5.26E+05 ± 8.23E+04^{a}	-4.47^{bB}	-3.88^{bB}	0.000004	5.370729
p (Food matrix)	0.645485		<0.000001	<0.000001		
Faecalibacterium prausnitzii						
RRSO	1.06E+07	2.22E+07 ± 7.79E+06	0.59^{A}	0.93^{A}	0.272796	0.564162
GRSO	2.52E+07	2.22E+07 ± 7.79E+06	-0.32^{B}	-1.60^{B}	0.098563	1.006284
FRSO	2.59E+07	2.22E+07 ± 7.79E+06^{a}	-1.45^{bBC}	-2.50^{bB}	0.019097	1.719023
BC	2.71E+07	2.22E+07 ± 7.79E+06^{a}	-1.82^{bC}	-4.74^{cC}	0.011480	1.940064
p (Food matrix)	0.300806		0.001150	0.007717		

* Multivariate ANOVA model with category "time" on column and category "food matrix" on rows ($p < 0.05$); **BL raw values are the means of sextuplicates from each sample at the beginning of its fermentations; *§BL ± SD is BL mean ± standard deviation and is obtained from BL raw values; ^{A,B,C}Upperscore letter are for statistical difference within "food matrix" and ^{a,b,c}lowerscore letter are for statistical difference within "time", according to Duncan's Multiple Range post hoc test ($p < 0.05$). -Log_{10}(*p*) = Significance of Log_2(F/C); BL = baseline (3.48 h); T1 = intermediate time point (21.48 h); EP = endpoint (27.48 h); RRSO = raw red skinned onion; GRSO = grilled red skinned onion; FRSO = fried red skinned onion; BC = blank control.

3.5. Shift in selected opportunistic bacterial taxa.

Considering the opportunistic bacterial taxa, we have targeted the *Enterobacteriaceae*, *Clostridium* group I, the *Atopobium–Collinsella–Eggerthella* (ATOP) group, and *Escherichia coli* (**Table 8**). Within these groups we have found the most important features on the modulation of gut microbiota by onion fermentations. Indeed, *Enterobacteriaceae* at both time points were inhibited just by RRSO, but the other samples were able to limit their growth more than what happened in the BC. This similar trend was also detected by the resident *Escherichia coli* taxon. *Clostridium* group I was reduced almost in any case at any time point, except for the BC, but significantly just for RRSO at the EP ($p < 0.05$). Lastly, considering the ATOP group, slight increases were observed just in the BC, while the onion samples were able to largely reduce ATOP group loads up to growth inhibition, due to a mean reduction of 4.53-fold less for any onion substrates in respect to the BL. Additionally, the RRSO and GRSO were better performer in the action than FRSO. This group engages in the biotransformation of phenolic compounds, because *Atopobium parvulum* for example possess tannases[75], while *Atopobium* spp. Is correlated to protocatechuic acid production after colonic fermentation of berry phenolic compounds[76]. Although, form our results this scenario is oppositely described, giving to onion samples a high capability in contrasting opportunistic bacteria, such as *Atopobium* and *Eggerthella*. This feature is of high relevance since indicates that the consumption of RSO can protect the intestinal tract from colonization by opportunistics and pathogens, limiting possible dysbiosis.

Table 8. Absolute quantifications and changes in the abundances of opportunistic bacterial taxa of colonic fermentation of red-skinned onion, by qPCR.

Targets & Sample	Cells/mL		Log_2(F/C)		MANOVA*	
	BL raw values**	BL ± SD*§	T1	EP	*p* (Time)	-Log_{10}(*p*)
Enterobacteriaceae						
RRSO	1.42E+06	1.44E+06 ± 2.98E+05	-0.69^{B}	-0.87^{B}	0.199488	0.700083
GRSO	1.81E+06	1.44E+06 ± 2.98E+05^{b}	0.90^{aAB}	0.72^{aB}	0.014941	1.825625
FRSO	1.08E+06	1.44E+06 ± 2.98E+05^{b}	1.13^{abAB}	1.84^{aAB}	0.000012	4.914587
BC	1.44E+06	1.44E+06 ± 2.98E+05^{b}	2.61^{aA}	3.62^{aA}	0.000011	4.956323
p (Food matrix)	0.977079		<0.000001	<0.000001		
***Clostridium* group I (CLOS I)**						
RRSO	8.91E+03	1.51E+04 ± 9.51E+03	0.11	-1.35	0.142556	0.700083
GRSO	5.19E+03	1.51E+04 ±9.51E+03	-0.12	-0.02	0.844734	1.825625
FRSO	2.17E+04	1.51E+04 ±9.51E+03^{a}	-0.10^{b}	-0.81^{b}	0.038646	4.914587
BC	2.46E+04	1.51E+04 ± 9.51E+03^{b}	0.16^{b}	1.09^{a}	0.033585	4.956323
p (Food matrix)	0.262495		0.084737	0.086195		
***Atopobium-Collinsella-Eggerthella* (ATOP) group**						
RRSO	1.22E+05	5.08E+04 ± 5.24E+04	-5.17^{B}	-5.06^{B}	0.072155	1.141731
GRSO	5.77E+04	5.08E+04 ± 5.24E+04	-5.11^{B}	-5.26^{B}	0.071840	1.143634
FRSO	1.55E+04	5.08E+04 ± 5.24E+04	-2.40^{B}	-3.27^{B}	0.079729	1.098386
BC	7.85E+03	5.08E+04 ± 5.24E+04	0.19^{A}	1.18^{A}	0.152951	0.815447
p (Food matrix)	0.046520		0.000043	<0.000001		
Escherichia coli						
RRSO	8.70E+02	5.25E+03 ± 5.37E+03	0.30^{B}	0.02^{C}	0.829838	0.081006
GRSO	4.43E+02	5.25E+03 ± 5.37E+03	0.55^{B}	1.61^{B}	0.279017	0.554369
FRSO	1.09E+04	5.25E+03 ± 5.37E+03	2.81^{AB}	2.22^{B}	0.093314	1.030053
BC	8.82E+03	5.25E+03 ± 5.37E+03^{b}	3.92^{aA}	4.57^{aA}	0.005090	2.293282
p (Food matrix)	0.040281		0.000067	0.001695		

*Multivariate ANOVA model with category "time" on column and category "food matrix" on rows ($p < 0.05$); **BL raw values are the means of sextuplicates from each sample at the beginning of its fermentations; *§BL ± SD is BL mean ± standard deviation and is obtained from BL raw values; ^{A,B,C}Upperscore letter are for statistical difference within "food matrix" and ^{a,b,c}lowerscore letter are for statistical difference within "time", according to Duncan's Multiple Range post hoc test ($p < 0.05$); -Log_{10}(p) = Significance of Log_2(F/C); BL = baseline (3.48 h); T1 = intermediate time point (21.48 h); EP = endpoint (27.48 h); RRSO = raw red skinned onion; GRSO = grilled red skinned onion; FRSO = fried red skinned onion; BC = blank control.

3.6. qPCR Prebiotic Index

From the results for qPI (**Table 9**), the fermented substrate with the best prebiotic activity at the EP of fermentation was RRSO, followed by GRSO and FRSO. Thus, FRSO had the lowest prebiotic index, except that of the negative control (BC), although there were no significant differences among RSO samples The different prebiotic index of samples could be attributed to degradation of the structural composition of the onion fibers after cooking or by transformation of onion flavanols with prebiotic capacity. Similarly to most know prebiotics (e.g., fructooligosaccharides), it is recognized that soluble fibers are excellent substrates for production of postbiotics (*e.g.* short chain fatty acids) in the large intestine[24]. Additionally, in line with the latest definition of prebiotics[83], there is evidence that some phenolics could foster the growth of beneficial bacteria, thus owning a prebiotic nature[16]. Anyhow, the prebiotic potential generated by any onion samples could be more ascribed to the reduction and limitation of opportunistic growth, than to the fosterage of beneficial bacteria. A previous study

documented the prebiotic potential of onion, able to foster the growth of three main beneficial lactobacilli in just 12 h of faecal in vitro fermentation Date et al., 2014[75].The relevance of our results is therefore the confirmation of onion prebiotic activity even after cooking and intestinal digestion.

Table 9. qPI (quantitative PCR Prebiotic Index) of colonic fermentation of red-skinned onion.

Sample	qPCR Prebiotic Index (qPI)*		MANOVA**
	T1	EP	*p* (Time)
RRSO	0.821 ± 0.01^{bAB}	0.932 ± 0.05^{aA}	<0.000001
GRSO	0.840 ± 0.07^{A}	0.857 ± 0.041^{AB}	0.431179
FRSO	0.829 ± 0.09^{A}	0.814 ± 0.011^{AB}	0.084396
BC	0.749 ± 0.014^{aB}	0.664 ± 0.291^{bB}	0.000047
p (Food matrix)	<0.000001	0.000002	

*Normalized means; ** Multivariate ANOVA model with category "time" on column and category "food matrix" on rows ($p < 0.05$); ^{A,B,C}Upperscore letter are for statistical difference within "food matrix" and ^{a,b,c}lowerscore letter are for statistical difference within "time", according to Duncan's Multiple Range post hoc test ($p < 0.05$); BL = baseline (3.48 h); T1 = intermediate time point (21.48 h); EP = endpoint (27.48 h); RRSO = raw red skinned onion; GRSO = grilled red skinned onion; FRSO = fried red skinned onion; BC = blank control.

3.7. Correlations among phenolics and microbial taxa

Spearman rank correlations ($p < 0.05$), two-joining-way heatmaps, and Pearson cluster analysis were performed by comparison of two different normalized datasets, each derived from values of absolute quantification (microbes and phenolic compounds) (**Figure 2**). From the left-side dendrograms, two main clusters were acknowledged. The two key clusters were then sorted in two different subgroups obtained by the dendrograms related to phenolics. In the resulting joint cluster 1A the most significant positive correlations were held by *Bacteroides* and associated BPP group, by *Firmicutes* and associated Clostridium group IV and associated *F. prausnitizii*, in respect to five different phenolics. All of these taxa shared significant correlations to two common variables, i.e., hydroxybenzoic acid isomer 1 and 3-(4'-hydroxy-3'-methoxyphenyl)propanoic acid. In cluster 1B, the same phenolics variables had negative correlations, but little was the overall significance. In particular, ATOP had significant negative correlations just towards 4-hydroxy-3-methoxybenzoic acid, *E. coli* towards hydroxybenzoic acid isomer 1 and 3-(4'-hydroxy-3'-methoxyphenyl)propanoic acid, and *Enterobacteriaceae* towards these former two and 3-(3',4'-dihydroxyphenyl)propanoic acid. Suggestions indicate that all these metabolites putatively produced by beneficial *F. prausnitzii* had a central role in containment of opportunistic taxa, as happened in RRSO samples. On this regard, our outputs are in line to recent result demonstrating the antibiofilm and antiproliferative action of hydroxybenzoic acids versus *E. coli* and other *Enterobacteriaceae*[84]. Also in scientific literature, it is reported that species from *Clostridium* group IV are able also to hydrolyze ester bonds, *C*-ring cleavage, and dehydroxylation of anthocyanins and proanthocyanidins releasing hydroxybenzoic acids[70].

Cluster 2 accounted for a higher number of significant negative correlations in respect to cluster 1. Indeed, subcluster 2A was defined by nine variables with significant negative correlations differently shared among seven bacterial variables. In particular, *Firmicutes*, ClosIV group and *F. prausnitzii* were negatively correlated to four common variables, namely: 3-(3',4'-dihydroxyphenyl)acetic acid, isorhamnetin, 3',4'-dihydroxycinnamic acid, and 3-(4'-hydroxypheyl)acetic acid. Oppositely, in subcluster 2B, these four former phenolics showed positive correlations in respect to the family *Enterobacteriaceae*, and two of these molecules (3-(3',4'-dihydroxyphenyl)acetic acid and 3',4'-dihydroxycinnamic acid) also in respect to *E. coli*. It is possible that 3',4'-dihydroxycinnamic acid and 3-(4'-hydroxypheyl)acetic acid produced or transformed by *Enterobacteriaceae* could be the culprit of the depletion of *A. muciniphila* during fermentation of FRSO samples. Even if in literature is generally appreciated the concept that most phenolics protect gut epithelial barrier function and foster *A. muciniphila* and other beneficial microbes[79,80], it is also reported that hydroxycinnamic compounds, e.g cinnamaldehyde, produce side effect on commensals of the gut microbiota[81].

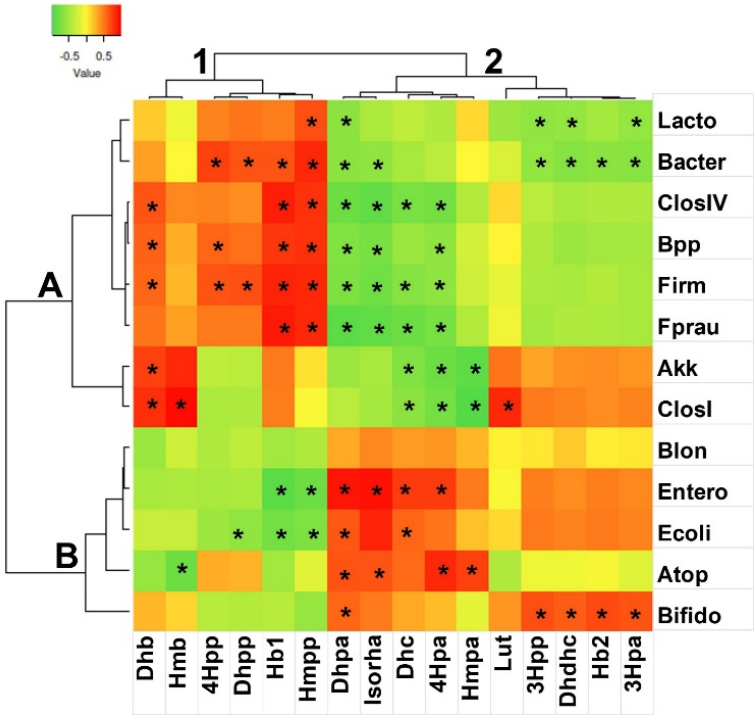


Figure 2. Two-way joining heatmap of Spearman-rank correlations. A,B,1,2**)** Cluster labels from Pearson dendrograms with centroid linkage. *) Significant correlations ($p > 0.05$). X axis labels: Hb1 = Hydroxybenzoic acid isomer 1; Hb2 = Hydroxybenzoic acid isomer 2; 3Hpa = 3'-Hydroxyphenyilacetic acid; 4Hpa = 4-Hydroxyphenylacetic acid; Dhb = 3,4-Dihydroxybenzoic acid; 3Hpp = 3-(3'-Hydroxyphenyl)propanoic acid; 4Hpp = 3-(4'-Hydroxyphenyl)propanoic acid; Hmb = 4-Hydroxy-3-methoxybenzoic acid; Dhpa = 3-(3',4'-Dihydroxyphenyl)acetic acid; Dhc = 3',4'-Dihydroxycinnamic acid; Dhdhc = Di-hydro-dihydroxycinnamic acid; Dhpp = 3-(3',4'-Dihydroxyphenyl)propanoic acid; Hmpa = 4'-Hydroxy-3'-methoxyphenylacetic acid; Hmpp = 3-(4'-Hydroxy-3'-methoxyphenyl)propanoic acid; Lut = Luteolin; Isorha = Isorhamnetin. Y label; Lacto = *Lactobacillales*; Bacter = *Bacteroidetes*; ClosIV = *Clostridium* IV group; BPP = *Bacteroides-Prevotella-Porphyromonas* group; Firm = *Firmicutes*; Fprau = *Faecalibacterium prausnitzii*; Akk = *Akkermansia muciniphila*; ClosI = *Clostridium* group I; Blon = *Bifidobacterium longum*; Entero = *Enterobacteriaceae*; Ecoli = *Escherichia coli*; ATOP = *Atopobium-Collinsella-Eggerthella* group; Bifido = *Bifidobacteriaceae*.

4. Conclusions

Heat treatments increased the extractability of red-skinned onion phenolic compounds and their release during in vitro gastro-intestinal digestion. Grilled and fried red-skinned onions presented the highest amount of phenolic compounds following in vitro gastro-intestinal digestion and colonic fermentation. Additionally, specific beneficial taxa of the colon microbiota used in these experiments were able to transform and modify the chemical structure of onion phenolic compounds employing specific metabolic pathways, and in turn gaining energy to multiply and foster in the colon niche. The degradation driven by colon microbiota of RSO flavonols resulted in the accumulation of three main metabolites, 3-(3'-hydroxyphenyl)propanoic acid, 3-(3'-hydroxyphenyl)acetic acid and 3-(3',4'-dihydroxyphenyl)acetic acid, with some qualitative and quantitative differences among the samples. Colonic fermentation of raw onions resulted in a substantial increase in beneficial taxa, larger in respect to the heat-treated onions, particularly on *Lactobacillales* and beneficial clostridia. Also, a higher level of inhibition of opportunistic bacteria was seen for raw onion samples, namely *Clostridium perfringens* group and *E. coli*. Eventually, the high flavonols content of onion raw samples, could be the responsible for such effect, also negatively seen for the depletion of *Bifidobacteriaceae*, that has been reported of being sensitive to quercetin, slowing metabolic pathways and cell wall synthesis[50].

Therefore, consuming raw onions would be preferable respect to consume the grilled or fried onions, in terms of amount of bioaccessible phenolic compounds and metabolites, which also have a prebiotic activity fostering the growth of beneficial colonic bacteria and containing the growth of opportunistic ones. Also, the cooked onions failed to maintain an eubiotic condition in the colon, triggering the raise in the F/B ratio to the level of a dysbiotic situation. Results reported in the present study showed that red-skinned onion, and especially the raw one, is an excellent dietary source of flavonols that are strongly metabolized by gut bacteria and able to modulate the gut microbiota promoting the growth of bacterial groups associated with a healthy status. However, more *in vivo* studies are necessary to validate the observed *in vitro* effect.

5. Supplementary materials

Table S1. Primers pairs employed for PCR and qPCR reactions and quantifications.

Bacterial taxa	Target	Sequence 3'-5'	Bp*	MT** (°C)	Reference
Eubacteria	*V3-V4*	Eub518-R: ATTACCGCGGCTGCTGG	147	57.6	82
	16S	Eub338-F: ACTCCTACGGGAGGCAG		63.5	
Firmicutes	*V3-V4*	Firm-F: GGAGYATGTGGTTTAATT	300	60.5	83
	16S	Firm-R: ACTCCTACGGGAGGCAG		63.5	
Bacteroidetes	*V3-V4*	Bact-F: GGARCATGTGGTTTAATT	250	58.9	83
	16S	Bact-R: AGCTGACGACAACCATG		59.4	
Lactobacillales	*V3-V4*	F-Lac: GCAGCAGTAGGGAATCT	340	59.8	84
	16S	R-Lac: GCATTYCACCGCTACACA		58.3	
Bifidobacteriaceae	*RecA*	RecA-F: CGTYTCBCAGCCGGAYA	220	60.3	85
		RecA-R: CCARVGCRCCGGTCATC		59.2	
Enterobacteriaceae	*V3-V4*	Enterb-F: TGCCGTAACTTCGGGAG	450	64.2	86
	16S	Enterb-R: TCAAGGACCAGTGTTCAG		60.3	
Clostridium group I	*V3-V4*	ClosI-F: TACCHRAGGAGGAAGCCAC	148	54.6	86
	16S	ClosI-R: GTTCTTCCTAATCTCTACGCAT		53.0	
Clostridium group IV	*V3-V4*	ClosIV-F: TTAACACAATAAGTWATC	400	58.1	87
	16S	ClosIV-R: ACCTTCCTCCGTTTTGTC		57.9	
ATOP group	*V3-V4*	ATOP-F: GGGTTGAGAGACCGACC	190	57.6	88
	16S	ATOP-R: CGGRGCTTCTTCTGCAG		59.4	
BPP group	*V3-V4*	BPP-F: GAGAGGAAGGTCCCCCA	140	60.5	89
	16S	BPP-R: CGCKACTTGGCTGGTTCA		59.9	
Escherichia coli	*FtsZ*	EcFtsZ-F: GGTATCCTGACCGTTGCT	250	59.4	90
		EcFtsZ-R: ATACCTCGGCCCAGAACT		57.3	
Akkermansia	*V3-V4*	AkM1: CAGCACGTGAAGGTGGG	327	63.5	91
muciniphila	*16S*	AkM2: CCTTGCGGTTGGCTTCA		59.4	
Bifidobacterium	*V3-V4*	Blon-F: GATTCTGGCTCAGGATGA	220	62.1	92
longum	*16S*	Blon-R: CTGATAGGACGCGACCC		61.4	
Faecalibacterium	*V3-V4*	Fprau223-F: GATGGCCTCGCGTCCGA	199	63.7	93
prausnitzii	*16S*	Fprau420-R: CCGAAGACCTTCTTCCTC		58.8	

*Base pairs, **Melting Temperature.

