Università degli studi del Piemonte Orientale
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Dipartimento di Scienze del Farmaco

Dottorato di ricerca in Food, Health and Longevity
XXXV ciclo – 2019-2022

Rice as natural functional food for a healthy nutrition: characterization, technological aspects and bioaccessibility

SSD CHIM/10

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Thesis

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Summary

Pigmented rice varieties are characterized by a specific color (red, black or purple) due to the presence of pigments in the outer layers of the bran and are therefore consumed in unmilled form. The black rice varieties are considered a sort of natural functional food due to their significant content of polyphenols, especially anthocyanins, and their antioxidant properties. The main black rice varieties cultivated in Italy are Artemide, Nerone and Venere. Polyphenols are a huge heterogeneous group of molecules belonging to different chemical classes able to exert a beneficial action on the human organism, which depends not only on their quantity present in the diet, but also on their bioavailability and bioaccessibility. So, it's important to study how technological (such as cooking) and digestive processes could impact on these healthy properties. A general introduction of the topic is given in **Chapter 1**.

The principal aims of this thesis work, reported in **Chapter 2**, are:

1) to characterize the chemical-nutritional profile of the pigmented Italian Artemide black rice;

2) to evaluate *in vitro* the impact of processing technologies, such as cooking, on the chemical composition and on the stability of bioactive molecules;

3) to evaluate the effect of the digestion process on cooked rice bioactive compounds, through the *in vitro* INFOGEST simulated digestion protocol;

4) to evaluate *in vivo* the impact of consumption of different rice varieties, cooked in various ways, on the health of people with pathological conditions, such as diabetes;

5) to subject these cooked rice varieties to simulated digestion and subsequent faecal fermentation in batches, to observe their effect on a healthy microbiota.

In **Chapter 3** the chemical and nutritional characterization of the Italian Artemide black rice was carried out. Then, the rice was subjected to four different cooking methods (boiling, microwaves oven, under pressure pot and risotto preparation) to evaluate the impact of cooking. Proteins and ashes content didn't change after cooking, while total dietary fiber, after all cooking methods, decreases probably because cooking processes led to the degradation of some fiber components. The risotto mode, with a toasting phase without oil, was confirmed as the best cooking method in preserving antioxidant capacity, polyphenols, and anthocyanin content, while boiling turned out to be the worst, due to the solubilization part of polyphenolic compounds in the exceeding cooking water.

In **Chapter 4** also innovative cooking methods, such as *sous vide* cooking, were considered and compared with traditional domestic techniques (risotto and pilaf). The *sous vide* mode at 89 °C and the risotto mode were the more efficient cooking methods to preserve anthocyanins, total polyphenols content and antioxidant capacity, while no particular differences between cooking methods were observed regarding their impact on the protein fraction. It is interesting to note that on one hand we have a traditional cooking method (risotto), easily reproducible at home, while on the other hand an innovative one (*sous vide* at 89 °C) that, even though not so easy at home, could be used in the canteens of schools, hospitals and Companies.

In **Chapter 5** the impact of the digestive process on the polyphenolic fraction of Artemide black rice, subjected to the risotto preparation, was studied. Cooked rice was subjected to the *in vitro* INFOGEST simulated digestion process and the concentrations of anthocyanins, catechins and phenolic acids were quantified through RP-HPLC-DAD, also calculating the digestibility and bioaccessibility indexes. Anthocyanins were found to be stable up to the gastric level, while their concentrations decreased significantly in the intestine, with a consequent reduction also in the bioaccessibility values. The phenolic acids free fraction showed a reduction in its concentration in the rice insoluble portion and a simultaneous increase in the soluble portion during the digestion, while the bound phenolic acids remain almost unchanged after digestion. The flavonoids fraction showed an increase in the concentration in free form, with a consequent probable increase in their bioaccessibility, during the digestion. In general, our results allowed to highlight the main changes in the polyphenolic composition of one variety of black rice after cooking and digestion.

In **Chapter 6** we want to compare the impact on the postprandial glycaemic trend in type 1 diabetic children and adolescents of two different types of rice (“Gigante Vercelli” white rice, and “Artemide” black rice), cooked in different modes (risotto vs boiled). The diabetic children use an advanced hybrid closed loop (AHCL) system (Tandem ControlIQ^{TM}) for insulin control. The different rice varieties and cooking methods impacted differently on the glycaemic trend. In particular, Artemide black rice gave the best post-prandial glycaemic values, while “Gigante Vercelli” white rice gave a lower glycaemic peak if cooked by boiling than risotto. This agrees with the minor content of soluble starch and dietary fiber determined in the boiled sample of white rice.

In **Chapter 7** a faecal batch fermentation of cooked and treated with simulated digestion black and white rice varieties was conducted. The aim was to evaluate rice impact on a

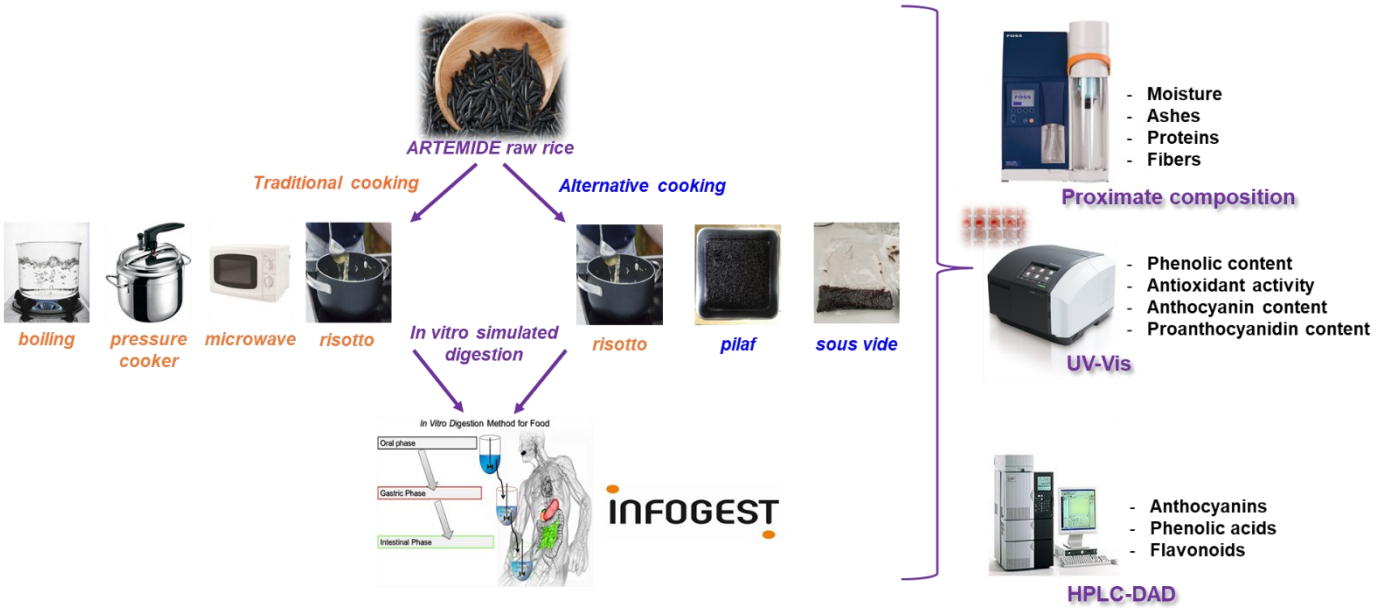
healthy microbiota, through the quantification via GC-FID of SCFAs (Short Chain Fatty Acids), positive and desired indicators of the microbiota metabolism. Significant differences in the SCFAs production between black and white rice fermentations were found. The elevated amounts of acetate and propionate detected in the Artemide black rice fermented sample, compared to the white rice samples, could be related to its higher content in total dietary fiber and polyphenols that are well known to influence the microbiota and thus to increase the production of SCFAs.

The conclusions and future perspectives of the study were proposed in **Chapter 8**, while in **Figure 1** a schematic representation of the aims and the obtained results of each Chapter is reported.

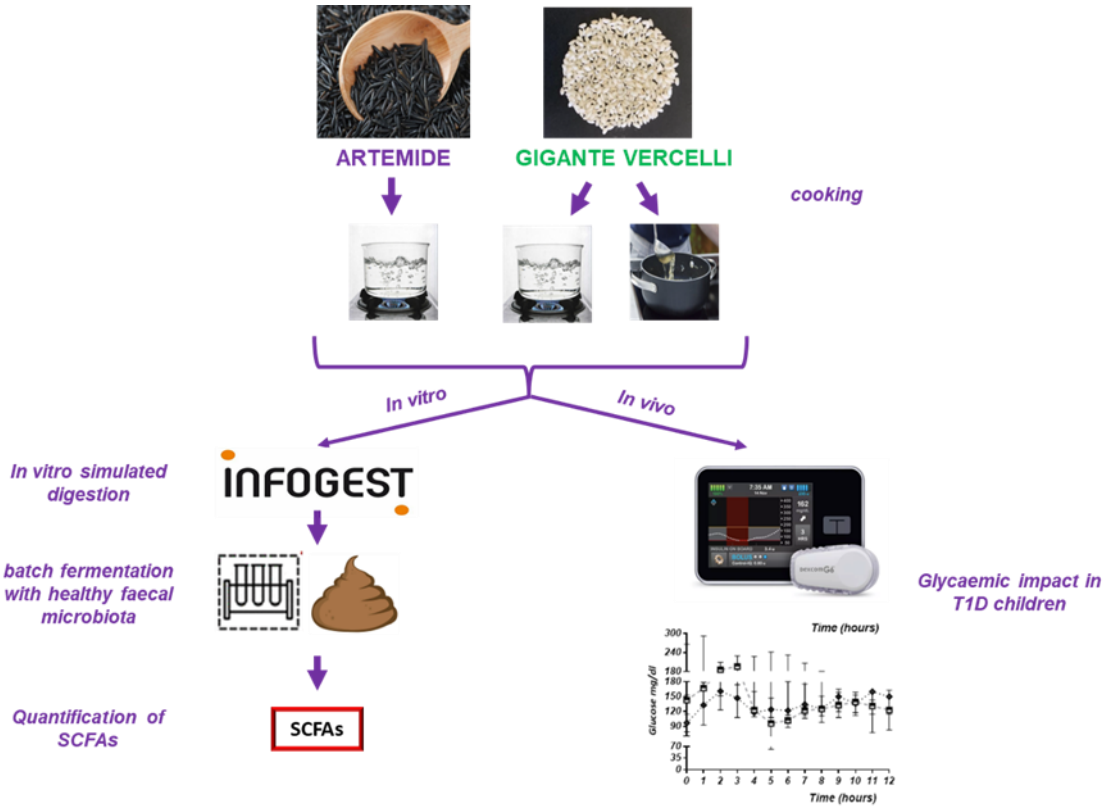
Rice as natural functional food for a healthy nutrition: characterization, technological aspects and bioaccessibility

Chapter 1	*General introduction*	
Chapter 2	*Aims of the thesis*	
Chapter 3	*Artemide black rice: impact of classic cooking methods on polyphenolic and nutritional profile*	*Risotto preparation (with a toasting phase) is the best cooking method to preserve polyphenols*
Chapter 4	*Artemide black rice: comparison between the impact of traditional and innovative cooking*	*Risotto preparation and sous vide at 89 °C are the most performing cooking methods*
Chapter 5	*Artemide black rice: impact of the digestive process on the polyphenolic fraction*	*Anthocyanins stable up to the gastric level. Other flavonoids increased their bioavailability during digestion.*
Chapter 6	*Postprandial glycaemic trend in type 1 diabetic children after the consumption of black or white rice cooked in different ways*	*Black rice -> best post-prandial glycaemic values. White rice -> better if cooked by boiling than risotto.*
Chapter 7	*The impact of cooked and digested rice on the gut microbiota through the determination of SCFAs production*	*Black rice -> higher production of SCFAs White rice -> no differences in SCFAs production between cooking methods*
Chapter 8	*Conclusions and future perspectives*	

Infographic (chapters 3, 4 and 5)



Infographic (chapters 6 and 7)



Sommario

I risi pigmentati sono varietà di riso caratterizzate da uno specifico colore (rosso, nero o viola) dovuto alla presenza di pigmenti negli strati esterni della cariosside, motivo per cui questi risi vengono consumati in forma integrale. Le varietà di riso nero sono considerate come una sorta di alimento naturalmente funzionale, grazie alle loro proprietà antiossidanti e al loro considerevole contenuto di polifenoli, soprattutto antociani. Le principali varietà di riso nero coltivate in Italia sono l'Artemide, il Nerone ed il Venere. I polifenoli sono un vasto gruppo di molecole, di diversa natura chimica, in grado di esercitare un'azione benefica sull'organismo umano, che dipende non solo dalla loro quantità presente nella dieta, ma anche dalla loro biodisponibilità e bioaccessibilità. Quindi, è importante studiare come i processi tecnologici (come la cottura) e quelli digestivi possano influire su queste proprietà salutari. Nel **Capitolo 1** si presenta un'introduzione generale dell'argomento.

Gli obiettivi primari del lavoro di tesi, riportati nel **Capitolo 2**, sono:

1) caratterizzare il profilo chimico-nutrizionale del riso nero Artemide;

2) valutare *in vitro* l'impatto dei processi tecnologici, come la cottura, sulla composizione chimica e sulla stabilità delle molecole bioattive;

3) valutare l'effetto del processo di digestione sui bioattivi del riso cotto, attraverso il protocollo INFOGEST di digestione simulata *in vitro*;

4) valutare *in vivo* l'impatto del consumo di diverse varietà di riso, cotto in vari modi, sulla salute di persone con condizioni patologiche, come il diabete;

5) sottoporre queste varietà di riso cotto a digestione simulata e successiva fermentazione fecale per osservarne l'effetto su un microbiota sano.

Nel **Capitolo 3** è stata effettuata la caratterizzazione chimica e nutrizionale del riso nero Artemide come riso crudo nella sua forma di commercializzazione. Successivamente, il riso è stato sottoposto a quattro diversi processi di cottura (bollitura, forno a microonde, pentola a pressione e risotto) per valutare l'impatto della stessa. Il contenuto di proteine e ceneri non è cambiato in seguito a cottura, mentre la fibra totale, con tutti i metodi di cottura, è diminuita, probabilmente perché la cottura ha degradato alcune componenti della fibra. La modalità risotto, con fase di tostatura a secco (senza l'aggiunta di lipide), è risultata il metodo di cottura più efficace nel preservare la capacità antiossidante, i polifenoli e il contenuto di antociani nel riso, mentre la bollitura si è rivelata la peggiore, in quanto parte dei composti polifenolici si sono solubilizzati nell'acqua di cottura che è stata eliminata.

Nel **Capitolo 4** sono stati considerati anche metodi di cottura innovativi, come la cottura *sous vide*, confrontati con le tradizionali tecniche domestiche (risotto e pilaf). La modalità sottovuoto a 89 °C e la modalità risotto sono state le migliori per preservare antociani, contenuto di polifenoli totali e capacità antiossidante, mentre non sono state osservate particolari differenze, tra le modalità di cottura, per quanto riguarda il loro impatto sulla frazione proteica. È interessante notare che da un lato abbiamo un metodo di cottura tradizionale (il risotto), facilmente riproducibile in casa, mentre dall'altro uno innovativo (*sous vide* a 89 °C) che, pur non essendo di semplice realizzazione in ambito domestico, potrebbe essere utilizzato nelle mense di scuole, ospedali e aziende.

Nel **Capitolo 5** è stato studiato l'impatto del processo digestivo sulla frazione polifenolica del riso nero Artemide, cotto nella modalità risotto. Il riso cotto è stato sottoposto al processo di digestione simulata *in vitro* INFOGEST e le concentrazioni di antociani, catechine e acidi fenolici sono state determinate tramite RP-HPLC-DAD, permettendo inoltre il calcolo degli indici di digeribilità e di bioaccessibilità. Le antocianine sono risultate stabili fino a livello gastrico, mentre le loro concentrazioni sono diminuite sensibilmente a livello intestinale, con conseguente riduzione anche dei valori di bioaccessibilità. La concentrazione degli acidi fenolici, nella loro forma libera, è diminuita nella frazione insolubile, mentre è aumentata in quella solubile, durante la digestione. Viceversa, gli acidi fenolici della componente legata sono rimasti pressoché invariati anche al termine del processo digestivo. Altri flavonoidi quantificati (quali catechina ed epicatechina) hanno mostrato un aumento della concentrazione nella componente libera, con conseguente aumento della loro bioaccessibilità, durante le fasi digestive. Nel complesso, i nostri risultati hanno permesso di caratterizzare i principali cambiamenti nella composizione polifenolica di una varietà di riso nero in seguito a cottura e digestione.

Nel **Capitolo 6** è stato confrontato l'impatto di due diverse tipologie di riso (riso bianco “Gigante Vercelli” e riso nero “Artemide”), cucinate in due diverse modalità (risotto vs bollito), sull'andamento glicemico postprandiale in bambini e adolescenti affetti da diabete di tipo 1, che fanno uso di un avanzato sistema ibrido a circuito chiuso (AHCL) di erogazione di insulina (Tandem ControlIQ^{TM}). Le diverse varietà di riso e i diversi metodi di cottura hanno influito in maniera differente sull'andamento glicemico. In particolare, il riso nero ha dato i migliori valori glicemici post-prandiali, mentre il riso bianco ha prodotto un picco glicemico inferiore in seguito a bollitura, rispetto alla cottura in modalità risotto. Ciò può essere spiegato dal minor contenuto di amido solubile e fibra alimentare riscontrato nel campione bollito di riso bianco.