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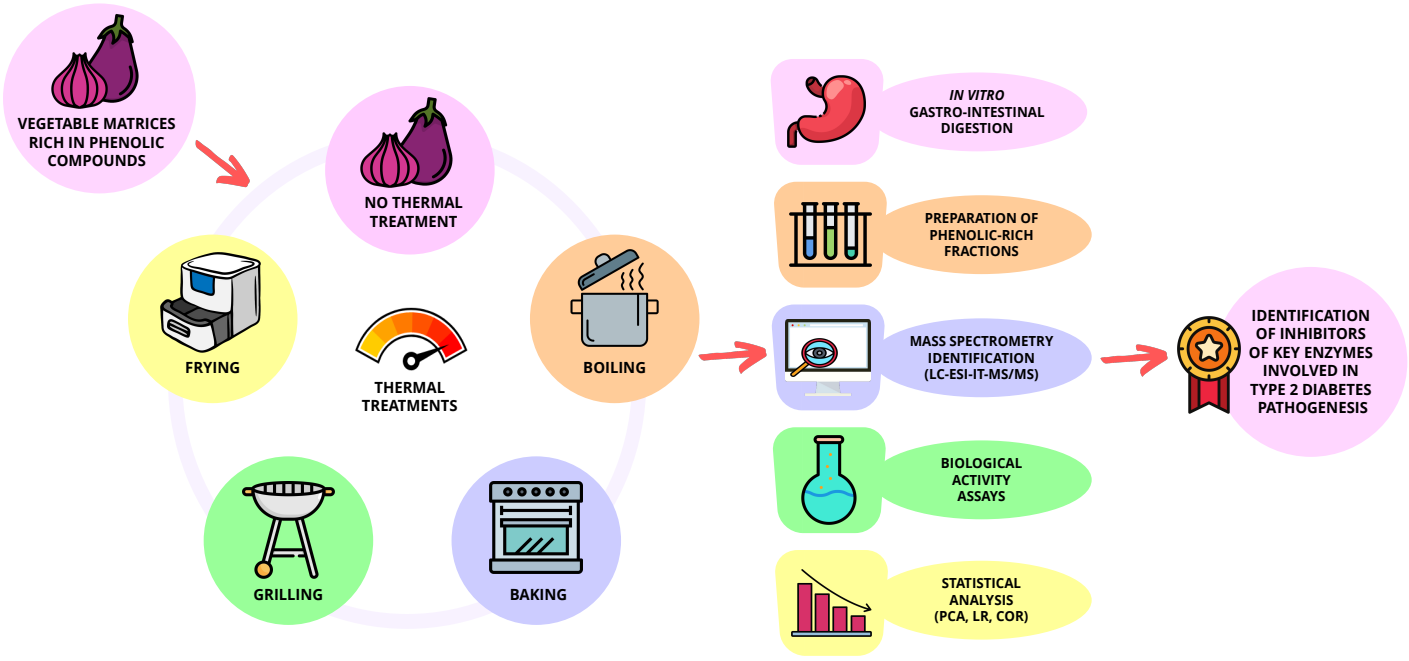
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COOKING AND IN VITRO DIGESTION MODULATE THE ANTI-DIABETIC PROPERTIES OF RED-SKINNED ONION AND DARK PURPLE EGGPLANT PHENOLIC COMPOUNDS

ABSTRACT

The intake of phenolic-rich foods is an emerging preventive approach for the management of type 2 diabetes, thanks to the ability of these compounds to inhibit some key metabolic enzymes. In this study, the influence of cooking and in vitro digestion on the α-glucosidase, α-amylase, and dipeptidyl-peptidase IV (DPP-IV) inhibitory activity of red-skinned onion (RSO) and dark purple eggplant (DPE) phenolic fractions was assessed. The applied cooking procedures had different influences on the total and individual phenolic compounds gastrointestinal bioaccessibility. DPE in vitro digested phenolic fractions displayed no inhibitory activity versus α-amylase and DPP-IV, whereas the fried DPE sample exhibited moderate inhibitory activity against α-glucosidase. This sample mainly contained hydroxycinnamic acid amides that can be responsible for the observed effect. Contrariwise, raw and cooked in vitro digested RSO phenolic fractions inhibited all three enzymes but with different effectiveness. Fried and raw RSO samples were the most active against them. Statistical analysis pointed out that quercetin mono-hexosides (mainly quercetin-4′-*O*-hexoside) were responsible for the inhibition of α-glucosidase, whereas quercetin di-hexosides (mainly quercetin-3-*O*-hexoside-4′-*O*-hexoside) were responsible for the DPP-IV-inhibitory activity of RSO samples. An accurate design of the cooking methods could be essential to maximize the release of individual phenolic compounds and the related bioactivities.

Keywords: type 2 diabetes; polyphenols; food processing; mass spectrometry; in vitro digestion; thermal treatments

1. Introduction

Type 2 diabetes (T2D) is a primary reason of death and was estimated to have caused around 4 million deaths worldwide in 2017[1]. Both T2D morbidity and mortality are increasing from year to year, affecting dramatically the health-care systems and quality of life[2-4]. This metabolic condition, characterized by elevated levels of blood glucose, can lead to development of chronic diseases affecting the cardiovascular, renal, and nervous systems[5-8]. Nowadays, the management of T2D aims to reduce blood glucose levels through both pharmacological and non-pharmacological (healthy lifestyle and balanced diet) treatments. To overcome the high costs and side effects of drug therapies, dietary supplementation with natural anti-diabetic agents is becoming increasingly crucial[9-12]. A plethora of researches have indicated that the intake of functional foods could help to prevent and moderate metabolic syndrome and T2D[13,14].

Phenolic compounds are secondary metabolites of plant origin that exert several beneficial effects on human health. They may act as natural antioxidants with anti-aging properties, are also able to decrease the risk of the onset of cardiovascular diseases and cancers, and also play a significant role against diabetes[13,15]. Phenolic compounds may exert their anti-diabetic activity, acting at different levels[15,16]. For example, polyphenols can reduce the absorption of dietary carbohydrates, ameliorate the function of β-cells, stimulate insulin secretion, and induce satiety[17]. They have shown to decrease post-prandial hyperglycemia by inhibiting the activity of amylolytic enzymes and preventing the hydrolysis of incretins through the inhibition of dipeptidyl-peptidase IV[18-20].

The enzymes α-amylase and α-glucosidase are involved in the gastrointestinal digestion of dietary carbohydrates to free monosaccharides. The enzyme α-amylase is located in the intestinal fluid and is able to cleave polysaccharides (such as starch and glycogen), releasing shorter oligosaccharides (dextrins, maltotriose, and maltose). In contrast, α-glucosidase is a digestive enzyme situated at the intestinal brush border that completes the hydrolysis of short oligosaccharides, releasing free monosaccharides, which are in turn absorbed by intestinal enterocytes. Various studies have demonstrated that α-amylase and α-glucosidase inhibitors from traditionally used plants can retard the digestion and absorption of complex polysaccharides, thus diminishing the post-prandial blood glucose level[21]. Oboh et al.[11] demonstrated the anti-diabetic effect of phenolic-rich aqueous extracts of purple and white onion and garlic. Similarly, Paoutsis et al.[22] reported high inhibitory effects of citrus, pomegranate, berries, and *Prunus* phenolic-rich extracts on α-amylase and α-glucosidase. Kwon et al.[10] indicated the positive effect of phenolic-rich extracts from two types of eggplant on hyperglycemia risk factors.

Dipeptidyl-peptidase IV (DPP-IV) is a serine protease placed in the brush-border membrane of intestinal enterocytes and responsible for the rapid inactivation of incretins hormones such as glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP)[18] . Incretins are able to lower the hematic levels of glucose by stimulating the release of insulin and inhibiting the release of glucagon[23]. As a consequence, DPP-IV inhibition may lead to a reduction of hyperglycemia by improving insulin secretion[24].

Although there are no in vivo studies, several in vitro researchers highlighted the role of phenolic compounds as DPP-IV inhibitors[20,25].

Most of the studies about the inhibitory effect of phenolic compounds on key enzymes involved in the regulation of blood glucose levels have been carried out on pure food extracts[10,11,18,19,22]. However, this is far from the real conditions of food consumption and phenolic compounds release in the gastrointestinal tract. Indeed, some vegetable foods, such as eggplant and onion, are mainly consumed after cooking. Several previous studies have suggested that cooking treatments play a significant role in modulating the phenolic compounds content of foods[26-28]. The effects of thermal processes on the stability of phenolic compounds are manifold. Some compounds may be degraded by high temperatures (e.g., anthocyanins) or released in the cooking medium (e.g., flavonols during boiling), while others may increase their extractability through the matrix softening effect[29,30]. This depends on several factors such as the food matrix, the phenolic structure, and technological parameters: among others, cooking time, temperature, and medium[30].

Another aspect to be considered is that phenolic compounds, to exert their anti-diabetic effects by inhibiting intestinal enzymes, should be bioaccessible, i.e., they should be released from the food material and stable over the gastrointestinal transit[26]. Testing the anti-diabetic properties of digested samples is pivotal, since solely the phenolic compounds released during digestion may interact with the enzymes present in the intestinal tract. To the best of our knowledge, no studies have been published on the anti-diabetic properties of vegetable food after cooking and in vitro gastrointestinal digestion.

Therefore, this study was designed to explore the in vitro anti-diabetic properties (i.e., inhibitory activity toward three key metabolic enzymes, DPP-IV, α-glucosidase, and α-amylase) of two vegetable matrices rich in phenolic compounds, dark purple eggplant (DPE) and red-skinned onion (RSO), subjected first to four different cooking processes (baking, boiling, frying, and grilling) and then to in vitro gastrointestinal digestion.

2. Materials and Methods

2.1. Materials

Chemicals and enzymes for in vitro digestion, α-glucosidase, α-amylase, and DPP-IV assays were obtained from Sigma-Aldrich (Milan, Italy). Solvents for phenolic extraction and MS/MS were supplied by Bio-Rad (Hercules, CA, USA). C18 columns for solid-phase extraction (500 mg, 50 µm, 60 Å) were purchased from Incofar (Modena, Italy). Dark purple eggplant and red-skinned onion samples were purchased in a local supermarket.

2.2. Cooking Treatments and In Vitro Gastrointestinal Digestion

Dark purple eggplant and red-skinned onion samples were subjected to four different types of cooking: boiling, baking, frying, and grilling, as described in Martini et al.[27] and Cattivelli et al.[26]. Each cooking procedure was carried out in triplicate. For the in vitro gastrointestinal digestion procedure, the method elaborated in the framework of the COST Action INFOGEST was applied[31]. As reported in Martini et al.[27] and Cattivelli et al.[26], raw and cooked dark purple eggplant and red-skinned onion samples were subjected to three consecutive passages: oral phase, gastric phase, and intestinal phase. At the end of the intestinal phase, an aliquot of sample was withdrawn and stored at −80 °C for further analysis. All the digestions were carried out in triplicate.

2.3. Preparation of the Phenolic-Rich Fractions

For the phenolic compounds extraction, C18 columns were pre-conditioned by passing 4 mL of methanol (acidified with 0.1% formic acid) and subsequently 5 mL of water (acidified with 0.1% formic acid). After column conditioning, 2 mL of in vitro digested samples were loaded at the top the column. The first elution step was performed with 3 mL of water (acidified with 0.1% formic acid) in order to remove the unbound material. Subsequently, phenolic compounds were detached from the column by eluting with 3 mL of acidified methanol (0.1% formic acid). Each sample was extracted in triplicate. After methanol removal, samples were re-suspended in distilled water and stored at −80 °C for further analysis.

2.4. Characterization of the Phenolics Profile of Phenolic-Rich Fractions by Liquid Chromatography Electrospray Ionization Ion Trap Mass Spectrometer (LC-ESI-IT-MS)

Individual phenolic compounds present in the different phenolic-rich fractions were identified and quantified through liquid chromatography-mass spectrometry analysis. The instrumental characteristics, the liquid chromatography parameters, and the ESI-MS (both positive and negative) conditions were the same as previously detailed[32,33]. Specific standard compounds were used for phenolic compounds quantification as previously reported[26,27,32,33].

2.5. Biological Activity Analysis

2.5.1. Measurements of α-Amylase Inhibitory Activity

The inhibitory activity of phenolic-rich fractions against α-amylase was established as described by Li et al.[34] with slight modifications. Briefly, 5 µL of α-amylase from porcine pancreas (2 U/mL) were combined with 45 µL of different concentrations of phenolic-rich fractions (5–200 µmol/L). After 20 min of incubation at 37 °C, 50 µL of 1% starch solution, prepared in 0.1 mol/L sodium phosphate buffer (pH 6.9 and containing 6.7 mmol/L of NaCl), were added and the reaction mix was further incubated for 10 min at 37 °C. After incubation, 50 µL of dinitrosalicylic acid solution were added to the mix, and samples were boiled for 15 min in water. Before reading, 450 µL of distilled water were added to the mixture, and 200 µL of each experiment were transferred to a 96-well plate and read at 540 nm using a microplate reader. The concentration of phenolic compounds required to inhibit α-amylase activity by 50% (IC_{50}) was calculated by correlating the percentage of α-amylase inhibition with the phenolic compounds concentration (base-10 logarithm). The concentration of phenolic compounds was calculated by LC-ESI-IT MS/MS analysis. Values were expressed as µmol of phenolic compounds per mL. Analyses were carried out in triplicate.

2.5.2. Measurements of α-Glucosidase Inhibitory Activity

The α-glucosidase assay was carried out as stated in Bellesia et al.[35] adapted to a microplate reader. For the inhibition assay, 66.7 µL of 0.1 mol/L potassium phosphate buffer (pH 6.8), 20 µL of different concentrations of phenolic-rich fractions (5–200 µmol/L), 3.3 µL of reduced glutathione (3.3 mmol/L), and 5 µL of yeast α-glucosidase solution (0.2 U/mL) were mixed in a 96-well plate and pre-incubated for 20 min at 37 °C. Then, 5 µL of 5 mmol/L *p*-nitrophenyl-glucose substrate (diluted in potassium phosphate buffer 0.1 mol/L, pH 6.8) were added to start the reaction, and the plate was further incubated (20 min; 37 °C). Finally, the reaction was stopped by adding 150 µL of 1 mol/L sodium carbonate. Then, the plate was read at 405 nm. The concentration of phenolic compounds required to inhibit α-glucosidase activity by 50% (IC_{50}) was calculated as reported in Section 2.5.1. Values were expressed as µmol of phenolic compounds per mL. As reported previously, three analytical replicates for each sample were performed.

2.5.3. Measurements of Dipeptidyl Peptidase IV (DPP-IV) Inhibitory Activity

The DPP-IV enzyme was extracted from rat intestinal acetone powder as reported in the protocol of Tagliazucchi et al.[36]. For the inhibition assay, 80 µL of different concentrations of phenolic-rich fractions (5–200 µmol/L), 205 µL of Tris-HCl buffer (0.1 mol/L; pH 8), and 10 µL of extracted enzyme (0.1 U/mL in the assay) were added to a 96-well plate and pre-incubated for 20 min at 37 °C. Then, the reaction was started by the addition of 5 µL of 6.4 mmol/L Gly-Pro-*p*NA. The quantity of released *p*-nitroanilide (*p*NA) was determined following 20 min of reaction at 37 °C by reading at 405 nm with a microplate reader. As reported above, the IC_{50} values were determined as reported in Section 2.5.1. Values were expressed as µmol of phenolic compounds per mL. For the enzymatic assay, three analytical replicates for each sample were carried out.

2.6. Statistics

Data are shown as mean ± standard deviation for three replicates for each sample. Univariate analysis of variance (ANOVA) coupled to Tukey's post hoc test was executed using Graph Pad prism 6.0 (GraphPad Software, San Diego, CA, USA.). The differences were evaluated significant when $p < 0.05$. Principal component analysis (PCA) was performed using Solo software (Eigenvector Research Inc., Manson, WA, USA), and data are presented as biplots. Before performing PCA, the mean data were normalized to neutralize the influence of any hidden factors. Correlation and non-linear regression analysis were carried out with Graph Pad Prism. The correlation, expressed as Pearson *r*-value, was considered significant when $p < 0.05$.

3. Results and Discussion

3.1. Phenolic Compounds Profile of Phenolic-Rich Fractions Extracted from Raw and Cooked In Vitro Digested Dark Purple Eggplant (DPE)

A total of 12, 15, 9, 14, and 20 individual phenolic compounds were identified and quantified in the phenolic-rich fractions of in vitro digested raw, baked, boiled, fried, and grilled DPE, respectively. The list of detected compounds, together with the quantitative information, is given in **Table 1**. From a quantitative point of view, in in vitro digested raw DPE phenolic-rich fraction, hydroxycinnamic acids made up the majority of the compounds present (96.7% of total phenolic compounds), while flavonols represented the remaining 3.3%. The most abundant compounds in the in vitro digested raw DPE phenolic-rich fraction were 5-*O*-caffeoylquinic acid *trans* and the oxidation products caffeoylquinic acid dehydrodymer isomers. The total amount of bioaccessible phenolic compounds quantified by mass spectrometry in in vitro digested raw DPE phenolic-rich fraction was 10.74 ± 0.25 µmol/100g.

Table 1. Concentration of phenolic compounds in in vitro digested raw and cooked dark purple eggplant phenolic-rich fractions. Results are reported as µmol of phenolic compounds/100 g of raw or cooked eggplant.

In Vitro Digested Dark Purple Eggplant Phenolic-Rich Fractions					
Compound	**Raw**	**Baked**	**Boiled**	**Fried**	**Grilled**
Hydroxycinnamic Acids					
Caffeoylshikimic acid	n.d.	n.d.	n.d.	n.d.	1.01 ± 0.08
3-*O*-Caffeoylquinic acid	0.31 ± 0.01 ^{c}	8.44 ± 0.03 ^{a}	6.03 ± 0.03 ^{b}	5.26 ± 0.03 ^{b}	8.12 ± 0.97 ^{a}
4-*O*-Caffeoylquinic acid	n.d.	3.10 ± 0.11 ^{b}	2.47 ± 0.03 ^{c}	3.01 ± 0.03 ^{b}	3.45 ± 0.04 ^{a}
5-*O*-Caffeoylquinic acid *trans*	2.86 ± 0.08 ^{d}	30.29 ± 0.50 ^{b}	19.25 ± 0.89 ^{c}	32.26 ± 0.56 ^{a}	33.17 ± 0.99 ^{a}
5-*O*-Caffeoylquinic acid *cis*	0.34 ± 0.00 ^{e}	10.13 ± 0.13 ^{c}	3.25 ± 0.12 ^{d}	12.01 ± 0.04 ^{b}	14.03 ± 0.58 ^{a}
3-*O*-Feruloylquinic acid *cis*	n.d.	0.53 ± 0.00 ^{a}	n.d.	n.d.	0.12 ± 0.00 ^{b}
3-*O*-Feruloylquinic acid *trans*	n.d.	1.49 ± 0.03 ^{b}	n.d.	n.d.	4.09 ± 0.10 ^{a}
5-*O*-Feruloylquinic acid *trans*	n.d.	2.08 ± 0.05 ^{b}	n.d.	1.22 ± 0.01 ^{b}	19.96 ± 1.13 ^{a}
4-*O*-Feruloylquinic acid	0.26 ± 0.00 ^{b}	0.68 ± 0.02 ^{a}	n.d.	n.d.	n.d.
5-*O*-Feruloylquinic acid *cis*	0.63 ± 0.00 ^{d}	5.66 ± 0.09 ^{b}	n.d.	18.36 ± 0.70 ^{a}	2.12 ± 0.03 ^{c}
1,5-Di-*O*-caffeoylquinic acid	n.d.	n.d.	n.d.	n.d.	2.23 ± 0.08
3,4-Di-*O*-caffeoylquinic acid	0.56 ± 0.01 ^{c}	n.d.	n.d.	17.68 ± 0.08 ^{a}	13.16 ± 0.20 ^{b}
4,5-Di-*O*-caffeoylquinic acid	n.d.	n.d.	n.d.	29.27 ± 0.86 ^{a}	8.58 ± 0.04 ^{b}
3,5-Di-*O*-caffeoylquinic acid	n.d.	n.d.	n.d.	24.52 ± 0.36 ^{b}	36.13 ± 0.83 ^{a}
Caffeoylquinic acid dehydrodimer isomer	1.61 ± 0.04 ^{b}	2.79 ± 0.05 ^{a}	0.79 ± 0.02^{d}	0.92 ± 0.02 ^{c}	1.65 ± 0.01 ^{b}
Caffeoylquinic acid dehydrodimer isomer	1.36 ± 0.04 ^{b}	1.34 ± 0.04 ^{b}	1.36 ± 0.10 ^{b}	n.d.	4.12 ± 0.09 ^{a}
Caffeoylquinic acid dehydrodimer isomer	0.99 ± 0.02 ^{d}	3.20 ± 0.09 ^{b}	1.60 ± 0.08 ^{c}	n.d.	5.32 ± 0.06 ^{a}
Caffeoylquinic acid dehydrodimer isomer	1.47 ± 0.04 ^{c}	5.48 ± 0.14 ^{a}	0.70 ± 0.05 ^{d}	n.d.	1.89 ± 0.06 ^{b}
Caffeoylquinic acid dehydrodimer isomer	n.d.	11.49 ± 0.15 ^{a}	1.86 ± 0.11 ^{b}	n.d.	2.03 ± 0.07 ^{b}
Eggplant					
Compound	**Raw**	**Baked**	**Boiled**	**Fried**	**Grilled**
Caffeoylquinic acid dehydrodimer isomer	n.d.	n.d.	n.d.	n.d.	0.87 ± 0.01
N^{1},N^{5}-Di-caffeoyl-spermidine	n.d.	6.15 ± 0.17	n.d.	n.d.	n.d.
N^{1},N^{10}-Di-caffeoyl-spermidine	n.d.	n.d.	n.d.	7.66 ± 0.03	n.d.
N^{1},N^{5}-Di-dihydrocaffeoyl-spermidine	n.d.	n.d.	n.d.	107.50 ± 4.02 ^{a}	11.58 ± 0.20 ^{b}
N^{1},N^{10}-Dihydrocaffeoyl-caffeoyl-spermidine	n.d.	n.d.	n.d.	37.40 ± 0.54	n.d.
Total hydroxycinnamic acids	**10.39 ± 0.24 ^{e}**	**92.87 ± 1.60 ^{c}**	37.32 ± 1.44 ^{d}	297.07 ± 7.28 ^{a}	173.63 ± 5.57 ^{b}
Flavonols					
Quercetin-di-*O*-hexoside	0.23 ± 0.00	n.d.	n.d.	n.d.	n.d.
Kaempferol-3-*O*-rutinoside	0.11 ± 0.00 ^{b}	n.d.	n.d.	0.87 ± 0.02 ^{a}	n.d.
Total flavonols	**0.35 ± 0.01 ^{b}**	**n.d.**	**n.d.**	**0.87 ± 0.02 ^{a}**	**n.d.**
Total	**10.74 ± 0.25 ^{e}**	**92.87 ± 1.60 ^{c}**	**37.32 ± 1.44 ^{d}**	**297.95 ± 7.29 ^{a}**	**173.63 ± 5.57 ^{b}**

Different letters within the same row mean significant different ($p < 0.05$) values. n.d. means that the compound was not detected in the sample.

As shown in **Table 1**, all heat treatments resulted in an increase in bioaccessible total phenolic compounds compared to the in vitro digested raw phenolic-rich fraction. Frying accounted for the largest increase in total phenolic compounds (2674.2%), which was followed by grilling (1516.7%), baking (764.71%) and boiling (247.5%). These results suggested that cooking is one of the key aspects in the release of phenolic compounds from the food matrix during digestion, as already stated by other authors[64-67]. This cooking-related impact may be a consequence of the matrix-softening effect promoted by the cooking methods that enhance the release and consequently phenolic compounds bioaccessibility[30,37]. As previously reported, distinct thermal treatments may have a different effect on the bioaccessibility of phenolic compounds[26,27,38-41].

The applied cooking methods also allowed a distinctive release in specific phenolic compounds resulting in a different phenolic profile in the in vitro digested phenolic-rich fractions (**Table 1**). Grilled, fried, and baked in vitro digested DPE phenolic-rich fractions showed the highest amount of caffeoylquinic acids, especially in the compounds 5-*O*-caffeoylquinic acid *trans* and *cis*. In addition, fried and grilled phenolic-rich fraction samples also displayed the highest amount of bioaccessible dicaffeoylquinic and feruloylquinic acids. Finally, the fried sample phenolic profile was dominated by hydroxycinnamic acid amides. The percentage of distribution of the different phenolic compounds grouped by class is shown in Figure 1. Caffeoylquinic acids represented the most important class of phenolic compounds in boiled (83.1% of total phenolic compounds) and baked (56.0% of total phenolic compounds) in vitro digested DPE phenolic-rich fractions. The grilled in vitro digested DPE phenolic-rich fraction was mainly composed of dicaffeoylquinic acids (34.4% of total phenolic compounds) and caffeoylquinic acids (34.2% of total phenolic compounds), whereas hydroxycinnamic acid amides (51.2% of total phenolic compounds) were predominant in the fried sample followed by dicaffeoylquinic acids (24.0% of total phenolic compounds).

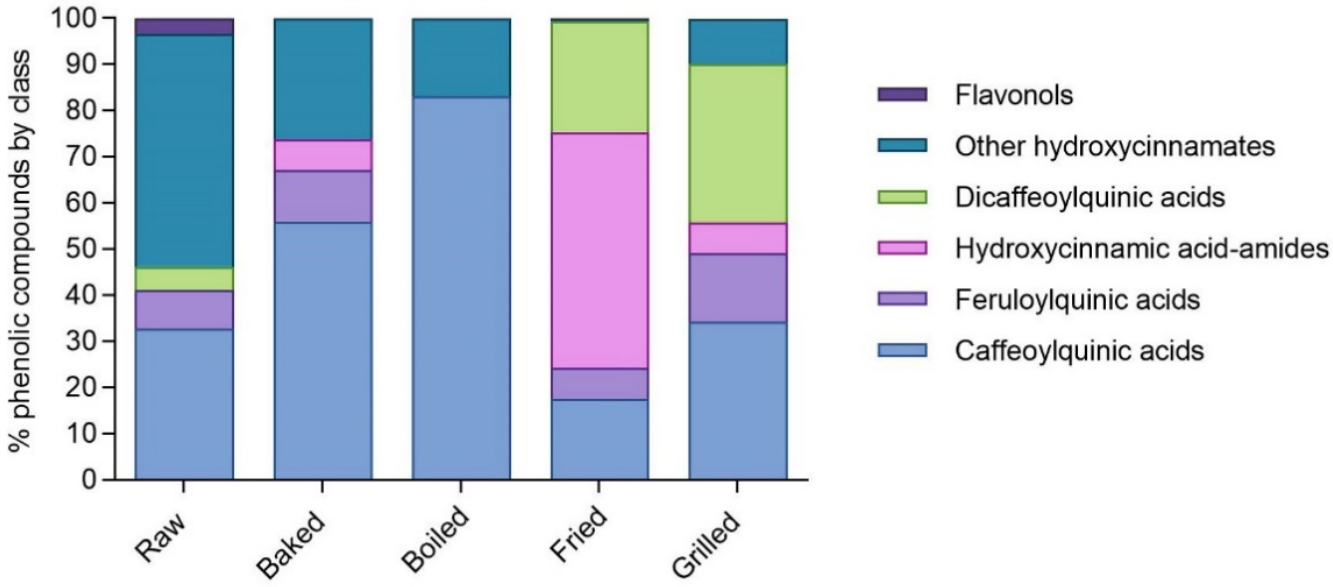


Figure 1. Percentage distribution of the main classes of phenolic compounds in raw and cooked in vitro digested dark purple eggplant phenolic-rich fractions. The percentage values are referred to the total concentration of phenolic compounds identified in the raw and cooked in vitro digested dark purple eggplant samples as reported in Table 1 and expressed as µmol of compound/100 g of raw or cooked eggplant.

3.2. Phenolic Compounds Profile of Phenolic-Rich Fractions Extracted from Raw and Cooked In Vitro Digested Red-Skinned Onion (RSO)

As reported in **Table 2**, the number of phenolic compounds identified in raw, baked, boiled, fried, and grilled in vitro digested RSO phenolic rich-fractions was 15, 10, 6, 9, and 11, respectively. In in vitro digested raw RSO phenolic rich-fraction, the class of phenolic compounds most commonly present was flavonols (10 compounds) followed by anthocyanins (3 compounds) and dihydro-flavonols (one compound). In the in vitro digested raw RSO sample, the quantitative phenolic profile was dominated by flavonols and in particular by quercetin mono-hexosides and quercetin di-hexosides. The total concentration of bioaccessible phenolic compounds quantified by mass spectrometry in the in vitro digested raw RSO phenolic-rich fraction was 85.49 ± 0.39 µmol/100g of onion. As reported in **Table 2**, heat treatments also caused an increase in bioaccessible total phenolic compounds in the in vitro digested phenolic-rich fractions of cooked RSO with respect to the raw sample, although it was less evident than in DPE. The greatest increase occurred with grilling treatment (95.5%), which was followed by baking (12.5%), boiling (2.4%), and frying (1.9%). A comparable trend was noted for the content of total flavonols as a function of the cooking treatment. The greatest augment in total bioaccessible flavonols content, compared to the raw sample, was found after grilling (391%), which was followed by baking (19.2%) and frying (8.5%). Boiling, on the other hand, is the only cooking procedure that brought about a reduction in the bioaccessible total flavonols content (43.6%) in comparison with the raw sample. This behavior after the different cooking methods and in vitro digestion was already reported by other studies. Specifically, the authors found that baking, grilling, and frying enhanced the bioaccessibility of

flavonols in green pepper, cactus cladodes, and onion, whereas the flavonols content decreased after boiling treatment[26,38,41]. Considering the individual phenolic compounds, the one with the greatest bioaccessibility in all of the samples was quercetin-3-*O*-hexoside-4'-*O*-hexoside followed by quercetin-4'-*O*-hexoside (**Table 2**). In addition, grilling was the only treatment able to preserve anthocyanins after in vitro digestion. All other treatments caused a complete loss of these compounds in in vitro digested RSO phenolic-rich fractions (**Table 2**).

Table 2. Concentration of phenolic compounds in in vitro digested raw and cooked red-skinned onion phenolic-rich fractions. Results are reported as µmol of phenolic compounds/100 g of raw or cooked onion.

In Vitro Digested Red-Skinned Onion Phenolic-Rich Fractions						
	Compound	**Raw**	**Baked**	**Boiled**	**Fried**	**Grilled**
	Hydroxybenzoic acids					
1	Protocatechuic acid-*O*-hexoside	n.d.	n.d.	42.80 ± 0.24 ^{b}	n.d.	51.35 ± 0.22 ^{a}
	Total hydroxybenzoic acids	***n.d.***	***n.d.***	***42.80 ± 0.24 ^{b}***	***n.d.***	***51.35 ± 0.22 ^{a}***
	Flavan-3-ols					
2	(Epi)catechin-3-*O*-hexoside isomer	n.d.	1.55 ± 0.01 ^{a}	n.d.	0.75 ± 0.01 ^{c}	1.02 ± 0.00 ^{b}
	Total flavan-3-ols	***n.d.***	***1.55 ± 0.01 ^{a}***	***n.d.***	***0.75 ± 0.01 ^{c}***	***1.02 ± 0.00 ^{b}***
	Dihydro-flavonols					
3	Taxifolin-*O*-hexoside isomer	0.09 ± 0.00 ^{b}	n.d.	n.d.	0.15 ± 0.00 ^{a}	n.d.
	Total dihydro-flavonols	***0.09 ± 0.00 ^{b}***	***n.d.***	***n.d.***	***0.15 ± 0.00 ^{a}***	***n.d.***
	Flavonols					
4	Quercetin-3-*O*-hexoside isomer	11.51 ± 0.08 ^{d}	18.70 ± 0.07 ^{b}	n.d.	15.17 ± 0.07 ^{c}	20.69 ± 0.06 ^{a}
5	Quercetin-4'-*O*-hexoside	22.76 ± 0.11 ^{a}	12.90 ± 0.10 ^{d}	10.27 ± 0.01 ^{e}	18.02 ± 0.07 ^{b}	15.94 ± 0.01 ^{c}
6	Quercetin-3-*O*-glucoside	0.91 ± 0.01 ^{d}	1.72 ± 0.02 ^{b}	3.81 ± 0.08 ^{a}	1.42 ± 0.01 ^{c}	3.71 ± 0.05 ^{a}
7	Quercetin-3-*O*-hexoside-7-*O*-hexoside	n.d.	1.83 ± 0.00	n.d.	n.d.	n.d.
8	Quercetin-7-*O*-hexoside-4'-*O*-hexoside	1.78 ± 0.00 ^{c}	2.12 ± 0.01 ^{a}	n.d.	n.d.	1.83 ± 0.00 ^{b}
9	Quercetin-3-*O*-hexoside-4'-*O*-hexoside	34.36 ± 0.10 ^{d}	50.49 ± 0.10 ^{b}	29.45 ± 0.05 ^{e}	43.54 ± 0.16 ^{c}	62.08 ± 0.81 ^{a}
10	Quercetin-tri-*O*-hexoside isomer	0.92 ± 0.01 ^{c}	1.29 ± 0.00 ^{a}	0.21 ± 0.00 ^{d}	0.94 ± 0.01 ^{b}	1.28 ± 0.00 ^{a}
11	Kaempferol-7-*O*-hexoside isomer	0.13 ± 0.00	n.d.	n.d.	n.d.	n.d.
12	Kaempferol-3-*O*-hexoside isomer	0.23 ± 0.0	n.d.	n.d.	n.d.	n.d.
13	Isorhamnetin-3-*O*-hexoside isomer	n.d.	2.33 ± 0.01 ^{a}	n.d.	1.73 ± 0.00 ^{b}	1.60 ± 0.00 ^{c}
14	Isorhamnetin-4'-*O*-hexoside	4.45 ± 0.04 ^{b}	n.d.	n.d.	5.37 ± 0.06 ^{a}	n.d.
15	Isorhamnetin-3-*O*-hexoside-4'-*O*-hexoside	2.37 ± 0.00 ^{c}	3.28 ± 0.01 ^{b}	1.04 ± 0.01 ^{d}	n.d	3.35 ± 0.00 ^{a}
	Total flavonols	*79.42 ± 0.36 ^{d}*	*94.66 ± 0.33 ^{b}*	*44.78 ± 0.15 ^{e}*	*86.19 ± 0.37 ^{c}*	*110.49 ± 1.08 ^{a}*
	Anthocyanins					
16	Cyanidin-3-*O*-hexoside	n.d.	n.d.	n.d.	n.d.	0.73 ± 0.00
Red-skinned onion						
	Compound	**Raw**	**Baked**	**Boiled**	**Fried**	**Grilled**
17	Cyanidin-*O*-malonyl-hexoside isomer	2.02 ± 0.02	n.d.	n.d.	n.d.	n.d.
18	Cyanidin-*O*-hexoside-*O*-hexoside isomer	1.24 ± 0.00 ^{b}	n.d.	n.d.	n.d.	1.90 ± 0.01 ^{a}
19	Cyanidin-*O*-hexoside-*O*-hexoside isomer	0.79 ± 0.00	n.d.	n.d.	n.d.	n.d.
20	Cyanidin-*O*-hexoside-*O*-malonyl-hexoside isomer	1.92 ± 0.00 ^{a}	n.d.	n.d.	n.d.	1.62 ± 0.01 ^{b}
	Total anthocyanins	***5.98 ± 0.03 ^{a}***	***n.d.***	***n.d.***	***n.d.***	***4.25 ± 0.02 ^{b}***
	Total	***85.49 ± 0.39 ^{d}***	***96.21 ± 0.34 ^{b}***	***87.58 ± 0.39 ^{c}***	***87.09 ± 0.38 ^{c,d}***	***167.11 ± 1.18 ^{a}***

Different letters within the same row mean significant different ($p < 0.05$) values. n.d. means that the compound was not detected in the sample.

Additionally in RSO, the different cooking methods modified not only quantitatively but also qualitatively the phenolic profile after in vitro digestion, as already stated for DPE (**Figure 2**). Raw, baked, and fried in vitro digested RSO phenolic-rich fractions contained mainly quercetin-mono hexosides (41.2%, 34.6%, and 39.7% of total phenolic compounds, respectively) and quercetin-di-hexosides (42.3%, 56.6%, and 50.0% of total phenolic compounds, respectively). On the contrary, the predominant compounds in boiled in vitro digested RSO phenolic-rich fraction were hydroxybenzoic acids (48.9% of total phenolic compounds).

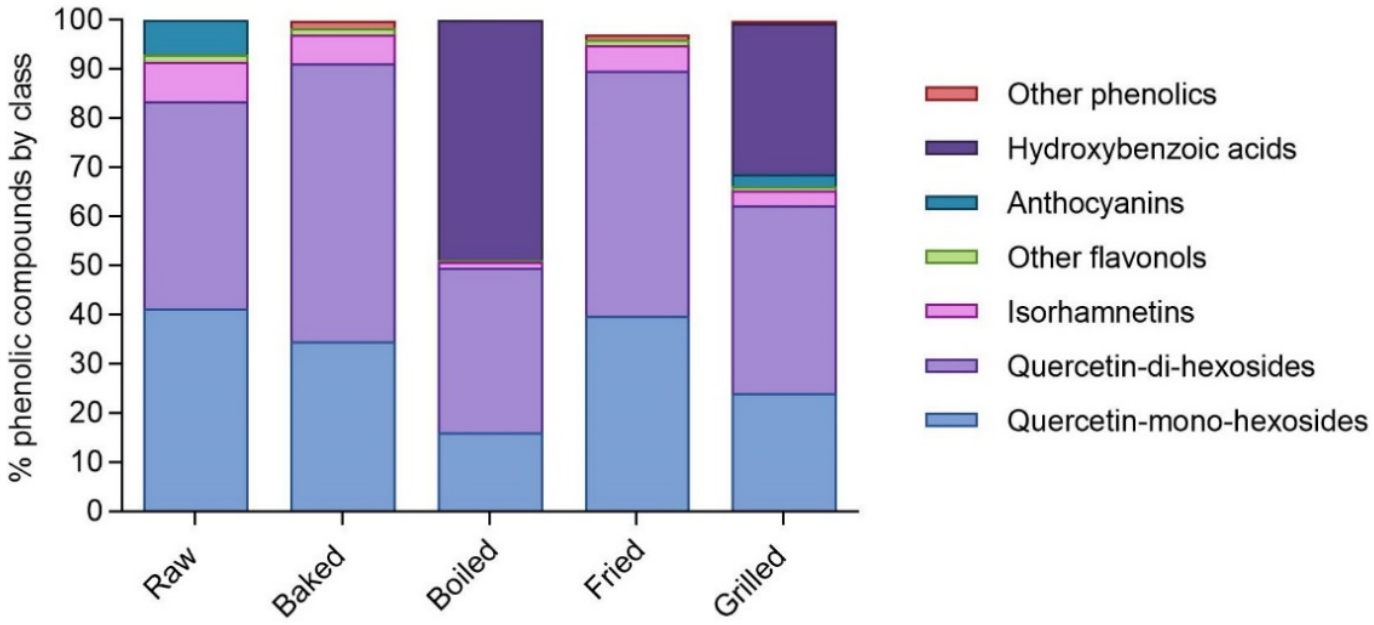


Figure 2. Percentage distribution of the main classes of phenolic compounds in raw and cooked in vitro digested red-skinned onion phenolic-rich fractions. The percentage values are referred to the total concentration of phenolic compounds identified in the raw and cooked in vitro digested red-skinned onion samples as reported in Table 2 and expressed as µmol of compound/100 g of raw or cooked onion.