Nel **Capitolo 7** i campioni di riso nero e bianco, precedentemente cotti e trattati con digestione simulata, sono stati sottoposti ad una fermentazione fecale *in batch*. Lo scopo è stato quello di valutare il loro impatto su un microbiota sano, attraverso il rilevamento tramite GC-FID delle concentrazioni di di acidi grassi a catena corta (SCFAs), noti indicatori positivi e desiderati del metabolismo di un microbiota sano. Sono state riscontrate differenze significative nella produzione di SCFAs tra le fermentazioni del riso bianco e del riso nero. Le elevate quantità di acetato e propionato rilevate nel campione da fermentazione fecale di riso nero Artemide, rispetto ai campioni di riso bianco, si pensano correlate ad un contenuto più elevato in fibra e polifenoli, bioattivi ben noti per le loro proprietà di influenza sul microbiota e quindi validi per produrre un aumento di SCFAs.

Infine, nel **Capitolo 8** sono riportate le conclusioni e le prospettive future. Per una visualizzazione complessiva del progetto seguito, in **Figura 1** è riportata una rappresentazione schematica degli obiettivi e dei risultati ottenuti in ciascun capitolo.

Il riso come alimento funzionale per una nutrizione salutistica: caratterizzazione, aspetti tecnologici e bioaccessibilità

Capitolo 1	*Introduzione generale*	
Capitolo 2	*Obiettivi della tesi*	
Capitolo 3	*Riso Artemide: impatto di metodi di cottura classici sul profile nutrizionale e polifenolico*	*Il risotto (con fase di tostatura a secco) è il miglior metodo per preservare I polifenoli*
Capitolo 4	*Riso Artemide: confronto tra l'impatto di cotture tradzionali e innovative*	*Il risotto e la cottura sous vide a 89 °C sono i metodi più performanti per i parametri salutistici*
Capitolo 5	*Riso Artemide: impatto del processo digestivo sulla frazione polifenolica*	*Antocianine stabili fino a livello gastrico. Altri flavonoidi aumentano la loro biodisponibilità durante la digestione*
Capitolo 6	*Andamento glicemico post-prandiale in bambini con diabete di tipo 1, dopo il consume di riso nero o bianco, cotto in diverse modalità*	*Riso nero -> migliori valori di glicemia* *Riso bianco -> meglio bollito che risotto*
Capitolo 7	*Impatto del riso cotto e digerito sul microbiota: determinazione della produzione di acidi grassi a corta catena (SCFAs)*	*Riso nero bollito -> maggior produzione di SCFAs* *Riso bianco -> nessuna differenza nella produzione di SCFAs tra risotto e riso bollito*
Capitolo 8	*Conclusioni e prospettive future*	

Chapter 1

General introduction

1.1. Functional foods and ingredients, diets, and healthy nutrition

The human nutrition is a fundamental prerequisite for a healthy life. The selection (and the use) of safe and healthy foods in common diet can trigger positive effects both regarding the wellbeing and the reduction of food-related chronic diseases.

During the last decades, the concept of "functionality" in nutrition, moving from the functional "rheological or technological" properties of ingredients meaning to an overall "healthy-related" concept, become a standard reference worldwide. Despite the presence of well-recognized classes of healthy foods (e.g. foods added with vitamins, minerals and other compounds with physiological role, as described in Reg. EC 1925/2006), the concept of "functional food" is currently not covered by a specific regulatory framework in Europe, even if some international consensus-based definitions are available.

Functional foods can be defined as "*foods that have a potentially positive effect on health beyond basic nutrition, helping the promotion of optimal health conditions and reducing the risk of non-communicable diseases*" **[1]**.

The definition of "food" and "food ingredient" are reported in Reg. 1169/2011, anyway, this Regulation, focused on food labelling and information to the consumers, does not consider the definition of "functional ingredient".

Functional ingredients are "*the standardized and characterized preparations, fractions or extracts containing bioactive compounds of varying purity, that are used as ingredients by manufacturers in the food*" [2].

The natural foods containing molecules with healthy benefits (commonly reported as bioactive compounds) are not strictly functional foods, but they can be considered as a sort of "natural" functional foods. Salmon rich in natural DHA-rich lipids, or berries naturally reach in antioxidant anthocyanins are clear examples of these foods.

Moreover, isolating the healthy contributing fraction of these natural foods (often referring to "nutraceuticals", a "portmanteau" between nutrition and pharmaceuticals terms, concerning extracts from foods with healthy properties in concentrated form, as introduced by Stephen De Felice), and adding it to another food to enhance health positively, a functional food could be developed **[2]**. As anticipated, the class of food enriched with vitamins, minerals or other ingredients with physiological role can be considered a sub-class of functional foods.

A limit is related to the use of forbidden ingredients or ingredients used at not permitted quantity (as reported in Reg. EC 1169/2011). Another regulatory limit, in Europe, is represented by the use of Novel foods (and novel ingredients), as defined in Reg. 2283/2015.

For a long time various food ingredients, including plants, berries, herbs and fruits, have been used around the world to prevent or treat different diseases **[3],** particularly concerning the “traditional medicine”. Anyway, a concept must be highlighted: all foods (and so, also food supplements, enriched foods, and other food for special uses) are not allowed to treat diseases or to be defined as “agent” able to prevent pathologies, avoiding to be classified as “medicines” or “drugs”: food must to remain food, even if and when characterized by positive properties well established by in vivo scientific researches.

From the introduction of the term (and concept) of “functional food” (FOSHU, “Food for Specified Health Use”) in Japan in the 1980s, a lot of books, articles and chapters were published, demonstrating how much attention this topic has attracted **[4,5]**. Concerning the “natural containing bioactive molecules”, there are numerous functional foods and ingredients worldwide, belonging to different classes: dairy products, cereals, flaxseed, tea, fruits, vegetables, seafood, meat, egg, herbs and vitamin based **[2]**.

The development of functional ingredients, as well as the chemical-nutritional characterization of functional foods with a “healthy” connotation, is the basis of the new trend in healthy nutrition, even if the production of “novel foods” (also including the application of alternative new challenging technological approaches to “conventional” foods) represents a critical limit. The development of bio-accessible (and hopefully, bioavailable) formulations of ingredients specifically designed to improve the nutritional profile of foods dedicated to the healthy population (and also to subjects in a pathological status, even if always considered foods) will lead in the future toward the concept of the “personalized” nutrition, capable to improve a state of good health and wellness **[6,7]**.

In the last years the fundamental role of microbiota for human health is becoming increasingly evident and we are witnessing in what, in medicine, is called “microbiota revolution”. In fact, it is evident the correlation between the dysregulation of the gut microbiota composition (dysbiosis) and several diseases, which includes gastroenterological diseases such as inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), colon cancer, celiac disease, diarrhoea and colitis caused by *Clostridium difficile* infection (the only disorder for which it’s currently approved the microbial transplant),

but also inflammatory-based diseases like metabolic syndrome, obesity and diabetes or nephrological, gynaecological, urological, oncological, autoimmune, neurological (Parkinson and Alzheimer) and psychiatric (schizophrenia, autism, depression) disorders **[8-11]**.

As well known, the diet plays a key role in the modulation of the gut microbiota, which has been recently described as a key driver of the human health. The gut's microbiota composition and profile strictly depend on various factors such as diet, lifestyle, age, stress, use of drugs and pathological conditions (the so called "exposome": the sum of external factors that a person is exposed during lifetime) **[12,13]**. In regard to diet, it is known that Mediterranean diet leads an increase of some specific bacterial species, like *Faecalibacterium prausnitzii* and *Roseburia hominis*, contrarily promoting a decrease of other ones, such as *R. gnavus*, *R. torques* and *Bacteroides stercoris*. This modulation is correlated with an improvement of the microbial profile in the gut: so, a dietary intervention allying a Mediterranean diet may boost the development of a "healthy" microbiome **[14,15,16]**. We may therefore suppose that also other diets could be effective in the modulation of gut microbiota and consequently on human health; for example, diets rich in polyphenols compounds, such as anthocyanins, phenolic acids and flavonoids, or diet rich in lactic acid (from fermented foods, particularly fermented milks). Anyway, the resilience of the gut microbiota profile (apparently fixed at 3-4 years of age in humans) must be considered **[17]**. Moreover, as well established during the last years of research, the modulation of the gut must be strongly affected by the ingestion in the diet of prebiotics compounds (fructo-oligosaccharides, FOS; galacto-oligosaccharides, GOS; fructanes; xylo-oligosaccharides; human milk oligosaccharides, HMOs; bovine milk oligosaccharides, BMOs) allowing the release of short chain fatty acids SCFA, as propionic and butyric acids) able to work as mediator of signalling, also including the gut-brain axis **[18,19]**.

1.2. The interplays between polyphenols and gut microbiota

The metabolism of polyphenolic compounds strictly depends on the gut microbiota composition, which also determine their functionality in terms of modulation of the bioavailability. In fact, in some cases, the metabolization of polyphenols operated by the gut microbiota, generates more active metabolites. A clear example is related to the production of equol from the soya-isoflavone daidzein **[20]**. Equol is the active compounds in the gut; this kind of "metabolic activation" is common within the polyphenol class, but the same mechanisms can trigger negative effects, sometime reducing the activity of the "precursor" phenolic. On the other hand, as clear example of "other side of the medal", also the microbiota composition strictly depends on the action of polyphenols, as reported later. This bi-directional interplay is important to contribute to the gut's microbiota balance, finally affecting the human health **[21]**.

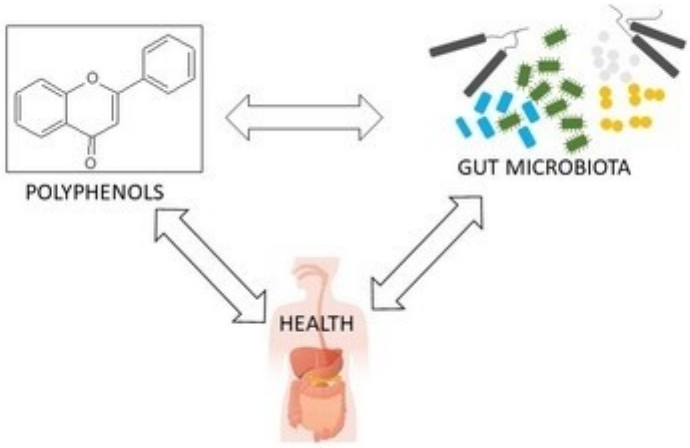


Figure 1. *Schematic representation of the Interaction between polyphenols, microbiota and human health* ***[22]****.*

Polyphenols are a huge heterogenous group of molecules belonging to different chemical classes: flavonoids, phenolic and cinnamic acids, tannins, lignans, stilbenes and coumarins. The ingestion of polyphenols in the human diet derives mainly from fruits, vegetables, tea, wine, coffee, cocoa, and cereals. However, polyphenols are not always able to exert a beneficial action, which depends not only on their quantity present in the diet, but also on their bioavailability and bioaccessibility, as well as on the biological activity of the metabolites that are formed during the digestion. Sometime, the bioavailability of the beneficial polyphenols is very limited. In this sense, the uniqueness of gut microbiota composition is

crucial. The same polyphenolic molecules could exert an effect (positive or negative) in one individual, while in another human there will be no effect **[22]**.

1.2.1. Flavonoids

Flavonoids represent a fundamental class of polyphenols compounds in foods. The impressive number of natural flavonoid structures, starting from a C6-C3-C6 skeleton, represents well the food biodiversity. Flavonols, flavones, isoflavones, flavanols and anthocyanins are different phenolics belonging to the flavonoids group. Depending on their chemical structure, each class of flavonoids differently interacts with the human gut microbiota. The different flavonoid classes have in common a basic chemical structure formed by two benzene rings (A and B), connected through a pyrone ring (C). Most flavonoid metabolism by the gut microbiota involves a cut on the C ring, and in **Figure 2** the possible cleavage points of flavonols, isoflavones, flavan-3-ols and anthocyanins are reported **[22]**.

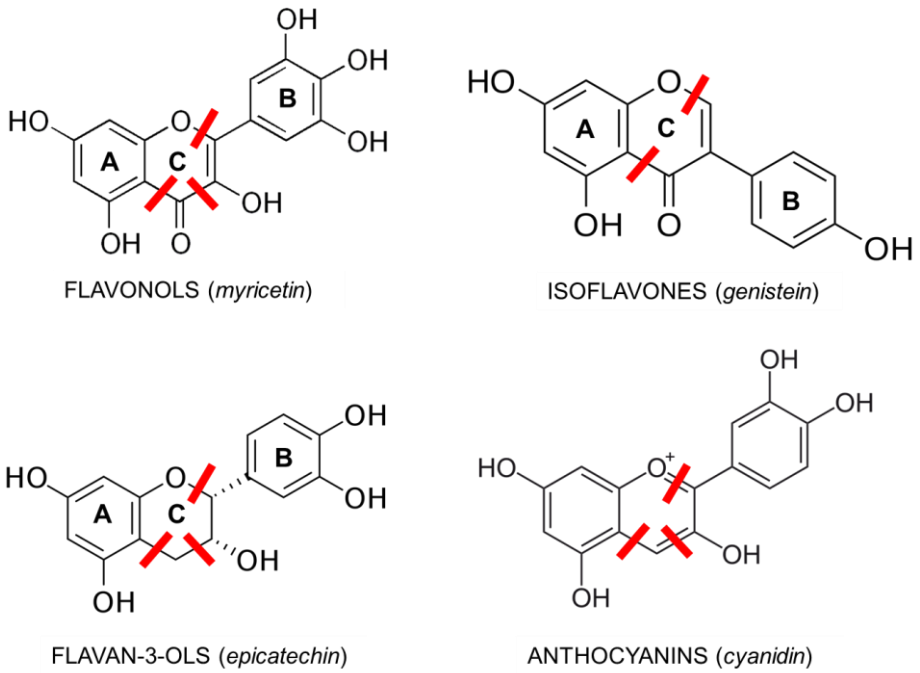


Figure 2. *General chemical structure of flavonols, isoflavones, flavan-3-ols and anthocyanins, and possible positions of cleavage on the C ring (modified from* ***[21]****).*

1.2.1.1. Flavonols

Flavonols are very common in plant food: broccoli (*Brassica oleracea*), asparagus (*Asparagus officinalis*), onions (*Allium cepa*), ginger (*Zingiber officinale*), leafy greens, tea and grape/red wine are foods particularly rich in flavonols. Myricetin, quercetin and kaempferol are the most commonly known flavonols recovered in foods **[22,23]**; they are often present in the form of glycosides. After the ingestion, their metabolism first involves the action of the microbial β-glucosidase, which leads to the formation/release of the aglycone. The metabolic pathway continues with the cut on the C ring of the structure, with the consequent delivery of phenols and benzoates in the environment. As referring example, the metabolism of the myricetin, which contains a tri-hydroxy ring-B, yields 2-(3-dihydroxyphenyl) acetic acid, 2-(3-hydroxyphenyl) acetic acid and 2-(3,4,5-trihydroxyphenyl) acetic acid **[24]**.

Different studies were conducted to investigate the impact of flavonols on the gut microbiota. Yan *et al.* investigated the effect of a green microalgae (*E. prolifera*) flavonols-rich fraction on the microbiota composition of diabetic mice. The Authors highlighted a significant increase in the relative abundance of *Alisties* spp, an important SCFAs-producing bacteria, and a concomitant improvement of glucose tolerance and a prevention of kidney and liver damage due to the anti-inflammatory action **[25]**. This example clearly shows how a flavonols-rich diet impacts on the microbiota profile in animals.

Cuervo *et al.* studied the flavonols effect on women suffering from systemic lupus erythematosus, fed with an orange and apple-rich diet (standardised to have 300 mg/day of flavonols). A general increase in the relative abundance of *Lactobacilli spp.* and *Bifidobacteria spp.* was observed in the microbiota biodiversity, with a consequent beneficial effect for patients, also modulating the host immune response **[26]**.

The *in vitro* human faecal batch fermentation of green tea flavonols, performed by Rha *et al.*, highlighted an increase of *Lactobacilli* spp. and *Bifidobacteria* spp., as well as an increase of SCFAs, positive indicators of the gut microbiota ecosystem **[27]**. All these examples confirm, first of all, the usefulness of the *in vitro* (and *in vivo*) investigations aimed to determine the effect of polyphenolic fraction on the gut microbiota, trying to correlate the change to positive effects on human health.

1.2.1.2. Isoflavones

Isoflavones are mainly present in legumes and pulses group, such as soy (*Glycine max*) and beans (*Phaseolus vulgaris*), as well as in several vegetables. Their chemical structure is similar to that of non-steroidal estrogens. There is a lot of evidence about the healthy properties of these phenolic class and, being SERMs (selective estrogen receptor modulators), they are used in hormonal disorders-dependent diseases, such as breast and prostate cancer, cardiovascular diseases and menopausal symptoms **[22,28,29]**.