3.3. Inhibitory Effect of In Vitro Digested Phenolic Rich-Fractions on α-Amylase, α-Glucosidase, and Dipeptidyl Peptidase-IV (DPP-IV) Activities

In vitro digested phenolic-rich fractions of DPE and RSO were tested for their capacity to inhibit α-amylase, α-glucosidase, and DPP-IV, which are the three key enzymes considered the pharmacological targets in the anti-diabetic therapy. The in vitro digested phenolic-rich fractions of DPE displayed no inhibitory effect versus α-amylase and DPP-IV enzymes, independently of the cooking treatment. The only exception was the in vitro digested fried DPE sample, which showed moderate inhibitory activity toward the enzyme α-glucosidase with an IC_{50} value of 33.28 ± 1.02 µmol of phenolic compounds/L.

Differently from the others, an in vitro digested DPE fried sample contained predominantly hydroxycinnamic acid-amides (51.2% of total phenolic compounds, **Figure 1**) and especially N^1,N^5-di-dihydrocaffeoyl-spermidine and N^1,N^{10}-dihydrocaffeoyl-caffeoyl-spermidine (49.9% of total phenolic compounds in the sample). The other two representative classes of phenolic compounds in the in vitro digested DPE fried

sample were dicaffeoylquinic acids and caffeoylquinic acids. Previous studies showed that caffeoylquinic acids did not present α-glucosidase inhibitory activity[42-44]. Indeed, boiled and baked samples contained a high percentage of caffeoylquinic acids and were not active against α-glucosidase. Contrariwise, dicaffeoylquinic acids were found to be able to inhibit α-glucosidase but with IC_{50} values in the order of hundreds of μmol/L[42-44]. Moreover, the in vitro digested grilled sample had a higher percentage of dicaffeoylquinic acids compared to the fried sample but no inhibitory activity. Therefore, hydroxycinnamic acid-amides can be considered the α-glucosidase-inhibitory active compounds present in the in vitro digested fried sample. Furthermore, there is strong evidence that hydroxycinnamic acid spermidine derivatives and, more in general, phenol amides exhibit a broad range of bioactivities including antioxidant, anti-microbial, and anti-cancer activities as well as protective effects against cardiovascular and metabolic syndrome-related diseases[45].

As reported in Figure 3, the phenolic-rich fractions of in vitro digested RSO were able to inhibit all three key enzymes. Samples that showed the greatest inhibitory activity against the α-amylase enzyme (**Figure 3A**) were fried, raw, and baked in vitro digested RSO samples (IC_{50} = 65.76 ± 1.00 μmol/L, 66.98 ± 1.00 μmol/L and 68.46 ± 1.01 μmol/L, respectively). No significant differences ($p > 0.05$) were found between the α-amylase-inhibitory activity of these samples. The in vitro digested boiled sample differed slightly but significantly ($p < 0.05$) from raw, baked, and fried in vitro digested RSO samples (IC_{50} = 73.15 ± 1.00 μmol/L), while the in vitro digested grilled sample showed the lowest inhibitory activity (IC_{50} = 125.10 ± 1.02 μmol/L). As shown in **Figure 3B**, the RSO samples differed in their inhibitory activity toward the α-glucosidase enzyme. In this case, the in vitro digested raw RSO sample was the most active (IC_{50} = 16.93 ± 1.09 μmol/L), which was followed by the in vitro digested fried, baked, grilled, and, lastly, boiled sample (IC_{50} = 20.92 ± 1.07 μmol/L, 24.59 ± 1.05 μmol/L, 38.54 ± 1.03 μmol/L, and 56.15 ± 1.08 μmol/L, respectively).

Although there are no studies on cooked and digested samples, the α-glucosidase and α-amylase inhibitory effects of red-skinned onion extracts have been confirmed by other researchers[11,46,47]. Accordingly, in all these studies, the red-skinned onion extracts were most active against α-glucosidase with respect to α-amylase. The inhibitory effects of the red-skinned onion extracts versus α-glucosidase and α-amylase have been assigned to the occurrence of phenolic compounds and in particular flavonols and anthocyanins, which displayed enzymes inhibitory activity[19,48,49].

As depicted in **Figure 3C**, all of the in vitro digested RSO phenolic-rich fractions were able to inhibit DPP-IV activity with grilled and fried samples showing the highest inhibitory activity (IC_{50} = 34.14 ± 1.05 and 43.23 ± 1.04 μmol/L, respectively). A significantly lower amount of activity was observed for the in vitro digested raw sample (IC_{50} = 56.85 ± 1.05 μmol/L) as well as for boiled and grilled samples (IC_{50} = 59.01 ± 1.10 and 59.45 ± 1.03 μmol/L, respectively). To the best of our knowledge, there are no studies in the literature on the DPP-IV-inhibitory activity of red-skinned onion extracts. Nevertheless, Kim et al. demonstrated that some flavonol

glycosides were potent DPP-IV inhibitors[50]. Therefore, it can be speculated that flavonol glycosides are the active compounds in red-skinned onion in vitro digested samples able to inhibit DPP-IV activity.

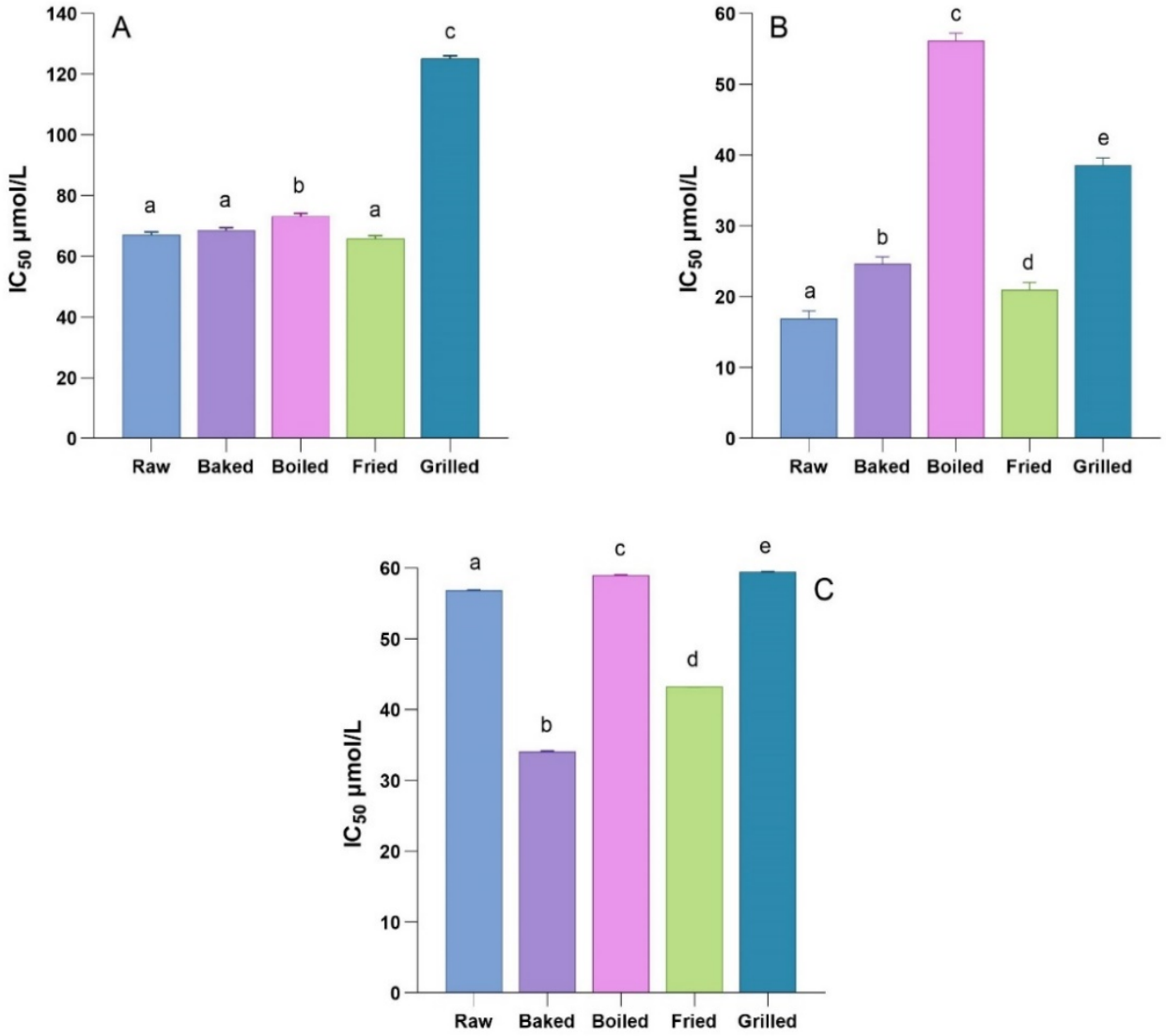


Figure 3. Effect of in vitro digested red-skinned onion phenolic-rich fractions on the activity of key metabolic enzymes. (**A**) Inhibitory activity against α-amylase. (**B**) Inhibitory activity against α-glucosidase. (**C**) Inhibitory activity against dipeptidyl-peptidase IV. The concentration of phenolic compounds (expressed as µmol of phenolic compounds per mL) required to inhibit the enzymatic activity by 50% (IC50) was calculated by plotting the percentage of the enzyme inhibition with the phenolic compounds concentration (base-10 logarithm). The concentration of phenolic compounds was determined by LC-ESI-IT MS/MS analysis and detailed in Table 2.

3.4. Statistical Analysis

3.4.1. Principal Component Analysis

To further understand the relationship between cooking treatments and the biological activities and to identify potential active compounds in red-skinned onion, principal component analysis (PCA) was carried out (**Figure 4**). Two principal components account for 78.18% of the total variance. The bi-plot PC1 vs. PC2 displayed an evident splitting of the treatments. Boiled and grilled samples had a negative PC1 score and formed the first group, which was dominated by the presence of hydroxybenzoic acids. The other group consisted of the raw, baked, and fried samples, which were positively related to PC1. PC2 was mainly associated with different classes of compounds and had a positive loading for total hydroxybenzoic acids, total anthocyanins, total dihydro-flavonols, and total isorhamnetins, while total flavonols and flavan-3-ols had a negative loading value.

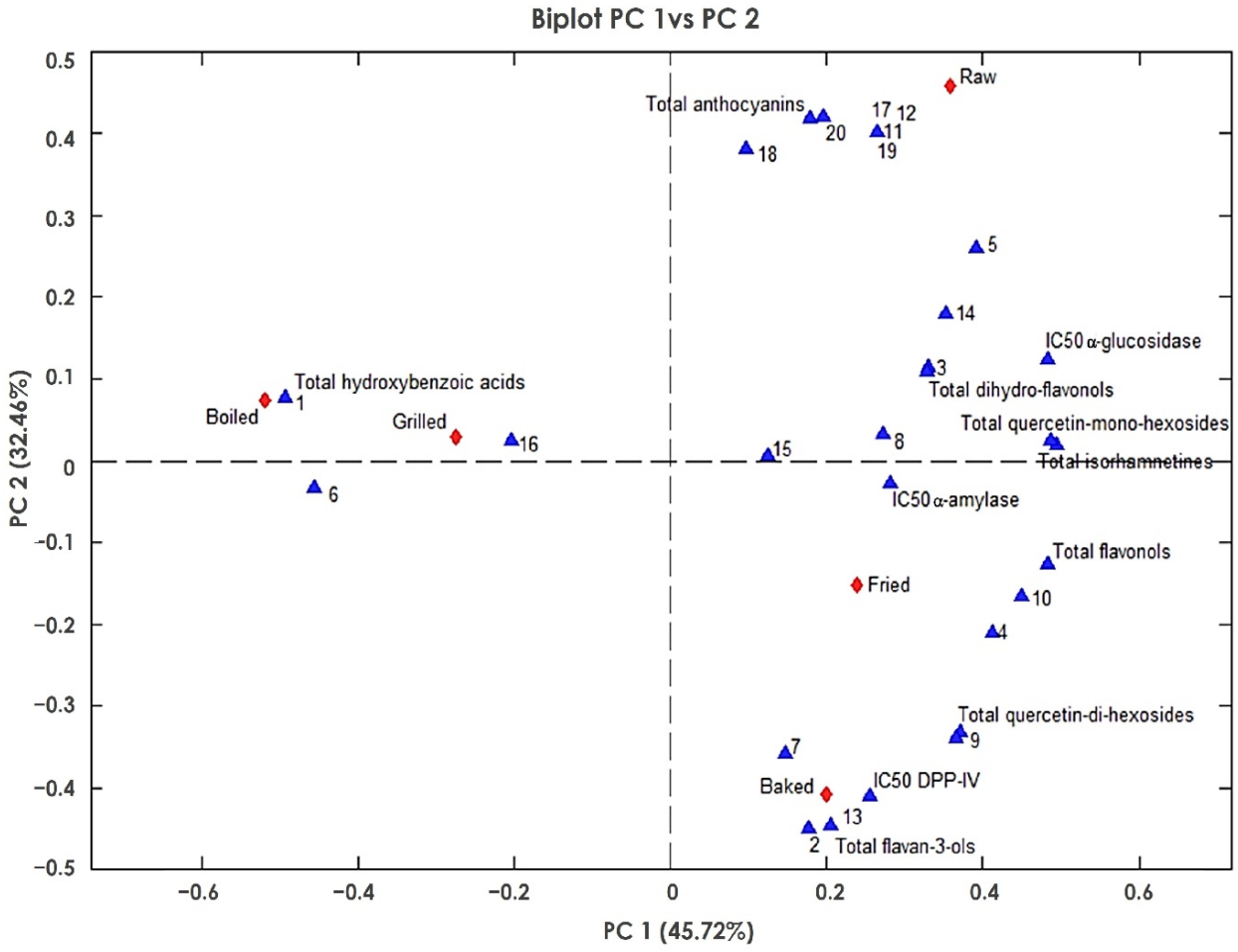


Figure 4. Principal component analysis (PCA). Loading plot of PC1 vs. PC2. The code number of compounds is reported in Table 2. The symbol ▲ identifies compounds, while the symbol ♦ identifies different cooking treatments. The individual compounds are reported with the same number as listed in Table 2.

By focusing on PC1 and PC2, it is possible to identify the most active compounds and thus discriminate between different heat treatments. Baked and fried samples had a negative score on PC2 and were positively correlated with the IC_{50} of DPP-IV. In addition, a positive correlation between the IC_{50} of DPP-IV and the presence of quercetin-di-hexosides was observed. In contrast, raw, baked and fried samples, which had a positive score on PC1, were positively correlated with the IC_{50} of α-glucosidase and the amount of quercetin-mono-hexosides and total isorhamnetins. Finally, no clear relationship was observed between the α-amylase inhibition and specific classes of compounds.

3.4.2. Correlation and Linear Regression Analysis

To gain more detailed information about the possible associations among specific classes of compounds and the related inhibitory activity toward the three key enzymes, correlation and linear regression studies were also carried out. As reported in **Figure 5A**, a statistically significant inverse correlation *($p < 0.05$)* between the concentration of quercetin-mono-hexosides in a specific sample and the IC_{50} values against α-glucosidase was observed ($R^2 = 0.9756$ and Pearson $r = -0.9877$). No further correlations were found between the α-glucosidase-inhibitory activity and other specific classes of phenolic compounds. These results confirmed the PCA results and strongly identified quercetin-mono-hexosides as the compounds responsible for the α-glucosidase-inhibitory activity in onion samples. Previously, quercetin-4'-*O*-glucoside was identified as the compound responsible for the α-glucosidase-inhibitory activity of hot water extracts of peel from different onion varieties, whereas quercetin-3-*O*-hexoside-4'-*O*-hexoside was not active[51]. In another study, kaempferol-3-*O*-glucoside was found to be an effective inhibitor of α-glucosidase[52]. Moreover, some other investigations generically identified flavonols as the active compounds against α-glucosidase in plant extracts[19]. Previous structure–activity relationship studies highlighted that the catechol moieties in the A- and B-rings as well as the OH group at the C3 position were of paramount importance for the inhibitory activity of flavonoids against α-glucosidase[53,54]. However, the supposed active compounds identified in in vitro digested red-skinned onion were glycosylated at the C4' and/or C3 position. We can suppose that the hexose moiety present in the structure of these compounds may interact with the active site of the enzyme in analogy with the substrate (maltose).

Correlation and linear regression analysis, carried out considering the IC_{50} values against DPP-IV and the different classes of phenolic compounds identified in the in vitro digested onion samples, showed quercetin-di-hexosides as the principal active compounds against DPP-IV. As depicted in Figure **5B**, a strong inverse correlation ($R^2 = 0.9189$ and Pearson $r = -0.9586$; $p < 0.05$) was ascertained between quercetin-di-hexosides and IC_{50} values versus the enzyme DPP-IV. Very few studies have been performed regarding the interaction between flavonols and DPP-IV. In a recent article, the authors identified quercetin-3-*O*-glucoside-7-*O*-rhamnoside as a potent inhibitor of DPP-IV[55]. In addition, kaempferol-3-*O*-glucoside-*O*-galactoside was able to inhibit efficiently DPP-IV activity by binding the DPP-IV S1 pocket with the flavone core structure and the

DPP-IV S2 pocket with the disaccharide moiety[50]. On the contrary, flavonol-mono-glucosides have been characterized as weak DPP-IV inhibitors[56,57]. All together, these results suggest that quercetin-di-hexosides are potent DPP-IV inhibitors.

Finally, no correlation was found between specific compounds and the α-amylase inhibitory activity of in vitro digested red-skinned onion. Previous studies found that the hydroxylation of flavonoids favored the inhibitory activity, whereas glycosylation decreased it[58]. These data can explain why in vitro digested RSO phenolic-rich extracts were more effective against α-glucosidase with respect to α-amylase.

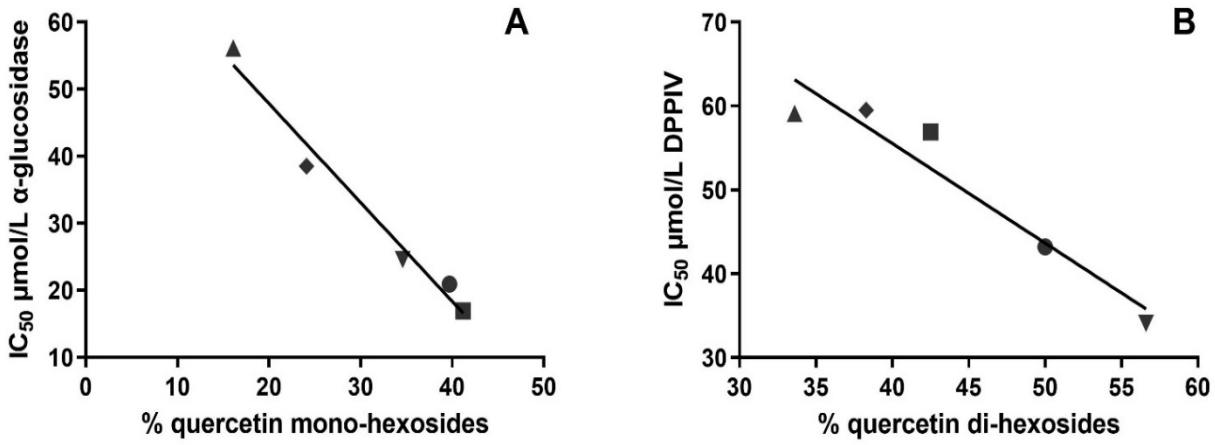


Figure 5. Linear regression analysis. (**A**) Relationship between the content of quercetin mono-hexosides in red-skinned onion samples and the IC50 values against the enzyme α-glucosidase. (**B**) Relationship between the content of quercetin di-hexosides in red-skinned onion samples and the IC50 against the enzyme di-peptidyl-peptidase-IV (DPP-IV). The symbol ▲ identifies boiled samples, ♦ grilled samples, ● fried samples, ■ raw samples, and ▼ baked samples.

4. Conclusions

This study demonstrates that different heat treatments associated with the in vitro gastrointestinal digestion changed the phenolic composition of phenolic-rich vegetables such as dark purple eggplant and red-skinned onion. In fact, some compounds were more bioaccessible, whereas others were degraded following cooking and in vitro digestion. More interestingly, by tuning the phenolic profile after digestion, heat treatments modulate the bioactive profiles of phenolic-rich vegetables. The results presented in the study proved that all the red-skinned onion samples presented some inhibitory activity against the three key enzymes involved in the diabetes pathogenesis but to a different extent depending on the cooking method and phenolic profile. The class of flavonols is the one that has been found to be most active especially against α-glucosidase and DPP-IV enzymes. Furthermore, statistical analysis confirmed a significant correlation between the amount of quercetin mono-hexosides (mainly quercetin-4'-*O*-hexoside) and the IC_{50} of α-glucosidase and the amount of quercetin di-hexosides (mainly quercetin-3-*O*-hexoside-4'-*O*-hexoside) and the IC_{50} of DPP-IV. This is of paramount importance, as the regular intake of foods rich in flavonols (such as red-skinned onion) could help prevent the metabolic syndrome. In addition, an accurate design of the cooking methods may be pivotal in suggesting the most suitable process to optimize the release of specific phenolic compounds and the alleged bioactivities. However, additionally, in vivo studies are strongly required to corroborate the physiological relevance of the in vitro results, especially related to the cooking impact on phenolic compounds absorption, metabolism, and health effect, which is a field of research not yet sufficiently considered and explored.

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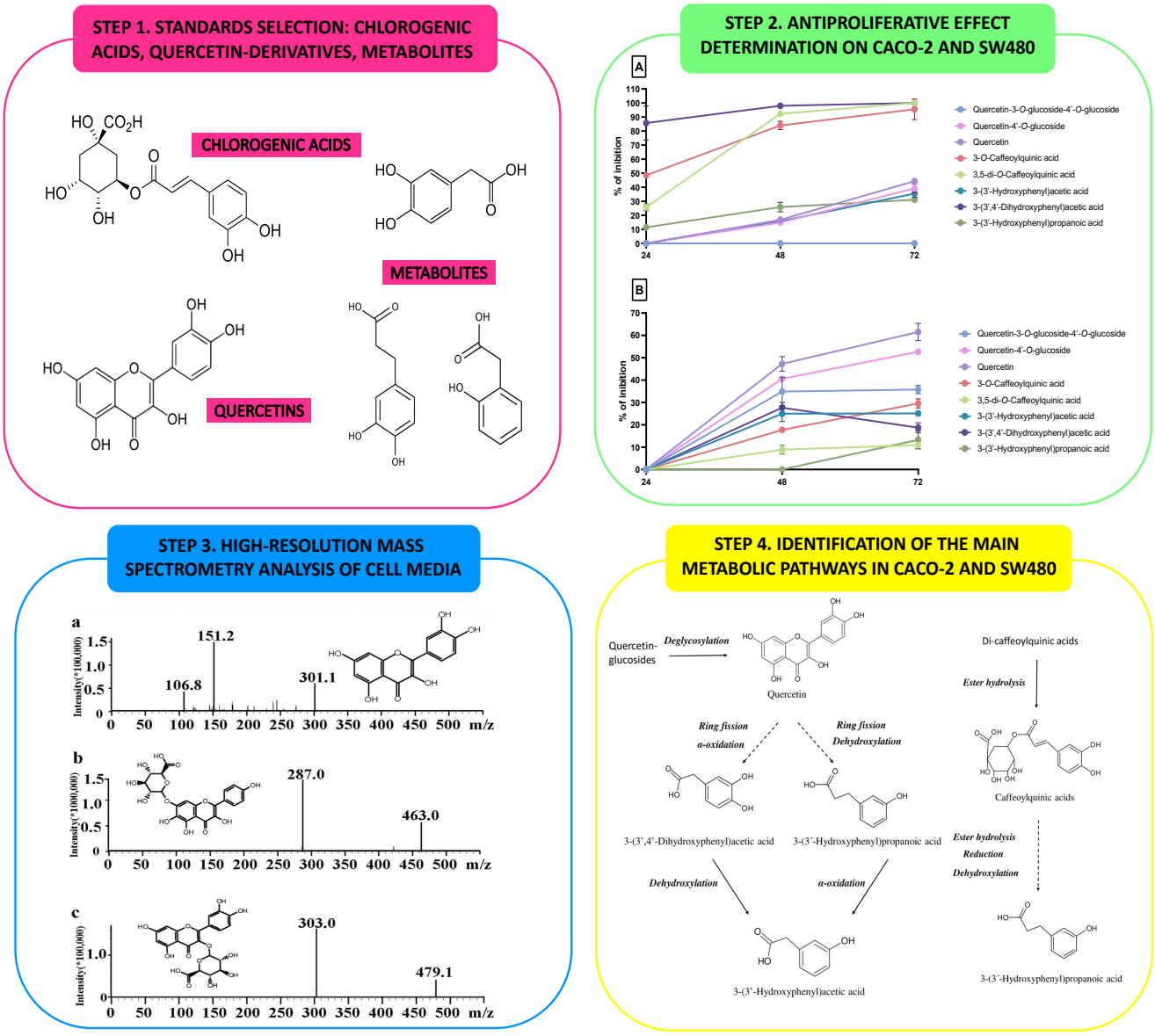
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A. Cattivelli, A. Conte, D. Tagliazucchi. Quercetins, chlorogenic acids and their colon metabolites inhibit colon cancer cell proliferation at physiologically relevant condition. Submitted to *International Journal of Molecular Sciences*.

QUERCETINS, CHLOROGENIC ACIDS AND THEIR COLON METABOLITES INHIBIT COLON CANCER CELL PROLIFERATION AT PHYSIOLOGICALLY RELEVANT CONDITION

ABSTRACT

Several studies suggested that a phenolic-rich diet may be protective against the onset of colon cancer. Most of the phenolic compounds introduced with the diet are not absorbed in the small intestine and reach the colon where they can be metabolized by gut microbiota in simple phenolic acids. In this study the anti-proliferative activity of dietary quercetins, chlorogenic acids, their colon metabolites and selected mixtures of parent compounds/metabolites was assessed by using two different colon cancer cell line models (Caco-2 and SW480) at physiologically relevant concentrations. Chlorogenic acids, quercetin, and the metabolite 3-(3',4'-dihydroxyphenyl)acetic acid) were the most active against Caco-2 cells, whereas quercetin-derivatives, and the metabolites 3-(3'-hydroxyphenyl)acetic acid and 3-(3',4'-dihydroxyphenyl)acetic acid) were the most active against SW480. The mixtures parent compounds/metabolites that mimic the colon human metabotypes that metabolize slowly or rapidly the parent compounds (low-producers vs high-producers) similarly inhibited cell growth. SW480 cells metabolizes parent phenolic compounds more rapidly and extensively than Caco-2, whereas colon metabolites were generally more stable. These results suggest that dietary phenolic compounds exert remarkable anti-proliferative effect against human colon cancer cells that can be further sustained by the produced colon metabolites which retained the anti-proliferative effect. Therefore, gut microbiota metabolism of phenolic compounds may be of paramount importance in explaining the protective effect of phenolic-rich foods against colon cancer.

Keywords: phenolic compounds; mass spectrometry; colon cancer; anti-proliferative activity; gut microbiota; metabolism

1. Introduction

Colorectal cancer is one of the most frequent forms of cancer playing an important role in mortality, lifestyle, and economic costs worldwide (Sung et al., 2021). Several epidemiological and observational studies highlighted the strong impact of the diet on the risk of colorectal cancer (Deng et al., 2021; Papadimitriou et al., 2022; Sawicki et al., 2021; Thanikachalam & Khan, 2019). Dietary factors and the type of diet are among the main determinants in the development of colorectal cancer (Deng et al., 2021). For example, diets rich in fibers, calcium, dairy products as well as a plant-based diet rich in phenolic compounds can decrease the risk of developing colorectal cancer (Amintas et al., 2022; Deng et al., 2021; Papadimitriou et al., 2022; Schwingshackl et al., 2018). Vice versa, a fiber-low diet associated with a high intake of red and processed meat may increase the risk of the onset of colorectal cancer (Amintas et al., 2022; Deng et al., 2021; Papadimitriou et al., 2022; Schwingshackl et al., 2018).

Among the different dietary patterns, Mediterranean diet is widely recognized as a healthy diet that may be protective against the onset of several chronic diseases including cancer (Farinetti et al., 2017; Finicelli et al., 2022). For example, population with high adherence to the Mediterranean diet displayed a lower incidence of colorectal cancer, suggesting that Mediterranean diet may be chemo-preventive against this type of cancer (Farinetti et al., 2017; Mahmod et al., 2022; Morze et al., 2021). Indeed, Mediterranean diet is characterized by a high intake of vegetable foods, fiber-rich foods, fresh fruits, and legumes parallel to a low intake of red and processed meat (Finicelli et al., 2022).

The beneficial consequences of Mediterranean diet and, more in general, of a diet rich in vegetables and fruits in preventing the onset of colorectal cancer may be in part ascribable to the high content in phenolic compounds of these foods (Amintas et al., 2022; Farinetti et al., 2017). For example, Zamora-Ros et al. (Zamora-Ros et al., 2018) found a positive association between the intake of phenolic acids (mainly hydroxycinnamic acids) and the reduction of colon cancer whereas Chang et al. (Chang et al., 2018) suggested that a high intake of flavonoids (especially flavonols) may decrease the onset of colon cancer.

Hydroxycinnamic acids are an important class of phenolic compounds which are mainly found in nature esterified with one or more quinic acid moieties giving the so-called chlorogenic acids (Clifford et al., 2017). The most important chlorogenic acids found in nature are caffeoylquinic, feruloylquinic and dicaffeoylquinic acids (Bento-Silva et al., 2020). In the context of the Mediterranean diet, they may account for the 25-35% of total phenolic compounds intake (Godos et al., 2017; Vázquez-Ruiz et al., 2022). The main dietary sources of chlorogenic acids in the Mediterranean diet were coffee, vegetables (such as eggplant and carrots) and fruits (such as cherries and apples) (Martini et al., 2017, 2021; Vázquez-Ruiz et al., 2022).

Flavonols are another widespread class of dietary phenolic compounds that are mainly found in nature in the form of glycoside-derivatives (Del Rio et al., 2013). Flavonols may account for the 7-15% of total phenolic compounds in the Mediterranean diet where the main sources were fruits and vegetables foods and in particular onions, apple, berries and red wine (Cattivelli et al., 2021; Godos et al., 2017). The principal

flavonols identified in vegetable foods are glycosylated quercetin- derivatives such as querctin-3-*O*-glucoside and quercetin-3-*O*-rutinoside as well as quercetin-4'-*O*-glucoside and quercetin-3-*O*-glucoside-4'-*O*-glucoside in onion (Cattivelli et al., 2021; Del Rio et al., 2013).

Several meta-analysis of epidemiological studies as well as animal studies supported the protective effect of flavonols and hydroxycinnamic acids against colorectal cancer (Chang et al., 2018; Coman & Vodnar, 2020; Li et al., 2018; Mihanfar et al., 2021). Nevertheless, the data extracted from human intervention studies are still inconclusive owing to the considerable inter-individual variability largely due to the different gut microbiota metabolism of phenolic compounds among individuals.

Quercetin and quercetin-derivatives reaching the colon are subjected to microbiota metabolism that involves deglycosylation reaction, followed by C-ring fission and β-oxidation generating some low-molecular weight phenolic acids, mainly 3-(3'-hydroxyphenyl)propanoic acid, 3-(3'-hydroxyphenyl)acetic acid and 3-(3',4'-dihydroxyphenyl)acetic acid (Serra et al., 2012) (Cattivelli et al., 2023). Similarly, the colon metabolism of chlorogenic acids starts with the removal of the quinic acid moiety followed by reduction and de-hydroxylation which results in the accumulation of 3-(3'-hydroxyphenyl)propanoic acid (Juániz et al., 2017; Nissen et al., 2022). However, the amount and the type of produced metabolites greatly differed among individuals depending on the microbiota composition (Almeida et al., 2018; Bento-Silva et al., 2020). Therefore, both the parent compounds and the microbial metabolites co-exist in the colon and the ratio parent compounds/metabolites strongly depend on the individual microbiota composition and on the ability to produce high or low amounts of phenolic metabolites and, therefore, on the inter-individual variability and metabotype (Cortés-Martín et al., 2020; Iglesias-Aguirre et al., 2021). In the low-producers metabotype (i.e. individual that produced low amount of colon metabolites) the ratio parent compounds/metabolite is strongly shifted towards the parent compounds whereas, in the high-producers metabotype (i.e. individual that produced high amount of colon metabolites) the equilibrium is directed towards a higher amount of colon metabolites. Thus, the aim of this work was to study the anti-proliferative activity and the cell metabolism of selected quercetin-derivatives (quercetin, quercetin-4'-O-glucoside and quercetin-3-O-glucoside-4'-O-glucoside), chlorogenic acids (3-O-caffeoylquinic acid, 5-O-caffeoylquinic acid and 3,5-dicaffeoylquinic acid) as well as their most important colon metabolites (3-(3'-hydroxyphenyl)propanoic acid, 3-(3'-hydroxyphenyl)acetic acid and 3-(3',4'-dihydroxyphenyl)acetic acid) using colon adenocarcinoma Caco-2 and SW480 cell lines. Indeed, to understand the possible role of inter-individual variability, six different mixes were prepared mimicking the low-producers (high amount of parent compounds and low amount of metabolites) and the high-producers (low amount of parent compounds and high amount of metabolites) metabotypes, in comparison with a third mix with equimolar amounts of parent compound and metabolites.

2. Materials and methods

2.1. Materials

Chemicals and materials for cell culture were purchased from VWR International (Milan, Italy). The MTS cell proliferation assay kit was from Promega (Milan, Italy). Mass spectrometry solvents were purchased from Bio-Rad (Hercules, CA, USA). The parent phenolic compounds 3-*O*-caffeoylquinic acid (purity ≥ 99%), 5-*O*-caffeoylquinic acid (purity ≥ 99%), 3,5-*O*-dicaffeoylquinic acid (purity ≥ 97%), quercetin (purity ≥ 99%), quercetin-4'-*O*-glucoside (purity ≥ 99%) and quercetin-3-*O*-glucoside-4'-*O*-glucoside (purity ≥ 98.5%) were obtained from Extrasynthese (Genay, France). Phenolic compounds metabolites 3-(3'-hydroxyphenyl)propanoic acid (purity ≥ 98%), 3-(3'-hydroxyphenyl)acetic acid (purity ≥ 99%) and 3-(3',4'-dihydroxyphenyl)acetic acid (purity ≥ 98%) were purchased from Thermo-Fisher Scientific (Waltham, MA, USA). All the other chemicals were purchased from Sigma-Aldrich (Milan, Italy).

2.2. Cell cultures, phenolic compounds preparation and anti-proliferative assay

Human colon cancer Caco-2 and SW480 cell lines were cultured and grown as previously reported (Martini et al., 2019). All the phenolic compounds were dissolved in dimethyl-sulfoxide (DMSO) at concentration of 50 mmol/L, diluted in the respective cell culture media at a concentration of 100 µmol/L (final DMSO concentration of 0.5%) and finally filtered at 0.2 µm.

For the anti-proliferative assay, cells were seeded in 96-well plates in the amount of 4000 and 8000 cells/cm^2 for Caco-2 and SW480, respectively. Cells were than left to adhere for 24 h before the addition of 200 µL of the phenolic compounds (100 µmol/L). Incubation was carried out for 24, 48 and 72 h. Data were compared with a control solution containing 0.5% DMSO in cell medium and representing the 100% of proliferation. In addition, six different mixes were prepared to simulate the simultaneous presence of parent phenolic compounds and metabolites by mimicking the possible human inter-individual variability in phenolic compounds colon metabolism. Two set of mixes were prepared to consider the colon metabolism of quercetins and chlorogenic acids. The quercetins mixtures (QUE mixes) contained different amount of quercetin-3-*O*-glucoside-4'-*O*-glucoside, quercetin-4'-*O*-glucoside and quercetin (the parent compounds) as well as of the respective metabolites (3-(3'-hydroxyphenyl)acetic acid, 3-(3',4'-dihydroxyphenyl)acetic acid and 3-(3'-hydroxyphenyl)propanoic acid). Instead, the chlorogenic acids mixtures (CGA mixes) were prepared by mixing the respective parent compounds (3-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid and 3,5-*O*-dicaffeoylquinic acid) with the metabolite 3-(3'-hydroxyphenyl)propanoic acid. For each set of mixtures (QUE and CGA mixtures) three different mixes were formulated. One set of mixes mimicked the low-producers metabotype and contained 90% of parent compounds and 10% of metabolites (mixes QUE-LP and CGA-LP). Another set of mixes was prepared to simulate the high-producers metabotype and contained 10% of parent compounds and 90% of metabolites (mixes QUE-HP and CGA-HP). Finally, a third set of mixes were prepared

with equimolar amount of all the compounds (mixes QUE-EQ and CGA-EQ). The anti-proliferative activity of the mixes was determined by using the final concentrations of 100 and 200 µmol/L (sum of the concentrations of the compounds present in the mixes). The composition of each mix is reported in **Table 1**.

Table 1. Composition of the different mixes tested for their anti-proliferative effect against human colon carcinoma cell lines.

Compound	***QUE-LP***	***QUE-EQ***	***QUE-HP***	***CGA-LP***	***CGA-EQ***	***CGA-HP***
Quercetin-3-*O*-glucoside-4'-*O*-glucoside	60 µmol/L	33.3 µmol/L	6.7 µmol/L	/	/	/
Quercetin-4'-*O*-glucoside	60 µmol/L	33.3 µmol/L	6.7 µmol/L	/	/	/
Quercetin	60 µmol/L	33.3 µmol/L	6.7 µmol/L	/	/	/
3-*O*-Caffeoylquinic acid	/	/	/	60 µmol/L	50 µmol/L	6.7 µmol/L
5-*O*-Caffeoylquinic acid	/	/	/	60 µmol/L	50 µmol/L	6.7 µmol/L
3,5-di-*O*-Caffeoylquinic acid	/	/	/	60 µmol/L	50 µmol/L	6.7 µmol/L
3-(3'-Hydroxyphenyl)acetic acid	6.7 µmol/L	33.3 µmol/L	60 µmol/L	/	/	/
3-(3',4'-Dihydroxyphenyl)acetic acid	6.7 µmol/L	33.3 µmol/L	60 µmol/L	/	/	/
3-(3'-Hydroxyphenyl)propanoic acid	6.7 µmol/L	33.3 µmol/L	60 µmol/L	20 µmol/L	50 µmol/L	180 µmol/L

The composition is referred to the mixes prepared at the final concentration of 200 µmol/L considering all the added compounds. In the mixes prepared at the final concentration of 100 µmol/L the concentration of each compound was halved.

The cell proliferation was assessed by the MTS assay as previously described (Martini et al., 2019).

For the most active compounds, the IC_{50} values (defined as the phenolic compound concentration able to inhibit cell proliferation by 50%) were calculated by carrying out the anti-proliferative assay with different concentrations of the specific phenolic compounds (range from 1.5 to 200 µmol/L). The IC_{50} values were calculated through non-linear regression analysis by plotting the base-10 logarithm of the phenolic compound concentration vs the percentage of inhibition.

2.3. High-resolution mass spectrometry analysis of cell media

To analyze the phenolic compounds metabolism by Caco-2 and SW480 cells, the cell culture media were withdrawn after 0, 24, 48 and 72 h of incubation. Phenolic compounds were extracted from the culture media by following the procedure reported in Sala et al. (Sala et al., 2015). Briefly, 100 µL of cold methanol were mixed with 100 µL of cell media and vortexed for 1 min. The mixture was then centrifuged at 10000*g* (4 °C, 5 min) and the supernatant injected, after appropriate dilution, in the ultra-high performance liquid chromatography high-resolution mass spectrometry system (UHPLC-HR-MS).

The HR-MS system consisted of a UHPLC Ultimate 3000 separation module, equipped with a C18 column (Acquity UPLC HSS C18 reversed phase, 2.1 × 100 mm, 1.8 µm particle size, Waters, Milan, Italy) and coupled with a Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, San Jose, CA, USA). Mobile phases consisted of a mixture of water containing formic acid (0.1%) (mobile phase A) and acetonitrile containing formic acid (0.1%) (mobile phase B). The gradient began at 1% B to reach 40% B in 20 min. In order to wash the column, the mobile phase B concentration was raised to 99% in 6 min and kept at 99% for 3 min before returning to the initial conditions. The MS and MS/MS conditions are fully described in Martini et al. (Martini et al., 2020).