The importance of the interaction gut microbiota-isoflavones is particularly evident for the daidzein, a soy isoflavone (aglycone) that is more active when metabolized into *S*-equol. Daidzein is firstly metabolized by the human gut microbiota in dihydrodaidzein (DHD), and then in *S*-equol and *O*-desmethylangolensin (*O*-DMA), but only about 33% of the Western population and 50% of Asian population are *S*-equol producers (**Figure 3**). There are many scientific works demonstrating that the daidzein-equol conversion depends on the microbiota composition. In particular, the abundance of *Asaccharobacter celatus* and *Slackia isoflavoniconvertens* in the gut microbiota may play a significant role **[30-32]**.

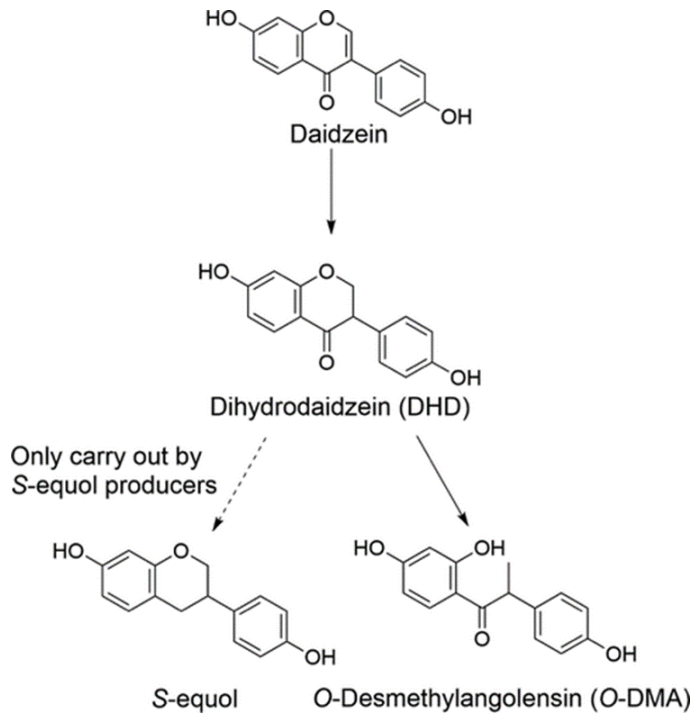


Figure 3. *Schematic representation of daidzen metabolism by human gut microbiota* ***[30]****.*

Isoflavones are able to modify the microbiota composition. For example, genistein inhibits the growth of pathogenic bacteria, such as *Helicobacter pylori* in the human gut **[24]**, while soy isoflavones in general have a certain effect on the relative abundance of *Intestinimonas, Parabacteroides* and *Faecalibacterium* spp, three groups of bacteria involved in obesity occurrence **[33]**.

1.2.1.3. Flavan-3-ols

The class of flavan-3-ols includes monomeric structure (called catechins) and oligo- or polymeric ones (proanthocyanidins). Catechin, epicatechin and epigallocatechin are only some of the wide range of compounds belonging to the class of monomeric flavan-3-ols. Proanthocyanidins (PACs) could belong to two different groups: A-type (e.g., A2-proanthocyanidin) and B-type (e.g., B2-proanthocyanidin), depending on the linkages position between the monomers. They are mainly present in fruits, tea, wine, berries and cocoa, but in minor quantity also in pigmented cereals **[34,35]**.

The flavan-3-ols metabolism is mainly based on the cleavage of C ring by the microbiota, yielding a very wide range of low molecular weight metabolites, such as phenylpropionic acids, *p*-hydroxyphenyl acetic acids, phenyl-γ-valerolactones or phenylvaleric acids **[22,36]**.

Flavan-3-ols interact with some bacterial species of the gut microbiota. For example, apple proanthocyanidins promote the growth of *Actinobacteria*; grape polyphenols increase the relative abundance of *Akkermansia muciniphila*, improving positively metabolic syndrome and of other potentially beneficial bacteria (*Lactobacillus spp.*). Blueberry proanthocyanidins were demonstrate able to increase the population of *Muribaculum intestinale*, important for the integrity of the intestinal barrier, while cranberry extract is efficient in the inhibition of *Bacteroide*s and *Prevotella* **[37-40]**.

1.2.1.4. Anthocyanins

Anthocyanins are a group of water-soluble pigments present in berries, fruits, vegetables, petals, pigmented cereals, and a lot of other coloured foods. Their basic structure, reported in **Figure 4**, is a flavylium cation, characterized at acid pH by a net positive charge. As well known, the colour of the anthocyanins is affected by the pH, moving to red (acid pH) to blue (neutral or basic pH, depending on the molecule, also passing from uncoloured structures). This is the reason why the use of anthocyanins as food colouring is often difficult to be stabilized in real food systems.

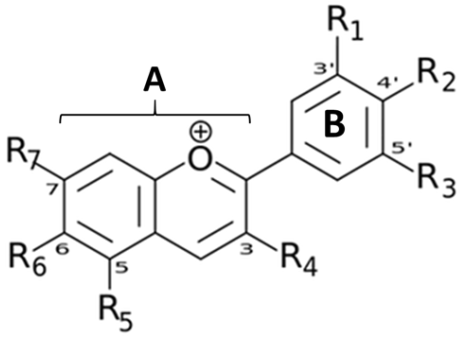


Figure 4. *Basic structural formula of the flavylium cation.*

Most of the anthocyanins isolated in nature present as aglycon (anthocyanidin) moiety one of the following 6 molecules: pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin (**Table 1**), which differ from each other in the number of hydroxyl or methoxy substituents present on the ring B of the flavylium ion **[41]**.

Table 1. *Substitution pattern of the main anthocyanidins identified in nature **[41]**.*

Anthocyanidin	Substitution pattern							Color*
	3	5	6	7	3'	4'	5'	
Pelargonidin	OH	OH	H	OH	H	OH	H	orange or salmon
Cyanidin	OH	OH	H	OH	OH	OH	H	magenta or crimson
Peonidin	OH	OH	H	OH	OCH_3	OH	H	magenta
Delphinidin	OH	OH	H	OH	OH	OH	OH	purple, mauv or blue
Petunidin	OH	OH	H	OH	OCH_3	OH	OH	purple
Malvidin	OH	OH	H	OH	OCH_3	OH	OCH_3	purple

* Depending on the environmental pH

In nature anthocyanins are present as glycosides, because their high instability in the aglyconic form. The main monosaccharides to which anthocyanins are linked are glucose,

galactose, rhamnose, arabinose and xylose (as 3-glycosides or 3,5-diglycosides), while rutinose is the most common disaccharide **[42]**.

The gut microbiota is involved in the anthocyanins metabolism through the deglycosylation and the breakdown of C-ring, yielding phloroglucinol derivates and benzoic acids. The main metabolites are 4-hydroxybenzoic acid from pelargonidin, protocatechuic acid from cyanidin, gallic acid from delphinidin, vanillic acid from peonidin, 3-methoxy-4,5-dihydroxybenzoic acid from petunidin and syringic acid from malvidin. Other catabolites are propionic acid, tyrosol, catechol and lactic acid **[21,42]**. Hanske *et al.* demonstrated that bacterial species such as *E. ramulus* and *C. saccharogumia* are responsible of the deglycosilation of cyanidin-3-O-glucoside in the gut **[43]**.

The literature suggests that anthocyanins interact with the gut microbiota promoting a healthy one. For example, they enhance the growth of *Lactobacillus* spp. and *Bifidobacterium* spp., bacteria associated with the production of SCFAs with positive effect for the intestine **[42]**. *Faecalibacterium prausnitzii* growth is stimulated by black raspberry anthocyanins, while *Enterococcus spp.* is inhibited **[44]**. Purple red rice bran anthocyanins (PRBA) are able to modulate the microbiota composition, promoting the growth of beneficial bacteria such as *Bacteroidaceae* and *Ruminococcaceae* **[45]** and black rice anthocyanins induced a significant enrichment in *Akkermansia muciniphila* **[46]**.

1.2.2. Phenolic acids

Phenolic acids accounts for about 30% of the total dietary phenolic compounds introduced daily. Based on their chemical structure, these antioxidants could belong to two different classes: the hydroxybenzoic and the hydroxycinnamic acids derivates (**Figure 5**). The first one includes hydroxybenzoic, gallic, syringic, protocatechuic, and vanilic acids as the main representatives, while coumaric, ferulic, caffeic, and sinapic acids are the most known of the second class. Whole grains, wine and berries contain the highest concentration of phenolic acids **[21,47]**.

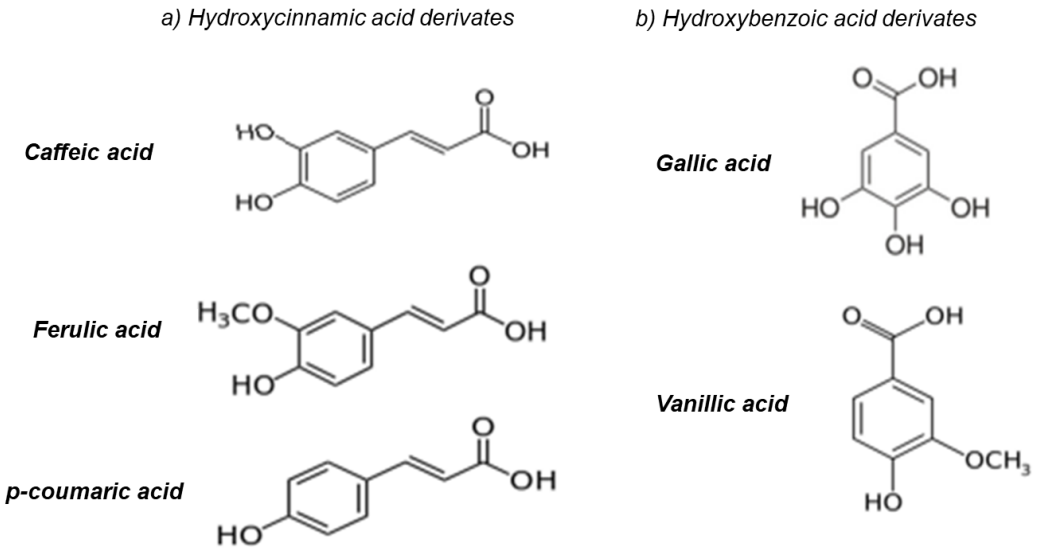


Figure 5. *Chemical structure of principal phenolic acids from the hydroxycinnamic (a) and hydroxybenzoic series* ***[47]****.*

As described before for the other phenolic classes, the gut microbiota can significantly affect the metabolism of phenolic acids and in turn these latter can influence the biodiversity of the microbial community. Russel *et al.* (2007) conducted a study on the polyphenolic fraction bound to the matrix following the extraction with ethyl acetate from blueberries. This fraction was incubated with faecal slurries from two human volunteers consuming an unrestricted Western-style diet. They observed that only few starting phenolic acids were found after faecal metabolism. Most of the detected compounds were hydrogenated, demethoxylated and dehydroxylated. The main metabolites detected were benzoic, phenylacetic and phenylpropionic acids derivates, such as methoxy-4-hydroxyphenylacetic, 4-hydroxy phenylpropionic and 3-methoxy-4-hydroxyphenylpropionic acid. The interesting fact is the difference in the concentrations of these metabolites found in the two different faecal donors. In one case, for many of the metabolites, approximately double values were observed compared to the other volunteer, highlighting the importance of the uniqueness of the gut microbiota composition **[48]**. Pyrocatechol, 3-(4-hydroxyphenyl) propionic acid, salicylic acid and *trans*-cinnamic acid are the mainly metabolites detected by Owolabi *et al.* after *in vitro* faecal fermentation of soaked and germinated purple rice phenolic acids **[49]**.

The positive effect of phenolic acids on the gut microbiota was confirmed by many studies reported in literature. Hidalgo *et al.* showed that gallic acid was positively correlated by the reduction of the pathogen *Clostridium histolyticum*, commonly associated with the inflammatory bowel disease **[50]**. Phenolic acids of germinated purple rice increased the

expression of *Lactobacilli* and *Bifidobacteria*, bacterial groups highly beneficial to human colon health, and inhibited the growth of *Clostridium* and *Bacteroides* **[49]**.

1.3. Static and dynamic *in vitro* simulated digestion models

The impact of digestive process on nutrients and bioactive compounds present in food is a topic which attracted particular interest in recent years. For this reason, various models capable to simulate *in vitro* the digestion process were developed. Among these models there are static and dynamic models, more or less complex depending on the protocol and on the devices required for their application.

The static models are certainly the most widespread and used, due to their cheapness and less complexity, respect to the dynamic ones. However, these models present some limitations, principally because they don't consider all the complex processes that occur in the gastro-intestinal tract. For example, it's really difficult to simulate the adsorption process, which is usually studied separately, using for example Caco-2 human epithelial cells or PAMPA model **[51,52]**. Furthermore, peristaltic movements, gastric emptying, microbiota contribution, age and the presence of diseases are not considered, and the variability of the conditions used in various laboratories is relevant **[53]**. For this reason, a standardized and harmonized protocol (INFOGEST) was defined to facilitate the comparison of results among different research groups. The INFOGEST protocol, update in 2019, is today the most used static model to simulate *in vitro* the digestion process **[54]**. Briefly, this protocol involves the use of SSF (Simulated Salivary Fluid) with salivary amylase, SGF (Simulated Gastric Fluid) with pepsin, SIF (Simulated intestinal fluid) with pancreatin and bile salt to simulate oral, gastric and intestinal digestion, respectively.

The application of this model is useful to start to clarify how much each single step (oral, gastric and intestinal digestions) impact on food matrix, permitting to analyse the food during the enzymatic processing sampling in each single phase. This model has some limits, depending on the very simple lay out as well as depending on the lack of possibility to include the microbiota effect at gut level.

The dynamic models, developed to overcome the shortcomings of static models, are more expensive and complex, but widely used in different research fields. The main dynamic models employed in food science are:

- DGM (Dynamic Gastric Model): dynamic gastric model developed at the Institute of Food Research (Norwich, UK), able to simulate mechanical and enzymatic mechanisms that lead to the digestion of gastric contents. However, it doesn't have compartments simulating the intestinal phase **[55]**;

- HGS (Human Gastric Simulator): dynamic gastric digestion model designed by Kong and Sing (2010) to reproduce the peristaltic movement continuum of the stomach wall. However, this model doesn't have the compartments simulating the intestine **[56]**;

- DIDGI System: system developed at the French National Institute for Agricultural Research, equipped with two compartments simulating the stomach and small intestine, sensors of temperature and pH, and peristaltic pumps, all controlled by a software **[57]**;

- TIM (TNO Gastro-Intestinal tract Model): multi-compartmental dynamic model developed by Minekus *et al.* **i**n the early 1990s. It allows to simulate the characteristic digestive conditions of various animal species (man, pig, dog), at different ages (new-born, adult, elderly), considering the presence of any pathologies and other parameters related to the type of diet **[58]**;

- SHIME® (Simulator of Human Intestinal Microbial Ecosystem): the method was developed in 1993 by Molly et al., and since 2010 its name has been jointly registered by ProDigest and Ghent University. This system includes several compartments simulating the stomach, small intestine, and different sections of the colon (ascending, transverse and descending). It is probably the most complete method, because the possibility to consider the action of the human microbiota, added through faecal samples. Furthermore, it's possible to compare simultaneously microbiotas from different people, for example one healthy and one sick (TWIN-SHIME®) **[59]**. Anyway, this method is not currently providing the mechanical digestion, typical in the natural gastric phase.

1.4. Processing, thermal and cooking impact on foods healthy properties

Having ascertained the importance of the interactions between polyphenols and microbiota, it is necessary to consider the impact of processing on food healthy properties. Both domestic and industrial food processing (e.g., cooking, drying, freezing, etc.) is one of the factors that influence the availability of polyphenols and that of other bioactive molecules **[60]**. Many of these treatments, basically related to thermal stress, cause a degradation of healthy compounds, but in some cases, on the contrary, they promote their bioavailability. For example, the tomatoes lycopene, a linear carotenoid, is made more available after cooking, because heating promotes the isomerization from *trans*-lycopene to *cis*-lycopene (which is more bioavailable than the *trans*-isomer), breaking down the cell walls, which weakens the bonding forces between lycopene and tissue matrix, thus making lycopene more bio-accessible **[61]**.

Thermal stress (particularly in presence of oxygen) usually triggers polyphenol loss or oxidation, as showed by cocoa roasting process **[62]**.

Anyway, the heat treatment is necessary to make edible certain foods, such as cereals or some vegetables The microbial stabilization, as well as the improvement of protein digestibility, are direct effect of thermal processes, also including cooking. So, the impact of cooking on foods healthy properties has a huge relevance. There are a lot of cooking methods that could be used: boiling, steaming, roasting, stewing, frying, grilling, microwave, pressure, and pilaf cooking, *etc.* Several studies were performed to investigate what are the best cooking methods to preserve the polyphenolic component in different foods. Ng et al. tested and compared four different cooking methods (boiling, steam, microwave, and pressure cooking) on five different mushrooms, in order to understand the best methods to preserve their antioxidant activity. The steam cooking resulted the best for 3 varieties (*F. velutipes*, *P. ostreatus* and *L. edodes*), while microwave cooking was preferred for *A. bisporus*, and pressure cooking for *P. eryngii* **[63]**. The vacuum-seal boiling was the cooking method able to better preserve the total polyphenolic content in purple potatoes, compared to normal boiling and steaming **[64]**. Hamed *et al.* observed that roasting considerably decreased the vitamin C content and the antioxidant activity on different peppers varieties, respect to the raw samples **[65]**.

In most of the cases, the boiling cooking method is the worst to preserve the healthy properties of food, especially regarding vitamins and polyphenols. The cause is probably the elimination of the cooking water, in which some water-soluble polyphenols could be

released, and so lost. Karigidi *et al.* demonstrated that boiled cocoyam (*Colocasia esculentum*) samples shown lower values of total polyphenols content and antioxidant activity, compared to the roasted samples **[66]**. In the work by Danowska-Oziewicz et al. the total phenolic content of green asparagus and broccoli had the greatest decrease after boiling, compared to steaming, microwave and oven cooking **[67]**. In Musilova et al. chlorogenic acid decreased of about 30% after boiling in sweet potato tubers, while after steaming no significant loss was observed. The *trans*-ferulic acid shown a decrease of about 15% after boiling and of 3% after steaming **[68]**.

In recent years innovative techniques for food cooking have become increasingly popular, including *sous vide* cooking, that consists in cooking raw foods under controlled time and temperature, under vacuum, in heat-resistant bags. Meat, fish, and vegetables are particularly suitable for this cooking method, because the low temperatures used in the *sous vide* cooking allow to have a minor shrinkage of the myofibrillar structure (in meat and fish), compared to traditional techniques, with consequent less water loss and tissue shrinkage, thus not leading to an increase in the hardness of the muscle tissues **[69]**. Furthermore, interesting results were obtained concerning the polyphenols preservation. Renna *et al.* studied chicory subjected to various types of cooking (boiling, steam cooking, *sous vide* and microwave cooking). They observed that the concentration of total polyphenols after *sous vide* cooking was not varied compared to the raw matrix, while after boiling a decrease of 46% of these was observed. Moreover, the antioxidant activity has had a partial reduction after boiling and steaming, while it has even increased after *sous vide* cooking **[70]**. In Nartea et al. an increase in the kaempferol-7-O-glucoside concentration in coloured cauliflowers was observed after *sous vide* cooking, while a significant decrease was detected after boiling **[71]**. Brussel sprouts phenolic content was better preserved by sous vide cooking rather than traditional cooking as steaming or boiling. For example, gallic acid shown values of 69.13 mg/kg (f.w.) after *sous vide* cooking, against the 62.45 and 51.17 mg/kg (f.w.) of boiling and steaming, respectively **[72]**.

It could be concluded that *sous vide* cooking has many advantages in terms of preserving the health properties of foods, when compared to some conventional techniques. It would therefore be interesting to apply this cooking approach also to foods for which it has not yet been used, such as for example pigmented cereals, particularly rich in polyphenolic antioxidants like anthocyanins.

1.5. Pigmented rice: an example of natural functional food

Rice (*Oryza sativa*) is a staple food for humans used in many geographical areas. Italy (particularly the triangle Vercelli, Novara, Pavia between Piedmont and Lombardia Regions) is the main producer of rice in Europe, with a lot of registered varieties, with different nutritional and technological characteristics. Despite the very broad cultivation, transformation and use of rice with white caryopsis, different pigmented varieties are produced.

The pigmented rice varieties (black, red and purple) are currently considered a sort of natural functional foods due to their significant content of phenolics and their antioxidant properties. For this reason, the consumption of pigmented rice has grown in recent years **[73,74]**. The pigments of these varieties are mainly found in the external layers of the bran, so these rice varieties are always consumed in unmilled form **[75,76]**.

The number of pigmented rice varieties existing worldwide is large, and their colour varies from red to black. It is hypothesized that the colour of the different varieties is related to the main localization of anthocyanins in the different components of the kernel: the purple varieties have the anthocyanins mainly located in the pericarp, the blue varieties in the aleurone, and the black variety both in the aleurone and in the pericarp. The black varieties are the richest in anthocyanins, while in some red varieties anthocyanins are absent **[77,78]**.

Some pigmented rice varieties are also rich in proanthocyanidins, able to improve their antioxidant properties.

Black rice is mainly produced in Southeast Asia, but in recent years its cultivation in Italy is gaining ground because of a greater culinary appreciation by the Italian population. The main varieties of black rice grown in Italy, and in particular in the Piedmont region, are Artemide, Venere and Nerone (**Figure 6**), but there are 21 black varieties registered in the varietal register 2022/2023 **[75]**.

Artemide rice	
TPC (mg GAE/kg rice d.w.)	**11872**
TAA (mmol TE/kg rice d.w.)	**55,1**
Cn-3-glc (mg/kg rice d.w.)	**1004**

Venere rice	
TPC (mg GAE/kg rice d.w.)	**6201**
TAA (mmol TE/kg rice d.w.)	**30,2**
Cn-3-glc (mg/kg rice d.w.)	**512**

Nerone rice	
TPC (mg GAE/kg rice d.w.)	**5507**
TAA (mmol TE/kg rice d.w.)	**37,2**
Cn-3-glc (mg/kg rice d.w.)	**593**

Figure 6. *Artemide, Venere and Nerone rices and their total phenolic content (TPC), total antioxidant activity (TAA) and cyanidin-3-O-glucoside content (Cn-3-glc). Modified from* ***[75]****.*

Black rice is mainly composed of carbohydrates (principally starch), proteins (glutelins, globulins, albumins and prolamins), dietary fiber, lipids (triglycerides, free fatty acids, diglycerides, and sterols), polyphenolic compounds, vitamins and mineral salts. Fiber is predominant in insoluble form (75% of total dietary fiber) and is important for preventing constipation. Potassium, calcium, and iron, particularly important for muscle function, are the main mineral salts, as well as phosphorus and magnesium **[76]**. As generally known about rice, rice proteins are not toxic for coeliac consumers. **Table 2** shows the chemical composition of Venere black rice.

Table 2. *Chemical composition and energy value per 100 g of edible portion. The values to raw Italian Venere black rice* ***[79]****.*

Nutrient	Value per 100 g of raw rice
Water (g)	12.5
Energy (kcal)	355
Proteins (g)	7.8
Lipids (g)	1.3
Carbohydrates (g):	80.4
Starch (g)	71.1
Total dietary fiber (g)	5.1
Phosphorus (mg)	336
Potassium (mg)	280
Magnesium (mg)	101
Calcium (mg)	13
Iron (mg)	1.1

As previously reported, black rice varieties have a great content of polyphenols, especially anthocyanins, phenolic acids and other flavonoids, such as catechins and proanthocyanidins. The most abundant anthocyanin in the Italian Artemide (ATM), Venere (VNR) and Nerone (NRN) black rice varieties is represented by cyanidin-3-O-glucoside, corresponding to 71.5%, 68% and 65.6% of the total anthocyanin content, respectively. ATM rice, compared to NRN and VNR, is the one that contains anthocyanins in higher quantities (**Figure 5**). Other anthocyanins of black pigmented rice are peonidin-3-O-glucoside (representing 8.8%, 7.6% and 7.6% of the total anthocyanin content in ATM, NRN and VNR, respectively) and cyanidin-3-O-rutinoside (4.5%, 4.2% and 4.5%, respectively). In addition, small amounts of malvidin-3-O-glucoside, peonidin-3-O-rutinoside and cyanidin-3-O-gentiobioside are present (each in relative amounts of less than 3% of the total anthocyanin content) **[75]**.

In accordance with the importance of food processing described above, many studies were conducted to evaluate the impact of cooking on the healthy compounds of pigmented rice **[78,80-83]**. In general, a different decrease based on variety and cooking methods was observed, but often the cooking was carried out in test tubes with cooking conditions not comparable to real domestic ones. Furthermore, a lot of alternative cooking techniques were not considered at all (for example *sous vide* cooking).

Moreover, only few studies were focused on the evaluation of the bioavailability of anthocyanins and phenolic acids in pigmented rice. The main results are about the anthocyanins. They seem to be stable up to the gastric level, while a significant decrease in

their concentration was observed in the intestinal tract **[84,85,86]**. In addition, most of the literature concerning the bioavailability of phenolics during the digestion is referred to raw samples or to anthocyanin or phenolics extracts, and not to cooked rice.

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Chapter 2

Aim of the Thesis

The digestibility of foods, in term of “functional” biochemical utilization of nutrients (also including non-caloric molecules with biological properties, like polyphenols) is largely affected by food processing and cooking methods, as previously reported. The evaluation of the bioaccessibility (particularly considering the matrix effect in foods) and consequent bioavailability is a cool target in food science.

In this Thesis, basing on the great importance of processing and digestion processes impact on the healthy properties of some foods, we intend

1) to characterize the chemical composition and the nutritional profile of the pigmented Italian Artemide black rice;

2) to evaluate *in vitro* the impact of processing technologies, such as cooking, i) on the chemical composition and ii) on the stability of bioactive molecules;

3) to evaluate the effect of the digestion process on cooked rice bioactive compounds, through the *in vitro* INFOGEST simulated digestion protocol;

4) to evaluate *in vivo* the impact of consumption of different rice varieties, cooked in various ways, on the health of people with pathological conditions, such as diabetes.

Finally, the last purpose was 5) to subject these cooked rice varieties to simulated *in vitro* digestion and subsequent batch faecal fermentation, to observe their effect on a healthy microbiota.

A schematic representation of the thesis outline is presented in **Figure 1**.

In **Chapter 3,** the chemical and nutritional characterization of the Italian Artemide black rice has been reported. Proximate composition (moisture, ashes, proteins, and total dietary fiber), antioxidant activity, total polyphenol and anthocyanin contents, and the determination of single anthocyanins, other flavonoids and phenolic acids, were performed. The rice was cooked comparing four different methods (boiling; microwaves oven; under pressure pot and risotto preparation) to evaluate the impact of cooking, particularly focusing on polyphenol fraction.

As described in **Chapter 4,** the evaluation of cooking impact on black rice was then expanded to innovative cooking methods, such as *sous vide* cooking, comparing with traditional domestic techniques (risotto and pilaf), in order to suggest its possible use in company, school and hospital canteens.

The aim of the study reported in **Chapter 5** was to evaluate how the digestive processes could change and impact on the polyphenolic fraction of Artemide black rice, subjected to the risotto preparation. Cooked rice was subjected to the *in vitro* INFOGEST simulated

digestion process, and the concentrations of anthocyanins, catechins and phenolic acids were detected through RP-HPLC-DAD. Furthermore, digestibility and bioaccessibility indexes of these compounds were calculated.

The aim of the study reported in **Chapter 6** was to evaluate the impact on the postprandial glycaemic trend in type 1 diabetic children and adolescents, considering two different varieties of rice ("Gigante Vercelli" white rice, and "Artemide" black rice), cooked in different modes (risotto vs boiled), using an advanced hybrid closed loop (AHCL) system (Tandem ControlIQ^{TM}).

Lastly, a faecal batch fermentation of cooked and pre-digested (INFOGEST) black rice is described in **Chapter 7**. The aim of this last study was to evaluate the impact of digested rice on a healthy gut microbiota sample, through the detection of SCFAs (Short Chain Fatty Acids), positive and desired indicators of the microbiota metabolism, via GC-FID.

Finally, some conclusions and future perspective are proposed in **Chapter 8**.

Functional foods and ingredients in healthy nutrtition: the importance of the food processing and interactions with the gut microbiota	
Chapter 3	*Chemical characterization of Artemide black rice and evaluation of the impact of classic cooking methods on polyphenolic and nutrtitional profile*
Chapter 4	*Comparison between the impact of classic and innovative cooking methods on nutritional profile of Artemide black rice*
Chapter 5	*Evaluation of the digestive process impact on the polyphenolic fraction of Artemide black rice*
Chapter 6	*Postprandial glycaemic trend in type 1 diabetic children after the consumption of black or white rice cooked in different ways*
Chapter 7	*Study of the impact of cooked and digested rice on the gut microbiota through the determination of SCFAs production*

Figure 1. *Schematic overview of the thesis outlines.*

Chapter 3

Cooking of Artemide Black Rice: Impact on Proximate Composition and Phenolic Compounds

This chapter is based on:

Colasanto, A., Travaglia, F., Bordiga, M., Monteduro, S., Arlorio, M., Coïsson, J. D., & Locatelli, M. (2021). Cooking of Artemide Black Rice: Impact on Proximate Composition and Phenolic Compounds. Foods, 10(4), 824.

Abstract:

The consumption of black rice has grown in recent years due to its particular organoleptic properties and high content of antioxidant polyphenols, which make it a sort of natural functional food. However, heat treatment applied during cooking can influence the content and the composition of antioxidant components, particularly anthocyanins, the main compounds of black rice, responsible for its color. The aim of this work was to evaluate the impact of different cooking techniques (boiling, microwaves oven, under pressure pot and risotto preparation) on the chemical and nutritional composition of the Italian Artemide black rice. Different cooking methods had significant and different impact on rice composition. Proximate composition was not affected by cooking, except for moisture, which increased, and fiber content, which decreased. Total polyphenols, total anthocyanin content, and antioxidant capacity were reduced; moreover, anthocyanins and phenolic acids determined by HPLC-DAD generally decreased, with the only exception of protocatechuic acid. The risotto preparation was the most useful cooking technique to preserve anthocyanins and antioxidant activity. Our results demonstrated the importance to study cooking methods and to evaluate their impact on rice characteristics, in order to preserve its nutritional and beneficial properties.

1. Introduction

Rice is one of the most produced and consumed cereals in the world. Rice crops belong to two different varieties: *Oryza glaberrima L.*, which is widespread in West Africa, and *Oryza sativa L.*, the most common variety in Asia and Europe. This last variety has two major subspecies: *Oryza sativa L. spp japonica*, rife in South-East Asia, Japan, Europe, and the U.S., and *Oryza sativa L. spp indica*, which is primarily consumed in India and Southern China. Italy is the main producer of rice (*Oryza sativa L.*) in Europe; approximately 90% of its production is concentrated in two Northern regions—Piedmont and Lombardy **[1]**. Even though white rice varieties are the most consumed, pigmented rice became more popular in the last few years due to their antioxidant properties and phenolic content, and the potential beneficial effects on the human health **[2]**. The pigments that give the characteristic color (red, purple or black) to rice are mainly contained in the bran fraction. The color is visible when the grains are dehulled, but it can be removed by polishing, revealing the white endosperm **[3]**. Pigmented rice varieties contain more nutrients than white rice, thus providing nutritional advantages **[4]**. For these particular characteristics pigmented rice, can be considered a “functional food” that, thanks to the presence of particular substances, can potentially induce health benefits in addition to the nutritional ones.

The origin of black rice is placed in the Asian continent, where it was widely used in traditional medicine **[5]**; it has been introduced in Italy only in the last 25 years because of the climate incompatibility, due to its photosensitivity and instability. The first variety of black rice grown in Italy was Venere rice, obtained from a hybridization process between a black rice from South-East Asia and a local variety. Although in Italy the production of pigmented rice is still limited, it is now increasing, due to the growing consumers’ appreciation **[6]**. At today, 23 pigmented varieties (black or red) are registered in the Italian Varietal Register (crop year 2019/2020). Among them, Artemide rice, which has been obtained through a natural hybridization process between Venere rice and a white indica rice variety (long and narrow grain), is characterized by high phenolic content, in particular anthocyanins, and antioxidant activity **[1]**.

The characteristic compounds of black rice, which determined its greatest spread, are anthocyanins. They are mainly located in the pericarp of black rice and are known to be influenced by various factors, including pH, temperature, light, oxygen, and metal ions **[7]**. Most of the studies reported in literature observed that cyanidin-3-glucoside is the main

anthocyanin in black rice, followed by peonidin-3-glucoside; in some cases, they represent more than 90% of the total anthocyanins determined **[8,9]**.

Most of the literature concerning antioxidant phenolic compounds in rice is referred to raw samples and concerns the analysis of antioxidant capacity, as well as the quantification of individual compounds **[2,10–12]**. In contrast, the effect of cooking on the antioxidant capacity and the quali-quantitative composition of phenolic compounds has been less studied. Considering that cereals must be consumed after cooking, it is important to analyze how heat treatments can influence the antioxidant components of these foods. Several studies conducted on different food matrices have shown that thermal treatment reduces the content of phenolic compounds and the antioxidant activity. In the case of rice, it was observed a different decrease based on variety and cooking methods. Finocchiaro et al. **[13]** reported a higher reduction of phenolic compounds in red rice than in white varieties. Several authors evidenced that cooking process is detrimental for the polyphenolic fraction, in particular anthocyanins **[14,15]**. Recently, Catena et al. **[16]** observed a loss of total anthocyanins in the range 70%–92% for Violet Nori rice, depending on different cooking method.

The aim of this work was to evaluate the impact of cooking on the chemical and nutritional composition of the Italian Artemide rice, evaluating, in particular, the proximate composition, and the antioxidant polyphenolic compounds. Four different domestic cooking techniques were applied: boiling, microwaves, under pressure and “risotto”.

2. Materials and Methods

2.1. Chemicals

Methanol, acetonitrile (all HPLC grade), and formic acid (50%, LC–MS grade) were purchased from Sigma–Aldrich (Milan, Italy). Ultrapure water (18.2 MΩ cm at 25 °C) was obtained by ELGA PURELAB Ultra system (M-medical, Cornaredo, Milan, Italy). Anthocyanins (Cyanidin-3-O-glucoside and Peonidin-3-O-glucoside) were obtained from LGC Standard srl (Sesto San Giovanni, Milan, Italy). All the other polyphenol reference compounds (gallic acid, protocatechuic acid, p-hydroxybenzoic acid, vanillic acid, coumaric acid, ferulic acid, catechin, myricetin), chemicals, and reagents were of analytical grade and purchased from Sigma–Aldrich (Milan, Italy).