Quantification was carried out by building external calibration curves with the available standard compounds.

2.4. Statistical analysis

All data are reported as mean ± standard deviation (SD) for three replicates for each prepared compound. Univariate analysis of variance (ANOVA) with Tukey post-hoc test was applied using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). The differences were considered significant with $P < 0.05$. The IC_{50} values were calculated by using GraphPad Prism 6.0.

3. Results

3.1. Anti-proliferative activity of phenolic compounds and metabolites

Tested parent phenolic compounds and metabolites at 100 µmol/L concentration exerted a time-dependent anti-proliferative activity against both the colon cancer cell lines, with the only exception of quercetin-3-*O*-glucoside-4'-*O*-glucoside that did not show any activity against Caco-2 cell proliferation at any time (**Figure 1A** and **B**).

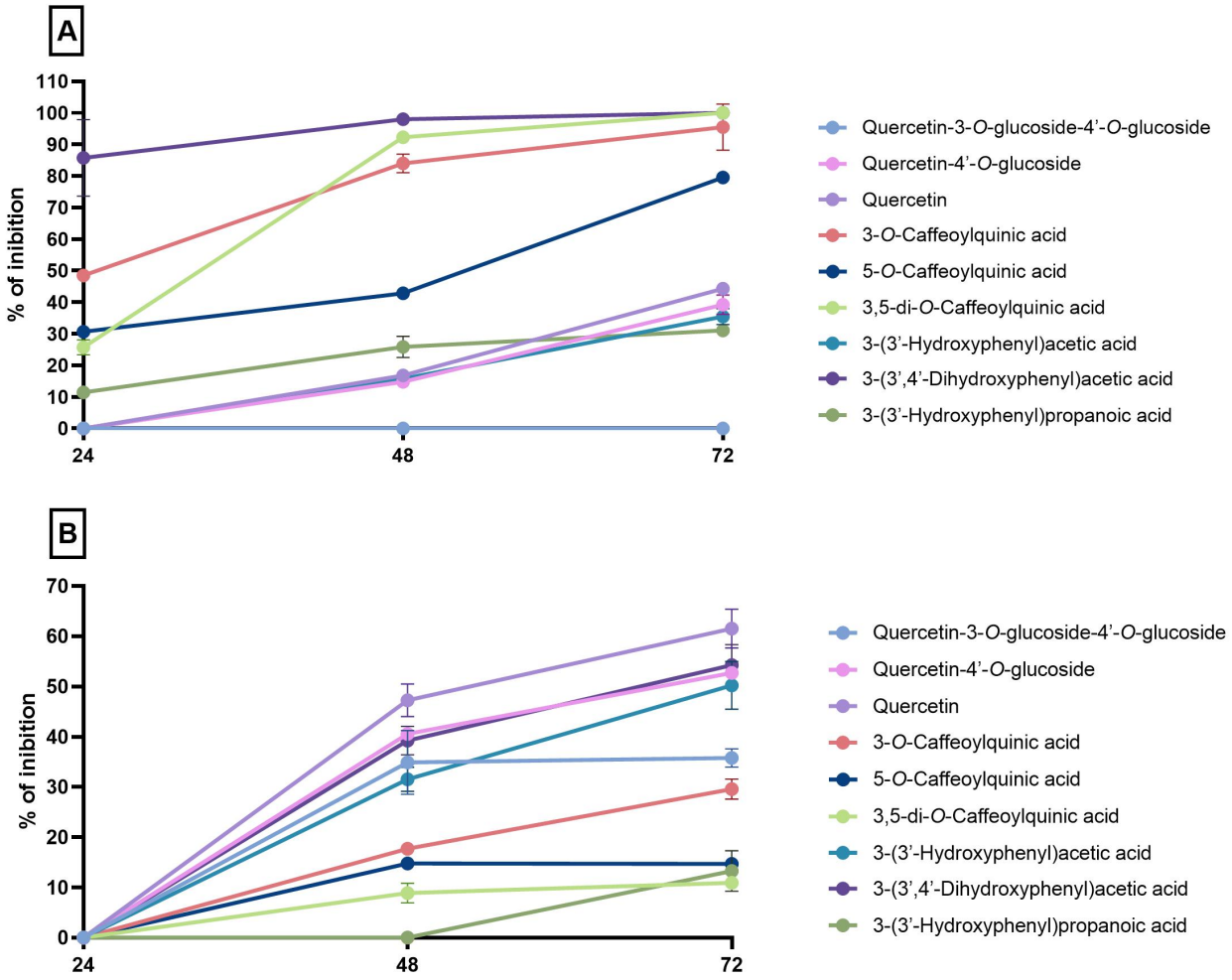


Figure 1. Antiproliferative activity of parent phenolic compounds and colon metabolites. All the compounds were tested at a final concentration of 100 µmol/L. (A) Percentage of inhibition of Caco-2 growth after 24, 48 and 72 h. (B) Percentage of inhibition of SW480 growth after 24, 48 and 72 h.

In general, quercetin-derivatives were more active against SW480 cell proliferation respect to Caco-2 proliferation. The anti-proliferative activity started to be detectable after 48 h of incubation and increased in both the cell lines after 72 h of incubation. Among quercetin-derivatives, the highest activity was found in both the cell lines for quercetin, followed by quercetin-4'-*O*-glucoside. For both the compounds the anti-proliferative effect was significantly higher ($P<0.05$) against SW480 respect to Caco-2 both after 48 and 72 h of incubation. As already mentioned, quercetin-3-*O*-glucoside-4'-*O*-glucoside displayed anti-proliferative

activity only against SW480 cell line although significantly lower (P<0.05) respect to quercetin and quercetin-4'-*O*-glucoside.

An opposite trend was observed for chlorogenic acids since they displayed a significantly higher (P<0.05) anti-proliferative activity against Caco-2 respect to SW480 cell lines at any time. In Caco-2 cells, 3,5-di-*O*-caffeoylquinic acid, 3-*O*-caffeoylquinic acid and 5-*O*-caffeoylquinic acid were active already after 24 h of incubation and then the anti-proliferative activity increased over the time reaching an almost complete inhibition after 72 h of incubation. The anti-proliferative activity of 5-*O*-caffeoylquinic acid was significantly lower (P<0.05) than that of its positional isomer 3-*O*-caffeoylquinic acid at any time. In the case of SW480 the maximum effect was observed after 72 h of incubation for both the compounds being 3-*O*-caffeoylquinic acid more active than 3,5-di-*O*-caffeoylquinic acid and 5-*O*-caffeoylquinic acid (29.6%, 10.9% and 14.7% of inhibition at 72 h, respectively).

A similar trend was observed also for the phenolic metabolites, which exerted a higher anti-proliferative effect (P<0.05) against Caco-2 respect to SW480 at any incubation time, with the exception of 3-(3'-hydroxyphenyl)acetic acid. The colon metabolite 3-(3',4'-dihydroxyphenyl)acetic acid was the tested compound that exerted the highest anti-proliferative effect among all the tested compounds against Caco-2 cell lines. The metabolite 3-(3'-hydroxyphenyl)propanoic acid was more effective than 3-(3'-hydroxyphenyl)acetic acid after 24 and 48 h of incubation with Caco-2 cell lines. However, no significant differences (P>0.05) were found in the anti-proliferative activity of these two colon metabolites after 72 h of incubation with Caco-2 cells. No anti-proliferative activity was observed in the SW480 cell line for all the tested colon metabolites after 24 h of incubation. After 48 and 72 h of incubation with SW480 cells no significant differences (P>0.05) were found between 3-(3',4'-dihydroxyphenyl)acetic acid and 3-(3'-hydroxyphenyl)acetic acid anti-proliferative activity. Among the tested metabolites, 3-(3'-hydroxyphenyl)propanoic acid displayed the lowest anti-proliferative activity against SW480 cells, with a recorded 13.3% of inhibition after 72 h of incubation.

The IC_{50} values were calculated for the most active compounds and data are reported in **Table 2**.

In the case of Caco-2 cells, the lowest IC_{50} values were calculated for the colon metabolite 3-(3',4'-dihydroxyphenyl)acetic acid, at any time of incubation. The IC_{50} values decreased according to the incubation time by 5.4 times from 24 to 48 h and by 4.9 times from 48 to 72 h. The IC_{50} values were also calculated for the three tested chlorogenic acids. At 24 h, 3-*O*-caffeoylquinic acid was more effective than 3,5-di-*O*-caffeoylquinic acid, whereas this last compound overcame 3-*O*-caffeoylquinic acid in its anti-proliferative effect after 48 and 72 h of incubation. The IC_{50} value of 3-*O*-caffeoylquinic acid decreased by 2.3 times passing from 24 to 48 h of incubation whereas no significant differences (P>0.05) were detected in the IC_{50} values between 48 and 72 h of incubation. The compound 5-O-caffeoylquinic acid displayed higher IC50 values (and therefore lower activity) than 3-O-caffeoylquinic acid at 24 and 48 h, however, at 72 h the calculated IC50 value of 5-O-caffeoylquinic acid was significantly lower (P<0.05) than that of 3-O-caffeoylquinic acid. The

decrease in the IC50 values for 5-O-caffeoylquinic acid was 1.7-times from 24 to 48 h and 3.2-times from 48 to 72 h. For 3,5-di-*O*-caffeoylquinic acid a 3.1-fold decrease in the IC_{50} value was recorded from 48 to 72 h of incubation.

In the case of SW480, the IC50 values were calculated only for two quercetin-derivatives and two metabolites. In the case of quercetin-derivatives the lowest IC50 values were calculated for quercetin both after 48 and 72 h of incubation with a recorded decrease of 1.5-fold passing from 48 to 72 h. A similar decrease in the IC50 values was observed for quercetin-4'-O-glucoside (1.4-fold) going from 48 to 72 h of incubation. The IC50 values calculated for the metabolites 3-(3',4'-dihydroxyphenyl)acetic acid and 3-(3'-hydroxyphenyl)acetic acid after 72 h of incubation were not significantly different (P>0.05).

Table 2. IC_{50} values (µmol/L) of the different tested compounds after 24, 48 and 72 h of incubation with Caco-2 and SW480 cell lines

Compound	*Caco-2 (µmol/L)*			*SW 480 (µmol/L)*		
	24 h	*48 h*	*72 h*	*24 h*	*48 h*	*72 h*
Quercetin-3-*O*-glucoside-4'-*O*-glucoside	n.a.	n.a.	n.a.	n.a.	> 200	~ 200
Quercetin-4'-*O*-glucoside	n.a.	> 200	> 200	n.a.	144.3 ± 6.6	102.2 ± 5.3$^{\#}$
Quercetin	n.a.	> 200	116.1 ± 4.9	n.a.	89.2 ± 3.1	58.2 ± 2.7
3-*O*-Caffeoylquinic acid	97.3 ± 2.9$^{\#}$	42.8 ± 1.7*	40.4 ± 2.0*	n.a.	> 200	> 200
5-*O*-Caffeoylquinic acid	169.5 ± 8.3	98.8 ± 3.0$^{\#}$	31.3 ± 2.1	n.a.	> 200	> 200
3,5-di-*O*-Caffeoylquinic acid	> 200	27.5 ± 1.0	8.8 ± 0.2	n.a.	> 200	> 200
3-(3'-Hydroxyphenyl)acetic acid	n.a.	> 200	> 200	n.a.	> 200	99.6 ± 4.8$^{\#}$
3-(3',4'-Dihydroxyphenyl)acetic acid	79.5 ± 1.4	14.7 ± 0.8	3.0 ± 0.1	n.a.	> 200	92.2 ± 6.9$^{\#}$
3-(3'-Hydroxyphenyl)propanoic acid	> 200	> 200	> 200	n.a.	n.a.	> 200

n.a. means not active compound
Number with the same superscript symbol were not significant different (*P*>0.05)
The maximum tested concentration was 200 µmol/L

3.2. Anti-proliferative activity of quercetin-derivatives/metabolites and chlorogenic acids/metabolites mixes

Exposure of Caco-2 and SW480 cell lines to the different mixes resulted in an anti-proliferative effect, whose intensity was dependent on the cell line, mix composition and concentration as well as on time of exposure (**Figure 2**). As regards the quercetin-derivatives/metabolites mixes, the trend in the anti-proliferative activity was opposite considering the two cell lines. In Caco-2 cell line the cell growth inhibitory effect increased passing from the mix containing higher amount of parent compounds (QUE-LP mix) to the mixture containing higher amount of metabolites (QUE-HP mix). This was particularly evident when the mixes were tested at 200 µmol/L concentration. In particular, the mix QUE-HP reached a 71% of cell growth inhibition after 72 of incubation at 200 µmol/L. An opposite behaviour was recorded with respect to the SW480 cell line where the anti-proliferative activity decreased as the concentration of the metabolites in the mix increased (from QUE-LP to QUE-HP). The mixes QUE-LP and QUE-EQ attained an anti-proliferative activity of 77% and 76% after 72 h of incubation at 200 µmol/L, respectively. Anyway, the mix QUE-HP was still able to inhibit SW480 proliferation by about 50% after 72 h of incubation at 200 µmol/L.

Anti-proliferative activity was also observed for the chlorogenic acids/metabolites mixtures in both the cell lines. In Caco-2 cell line the highest anti-proliferative effect was observed with CGA-LP and CGA-EQ that contained high amount of the parent compounds. The effect was already observed after 24 h of incubation and reached values more than 90% after 48 h of incubation. The mix with the high amount of the metabolite (CGA-HP) displayed lower inhibitor activity at any incubation time, with a maximum inhibitory activity of 47% after 72 h of incubation at 200 µmol/L.

Chlorogenic acids/metabolites mixtures did not showed anti-proliferative activity after 24 h of incubation with SW480 cells, whereas they achieved the maximum effect after 72 h of incubation. No significant differences were observed among the inhibitory activity of the three mixes after 72 h of incubation with SW480 cells.

3.3. Cell metabolism of phenolic compounds and metabolites

Most of the tested phenolic compounds underwent substantial biotransformation depending on their chemical structure and cell line.

In SW480 cell line, quercetin-3-*O*-glucoside-4'-*O*-glucoside was extensively metabolized such that its amount decreased by 14.4%, 26.0% and 53.6% after 24, 48 and 72 h of incubation (**Table 3**). Several new metabolites at *m/z* of 463.0888, 315.0513 and 301.0355 appeared already after 24 h of incubation. By comparing the *m/z* value, the fragmentation pattern, and the retention time with authentic standard, the three compounds were identified as quercetin-3-*O*-glucoside, isorhamnetin (and the corresponding isomer 4'-*O*-methyl-quercetin) and quercetin, respectively. Similarly, quercetin-4'-*O*-glucoside was strongly metabolized to quercetin, isorhamnetin and 4'-*O*-methyl-quercetin as well as quercetin metabolism resulted mainly in the formation of isorhamnetin and 4'-*O*-methyl-quercetin (**Table 3**). In these last two samples, a new compound

having *m/z* 380.9921 was detected and identified as quercetin-*O*-sulphate. Concerning Caco-2 cells, quercetin-3-*O*-glucoside-4'-*O*-glucoside was found to be quite stable without changing in its concentration during 72 h of incubation. Contrarywise, quercetin-4'-*O*-glucoside and quercetin cell medium concentrations strongly decreased during incubation with Caco-2 by 100% and 99.5%, respectively, already after 24 h of incubation (**Table 4**). Nevertheless, no newly formed compounds were detected in the Caco-2 cell media.

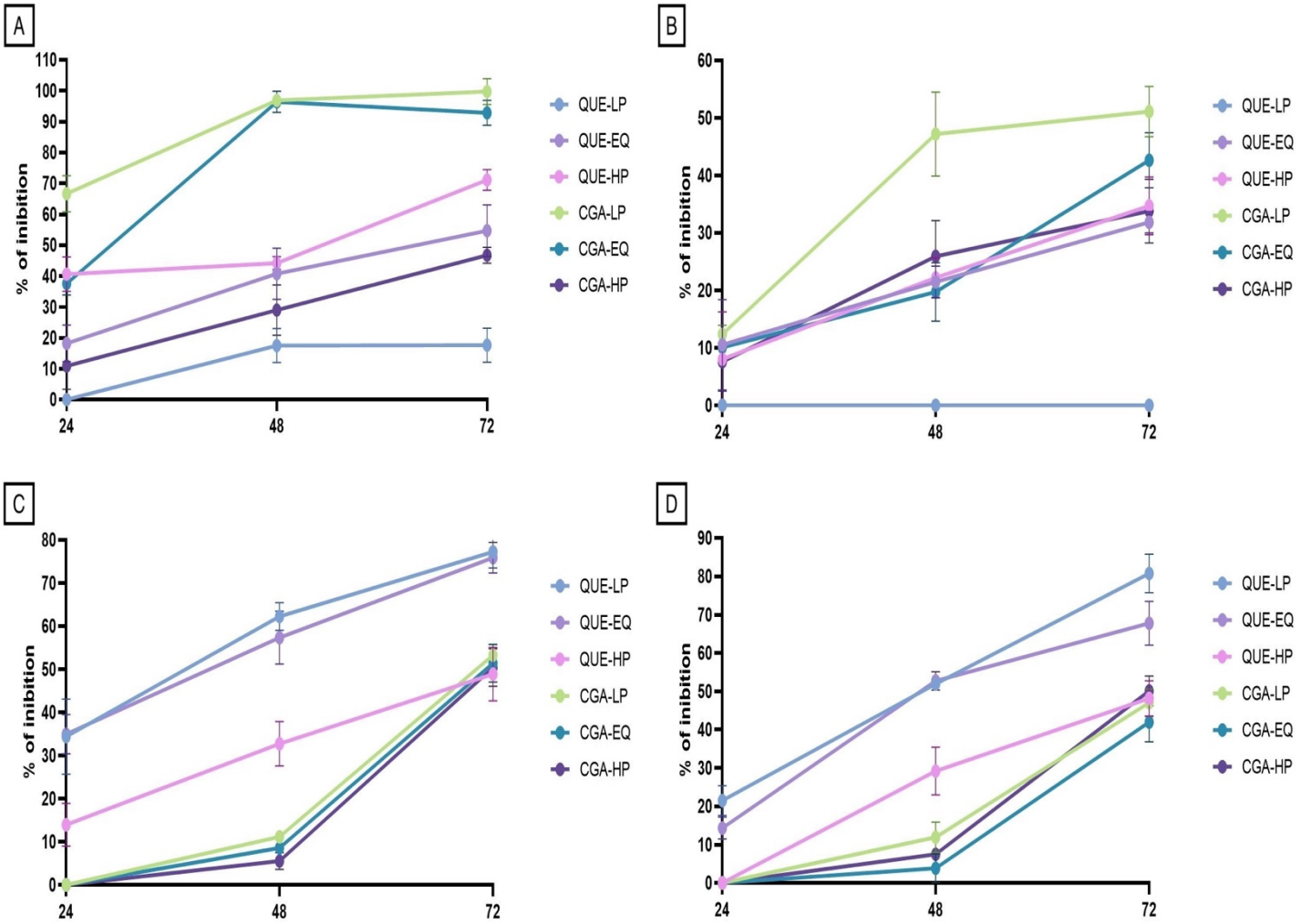


Figure 2. Antiproliferative activity of parent phenolic compounds/metabolites mixtures. (A) Percentage of inhibition of Caco-2 growth after 24, 48 and 72 h by the different mixtures at a concentration of 200 µmol/L. (B) Percentage of inhibition of Caco-2 growth after 24, 48 and 72 h by the different mixtures at a concentration of 100 µmol/L. (C) Percentage of inhibition of SW480 growth after 24, 48 and 72 h by the different mixtures at a concentration of 200 µmol/L. (D) Percentage of inhibition of SW480 growth after 24, 48 and 72 h by the different mixtures at a concentration of 200 µmol/L. Quercetin-derivatives/metabolites mixtures (QUE mixes) were prepared by mixing the parent compounds quercetin-3-*O*-glucoside-4'-*O*-glucoside, quercetin-4'-*O*-glucoside and quercetin as well as the colon metabolites 3-(3'-hydroxyphenyl)acetic acid, 3-(3',4'-dihydroxyphenyl)acetic acid and 3-(3'-hydroxyphenyl)propanoic acid. Chlorogenic acids/metabolites mixtures (CGA mixes) were prepared by mixing the parent compounds 3,5-di-*O*-caffeoylquinic acid, 3-*O*-caffeoylquinic acid and 5-*O*-caffeoylquinic acid as well as the colon metabolite 3-(3'-hydroxyphenyl)propanoic acid. The mixtures were formulated to mimic the metabotype low-producers of colon metabolites (QUE-LP and CGA-LP) and contained 90% of parent compounds and 10% of metabolites and to mimic the metabotype high-producers of colon metabolites (QUE-HP and CGA-HP) and contained 10% of parent compounds and 90% of metabolites. Mixes QUE-EQ and CGA-EQ contained equimolar amount of each compound. The exact composition of each mix is reported in **Table 1.**