2.2. Rice Samples and Cooking Procedures

The “Artemide” black rice was supplied by the local company “Azienda Agricola Luigi e Carlo Guidobono Cavalchini, tenuta La Mondina”, located in Casalbeltrame, Novara (Italy), in under-vacuum packages kept at room temperature.

Four different domestic cooking techniques were applied: boiling (BOI), microwave oven cooking (MW), cooking in a pressure cooker (PRES), and “risotto” (RIS). Microwave oven cooking was applied in two methods, different for the soaking pre-treatment of rice, the total cooking time and the total volume of water employed. The risotto mode was instead carried out in three different ways, with or without a preliminary toasting phase and, in the first case, with or without the addition of extra-virgin olive oil. Excepting for the microwave methods, all the cooking experiments were performed using an electric ceramic hob (Electrolux PQX320C, Stockholm, Sweden); the hob was always set to the power level “3”, equivalent to a slow/medium cooking. The different cooking conditions are detailed below and resumed in **Table 1**.

Table 1. Cooking conditions applied in the experiments.

Cooking Procedure	Ratio Rice/Water (g/mL)	Cooking Time (min)	Notes
Boiling (*BOI*)	200/1000	25	Time calculated from the boiling of water
Microwave oven (*MW-a*)	50/150	15	Previous soaking time: 1 h and 30 min
Microwave oven (*MW-b*)	50/200	20	Previous soaking time: 45 min
Pressure cooker (*PRES*)	500/1000	30	-
Risotto (*RIS-a*)	200/500	35	-
Risotto (*RIS-b*)	200/500	35	Time including 5 min toasting
Risotto (*RIS-c*)	200/500	40	Time including 5 min toasting in extra-virgin olive oil

Boiling (BOI). 200 g of black rice and 1 L of distilled water were placed in a steel pot and covered with a lid. The time required for proper cooking was calculated from the moment when cooking water started boiling and was determined as 25 min. During the cooking, the rice was occasionally mixed with a wooden spoon. At the end of cooking, the rice was drained, put in a container, and left to cool at room temperature (about 20 °C), covered by a film, away from light.

Microwave oven (MW). The MW cooking was done in two different ways:

MW-a: 50 g of black rice and 100 mL of distilled water were placed in a container suitable for the microwave cooking. The rice was left to soak for 1 h and 30 min, then it was cooked covered by a lid for 10 min at 600 W in a microwave oven (Dauer, model DM2). Afterward, 50 mL of water was added and the rice was cooked for further 5 min.

MW-b: 50 g of black rice and 150 mL of distilled water were placed in a container suitable for the microwave cooking. The rice was left to soak for 45 min, then it was cooked covered by a lid for 15 min at 600 W. Afterward, 50 mL of water was added and the rice was cooked for further 5 min.

For both the MW cooking methods, the water amount was determined in order to avoid the draining at the end of the cooking (water was completely absorbed by the rice or evaporated). The rice was put in a container and left to cool at room temperature (about 20 °C), covered by a film, away from light.

Pressure cooker (PRES). 500 g of black rice and 1000 mL of distilled water were placed in a pressure cooker operating at 112 °C and 55 kPa (Lagostina Itala Control, Italy). The total cooking time was 30 min. At the end of cooking all the water was absorbed by the rice or

evaporated. The rice was put in a container and left to cool at room temperature (about 20 °C), covered by a film, away from light.

Risotto (RIS). The risotto cooking was done in three different ways:

RIS-a: 200 g of black rice and 250 mL of distilled water were placed in a cooking pan, then cooking was started, occasionally stirring with a wooden spoon. After 15 min, the water was almost completely absorbed/evaporated, then other 250 mL of water were added. The cooking was continued for further 20 min.

RIS-b: 200 g of black rice were placed in a cooking pan and toasted for 5 min, continuously mixing with a wooden spoon. Then, 250 mL of distilled water was added and cooking was continued, stirring sometimes. After 15 min, the water was almost completely absorbed/evaporated, so others 250 mL of water were added; the rice was left to cook for further 15 min.

RIS-c: 200 g of black rice and three tablespoons of commercial extra-virgin olive oil were placed in a cooking pan and toasted for 5 min, continuously mixing with a wooden tablespoon. Then, 250 mL of distilled water was added and cooking was continued, occasionally stirring. After 15 min, the water was almost completely absorbed/evaporated, so others 250 mL of water were added to finish cooking in further 15 min.

For all the RIS methods, the water was completely absorbed by the rice or evaporated, and the rice was put in a container and left to cool at room temperature (about 20 °C), covered by a film, away from light.

2.3. Proximate Composition

The moisture content of raw and cooked rice was determined using a Sartorius MA30 thermo-balance (Sartorius AG, Goettingen, Germany). Due to the differences of moisture in raw grains and cooked samples, all the results were expressed on a dry weight (d.w.) basis. Prior to the other analyses, the cooked samples have been freeze-dried (Heto Drywin-ner 8, Copenhagen, Denmark) according to the following procedure: pre-freezing −25 °C for 1 h; primary drying −10 °C for 16 h and 0 °C for 16 h; secondary drying 10 °C for 30 h and 20 °C for 10 h. Finally, both raw grains and lyophilized cooked samples were ground to a fine flour using a laboratory blender (Sterilmixer 12, International PBI, Milan, Italy). The determination of proximate composition was performed as previously described in Giordano et al. **[17]**. The total nitrogen content and total protein content (conversion factor: 5.95) were obtained according to the Kjeldahl method, using Kjeltec system I (Foss Tecator AB,

Höganäs, Sweden). The ash content was determined after combustion of the organic material in a muffle furnace, according to the AOAC (1990) procedure. The total dietary fiber was determined by means of the Megazyme total dietary fiber analysis kit.

2.4. Extraction Procedure of Phenolics

100 mg of sample (raw grains or freeze-dried cooked rice, previously ground) were extracted with 1.7 mL of distilled water under agitation for 10 min at room temperature. The extraction was repeated for other two times with water and for other three times with ethanol. Each extraction step was followed by centrifugation at 9200 × g for 5 min, then the clear supernatants were combined. The extracts were divided in aliquots for the analyses and stored at −20 °C until use. For each rice sample the extraction was performed in triplicate.

2.5. Total Phenolic Content

The total phenolic content (TPC) was determined according to a modified version of the Folin–Ciocâlteu method **[18]**. Briefly, 100 µL of Folin–Ciocâlteu reagent and 350 µL of aqueous Na2CO3 (5% w/v) were added to an appropriate volume of rice hydroalcoholic extract and then the solution was diluted to a final volume of 2900 µL with distilled water. After 1 h, the absorbance was read at 760 nm, using an Evolution 60S spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Results were expressed as catechin equivalents (CE) through a calibration curve.

2.6. Total Anthocyanin Content

The content of TA (total anthocyanins) and TMA (total monomeric anthocyanins) was determined by the pH differential method, based on the protocol described by Lavelli, Harsha, and Spigno **[19]**. Different samples were opportunely diluted with potassium chloride buffer (0.025 M), pH 1.0, until the absorbance of the sample at 520 nm was within the linear range of the spectrophotometer. The same dilution factor (DF) was applied to the dilution with sodium acetate buffer (0.4 M), pH 4.5. Solutions at pH 1.0 were let to equilibrate for 5 min and those at pH 4.5 for 15 min; then the absorbance was measured at both 520 and 700 nm. The concentration of TA and TMA in the extracts was expressed as cya-nidin-3-O-glucoside (Cn-3-Glu) equivalents according to the equations:

$$\text{TMA (µg/mL)} = [(A_{520\ nm} - A_{700\ nm})_{pH\ 1} - (A_{520\ nm} - A_{700\ nm})_{pH\ 4.5}] \times MW \times d \times 1000/\varepsilon \quad (1)$$

$$\text{TA (µg/mL)} = [(A_{520\ nm} - A_{700\ nm})_{pH\ 1.0}] \times MW \times d \times 1000/\varepsilon \quad (2)$$

where MW is the molecular weight of cyanidin-3-glucoside (449.2 g/mol), d is the dilution factor, ε is the molar extinction coefficient of cyanidin-3-O-glucoside (26,900 M^{-1} cm^{-1}). Final results were then expressed based on rice weight (d.w.).

2.7. Antioxidant Activity (DPPH Radical Scavenging Assay)

The DPPH radical scavenging assay was performed according to the method vali-dated in Locatelli et al. **[20]**. Briefly, 700 µL of sample (rice extracts properly diluted with MeOH) or MeOH (control) were added to the same volume of methanolic solution of $DPPH^{\bullet}$ (100 µM). The solutions were shaken and left in the dark at room temperature for 20 min, then the absorbance was read at 515 nm. The antioxidant activity of rice extracts was expressed as inhibition percentage of the radical. The antioxidant activity was finally expressed as Trolox equivalents (TE) by means of a calibration curve.

2.8. RP-HPLC-DAD Analysis

A Shimadzu LC-20A Prominence chromatographic system equipped with a diode array detector (DAD detector SPD-M20A) was used. Separation was performed on a re-versed-phase Synergi TM 4 µm Max-RP 80 Å LC Column (250 × 4.6 mm i.d., with particle size of 4 µm) (Phenomenex, Torrance, CA, USA), protected by a guard column containing the same phase, at 30 °C. The mobile phase consisted of water/formic acid/acetonitrile (87:10:3, v/v) (eluent A) and water/formic acid/acetonitrile (40:10:50, v/v) (eluent B) using the following program gradient: from 6 to 20% B (20 min), from 20 to 40% B (15 min), from 40 to 60% B (5 min), from 60 to 90% B (5 min), isocratic 90% B (5 min), from 90 to 6% B (0.5 min), isocratic 6% B (22.5 min). Total run time was 73 min, at a constant flow rate of 500 µL/min. The injection volume was 5 µL. Cyanidin-3-O-glucoside and pe-onidin-3-O-glicoside were tentatively identified by comparison with retention times of in-dividual authentic standard molecules and their UV–Vis spectra; the quantification was performed on the basis of calibration curves obtained with the corresponding standards. Cyanidin-3-O-rutinoside and peonidin-3-O-rutinoside were identified based on chroma-tographic characteristics determined in our previous study **[1]** and were quantified as glucoside equivalents. In the Supplementary material file, the chromatogram of the standards and, as an example, the chromatogram of a “risotto” rice sample are reported. For each reference compound, calibration curves at six different concentration levels were obtained. Details concerning the method validation (concentration range, R2, LOD, LOQ) are reported in Bordiga et al. **[21]**. Polyphenolic rice extracts were centrifuged (14,000 rpm for 20 min, microcentrifuge 5417R, Eppendorf, Milan, Italy) prior to the injection in the chromatographic system.

2.9. Statistical Analysis

Results were expressed as mean ± standard deviation (SD) of at least three inde-pendent experiments. Differences were estimated by analysis of variance (ANOVA) fol-lowed by Tukey's honest significant difference test. The statistical significance level was set to 0.05. *All statistical analyses were performed using the free statistical software R 4.0.0 version* ***[22]****.*

3. Results and Discussion

The impact of cooking on the chemical and nutritional composition of the Italian Artemide black rice was evaluated by testing different domestic cooking techniques, in particular, boiling, microwaves (2 different conditions), under pressure and “risotto” (three different conditions). Cooking parameters were selected considering different points. First, the amounts of rice were chosen to simulate realistic homemade cooking, with quantities (from 50 g to 500 g) comparable to 1 or more portions. The ratios rice/water were optimized to allow a complete absorption of the water by the rice, except for boiling. Cooking times were selected in order to guarantee a similar texture of the rice, independently from the cooking method. For microwave cooking two different soaking times were used, in order to evaluate the impact of this pre-treatment. For risotto cooking toasting phase (5 min) was also considered, with and without the use of extra virgin olive oil.

All samples were placed into a container and left to cool at room temperature (about 20 °C), covered by an aluminum foil, to ensure the stabilization of the rice weight, and it was possible to proceed with the determination of moisture and lyophilization, after which the other analyses were carried out.

3.1. Proximate and Phenolic Composition of Uncooked Artemide Black Rice

In the first part of the work, the proximate and phenolic composition of uncooked black Artemide rice was determined. The moisture content was measured as 11.7%, total dietary fiber, proteins, and ashes were 10.8%, 10.5%, and 1.95% on dry weight (d.w.), respectively (**Table 2**). While black rice moisture content is comparable with that of both polished and whole grain white rice (about 12%), total dietary fiber and protein contents are higher in Artemide black rice. Indeed, considering that rice pigments are mainly located in the external layers of the grain (bran), in order to preserve the color, Artemide rice have to be consumed (and analyzed) in the whole grain form, thus maintaining high nutritional value. In particular, Artemide rice showed a fiber content higher than polished white rice (about 1.15% d.w.), than whole white (or brown) rice (2.16% d.w.), and also than Venere black rice (5.8 % d.w.) **[23]**. In the same manner, the protein content of Artemide rice is higher compared to polished white rice (7.6% d.w.), brown rice (8.5% d.w.), and Venere rice (8.9% d.w.) **[23]**.

Table 2. *Proximate composition, total phenolic content (mg CE/g d.w.), total antioxidant activity (mg TE/g d.w.), anthocyanins (mg Cn-3-Glu/g d.w.) and individual phenolic compounds (μg/g d.w.) determined in uncooked Artemide rice. The results are expressed as mean ± standard deviation.*

Composition of Uncooked Artemide Rice	
Moisture (%)	11.7 ± 0.4
Proteins (% d.w.)	10.5 ± 0.2
Total dietary fibre (% d.w.)	10.8 ± 1.4
Ashes (% d.w.)	1.95 ± 0.09
Total phenolic content (mg CE/g d.w.)	51.8 ± 2.2
Total anthocyanins (mg CnE/g d.w.)	6.99 ± 0.53
Total monomeric anthocyanins (mg CnE/g d.w.)	4.23 ± 0.45
Antioxidant activity (mg TE/g d.w.)	21.4 ± 0.1
Anthocyanins (μg/g d.w.)	
Cyanidin-3-O-glucoside	3623 ± 126
Cyanidin-3-O-rutinoside	107 ± 5
Peonidin-3-O-glucoside	323 ± 9
Peonidin-3-O-rutinoside	10.2 ± 0.2
Other phenolic compounds (μg/g d.w.)	
Gallic acid	86.5 ± 0.9
Protocatechuic acid	85.8 ± 0.8
p-hydroxybenzoic acid	4.80 ± 0.18
Vanillic acid	91.8 ± 0.2
Coumaric acid	7.29 ± 0.29
Ferulic acid	9.36 ± 0.76
Catechin	47.9 ± 2.6
Myricetin	8.52 ± 0.49

Whole grain cereals are also a good source of antioxidant compounds, mostly polyphenols **[24]**. Pigmented rice, from this point of view, is particularly interesting because its color is correlated to the presence of anthocyanins, and their content is highly correlated with the antioxidant capacity **[25,26]**. The anthocyanins' content in rice was analyzed in many studies, especially focused on black pigmented rice; in some red varieties, in fact, the pigmentation is not due to the presence of anthocyanins. Despite the wide number of black rice varieties characterized in the literature, their composition resulted quite similar. In fact, all the studies reports that cyanidin-3-O-glucoside is the main anthocyanin, followed by peonidin-3-glucoside **[1,8,9,25,27–29]**. This is confirmed for the Artemide black rice analyzed in this work; cyanidin-3-O-glucoside was the highest content anthocyanin (3623 ± 126 μg/g d.w.), accounting for about 89% of the identified most abundant anthocyanins, followed by peonidin-3-glucoside (323 ± 9 μg/g d.w.). Total phenolic content, total antioxidant activity, total and monomeric anthocyanins and phenolic compounds quantified

in this work are higher than those obtained in our previous studies **[30]**. Although the rice was obtained by the same producer and was cultivated in the same geographic area (Novara province, Northern Italy), changes related to cultivation year, climatic conditions, parasites or weed attach, and/or different agronomic practices, could have had an impact on the phenolic compounds' biosynthesis and their final concentration in rice.

3.2. Proximate Composition of Cooked Artemide Rice

In the second part of the work, the proximate composition of cooked Artemide rice was evaluated, in order to check the influence of different cooking methods on its nutritional characteristics. The results are summarized in **Table 3** and, except for moisture, expressed on a dry weight basis (d.w.). Moisture content increased in all the cooked samples, with an average rise of 3.84 times the value of the content in raw rice (11.7%), due to the water absorption during the cooking process. The values ranged from 52.7% (RIS-a) to 65.4% (PRES); the moisture content was lower than 60%, excepting for PRES, probably due to the higher temperature reached, resulting in more complete pasting of starch granules. The "risotto" modes showed the lowest values, while boiling and MW produced similar values; not significantly ($p > 0.05$) differences were observed based on the different soaking times prior to the MW cooking. In fact, the moisture absorption during soaking is generally described by a curve tending to saturation, in which the maximum moisture levels correspond to the maximum water sorption capacity **[31]**.

Concerning proteins and ashes content, no significant ($p > 0.05$) differences were found between cooking methods (average values of 10.4% and 1.89%, respectively) and the values were statistically similar to those observed in raw rice (10.5% and 1.95%, respectively), therefore suggesting that cooking methods do not involve variations in the number of proteins and ashes.

Regarding total dietary fiber, in all the cooked samples a significant ($p < 0.001$) decrease compared to the uncooked rice (10.8%) was observed, probably because cooking led to the degradation of some fiber components. The higher fiber content has been observed in RIS-c sample (9.23%), while the lower in RIS-b sample (7.16%).

Table 3. *Proximate composition of cooked Artemide rice; the results are expressed as mean ± standard deviation. Values with different letters in the same column are significantly different ($p < 0.05$).*

	Moisture (%)	**Proteins (% d.w.)**	**Total Dietary Fiber (% d.w.)**	**Ashes (% d.w.)**
BOI	57.0 ± 0.2b	10.2 ± 0.2a	7.91 ± 0.27bc	1.99 ± 0.10a
MW-a	56.8 ± 0.4b	10.4 ± 0.1a	8.54 ± 0.81bc	1.85 ± 0.06a
MW-b	56.2 ± 0.8b	10.2 ± 0.2a	8.28 ± 0.05bc	1.83 ± 0.09a
PRES	65.4 ± 0.2a	10.4 ± 0.1a	7.55 ± 0.50bc	1.99 ± 0.06a
RIS-a	52.7 ± 0.4d	10.6 ± 0.3a	7.24 ± 0.11bc	1.86 ± 0.06a
RIS-b	53.9 ± 0.4c	10.5 ± 0.7a	7.16 ± 0.80c	1.87 ± 0.07a
RIS-c	53.7 ± 0.03cd	10.3 ± 0.3a	9.23 ± 0.59ab	1.84 ± 0.03a

3.3. Phenolic Composition of Cooked Rice

3.3.1. Total Phenolic Content, Total Anthocyanins and Antioxidant Activity of Cooked Artemide Rice

The total phenolic content (TPC) of cooked rice samples was expressed as milligrams of catechin equivalents (CE) per gram of rice (dry weight, d.w.) (**Figure 1**, panel A). The results showed that the TPC of cooked samples is significantly ($p < 0.001$) lower than in raw black rice. The thermal treatment applied during cooking determined the degradation of most phenolic compounds, which on average decrease by about 83%. The greatest loss occurred in the boiling method (about 90% decrease). This type of cooking is the only one in which there is no complete absorption of the water; therefore, it can be assumed that polyphenolic compounds solubilized in the cooking water were lost in the discarded exceeding water, as previously observed by Melini et al. **[6]**. The other cooking methods showed more similar results among each other. Comparing the different RIS samples, RIS-a showed the greater loss of total phenolic content (−85% respect to the uncooked rice). This suggest that the toasting phase, carried out in the other RIS methods and typically performed in the traditional "risotto" preparation to reduce the starch release during cooking, could produce a sort of barrier on the grain, able to reduce also the loss of phenolic compounds. Moreover, RIS-c sample showed the higher TPC (corresponding to a reduction of 79% respect to raw rice), and this could be related to the use of extra virgin olive oil, a polyphenols rich matrix, during toasting.

The total anthocyanins content of cooked black rice was determined as both total anthocyanins (TA) and total monomeric anthocyanins (TMA) (**Figure 1**, panels C and D, respectively), and the values were expressed as cyanidin-3-O-glucoside equivalents (mg CnE/g d.w.). As for TPC, also for TA and TMA it was observed a significant ($p < 0.001$) decrease in cooked samples compared to raw rice (on average −77% for TA and −81% for TMA). Moreover, in this case, the greatest loss occurred in BOI sample: the TA values decreased from 6.99 μg CnE/g in raw black rice to 0.94 μg CnE/g in BOI, and from 4.23 μg CnE/g in raw black rice to 0.35 μg CnE/g in BOI for TMA content, thus recording a reduction of 87% and 92%, respectively. No statistical differences were found between MW-a, MW-b and PRES cooking, with an average decrease of TA and TMA of 79% and 83%, while RIS cooking provided the highest anthocyanins' content, with a decrement of TA and TMA on average of 71% and 77%, respectively. In a general way, TMA were subjected to a stronger degradation in respect to TA, which include all types of anthocyanins/anthocyanidins and polymeric forms; these results confirm our previous observations on Artemide rice, i.e., that monomeric anthocyanins are less stable to thermal treatment than non-monomeric forms **[31]** and are also in agreement with previous literature data **[32]**. Furthermore, while RIS-c sample showed the higher TA content (2.47 mg/g d.w., loss of 65%), RIS-b was the most TMA-rich sample (1.09 mg/g d.w., loss of 74%). We suggest that this difference could be related to a higher heating temperature in RIS-c due to the oil presence during toasting, thus resulting in a higher degradation of TMA in RIS-c than in RIS-b.

The antioxidant activity was evaluated by the $DPPH^{\bullet}$ radical scavenging assay, a common and easy method, useful to made comparison among samples, even if not exhaustive for the determination of the total antioxidant capacity. The results, expressed as Trolox equivalents (TE) (**Figure 1**, panel B), showed a trend quite similar to that observed for TMA content. RIS samples showed greater results (4.11, 5.59, and 4.60 mg TE/g d.w. for RIS-a, RIS-b, and RIS-c, respectively), with an average loss of 78% compared to raw black rice (21.4 mg TE/g d.w.); as expected, the most significant ($p < 0.001$) loss occurred in BOI (94% decrease). The high correlation evidenced between the antioxidant activity and TMA content ($p < 0.001$, $r = 0.973$), suggest a significant contribution of this class of phenolic compounds to the antiradical properties of rice.

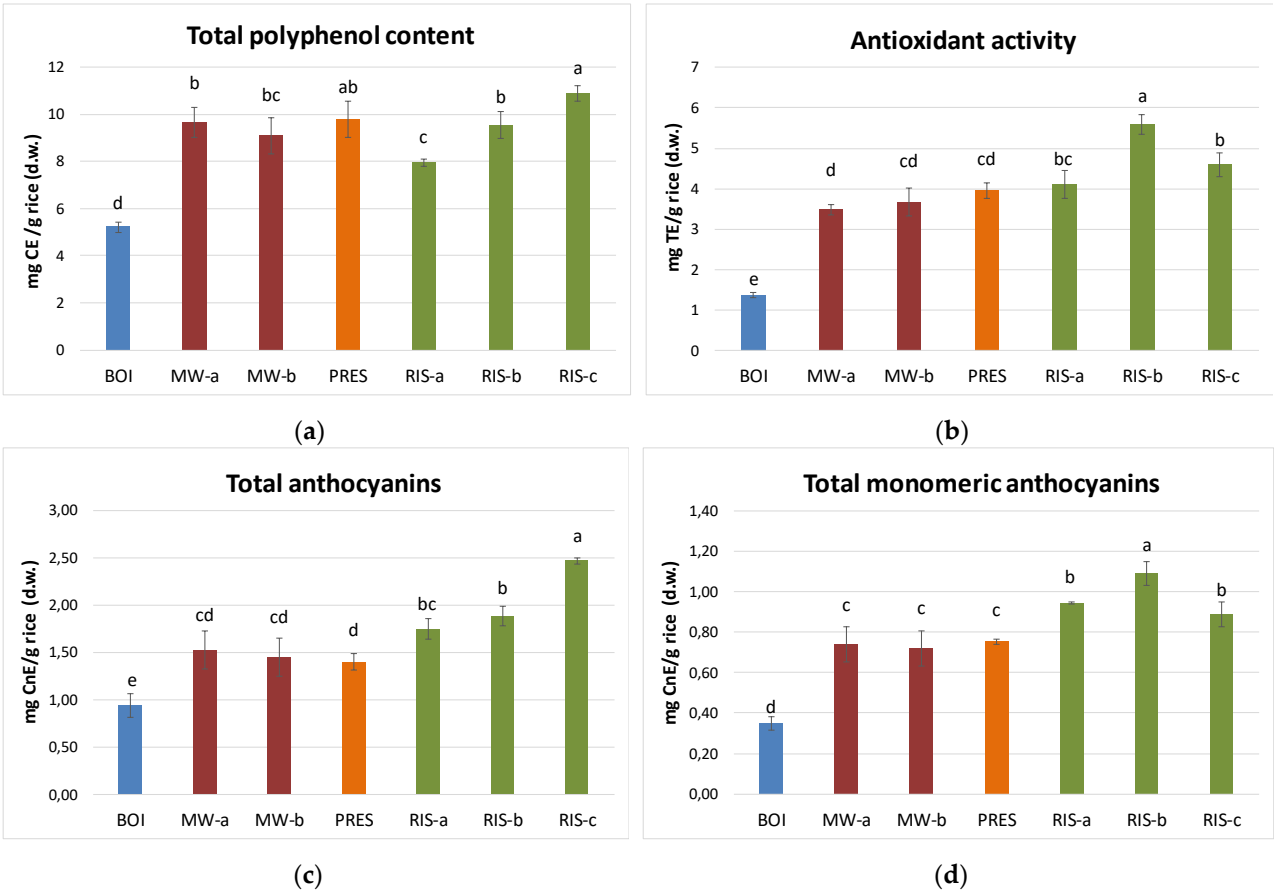

Figure 1. *(**a**)Total phenolic content (mg CE/g d.w.), (**b**) total antioxidant activity (mg TE/g d.w.), (**c**) total anthocyanins (mg CnE/g d.w.), and (**d**) total monomeric anthocyanins (mg CnE/g d.w.) quantified in cooked black rice, expressed as mean ± standard deviation. For each parameter, values with different letters are significantly different ($p < 0.05$). CE: catechin equivalents; CnE: cyanidin-3-O-glucoside equivalents; TE: Trolox equivalents.*

3.3.2. Individual Anthocyanins

The content of individual monomeric anthocyanins in cooked Artemide rice was determined through RP-HPLC-DAD analysis; results are reported in **Table 4** and expressed as μg/g (d.w.). Three main anthocyanins were identified through the comparison with the elution times of the standard molecules (in the supplementary materials, by way of example, the chromatograms of a rice sample are reported); the most abundant was cyanidin-3-O-glucoside, accounting for slightly less than 90% in all the samples, followed by peonidin-3-O-glucoside and cyanidin-3-O-rutinoside. Peonidin-3-O-rutinoside, determined only in minor quantities in raw rice (10.2 μg/g d.w.), was not quantified after cooking. BOI rice showed the lowest concentrations and was also characterized by the highest loss compared to raw rice (−97% for Cn-3-glc and Pn-3-glc and −96% for Cn-3-rut). These data agree with the spectrophotometric results, even if the relative reduction determined for the individual

compounds is higher than that observed for TMA. Differently to the spectrophotometric determinations, MW rice cooked in different conditions showed some significantly differences each other. In MW-a, the longer soaking time (1 h and 30 min against 45 min for MW-b) combined with a shorter cooking time (15 min against 20 min for MW-b) allowed to maintain higher values of both Cn-3-glc and Pn-3-glc content. PRES cooking produced results similar to MW-b cooking regarding the first two anthocyanins but evidenced a higher reduction of Cn-3-rut concentration (with a loss of 94% compared to raw black rice). In a general way, RIS cooking proved to be the best method for anthocyanins preservation, with a general average loss of 89%. Interesting results emerged comparing the different RIS methods; in particular, anthocyanins have been better preserved in RIS-b, with an average loss of 86%. As previously mentioned, we suggest that the toasting phase, a poorly investigated topic, in RIS-b can contribute to create a "barrier" reducing the loss of anthocyanins, while the adding of extra virgin olive oil in RIS-c could determine a major degradation of anthocyanins, due to a higher thermal impact.

Bhawamai et al. **[33]** showed for black rice a loss of about 55% of anthocyanins and about 67% of Cn-3-glc after cooking in a rice cooker for 25 min, measuring 1.0–1.2 mg anthocyanins and 238–296 μg Cn-3-glc content per gram in dry cooked rice. These authors observed a loss in anthocyanins lower than that observed in the present work; this could be due to the different cooking method, but also to the specific characteristics of black rice employed, in fact Artemide rice presented a higher anthocyanin content, both before and after cooking.

Table 4. *Anthocyanins (μg/g d.w.) identified in cooked black rice; the results are expressed as mean ± standard deviation. Values with different letters in the same column are significantly different ($p < 0.05$).*

	Cn-3-glc (μg/g d.w.)	***Pn-3-glc (μg/g d.w.)***	***Cn-3-rut (μg/g d.w.)***
BOI	118 ± 1 ^{e}	10.5 ± 0.3 ^{f}	4.73 ± 0.48 ^{c}
MW-a	293 ± 40 ^{c}	26.9 ± 1.4 ^{bc}	11.1 ± 0.1 ^{b}
MW-b	238 ± 7 ^{d}	21.2 ± 1.1 ^{de}	10.2 ± 1.0 ^{b}
PRES	218 ± 2 ^{d}	19.8 ± 0.9 ^{e}	6.53 ± 0.14 ^{c}
RIS-a	358 ± 7 ^{b}	31.2 ± 0.3 ^{b}	10.7 ± 0.8 ^{b}
RIS-b	472 ± 21 ^{a}	44.6 ± 2.2 ^{a}	15.0 ± 0.8 ^{a}
RIS-c	292 ± 7 ^{c}	25.2 ± 1.2 ^{cd}	9.87 ± 0.2 ^{b}

3.3.3. Phenolic Acids and Flavonoids

Beside anthocyanins, other phenolic compounds were identified and quantified in cooked rice; the results, expressed as μg/g (d.w.), are summarized in **Table 5**. In a general way, a decrease of concentrations compared to uncooked rice was observed also for individual phenolic compounds. The only one exception is protocatechuic acid, which showed a significant ($p < 0.001$) increase in almost all the cooked samples, resulting as the most abundant among the identified compounds. In fact, this phenolic acid can derive by the scission of flavylium cation of cyanidin-3-glucoside during cooking **[29]**. The same effect was observed after thermal treatment of microencapsulated anthocyanin-rich extracts, used as functional ingredient in baked model biscuits **[30]**. The higher increase was observed in PRES sample (+60% respect to uncooked rice), while the lower in MW samples, in particular in MW-b, for which the increase respect to the concentration in raw rice (+8%) was not statistically significant ($p > 0.05$). No differences were found between RIS-a, RIS-b and RIS-c samples, evidencing an average increase of 20%. Differently to the cooking methods involving the water absorption, the protocatechuic acid concentration in BOI rice was significantly ($p < 0.01$) lower (−61%) than in uncooked rice. It is important to note that the low values observed in BOI sample should not be related to a reduced degradation of cyanidin-3-glucoside; more probably, the protocatechuic acid formed from the anthocyanin scission was dissolved in the cooking water and was eliminated with it. In fact, all the identified phenolic compounds were determined in the lowest concentrations in BOI sample.

Following protocatechuic acid, gallic acid, vanillic acid, and catechin are the three phenolic compounds present in higher amount in all the cooked samples; RIS-b sample showed the highest concentrations of them: 27.0, 28.7, and 26.6 μg/g d.w., respectively.

Table 5. *Phenolic acids and flavonoids (µg/g d.w.) identified in cooked black rice; the results are expressed as mean ± standard deviation. Values with different letters in the same column are significantly different ($p < 0.05$). Values marked with # are not significantly different from the corresponding ones determined in uncooked rice. Gal, gallic acid; Proto, protocatechuic acid; Van, vanillic acid; Coum, coumaric acid; Fer, ferulic acid; Cat, catechin; Myr, myricetin.*

	Gal ***(µg/g d.w.)***	***Proto*** ***(µg/g d.w.)***	***Van*** ***(µg/g d.w.)***	***Coum*** ***(µg/g d.w.)***	***Fer*** ***(µg/g d.w.)***	***Cat*** ***(µg/g d.w.)***	***Myr*** ***(µg/g d.w.)***
BOI	6.80 ± 0.10 ^{d}	33.4 ± 0.1 ^{e}	6.67 ± 0.32 ^{e}	1.91 ± 0.16 ^{c}	-	9.22 ± 0.30 ^{d}	3.32 ± 0.29 ^{e}
MW-a	19.4 ± 0.8 ^{bc}	96.4 ± 1.2 ^{cd}	21.1 ± 0.005 ^{c}	2.01 ± 0.01 ^{b}	3.81 ± 0.02 ^{b}	15.2 ± 2.0 ^{cd}	5.20 ± 0.05 ^{d}
MW-b	18.5 ± 0.2 ^{c}	92.9 ± 2.0 ^{d}#	18.4 ± 0.5 ^{d}	1.98 ± 0.13 ^{b}	11.6 ± 0.3 ^{a}	13.6 ± 0.3 ^{cd}	6.28 ± 0.31 ^{c}
PRES	19.6 ± 0.6 ^{bc}	137 ± 1.9 ^{a}	24.1 ± 0.8 ^{b}	2.83 ± 0.01 ^{a}	4.34 ± 0.20 ^{b}	23.0 ± 3.0 ^{ab}	8.72 ± 0.25 ^{a}#
RIS-a	22.1 ± 0.2 ^{b}	101 ± 1.0 ^{bc}	22.9 ± 0.4 ^{bc}	2.12 ± 0.17 ^{b}	4.34 ± 0.01 ^{b}	17.6 ± 0.6 ^{bc}	6.44 ± 0.36 ^{bc}
RIS-b	27.0 ± 2.1 ^{a}	103 ± 3.2 ^{bc}	28.7 ± 0.7 ^{a}	1.96 ± 0.06 ^{b}	3.93 ± 0.13 ^{b}	26.6 ± 1.4 ^{a}	7.27 ± 0.15 ^{b}
RIS-c	20.0 ± 0.03 ^{bc}	104 ± 0.6 ^{b}	20.7 ± 1.1 ^{cd}	2.83 ± 0.19 ^{a}	4.42 ± 0.23 ^{b}	23.2 ± 1.1 ^{ab}	7.02 ± 0.15 ^{bc}

Interestingly, myricetin was the compound less affected by the thermal degradation. Excluding boiling (in which a loss due to water discarding necessarily occurred), reduction during cooking ranged from −39% in MW-a to −14% in RIS-b; in PRES sample the myricetin content did not statistically varied in respect to the cooked rice. In respect to these results, it would be interesting to know if part of myricetin could derive from the degradation of other compounds and/or the release from glycosylated forms during cooking (and the application of pressure in PRES cooking could justify this hypothesis), or if it is simply more stable than the other compounds. Myricetin was already identified in black rice as aglycone **[34]**, but also as the corresponding 7-O-glucoside derivative **[35]**.