Table 3. Changes in the concentration of tested phenolic compounds and in the newly formed metabolites after 24, 48 and 72 h of incubation with SW480 cell line

	Compound	*SW 480 (µmol/L)*			
		0 h	*24 h*	*48 h*	*72 h*
Substrate	Quercetin-3-*O*-glucoside-4'-*O*-glucoside	50	42.77 ± 0.10	37.04 ± 0.35	23.22 ± 0.22
Metabolite	Quercetin-3-*O*-glucoside		3.35 ± 0.01	8.94 ± 0.03	9.34 ± 0.05
	Quercetin		n.d.	0.04 ± 0.00	0.07 ± 0.00
	Isorhamnetin		n.d.	0.01 ± 0.00	0.01 ± 0.00
	4'-*O*-methylquercetin		n.d.	0.01 ± 0.00	0.01 ± 0.00
Substrate	Quercetin-4'-*O*-glucoside	50	2.98 ± 0.07	0.07 ± 0.00	0.01 ± 0.00
Metabolite	Quercetin		52.70 ± 2.24	36.89 ± 0.52	12.78 ± 0.41
	Isorhamnetin		n.d.	0.79 ± 0.03	1.03 ± 0.04
	4'-*O*-methylquercetin		n.d.	0.49 ± 0.01	0.71 ± 0.04
	Quercetin-*O*-sulphate		n.d.	0.06 ± 0.00	0.12 ± 0.01
Substrate	Quercetin	50	47.39 ± 1.22	40.37 ± 1.20	14.83 ± 0.84
Metabolite	Isorhamnetin		1.61 ± 0.01	3.46 ± 0.08	3.76 ± 0.11
	4'-*O*-methylquercetin		0.97 ± 0.04	1.65 ± 0.04	1.83 ± 0.25
	Quercetin-*O*-sulphate		0.26 ± 0.01	0.48 ± 0.01	0.61 ± 0.01
Substrate	3-*O*-Caffeoylquinic acid	50	23.34 ± 0.07	19.54 ± 0.07	19.83 ± 0.05
Metabolite	5-*O*-Caffeoylquinic acid		4.95 ± 0.04	7.37 ± 0.07	11.32 ± 0.43
	4-*O*-Caffeoylquinic acid		6.89 ± 0.06	8.07 ± 0.24	12.02 ± 0.24
	3-*O*-Caffeoylquinic acid *cis*		4.72 ± 0.04	2.51 ± 0.01	3.27 ± 0.17
	Caffeic acid		0.44 ± 0.01	0.43 ± 0.01	0.57 ± 0.01
	3-*O*-Feruloylquinic acid		0.08 ± 0.00	0.12 ± 0.00	0.17 ± 0.00
	4-*O*-Feruloylquinic acid		0.02 ± 0.00	0.04 ± 0.00	0.07 ± 0.00
	5-*O*-Feruloylquinic acid		0.03 ± 0.00	0.03 ± 0.00	0.05 ± 0.00
Substrate	5-*O*-Caffeoylquinic acid *trans*	50	16.76 ± 0.01	17.52 ± 0.08	13.96 ± 0.03
Metabolite	5-*O*-Caffeoylquinic acid *cis*		2.50 ± 0.01	0.97 ± 0.05	1.42 ± 0.01
	3-*O*-Caffeoylquinic acid *trans*		2.21 ± 0.05	5.14 ± 0.07	5.55 ± 0.09
	4-*O*-Caffeoylquinic acid *trans*		12.69 ± 0.12	14.80 ± 0.09	11.93 ± 0.03
	3-*O*-Caffeoylquinic acid *cis*		0.41 ± 0.05	0.36 ± 0.04	0.72 ± 0.03
	Caffeic acid		n.d.	0.08 ± 0.00	0.09 ± 0.01
	3-*O*-Feruloylquinic acid		0.06 ± 0.00	0.07 ± 0.00	0.05 ± 0.00
	4-*O*-Feruloylquinic acid		0.04 ± 0.00	0.05 ± 0.00	0.04 ± 0.00
	Ferulic acid		0.01 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
Substrate	3,5-di-*O*-Caffeoylquinic acid	50	7.13 ± 0.13	5.41 ± 0.07	6.17 ± 0.05
Metabolite	1,3-di-*O*-Caffeoylquinic acid		8.97 ± 0.04	8.87 ± 0.05	10.98 ± 0.02
	3,4-di-*O*-Caffeoylquinic acid		10.12 ± 0.10	8.04 ± 0.14	9.05 ± 0.15
	4,5-di-*O*-Caffeoylquinic acid		3.30 ± 0.05	2.20 ± 0.02	1.71 ± 0.03
	1,4-di-*O*-Caffeoylquinic acid		0.91 ± 0.03	0.66 ± 0.03	0.55 ± 0.01
	3-*O*-Caffeoylquinic acid		0.09 ± 0.01	0.13 ± 0.01	0.27 ± 0.01
	4-*O*-Caffeoylquinic acid		0.07 ± 0.01	0.12 ± 0.01	0.25 ± 0.01
	5-*O*-Caffeoylquinic acid		0.08 ± 0.01	0.14 ± 0.01	0.29 ± 0.01
	Caffeic acid		n.d.	n.d.	1.07 ± 0.01
Substrate	3-(3'-hydroxyphenyl)acetic acid	50	42.23 ± 0.55	42.81 ± 0.10	39.28 ± 0.85
Substrate	3-(3',4'-dihydroxyphenyl)acetic acid	50	21.62 ± 0.48	15.84 ± 0.09	10.30 ± 0.36
Substrate	3-(3'-hydroxyphenyl)propanoic acid	50	52.74 ± 0.44	50.70 ± 0.11	49.95 ± 0.37

n.d. means not detected

Table 4. Changes in the concentration of tested phenolic compounds and in the newly formed metabolites after 24, 48 and 72 h of incubation with Caco-2 cell line

	Compound	*CACO-2 (µmol/L)*			
		0 h	*24 h*	*48 h*	*72 h*
Substrate	Quercetin-3-*O*-glucoside-4'-*O*-glucoside	50	53.05 ± 0.44	50.05 ± 0.32	50.18 ± 0.66
Substrate	Quercetin-4'-*O*-glucoside	50	n.d.	n.d.	n.d.
Substrate	Quercetin	50	0.61 ± 0.01	0.29 ± 0.01	0.52 ± 0.02
Substrate	3-*O*-Caffeoylquinic acid	50	2.80 ± 0.16	0.04 ± 0.00	0.02 ± 0.00
Metabolite	5-*O*-Caffeoylquinic acid		2.71 ± 0.09	0.02 ± 0.00	0.01 ± 0.00
	4-*O*-Caffeoylquinic acid		2.93 ± 0.10	0.03 ± 0.00	0.02 ± 0.00
	3-*O*-Caffeoylquinic acid *cis*		0.26 ± 0.01	0.02 ± 0.00	0.02 ± 0.00
Substrate	3,5-di-*O*-Caffeoylquinic acid	50	0.15 ± 0.00	0.08 ± 0.00	0.18 ± 0.00
Metabolite	1,3-di-*O*-Caffeoylquinic acid		0.33 ± 0.01	0.16 ± 0.00	0.39 ± 0.00
	3,4-di-*O*-Caffeoylquinic acid		0.32 ± 0.00	0.18 ± 0.00	0.36 ± 0.00
	4,5-di-*O*-Caffeoylquinic acid		0.06 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
	1,4-di-*O*-Caffeoylquinic acid		n.d.	n.d.	0.10 ± 0.00
Substrate	3-(3'-hydroxyphenyl)acetic acid	50	48.83 ± 0.85	47.02 ± 0.39	43.17 ± 0.06
Substrate	3-(3',4'-dihydroxyphenyl)acetic acid	50	n.d.	n.d.	n.d.
Metabolite	Hydroxybenzoic acid		n.d.	3.51 ± 0.07	15.73 ± 0.37
Substrate	3-(3'-hydroxyphenyl)propanoic acid	50	45.51 ± 0.41	46.27 ± 0.58	46.28 ± 0.76
Metabolite	Hydroxybenzoic acid		n.d.	0.31 ± 0.01	0.30 ± 0.01

n.d. means not detected

As reported in **Table 3** and **Table 4**, 3,5-di-*O*-caffeoylquinic acid, 3-*O*-caffeoylquinic acid and 5-*O*-caffeoylquinic acid underwent substantial isomerization during incubation with both the cell lines. Several positional isomers were identified already after 24 h of incubation of 3,5-di-*O*-caffeoylquinic acid with both the cell lines. The two newly formed isomers detected in the highest amount were 3,4-di-*O*-caffeoylquinic and 1,3-di-*O*-caffeoylquinic acids. Besides the isomerization, after incubation of 3,5-di-*O*-caffeoylquinic acid with both the cell lines newly formed metabolites were detected and in particular the different positional isomers of caffeoylquinic acids (i.e. 5-*O*-caffeoylquinic acid, 3-*O*-caffeoylquinic acid and 4-*O*-caffeoylquinic acid) deriving from the hydrolysis of a caffeoyl moiety from the di-*O*-caffeoylquinic acids. Similarly, to 3,5-di-*O*-caffeoylquinic acid, also 3-*O*-caffeoylquinic acid and 5-*O*-caffeoylquinic acid were partially transformed in the corresponding positional isomers as well as suffered of *trans-cis* isomerization (**Table 3** and **Table 4**). After incubation with SW480 additional metabolites were detected such as caffeic acid (deriving from the hydrolysis of the quinic acid moiety from caffeoylquinic acids) and the different isomers of the methylated metabolites feruloylquinic acids (**Table 3**). In addition, ferulic acid (deriving from the hydrolysis of the quinic acid moiety from feruloylquinic acids or from the methylation of caffeic acid) was detected after incubation of 5-*O*-caffeoylquinic acid with SW480 cells. No newly formed compounds were observed after incubation with Caco-2 cells.

Regarding the three tested colon metabolites, 3-(3'-hydroxyphenyl)acetic acid and 3-(3'-hydroxyphenyl)propanoic acid were quite stable after incubation with both the cell lines (**Table 3** and **Table 4**). Contrariwise, a decrease in 3-(3',4'-dihydroxyphenyl)acetic acid concentration was observed after incubation with both the cell lines with the values collapsing to zero already after 24 h of incubation with Caco-2. No newly formed compounds were observed in SW480 cell medium whereas a strong production of hydroxybenzoic acid was detected after incubation with Caco-2 (**Table 4**). Small amount of hydroxybenzoic acid were also revealed after incubation of 3-(3'-hydroxyphenyl)propanoic acid with Caco-2 (**Table 4**).

4. Discussion

Flavonols (such as glycosylated quercetin-derivatives) and hydroxycinnamic acids (such as chlorogenic acids) are among the most widespread phenolic compounds in foods and among the most important phenolic compounds introduced with the diet (Godos et al., 2017). For example, onion, apple, tea, and red wine are rich in glycosylated quercetin-derivatives whereas coffee, eggplant, artichoke, apple and cherry contain high amount of chlorogenic acids (Almeida et al., 2018; Cattivelli et al., 2021; Domínguez-Fernández et al., 2022; Feng et al., 2021; Martini et al., 2017; Martini et al., 2021; Stalmach et al., 2010).

A plethora of studies suggested that dietary phenolic compounds, such as glycosylated quercetin-derivatives and chlorogenic acids, are poorly absorbed in the stomach and small intestine (Domínguez-Fernández et al., 2022; Hollman et al., 1995; Olthof et al., 2000; Stalmach et al., 2009). For example, only the 20-30% of hydroxycinnamic acids present in coffee or other foods such as yerba mate and artichoke were absorbed in the small intestine and appeared in plasma mainly as phase II conjugated metabolites (Domínguez-Fernández et al., 2022; Gómez-Juaristi et al., 2018; Stalmach et al., 2009; Stalmach et al., 2010). The absorption of quercetin-derivatives in the small intestine was dependent on the chemical structure of the compound itself. For example, the quercetin aglycone was poorly absorbed (about 20%) whereas quercetin-mono-glucosides were better absorbed reaching the 50% of the ingested dose (Hollman et al., 1995; Olthof et al., 2000). Therefore, unabsorbed phenolic compounds reach the colon, where the gut microbiota may metabolize the parent phenolic compounds producing a broad assortment of low-molecular weight phenolic acids, mainly hydroxyphenylacetic, hydroxyphenylpropanoic and hydroxybenzoic acids (Carregosa et al., 2022).

Colon metabolism of glycosylated quercetin-derivatives begins with the hydrolysis of the glucoside moieties, releasing the corresponding aglycone that undergoes C-ring fission generating 3-(hydroxyphenyl)cinnammic acids that can be further reduced to 3-(hydroxyphenyl)propanoic acids. These last compounds can be further oxidized to generate 3-(hydroxyphenyl)acetic acids and hydroxybenzoic acid (**Figure 3A**) (Cattivelli et al., 2023; Di Pede et al., 2020; Serra et al., 2012). Similarly, the colon metabolism of chlorogenic acids starts with the hydrolysis of the ester bond between quinic acid and hydroxycinnamic acids and these last intermediates are further reduced to 3-(hydroxyphenyl)propanoic acids (**Figure 3B**) (Serra et al., 2012; Nissen et al., 2022). Thus, the most important metabolites identified after colon fermentation of glycosylated quercetin-derivatives where quercetin aglycone, 3-(3’,4’-dihydroxyphenyl)acetic acid, 3-(3’-hydroxyphenyl)acetic acid and 3-(3’-hydroxyphenyl)propanoic acid whereas colon metabolism of chlorogenic acids mainly results in the accumulation of 3-(3’-hydroxyphenyl)propanoic acid (Cattivelli et al., 2023; Di Pede et al., 2020; Juániz et al., 2016; Nissen et al., 2022; Serra et al., 2012).

Therefore, it can be inferred that both parent phenolic compounds and their colon metabolites co-exist in the colon. Moreover, the relative amount of parent phenolic compounds and produced metabolites is greatly influenced by the individual composition of gut microbiota showing great interindividual variability (Almeida et al., 2018; Iglesias-Aguirre et al., 2021).

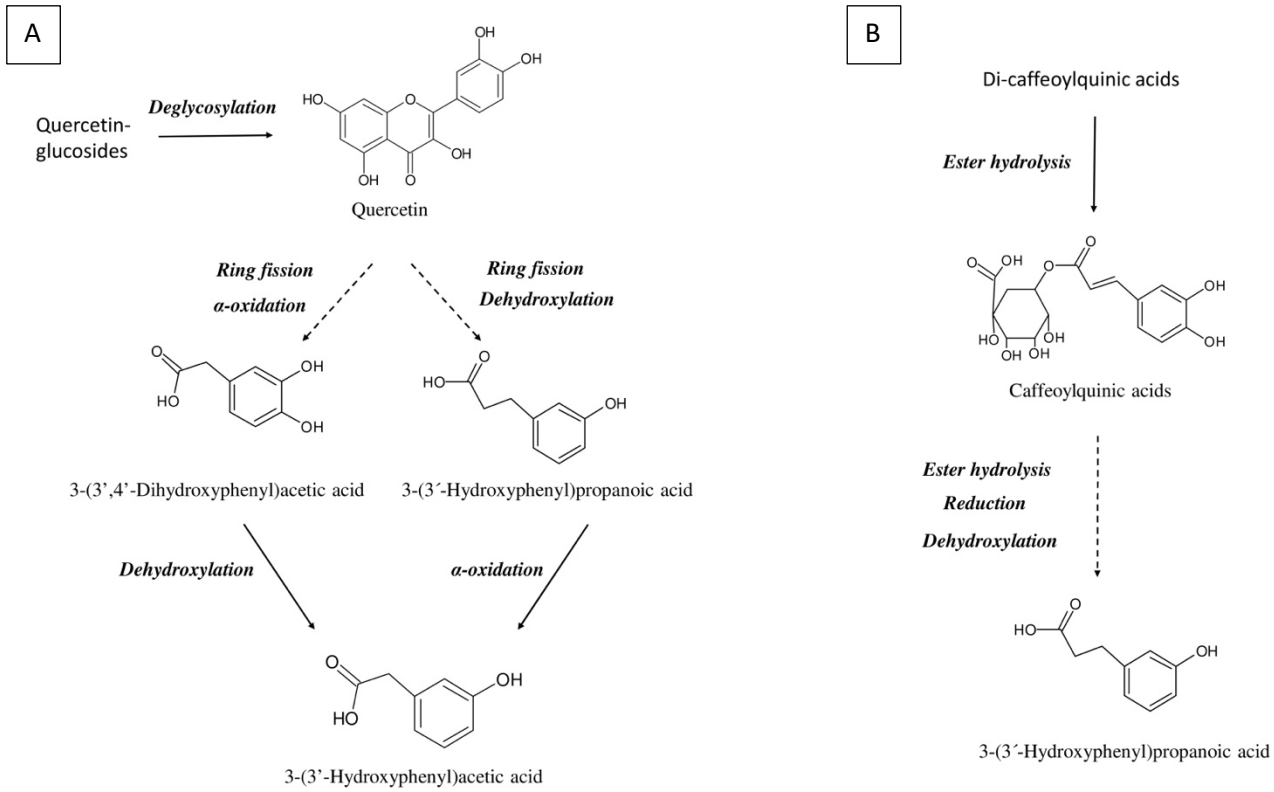


Figure 3. Simplified view of the colon metabolism of glycosylated quercetin-derivatives and chlorogenic acids

A comparison of the data previously reported in *in vitro* studies of colonic fermentation of quercetin and quercetin-derivatives highlighted this great interindividual variation. For example, Juániz et al. (2016) and Cattivelli et al. (2023) identified 3-(3'-hydroxyphenyl)propanoic acid as the main metabolite produced after faecal fermentation of glycosylated quercetins in green pepper and red-skinned onion. On the other hand, Di Pede et al. (2020) pointed out 3-(3',4'-dihydroxyphenyl)acetic acid as the metabolite generated in higher amount after quercetin fermentation by gut microbiota. Instead, Serra et al. (2012) detected 3-(3'-hydroxyphenyl)acetic acid as the major colon metabolite after fermentation of quercetin and quercetin-rhamnoside and almost equimolar amounts of 3-(3'-hydroxyphenyl)acetic acid and 3-(3',4'-dihydroxyphenyl)acetic acid after fermentation of quercetin-rutinoside. In another study Jaganath et al. (2009) compared the colon metabolism of quercetin-rutinoside among three individual donors. Only one donor was able to produce both 3-(3'-hydroxyphenyl)acetic acid and 3-(3',4'-dihydroxyphenyl)acetic acid whereas the other two donors generated only 3-(3',4'-dihydroxyphenyl)acetic acid. There were also differences in the rate of quercetin metabolism. In some studies quercetin aglycone was still present in high concentration after 5-6 hours of fermentation whereas in others quercetin completely disappeared after few hours of faecal fermentation (Aura et al., 2002; Cattivelli et al., 2023; Di Pede et al., 2020; Jaganath et al., 2009; Juániz et al., 2016). Therefore, interindividual variability determined not only the type and concentration of metabolites but also the rate of bioconversion of quercetin. Differently chlorogenic acid metabolism seemed to be less sensitive to interindividual variability being 3-(3'-hydroxyphenyl)propanoic

acid identified as the main metabolites in the majority of the studies (Gonthier et al., 2006; Juániz et al., 2017; Nissen et al., 2022). However, also in this case there were differences in the metabolism rate. In some studies, chlorogenic acids totally disappeared after few hours of incubation with colon bacteria whereas in other studies they were found still present after 10 or 24 h of incubation (Gonthier et al., 2006; Juániz et al., 2017; Ludwig et al., 2013; Mills et al., 2015; Nissen et al., 2022).