4. Conclusions

Four different types of cooking (boiling, microwaves, under pressure and risotto preparation) were tested in this work to evaluate their impact on the chemical and nutritional composition of Italian Artemide black rice. Cooking procedures were optimized and standardized in the laboratory, in order to simulate realistic homemade cooking. The risotto mode was the best cooking method to preserve antioxidant capacity, polyphenols, and anthocyanin content, while boiling turned out to be the worst, due to the fact that part of polyphenolic compounds remained in the exceeding cooking water.

The main novelty in our work is to improve the knowledge about pigmented rice, such as Artemide rice, which requires a longer cooking procedure than white rice. The data obtained were not limited to polyphenols variations **[36]**, but we have also improved the information about the cooking impact on the main nutrients.

In conclusion, different cooking methods had significant and different impact on rice composition, suggesting the importance of evaluating the treatment of rice before consumption, in order to limit the loss of antioxidant phenolic compounds. Based on these results, it would be interesting to evaluate also other cooking methods and treatments of rice, at both domestic and industrial level. Other future research could be focused on rice digestive process, in particular exploiting in vitro simulated methods, in order to evaluate the fate of polyphenolic and anthocyanic compounds along the oro-gastrointestinal tract, and to estimate their bioaccessibility and bioavailability.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, **Figure S1**: Chromatogram of anthocyanins standard (cyanidine-3-O-glucoside and peonidin-3-O-glucoside) obtained by RP-HPLC-DAD (wavelength of detection: 520 nm), **Figure S2**: Chromatogram of anthocyanins obtained by RP-HPLC-DAD from a “risotto” rice sample (wavelength of detection: 520 nm). Cyanidin-3-O-rutinoside and peonidin-3-O-rutinoside were identified on the basis of chromatographic characteristics determined in our previous study [1]; peonidin-3-O-glucoside was not detected in cooked sample.

Author Contributions: Conceptualization, M.L. and J.D.C.; methodology, M.L, S.M., M.B., and F.T.; formal analysis, A.C., S.M., and F.T.; data curation, A.C. and M.L.; writing—original draft, A.C., S.M., and M.L.; writing—review and editing, M.B., M.A., M.L., and J.D.C.; supervision, M.L. and J.D.C.; project administration, M.L.; funding acquisition, M.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Università degli Studi del Piemonte Orientale, Dipartimento di Scienze del Farmaco, through the grant “Fondi di Ricerca Locale-FAR 2019”. A.C. is the recipient of a fellowship grant within the PhD program in “Food, Health and Longevity” (Università degli Studi del Piemonte Orientale) (MINDFUL project).

Data Availability Statement: Data sharing not applicable.

Acknowledgments: The authors would like to thank Giulia Delzanno for her help in the preliminary stages of the research (development of analytical methods), and Cristina Brizzolari (Azienda Agricola Luigi e Carlo Guidobono Cavalchini, tenuta La Mondina) for having provided rice samples.

Conflicts of Interest: The authors declare no conflicts of interest.

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Supplementary material

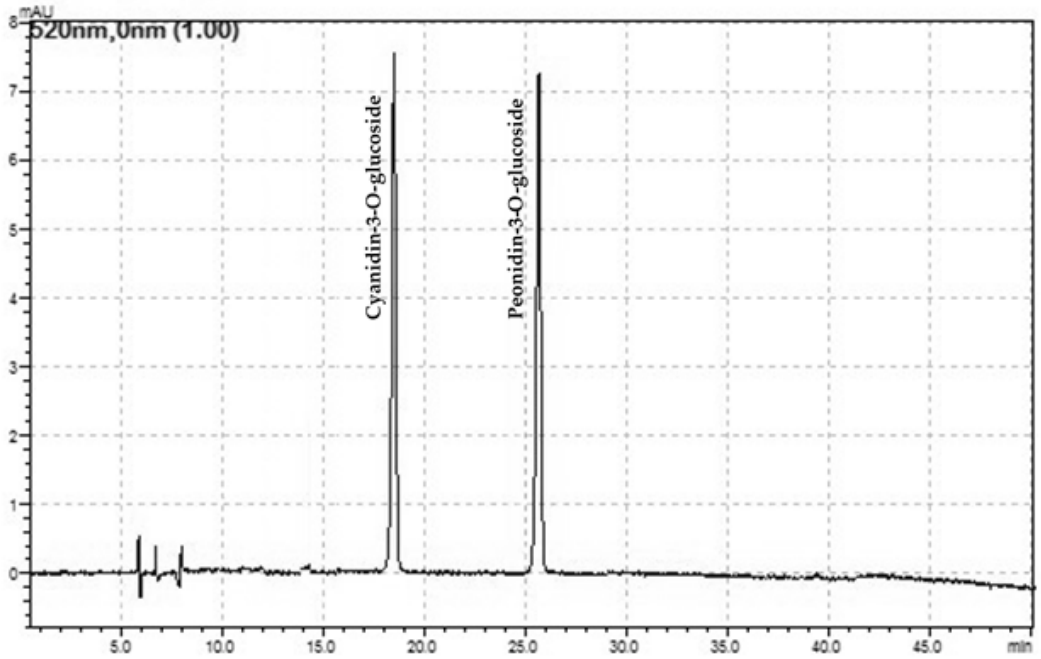

Figure S1. *Chromatogram of anthocyanins standard (cyanidine-3-O-glucoside and peonidin-3-O-glucoside) obtained by RP-HPLC-DAD (wavelength of detection: 520 nm).*

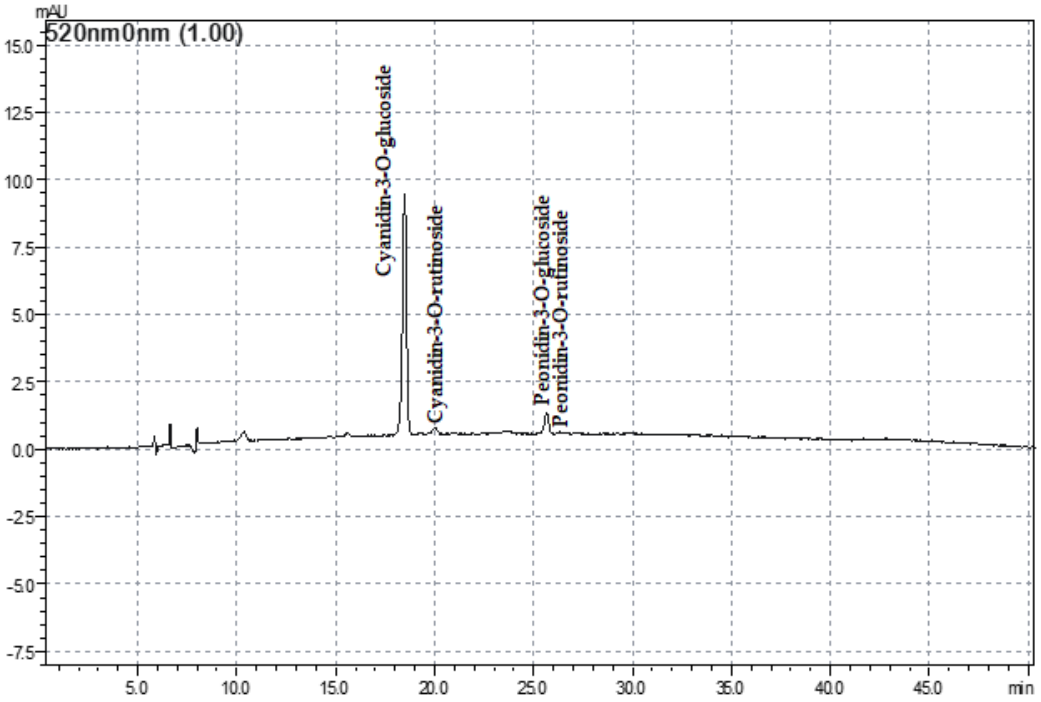

Figure S2. *Chromatogram of anthocyanins obtained by RP-HPLC-DAD from a "risotto" rice sample (wavelength of detection: 520 nm). Cyanidin-3-O-rutinoside and peonidin-3-O-rutinoside were identified on the basis of chromatographic characteristics determined in our previous study [1]; peonidin-3-O-glucoside was not detected in cooked sample.*

[1] Bordiga, M.; Gomez-Alonso, S.; Locatelli, M.; Travaglia, F.; Coïsson, J.D.; Hermosin-Gutierrez, I.; Arlorio, M. Phenolics characterization and antioxidant activity of six different pigmented Oryza sativa L. cultivars grown in Piedmont (Italy). Food Res. Int. 2014, 65, 282-290, https://doi.org/10.1016/j.foodres.2014.03.007.

Chapter 4

The impact of cooking on the composition of Artemide black rice: comparison between traditional and innovative cooking techniques

This chapter is based on:

Colasanto, A., Travaglia, F., Arlorio, M., Coïsson, J. D., & Locatelli, M. (2023). The impact of cooking on the composition of Artemide black rice: comparison between traditional and innovative cooking techniques. In preparation for the submission to Food Chemistry.

Abstract

The black rice varieties play an important role in the healthy nutrition, due to their high content of polyphenols, particularly anthocyanins. However, these antioxidant compounds are influenced by heat treatments required for the rice consumption. The aim of this work was to investigate the impact of cooking on the Artemide black rice composition, comparing innovative cooking methods, such as *sous vide* cooking, with traditional domestic techniques (risotto and pilaf).

In general, all the cooking methods had a different impact on the composition of Artemide black rice. Proteins and ashes were not affected by cooking, except for pilaf rice, where an ashes decrease was observed. The measured fiber content increased after all cooking methods. Antioxidant activity and total polyphenols, anthocyanins and proanthocyanidins content were reduced. Individual anthocyanins, phenolic acids and flavonoids determined through RP-HPLC-DAD generally decreased, with some exceptions. The risotto preparation and the *sous vide* cooking at 89 °C resulted the best performing cooking methods for preserving antioxidant polyphenols. It is interesting to note that the first preparation refers to a traditional cooking (risotto), easily reproducible at home, while the second one (*sous vide* at 89 °C) could be used in the canteens of schools, hospitals and companies.

1. Introduction

Due to its proteins, carbohydrates, vitamins and essential elements content, rice (*Oryza sativa* L.) is probably the most important food crop in the world. For more than half of the world population it is considered a staple food, particularly in Europe, America, and Asia **[1]**. The greatest production of rice in Europe comes from Italy, especially from two regions (Piedmont and Lombardy), in which more than 90% of the Italian rice is produced **[2]**. Brown rice is the whole-grain rice; the bran layers have not been removed by polishing, so brown rice is richer in fiber, lipids and bioactive phytochemicals, such as phytosterols and phenolic compounds, compared to dehulled one. White rice varieties are currently the most cultivated and consumed, contrary to pigmented ones, whole cultivation is limited to restricted areas of the globe (including Italy, France and north Africa). However, pigmented rice varieties in the last few years have received an increased attention due to their phenolic content, antioxidant activity and potential beneficial effects on human health **[3,4]**.

Pigmented varieties from different cereals are considered as health-promoting foods for their high content of anthocyanins, the pigments mainly responsible of the blue/red/black rice colour. They are located primarily in the bran, reason why these varieties are consumed preferably as whole rice. Cyanidin-3-O-glucoside is the most abundant anthocyanin in black rice, followed by peonidin-3-O-glucoside. The Italian Artemide black rice, a natural hybrid obtained from Venere rice and a white *indica* rice variety, is particularly rich in these healthy compounds **[5,6]**.

Notwithstanding anthocyanins have numerous beneficial effects on human health, they are highly unstable compounds, in fact they are influenced by pH, light, oxygen, enzymes and thermal treatments **[5]**. Since cereals must be consumed after cooking, it is important to investigate how heat treatments could influence the phenolic profile of these foods. A lot of techniques can be used to cook black rice; the methods vary depending on the culture and habits of the various countries, but the most common are boiling, in which rice is boiled in an excess of water, and other methods, in which rice is cooked in an amount of water that should be completely adsorbed, such as “risotto” and “pilaf” modes. In addition, microwave, pressure cooker and steam cooking are largely used (the last one particularly in Indonesia) **[7-9]**.

In recent years, innovative techniques for food cooking have become increasingly popular, including *sous vide* cooking, that consists in cooking raw foods under controlled time and temperature, under vacuum, in heat-resistant bags. Since the French chef Georges Pralus

tested it in the mid 70's, many applications were performed in both restaurants and food industries **[10]**. This technique is principally applied to cook meat, fish, and vegetables. The low temperatures generally used in the *sous vide* cooking determine in meat and fish a minor shrinkage of the myofibrillar structure, comparing to traditional techniques, with a consequent reduction of water loss and tissue shrinkage, thus also reducing the typical increase in the hardness of the muscle tissues after conventional cooking **[11]**. Some Authors have also observed that the impact of this technique on lipid oxidation and hydrolysis, as well as on the loss of mineral salts, is lower than other traditional techniques. Rasinska *et al.* demonstrated that the peroxide value in rabbit meat subjected to sous vide cooking (10.8 meq O_2/kg of lipid) is lower than that observed following boiling (13.6 meq O_2/kg of lipid) and roasting (22.2 meq O_2/kg of lipid) **[12]**. Nieva-Echevarría *et al.*, following NMR analysis, did not observe any presence of species deriving from either lipid oxidation or hydrolysis in *sous vide* cooked European sea bass **[13]**. Da Silva *et al.* observed that the loss of mineral salts in *sous vide* cooked bovine liver was almost nil, while they saw that their loss was significant after boiling **[14]**. Interesting results were obtained also on polyphenolic compounds. In their study on chicory subjected to various types of cooking (boiling, steam cooking, *sous vide* and microwave cooking), Renna *et al.* observed that the concentration of total polyphenols after *sous vide* cooking did not vary compared to the raw matrix, while after boiling a 46% decrease of these compounds was observed. Moreover, the antioxidant activity had a partial reduction after boiling and steaming, while it increased after *sous vide* cooking **[15]**.

Many works investigated the impact of cooking on bioactive compounds of black rice **[16-20]**. It was demonstrated demonstrate, for example, that boiling is the worst cooking method to preserve polyphenols **[16,17]**. However, none of these works has considered alternative cooking techniques to cook black rice. This is the reason why in this work we have investigated the impact of *sous vide* cooking, a method poorly applied to cereals, but highly used in other food matrices to minimize the polyphenolic degradation, on the chemical and nutritional composition of Artemide black rice, in comparison to other usual techniques (risotto and pilaf).

2. Materials and methods

2.1. Chemicals

Anthocyanins (cyanidin-3-O-glucoside, peonidin-3-O-glucoside, cyanidin-3-O-rutinoside and peonidin-3-O-rutinoside) were purchased from LGC Standard srl (Sesto San Giovanni, Milan, Italy). All the other polyphenol reference compounds (protocatechuic acid, ferulic acid, *p*-hydroxybenzoic acid, gallic acid, vanillic acid, coumaric acid, myricetin, catechin), chemicals, and reagents were of analytical grade from Merck KGaA (Darmstadt, Germany). Acetonitrile, methanol (all HPLC grade), and formic acid (50%, LC–MS grade) were obtained from Sigma–Aldrich (Milan, Italy). Ultrapure water (18.2 MΩ cm at 25 °C) was produced by ELGA PURELAB Ultra system (M-medical, Cornaredo, Milan, Italy).

2.2. Rice samples and cooking procedures

The local company “Azienda Agricola Luigi e Carlo Guidobono Cavalchini, tenuta La Mondina”, located in Casalbeltrame, Novara (Italy), kindly supplied the “Artemide” black rice (harvest year: 2018) in under-vacuum commercial packages kept at room temperature.

Rice was analyzed raw, previously ground in a laboratory blender (Sterilmixer 12, International PBI, Milan, Italy) and then reduced to a fine flour using a mixer mill (MM 400, Retsch GmbH, Haan, Germany), and after cooking.

Three cooking techniques were applied: “risotto” (Ris), “pilaf” (Pil) and *sous-vide* (SV).