In this study we found that both parent compounds (i.e., glycosylated quercetin-derivatives, quercetin aglycone and chlorogenic acids) as well as their most important metabolites were able to inhibit colon cancer cell proliferation at concentration of 100 µmol/L. The tested concentration of 100 µmol/L is relevant from a physiological point-of-view, especially for the colon metabolites.

Previous studies reported on human faecal water or matrix reported a broad range of concentration for the tested colon metabolites, according to the interindividual variability. Karlsson et al. (2005) reported a concentration of 61 ± 34 µmol/L in human faecal water whereas Jenner et al. (2005) stated a mean value of 68 ± 93 µmol/L (range 0.72-209 µmol/L) for the colon metabolite 3-(3'-hydroxyphenyl)propanoic acid. Higher values were described by Knust et al. (2006) ranging from 15 to 1076 µmol/L in faecal matrix. Lower concentrations were found for the colon metabolite 3-(3'-hydroxyphenyl)acetic acid (mean values from 30 to 110 µmol/L), with a wide range among individuals (from 4 to 294 µmol/L) and for 3-(3',4'-dihydroxyphenyl)acetic acid (mean values from 7 to 64 µmol/L) with an individual range from 1 to 277 µmol/L (Jenner et al., 2005; Karlsson et al., 2005; Knust et al., 2006). It is important to note that the discussed studies have been carried out without any dietary restriction or supplementation representing values commonly found in a normal dietary regimen. Supplementation of phenolic-rich foods such as raspberry may further increase the amount of phenolic metabolites as observed by Gill et al. (2010) that found maximum values of 1810, 442 and 327 µmol/L of 3-(3'-hydroxyphenyl)propanoic acid, 3-(3',4'-dihydroxyphenyl)acetic acid and 3-(3'-hydroxyphenyl)acetic acid. No available data were present in ,literature about the faecal concentration of quercetin and its derivatives as well as of chlorogenic acids. However, as reported above, *in vitro* studies suggested their presence in colon, in co-existence with their colon metabolites, especially following repeated ingestion of quercetin-rich or chlorogenic acids-rich foods.

The anti-proliferative effect was time-dependent and increased in conjunction with increasing incubation time. The parent compounds 3,5-di-*O*-caffeoylquinic acid, 3-*O*-caffeoylquinic acid and 5-*O*-caffeoylquinic acid as well as the metabolite 3-(3',4'-dihydroxyphenyl)acetic acid were the most active against Caco-2 cell proliferation at any incubation time. On the contrary, the parent compounds quercetin, quercetin-4'-*O*-glucoside and quercetin-3-*O*-glucoside-4'-*O*-glucoside as well as the metabolites 3-(3',4'-dihydroxyphenyl)acetic acid and 3-(3'-hydroxyphenyl)acetic acid were the most active against SW480 cell proliferation.

In Caco-2 cells the extent of the anti-proliferative effect seemed related to the presence of specific structural motifs in the analysed compounds. The most active molecules, i.e., 3,5-di-*O*-caffeoylquinic acid, 3-*O*-

caffeoylquinic acid, 5-*O*-caffeoylquinic acid, 3-(3',4'-dihydroxyphenyl)acetic acid and quercetin displayed in their structure a catechol-type moiety, which was fundamental for exerting an anti-proliferative effect. For example, 3-(3',4'-dihydroxyphenyl)acetic acid bearing a catechol moiety was clearly more active than the mono-hydroxylate 3-(3'-hydroxyphenyl)acetic acid. Similarly, quercetin showed a higher anti-proliferative activity than the glycosylated derivatives quercetin-4'-*O*-glucoside and quercetin-3-*O*-glucoside-4'-*O*-glucoside where one of the two hydroxyl groups of the catechol moiety is esterified with a glucose molecule. Also, the presence of one or more glucose residues hampered the anti-proliferative effect since quercetin showed a significant higher effect than quercetin-4'-*O*-glucoside whereas quercetin-3-*O*-glucoside-4'-*O*-glucoside was not active at any time. The effect of the tested compounds was quite different when incubated with SW480. In this case quercetin and its glycosylated derivatives together with the metabolites 3-(3',4'-dihydroxyphenyl)acetic acid and 3-(3'-hydroxyphenyl)acetic acid were the most active compounds whereas the tested chlorogenic acids, although they present a certain degree of inhibition, were less effective in inhibit SW480 proliferation.

In this case quercetin and its glycosylated derivatives were the most active compounds whereas the two tested chlorogenic acids and the metabolites, although they present a certain degree of inhibition, were less effective in inhibit SW480 proliferation.

Several previous studies reported the anti-proliferative effect of chlorogenic acids against human colon cancer cells. For example, 3-*O*-caffeoylquinic acid was found to reduce the proliferation of Caco-2 and HT29 colon cancer cells whereas dicaffeoylquinic acids inhibited the growth of HT29 and DLD-1 cancer cells (Ekbatan et al., 2018; Glei et al., 2006; LS & NJA, 2016). Moreover, 3-(3',4'-dihydroxyphenyl)acetic acid was found active against the proliferation of human colon cancer cells HCT116 and SNUC4 (Gao et al., 2006; Kim et al., 1999). Furthermore, quercetin was already found to be active against Caco-2 proliferation at concentrations similar to that tested in this study as well as in other colon cancer cell models (Dihal et al., 2006; Neamtu et al., 2022).

As already mentioned, parent phenolic compounds and metabolites may co-exist in the colon and the ratio parent phenolic compounds/metabolites is strongly dependent on the inter-individual variability related to the ability to metabolize slowly or rapidly the parent phenolic compounds. To better understand the potential impact of inter-individual variability different mixtures of parent compounds and metabolites were prepared to mimic the low-producers and high-producers metabotypes. The quercetin-derivatives/metabolites mixes behaved differently depending on the tested cell line. The cell growth inhibition increased passing from the mix with the low amount of metabolites (i.e. low-producers) to the mix with the highest amount of metabolites (i.e. high-producers) after incubation with Caco-2 cell line whereas the trend was opposite for SW480 cell line. However, the high-producers mix was still able to inhibit SW480 cell proliferation with values near to 50% at 200 µmol/L concentration. For the chlorogenic acids/metabolites mixes, the effect was more pronounced for the low-producers mix respect to the high-producers mix in Caco-

2 cells, nevertheless the inhibitory effect of high-producers mix was near to 50% at 200 µmol/L concentration. No difference in the anti-proliferative activity was observed in SW480 cell line with the three chlorogenic acids/metabolites mixes. Therefore, our data supported the hypothesis that parent phenolic compounds (i.e. quercetin-derivatives and chlorogenic acids), once arrived at the colon after the consumption of phenolic-rich vegetables, may exert anti-proliferative activity against colon cancer cells. Later, when they begin to be metabolized, the formed colon metabolites may sustain themselves and prolong the inhibitory effect towards the proliferation of cancerous cells.

The different tested phenolic compounds and metabolites displayed different stability in the cell media and were differently metabolized accordingly to their structure and the specific cell line. Quercetin and glycosylated quercetin-derivatives were strongly metabolized by SW480 cells whereas no newly formed metabolites were detected after incubation with Caco-2 cells. In SW480 cells, quercetin-3-*O*-glucoside-4'-*O*-glucoside underwent extensive de-glycosylation suggesting the presence in these cells of a membrane-bound β-glucosidase. Moreover, the appearance of only the 3-O-glucoside derivative of quercetin suggested a strong preference of this glucosidase for the glycosidic linkage at 4'-*O* position rather than 3-*O* position. This specificity was confirmed also by the data about quercetin-4'-*O*-glucoside metabolism by SW480. This compound was rapidly hydrolyzed after 24 h in an equimolar amount of the corresponding aglycone quercetin. Furthermore, the fastest de-glycosylation of quercetin-4'-*O*-glucoside respect to quercetin-3-*O*-glucoside-4'-*O*-glucoside indicated that the presence of a 3-*O*-glucoside moiety in the molecule hampered the hydrolyzing ability of SW480 β-glucosidase. The appearance of small amount of quercetin after 48 h of incubation of quercetin-3-*O*-glucoside-4'-*O*-glucoside pointed out the presence also of a β-glucosidase with specificity towards the 3-*O*-glucosidic linkage or the ability of the same β-glucosidase to hydrolyze with less efficacy also the 3-*O*-glucosidic linkage. Additional metabolites, and in particular isorhamnetin, 4'-*O*-methyl quercetin and quercetin-*O*-sulphate were detected after incubation of quercetin and its derivatives with SW480 cell lines suggesting the presence in this cell line also of a catechol-*O*-methyltransferase and a sulfotransferase. The appearance of higher amount of isorhamnetin respect to 4'-*O*-methyl quercetin pointed out a preference for the OH in position C3 of the catechol-*O*-methyltransferase. Due to the lack of authentic standard, it was not possible to identify the specific isomer of quercetin-sulphate. On the contrary, no β-glucosidase as well as catechol-*O*-methyltransferase and sulfotransferase activity was instead observed in the Caco-2 cells after incubation with quercetin and glycosylated quercetin-derivatives. In this cell line, quercetin-3-*O*-glucoside-4'-*O*-glucoside was stable in the cell media with a 100% recovery also after 72 h of incubation whereas quercetin and quercetin-4'-*O*-glucoside rapidly disappeared already after 24 h of incubation. Since no newly formed metabolites neither the known quercetin oxidation products were detected in the cell media the most plausible explanation was that quercetin and quercetin-4'-*O*-glucoside were rapidly absorbed by Caco-2 cells. Previous studies demonstrated active absorption of quercetin and quercetin-4'-*O*-glucoside by Caco-2 cells (Walgren et al., 2000; Zhang et al., 2020). The same consideration

can be made for SW480 cells where the final recovery of quercetin and its metabolites after 72 h of incubation of quercetin and quercetin-4'-*O*-glucoside was below the 100%.

The cell metabolism of glycosylated quercetin may have an impact on their anti-proliferative activity. In SW480, quercetin was the most active compound and can be considered also responsible for the anti-proliferative activity of quercetin-4'-*O*-glucoside since this last compound is rapidly hydrolysed to the corresponding aglycone. Similarly, quercetin-3-*O*-glucoside-4'-*O*-glucoside was hydrolysed to quercetin-3-*O*-glucoside, which can be responsible for the anti-proliferative effect recorded after incubation of SW480 with quercetin-3-*O*-glucoside-4'-*O*-glucoside. The anti-proliferative activity of quercetin-3-*O*-glucoside was already reported by several authors (C. Maiyo et al., 2015; Ju You et al., 2010).

Likewise to what noted for quercetins, also chlorogenic acids were extensively metabolized by SW480 whereas no metabolites were found after incubation with Caco-2. In addition to the obvious isomerization (acyl migration) which is dependent on the pH and not on the presence of cells (Gómez-Juaristi et al., 2020), both 3,5-di-*O*-caffeoylquinic acid and the two caffeoylquinic acids (and their isomers) were hydrolysed to the corresponding caffeoylquinic acids and caffeic acid, suggesting the presence of esterase activity in SW480 cells (Martini et al., 2019). Moreover, caffeoylquinic acids were methylated by SW480 catechol-*O*-methyltransferase producing different isomers of feruloylquinic acids. In Caco-2 cell line, 3,5-di-*O*-caffeoylquinic acid, 3-*O*-caffeoylquinic acid and 5-*O*-caffeoylquinic acid almost totally disappeared from the cell media already after 24 h of incubation. Some studies suggest that caffeoylquinic and dicaffeoylquinic acids can be transported in Caco-2 cells (Gómez-Juaristi et al., 2020; Konishi & Kobayashi, 2004).

Concerning the phenolic metabolites, both 3-(3'-hydroxyphenyl)acetic acid and 3-(3'-hydroxyphenyl)propanoic acid were stable after incubation with Caco-2 and SW480 with recoveries of more than 80% also after 72 h of incubation. On the contrary, 3-(3',4'-dihydroxyphenyl)acetic acid concentration dropped to zero already after 24 h of incubation with Caco-2 cells. The only identified metabolites was hydroxybenzoic acid, which accounted for about the 30% of the initial amount of 3-(3',4'-dihydroxyphenyl)acetic after 72 h of incubation. Possibly this metabolite was formed by two consecutive reactions of de-hydroxylation and α-oxidation (or vice versa), which implied the uptake of 3-(3',4'-dihydroxyphenyl)acetic in Caco-2 cells, as already suggested (Konishi, 2005).

5. Conclusion

For the first time, the anti-proliferative activity of the dietary most relevant chlorogenic acids, quercetin-derivatives, and their colon microbial metabolites against two different human colon cancer cell models was tested and compared. Both the parent compounds and the metabolites were able to inhibit cell proliferation at physiologically relevant concentrations, attainable in the colon after the consumption of phenolic-rich foods. The anti-proliferative activity against Caco-2 was related to the presence of a catechol moiety in the structure of the molecules whereas quercetin and glycosylated quercetin-derivatives were the most active against SW480. Generally, reported data demonstrate that *in vivo* pertinent mixtures of parent compounds and metabolites at concentrations attainable in human colon following the consumption of phenolic-rich vegetable foods exert anti-proliferative effects against colon cancer adenocarcinoma cell lines. Since the anti-proliferative activity was nearly comparable between mixes that mirror the low-producers and high-producers human metabotypes, it can be speculate that inter-individual variability does not substantially affect the potential *in vivo* anti-cancer activity of phenolic compounds.

Tested compounds were metabolized by SW480 more extensively and rapidly than Caco-2 cell line. Sometimes, the cell metabolism may be related to the anti-proliferative effect such as in the case of quercetin-3-O-glucoside-4'-O-glucoside and quercetin-4'-O-glucoside. These results suggest that gut microbiota metabolites may sustain the anti-proliferative effects observed for the parent compounds, playing an important role in the protective effect of phenolic compounds against colon cancer.

6. References

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CONCLUSION

Conclusion

Red-skinned onion and dark purple eggplant, as two typical foods of the Mediterranean diet and rich in phenolic compounds, have been studied for their characteristics and health properties. These foods can be considered functional foods as they can act in the prevention of noncommunicable diseases. This study showed that for both red-skinned onion and dark purple eggplant, heat treatments have an important impact on the stability and release of phenolic compounds during digestion. For example, for eggplant, excluding frying all other cooking methods caused degradation of individual phenolic compounds. Certain classes of compounds (such as di-caffeoylquinic acids and hydroxycinnamic acids-amides) were found to be more easily degraded by cooking while others were more stable. However, cooking was crucial for the release of phenolic compounds during digestion. In addition to increasing the bioaccessibility of phenolic compounds during digestion, different cooking techniques greatly influenced the phenolic profile of the sample. Frying, for example, promoted the release of di-caffeoylquinic acids and hydroxycinnamic acid-amides while other treatments that of caffeoylquinic acids. Moreover, either cooking or digestion of dark purple eggplant induced isomerization reactions of caffeoylquinic and di-caffeoylquinic acids, which determined changes among the several stereoisomeric forms. These differences among the different samples indicate that, depending on the type of cooking, as the phenolic profile of the sample changes, the biological effects exerted after consumption could be different. The same results were also found with the onion samples. In fact, again, based on the initial fresh weight, all treatments, apart from frying, caused a decrease in phenolic compounds compared with the raw sample. After *in vitro* digestion, baking for both the onion varieties (yellow-skinned and red-skinned onion) and grilling for red-skinned onion significantly increased the bioaccessibility of onion phenolic compounds, fostering their delivery from the onion matrices. For both the onion varieties, baking was the thermal treatment that provided the highest amount of phenolic compounds after *in vitro* digestion. In addition, baking and grilling caused an increase in the bioaccessibility of quercetin-3-*O*-glucoside-4'-*O*-glucoside, which has known antiproliferative properties against colonic adenocarcinoma cell lines.

Studies on colonic fermentation then showed that the different cooking procedures affects the intestinal microbiota differently. For dark purple eggplant, the gut microbiota showed considerable metabolic activity that led to a profound change in the chemical structure of the phenolic compounds in eggplant.

The study of colonic metabolism made it possible to identify the main colonic metabolic pathways of the different compounds present in dark purple eggplant, which all converged toward the production of 3-(3'-hydroxyphenyl)propanoic acid, regardless of the samples. Fried dark purple eggplant showed the highest amount of metabolites. In addition, colonic fermentation of fried dark purple eggplant led to a significant increase in many beneficial taxa. A positive correlation between the most important phenolic metabolites

and *Bifidobacteriaceae*, *B. longum* and Clostridium group IV suggests their involvement in colonic metabolism of hydroxycinnamic acids.

Regarding the red-skinned onion samples, the degradation driven by colon microbiota of flavonols, mainly quercetins, resulted in the accumulation of three main metabolites, 3-(3'-hydroxyphenyl)propanoic acid, 3-(3'-hydroxyphenyl)acetic acid and 3-(3',4'-dihydroxyphenyl)acetic acid, with some qualitative and quantitative differences among the samples. Colonic fermentation of raw onions resulted in a substantial increase in beneficial taxa, larger in respect to the heat-treated onions, particularly on *Lactobacillales* and beneficial clostridia. Moreover, in addition to promoting the development of beneficial taxa, raw onion promoted the inhibition of some opportunistic taxa such as *Clostridium perfringens* group and *E. coli*. In contrast, cooked onions triggered the increase in F/B ratio at the level of a dysbiotic situation.

Therefore, the composition of the gut microbiota and its metabolic activity may be modulated not only by cooking treatments but also by the type of food and the profile of phenolic compounds. Therefore, further studies are needed to find out the effect of different foods in combination with various cooking techniques on the diversity of the gut microbiota and the metabolism of phenolic compounds.

Furthermore, since the amount and type of phenolic metabolites and the composition of the colonic microbiota influence human health, the results presented suggest that the application of different cooking methods may have distinctive effects on human health.

In the fifth study, once again, it was shown how crucial cooking can be in modulating the bioactive profile of a food. The results proved that all the red-skinned onion samples presented some inhibitory activity against the three key enzymes involved in the diabetes pathogenesis but to a different extent depending on the cooking method and phenolic profile. For example, the flavonol class was the most active against two of the three key enzymes (α-glucosidase and DPP-IV). Furthermore, through statistical analysis, a significant correlation was identified between the amount of quercetin mono-hexosides (mainly quercetin-4'-*O*-glucoside) and the IC_{50} value against α-glucosidase and the amount of quercetin di-hexosides (mainly quercetin-3-*O*-glucoside-4'-*O*-glucoside) and the IC_{50} against DPP-IV. From here, it is evident that a careful design of the cooking methods can be critical in suggesting the most suitable process to optimize the release of specific phenolic compounds and presumed bioactivities.

Finally, we decided to test and compare the anti-proliferative activity of the dietary most relevant chlorogenic acids, quercetins (the classes of compounds found in higher amounts in onion and eggplant samples) and their colon microbial metabolites against two different human colon cancer cell models Caco-2 and SW480. Results showed that both the original compounds and metabolites inhibit cell proliferation at physiologically relevant concentrations, attainable in the colon after the consumption of phenolic-rich foods. The anti-proliferative activity against Caco-2 was related to the presence of a catechol moiety in the structure of the molecules whereas quercetin and glycosylated quercetin-derivatives were the most active against SW480.

Later we also decided to study cellular metabolism. The results showed that the SW80 cell line was able to metabolize compounds much faster than the Caco-2 cell line. Furthermore, it has been seen that sometimes the antiproliferative effect is related to cell metabolism (e.g., in quercetin-3-*O*-glucoside-4'-*O*-glucoside and quercetin-4'-*O*-glucoside). These results suggest that gut microbiota metabolites may support the antiproliferative effects observed for the parent compounds, playing an important role in the protective effect of phenolic compounds against colon cancer.

Although *in vivo* studies are strongly needed and requested, the data presented in this thesis support the conclusion that different cooking procedure may modulate the phenolic profile of vegetable foods as well as their release during digestion and the studied bioactivities. Therefore, food cooking is important not only from a nutritional and social point of view but may be of paramount importance also in determining the *in vivo* healthy properties of phenolic-rich vegetable foods.

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