Risotto (RIS)

100 g of black rice was placed in a cooking pot and set on the largest plate of the electric hob “Electrolux PQX320C” (Stockholm, Sweden). The plat was set to the power level “4”, corresponding to medium/high cooking, and the rice was toasted for 3 minutes, mixing continuously with a steel spoon. The start of the toasting phase coincides with the start of the cooking time. Then, 100 mL of distilled hot water (previously heated on a plate at 80 °C) was added and the pot was closed with a lid. After the almost complete adsorption of the water by the rice (5 minutes), eight aliquots of 50 mL of distilled hot water were added, at 3 minutes and 30 seconds from each other, for a total volume of 500 mL. The total cooking time was 35 minutes (in the last 1 minute the lid was removed), then the rice, maintained in the pot covered by the lid, was left to cool at room temperature (20 °C). At the end of the cooking the water was completely absorbed by the rice or evaporated. The temperature of rice during cooking, measured with a kitchen thermometer (Habor HCP1, Dongguan, Guangdong, China) varied in the range 98-100 °C.

Pilaf (PIL)

100 g of black rice was placed in a thin layer in an aluminium pan and 150 mL of boiling distilled water was added. The pan was covered by an appropriate lid and then was put in an oven (Candy FCS201X, Brugherio, Monza e Brianza, Italy) previously preheated at 175 °C (static mode). The cooking was conducted for 30 minutes, after which the rice was left to cool at room temperature (20 °C), in the pan covered by the lid.

***Sous-vide* (SV)**

100 g of black rice was placed in a plastic bag suitable for *sous vide* cooking and 100 mL of distilled water was added. Vacuum packaging was carried out using the chamber vacuum packer "LAVEZZINI UNICA" (UNIVAC Group S.r.l., Fiorenzuola d'Arda, Monza e Brianza, Italy). The black rice contained in the hermetically sealed bags was cooked at 89 °C (SV89) or 99 °C (SV99) for 1 hour in a cooking bath (SEVERIN SV 2447, SEVERIN Elektrogeräte GmbH, Sundern, Germany) able to maintain the set temperature in a range of ± 1 °C. The rice was left to cool at room temperature (20 °C), inside the bags and away from light.

In **Table 1** the different cooking conditions are reported.

Table 1 Cooking conditions applied

Cooking procedure	Ratio rice/water (g/mL)	Cooking time (min)	Temperature (°C)	Notes
Risotto (RIS)	100/500	35 (including 3 min toasting)	100	Temperature of the rice during cooking
Pilaf (PIL)	100/150	30	175	Temperature of the oven
Sous-vide 99 °C (SV99)	100/100	60	99	Temperature of the cooking bath
Sous-vide 89 °C (SV89)	100/100	60	89	Temperature of the cooking bath

Prior to the analyses, cooked rice samples were freeze-dried (Heto Drywinner 8, Copenhagen, Denmark) according to the following procedure: pre-freezing -25 °C for 1 h; primary drying -10 °C for 16 h and 0 °C for 16 h; secondary drying 10 °C for 30 h and 20 °C for 10 h. Finally, as raw grains, lyophilized cooked samples were ground (Sterilmixer 12, International PBI, Milan, Italy) and reduced to a fine flour (MM 400, Retsch GmbH, Haan, Germany).

2.3. Proximate composition

The determination of the proximate composition was performed as previously described in Colasanto *et al.* **[16]**. The thermo-balance Sartorius MA30 (Sartorius AG, Goettingen, Germany), the Kjeltec system I (Foss Tecator AB, Höganäs, Sweden) and the Megazyme total dietary fiber analysis kit were used to determine moisture, total protein (conversion factor: 5.95) and total dietary fiber contents, respectively. The ash content was determined according to the AOAC (1990) procedure **[21]**.

2.4. Proteins characterization

2.4.1. Extraction of proteins

For the protein extraction the procedure described by Furukawa *et al.* **[22]** was employed, with some modifications; 3 different protein fractions (albumin and globulin, glutelin and prolamin) were obtained.

2.4.1.1. Extraction of albumin and globulin

In a 1.5 mL test tube 100 mg of ground sample was added with 0.5 mL of 0.5 M NaCl solution and mixed using a vortex mixer. This suspension was centrifuged at 12,000 rpm for 30 minutes at 20 °C (centrifuge *Eppendorf* 5417 R, Eppendorf, Hamburg, Germany). The supernatant was collected in a 15 mL test tube, while the solid residue was re-extracted with 0.5 mL of 0.5 M NaCl. This operation was repeated other 2 times, recovering the finally solid residue for the glutelin extraction; the collected supernatant was added with 300 µL of trichloroacetic acid 100% *w/w* and left in the freezer at -20 °C for 30 minutes. Then it was centrifuged at 14,000 rpm for 20 minutes at 20 °C and the supernatant was eliminated, while the residue was mixed with 0.5 mL of iced acetone. This solution was again centrifuged at 14,000 rpm for 20 minutes at 20 °C and the supernatant was eliminated. This operation was repeated for a total of 3 times. The solid residue represents the fraction containing albumin and globulin.

2.4.1.2. Extraction of glutelin

The residue obtained from the extraction of albumin and globulin was extracted with 0.5 mL of lactic acid 1% *v/v*, mixing with a vortex. This suspension was centrifuged at 12,000 rpm for 30 minutes at 20 °C and the supernatant was collected in a 15 mL test tube; the solid residue was re-extracted with 0.5 mL of lactic acid 1% *v/v*. The operation was repeated for a total of 3 times; the final supernatant was added with 300 µL of trichloroacetic acid 100% *w/w* and left in the freezer at -20 °C for 30 minutes. The solid residue representing the

glutelin fraction was obtained following the same procedure described for albumin and globulin.

2.4.1.3. Extraction of prolamin

The precipitate obtained after glutelin was subjected to extraction using 0.5 mL of ethanol 70% *v/v*. This suspension was vortex-mixed and centrifuged at 12,000 rpm for 30 minutes at 20 °C, then the supernatant was collected in a 15 mL test tube, and the precipitate was re-extracted with 0.5 mL of ethanol 70% *v/v*. The solid residue representing the prolamin fraction was obtained following the same procedure described for albumin and globulin.

2.4.2. Determination of protein fingerprint (SDS-PAGE)

All the protein fractions were subjected to electrophoretic separation (SDS-PAGE; Mini-PROTEAN II Cell, Bio-Rad, Richmond, CA) using the conditions described by Arlorio *et al.* **[23]** with 12% acrylamide resolving and 4% acrylamide stacking gel. A molecular weight marker (Precision plus protein™ standards, Bio-Rad, Richmond, CA) was used to identify the molecular weight of protein bands. Following electrophoresis, the gels were stained with 0.1% Coomassie blue R-250 *w/v* (for the albumin and globulin, and glutelin fractions) or with silver staining procedure (for the prolamin fraction, which is not visible with the Coomassie blue R-250 stain) **[24]**.

2.5. Phenolic characterization

2.5.1. Extraction of phenolics

All the samples, both raw and freeze-dried cooked black rice, underwent an extraction procedure for the characterization of both free and bound phenolics, following the protocol described by Giordano *et al.* **[25]**, with some modifications.

100 mg of ground sample was extracted with 1.5 mL of a 50% *v/v* hydroalcoholic mixture in ultrasonic bath for 2 minutes at room temperature. Then, the test tube was centrifuged (Eppendorf Centrifuge 5417 R, Hamburg, Germany) at 14,000 rpm for 2 minutes and the upper phase was collected in a 15 mL test tube. The extraction was repeated two times on the solid residue, collecting every time the upper phase in the 15 mL test tube. This phase represents the free fraction of phenolics, while the solid lower phase was used for the extraction of bound phenolics. The total extract was divided in aliquots for the analyses and stored at -20 °C until use. For each rice sample the extraction was performed in triplicate.

The solid residue from the extraction of free phenolics was quantitatively transferred in a 50 mL Erlenmeyer flask using a total volume of 10 mL of NaOH 4M. The flask was placed on a magnetic stirring plate and mixed for 3 h and 30 minutes, after which the suspension was acidified with HCl 6M to a pH of 2.30. Then, the flask content was transferred into a separating funnel and three extractions with ethyl acetate, using 30, 20 and 10 mL, sequentially, were carried out. Following each extraction step, the organic phase was collected in a 100 mL flask and finally the solvent was removed by a rotary evaporator (Rotavapor® BUCHI R-210, Flawil, Switzerland). The dry extract obtained was dissolved in 2 mL of HPLC grade methanol, filtered on a 0.45 µm filter (Spartan^{TM} 30/0.45 RC) and transferred in a 2 mL test tube, representing the bound fraction of phenolics. The extract was stored at -20 °C until use. For each cooked rice sample the extraction was performed in triplicate.

2.5.2. Spectrophotometric analyses

Spectrophotometric analyses were performed on the hydroalcoholic extracts (free phenolic fraction).

2.5.2.1. Antioxidant activity

The antioxidant activity (AA) was determined as DPPH radical scavenging. In accordance with the method described by Locatelli *et al.* **[26]**, 700 µL of sample opportunely diluted in MeOH (or 700 µL of MeOH for the control) was added to the same volume of DPPH$^{\bullet}$ methanolic solution (100 µM). The solution was shaken and left in the dark at room temperature for 20 min, then the absorbance was read at 515 nm. The antioxidant activity of rice extracts was calculated as inhibition percentage of the radical and, finally, expressed as Trolox equivalents (TE) by means of a calibration curve.

2.5.2.2. Phenolic content

According to a modified version of the Folin-Ciocâlteu method **[27],** the phenolic content (PC) was determined. An appropriate volume of hydroalcoholic rice extract was added to 50 µL of Folin-Ciocâlteu reagent and 175 µL of aqueous Na_2CO_3 (5% *w/v*) and then the final solution volume was brought to 1450 µL with distilled water. After 1 h of incubation the absorbance was read at 760 nm, using a SHIMADZU UV-1900 spectrophotometer (Shimadzu, Tokyo, Japan). Results were expressed as catechin equivalents (CE) through a calibration curve.

2.5.2.3. Anthocyanin content

The pH differential method described by Lavelli *et al.* **[28]** was used to determine the content of anthocyanins (AC) and monomeric anthocyanins (MAC). Appropriate dilutions of the samples were prepared with potassium chloride buffer (0.025 M), pH 1.0 and the same dilution factor was applied with sodium acetate buffer (0.4 M), pH 4.5. Solutions at pH 1.0 were let to equilibrate for 5 min and those at pH 4.5 for 15 min; then the absorbance was measured at both 520 and 700 nm (SHIMADZU UV-1900 spectrophotometer, Shimadzu, Tokyo, Japan). The concentration of TA and TMA in the extracts was expressed as cyanidin-3-O-glucoside (Cn-3-Glu) equivalents according to the equations:

$$MAC\ (\mu g/mL) = [(A_{520\ nm} - A_{700\ nm})_{pH\ 1} - (A_{520\ nm} - A_{700\ nm})_{pH\ 4.5}] \times MW \times d \times 1000/\varepsilon \quad (1)$$

$$AC\ (\mu g/mL) = [(A_{520\ nm} - A_{700\ nm})_{pH\ 1}] \times MW \times d \times 1000/\varepsilon \quad (2)$$

where MW is the molecular weight of cyanidin-3-glucoside (449.2 g/mol), d is the dilution factor, ε is the molar extinction coefficient of cyanidin-3-O-glucoside (26,900 M^{-1} cm^{-1}). Results were then expressed based on rice weight (d.w.).

2.5.2.4. Proanthocyanidin content

For the determination of proanthocyanidin (PAC) content, the protocol described by Prior *et al.* **[29]** was used, with some modifications. Phenolic extracts were opportunely diluted with a mixture of acetone, water and acetic acid (75:24.5:0.5, *v/v*). Then, 280 µL of diluted extract (or 280 µL of solvent for the control) were added to 840 µL of DMAC (4-(dimethylamino) cinnamaldehyde) ethanolic solution (0.01% *w/v*). The solution was left to react until the maximum absorbance value was reached (20 min), at 20 °C and away from the light, then the absorbance was read at 640 nm (SHIMADZU UV-1900 spectrophotometer, Shimadzu, Tokyo, Japan). Results were expressed as catechin equivalents (CE) through a calibration curve.

2.5.3. Chromatographic analysis

Chromatographic analysis was performed on both the hydroalcoholic (free fraction) and hydrolysed (bound fraction) phenolic extracts.

For the RP-HPLC-DAD analysis the protocol described by Colasanto *et al.* **[16]** was applied. Using a Shimadzu LC-20A Prominence chromatographic system equipped with a diode array detector (DAD detector SPD-M20A), the separation was performed on a reversed-phase Synergi TM 4 µm Max-RP 80 Å LC Column (250 x 4.6 mm i.d.) (Phenomenex, Torrance, CA, USA), protected by a guard column containing the same phase, at 30 °C.

Eluent A of the mobile phase consisted of water/ formic acid/ acetonitrile (87:10:3, *v/v*), while eluent B consisted of water/ formic acid/ acetonitrile (40:10:50, *v/v*). The program gradient was the following: from 6 to 20% B (20 min), from 20 to 40% B (15 min), from 40 to 60% B (5 min), from 60 to 90% B (5 min), isocratic 90% B (5 min), from 90 to 6% B (1 min), isocratic 6% B (29 min), with a total run time of 80 minutes. The flow rate and the injection volume were 400 µL/min and 7 µL, respectively. Polyphenolic rice extracts were centrifuged (14,000 rpm for 20 min, microcentrifuge 5417R, Eppendorf, Milan, Italy) prior to the injection in the chromatographic system. Cyanidin-3-O-glucoside, peonidin-3-O-glicoside, cyanidin-3-O-rutinoside and peonidin-3-O-rutinoside were tentatively identified by comparison with retention times of individual authentic standard molecules and their UV–Vis spectra; the quantification was performed on the basis of calibration curves obtained with the corresponding standards. Cyanidin-3-O-gentiobioside, for which the commercial standard compound was not available, was identified based on the characteristics reported in the literature **[2]**. Its concentration was expressed as equivalent of cyanidin-3-O-glucoside.

2.6. Statistical analysis

All the statistical analyses were performed using the free statistical software R 4.1.1 version **[30]** and results were expressed as mean ± standard deviation (SD) of at least three independent experiments. Differences were estimated by analysis of variance (ANOVA) followed by Tukey's honest significant difference test. The statistical significance level was set to 0.05.

3. Results and discussion

Different cooking techniques (risotto, pilaf and *sous vide*) were applied to the Artemide Italian black rice variety to evaluate their impact on chemical and nutritional composition. The “risotto” is a traditional preparation of the northern Italian tradition; the “risotto” preparation includes a toasting phase that induce the reaction between starch and proteins on the surface of the grain, causing a structural change of rice and leading to the formation of a harder external layer in the caryopsis, which becomes more resistant to high temperatures, and inducing a slower release of the starch during cooking **[7]**. The toasting phase can be carried out with or without the addition of lipids (generally extra virgin olive oil, or butter). Due to the fact that in our previous work **[16]** the risotto preparation without any lipids resulted in a better preservation of polyphenolic fraction, in this work, we stated not to use a fat during the toasting phase. The “pilaf” is a typical Middle Eastern cooking technique, used especially for Basmati rice, considered a hybrid between boiling and “risotto” cooking, but with two main differences: the rice is oven-cooked in a pan, allowing a more homogeneous diffusion of heat, and it is not mixed during the entire cooking time **[31,32]**. The principle of the *sous vide* cooking technique is to cook raw food, vacuum-packed in special heat-resistant bags, at controlled temperatures (generally lower than usual) and times (generally longer), in order to improve its texture and allow, in some cases, a lower degradation of bioactive substances **[10,33,34]**. The chosen cooking parameters (rice amounts, cooking times and ratios rice/water) derived from previous tests, carried out to guarantee a similar texture of the rice, independently from the cooking method. 100 g of rice, comparable to 1 portion, was chosen as rice amount, and the ratios rice/water were optimized to allow a complete absorption of the water by the rice. Risotto mode included a 3 min toasting phase without any fat addition, this having turned out to be the most performing mode in our previous work **[16]**. Two temperatures were applied to *sous vide* cooking (89 and 99 °C), to evaluate the impact of this parameter. Each cooking was carried out in triplicate, in order to consider the eventual variability of preparations.

3.1. Proximate composition

The proximate composition of both raw and cooked rice was evaluated; the results are summarized in **Table 2**, and expressed on a dry weight (d.w.) basis, except for the moisture content. The moisture percentage of raw rice (11.52%) is in line with the average moisture values of black rice varieties (12.50%) **[35]** and with the value observed in a previous work on “Artemide” black rice (11.70%) **[16]**. After cooking, a moisture percentage increment was

observed, due to the water absorption by the rice grains, thus swelling, softening and becoming edible. Cooked rice showed comparable moisture values, with the exception of PIL, which presented a significant lower humidity (45.30%). This value could be due to the non-hermetic closure of the lid and/or high temperature applied during oven-cooking, thus determining a major drying of the grain during the pilaf cooking in respect to the other methods.

Table 2. Proximate composition of raw and cooked Artemide rice; the results are expressed as mean ± standard deviation. Values with different letters in the same column are significantly different ($p < 0.05$). Values between brackets indicate significant percentage variations in respect to raw rice.

	Moisture (%)	**Ashes (% d.w.)**	**Proteins (% d.w.)**	**Total dietary fiber (% d.w.)**
RAW	11.52 ± 0.26^{c}	1.49 ± 0.08^{a}	8.96 ± 0.01^{a}	6.10 ± 0.13^{d}
RIS	61.48 ± 1.10^{a} (+ 434%)	1.49 ± 0.07^{a}	9.02 ± 0.14^{a}	7.84 ± 0.46^{a} (+ 29%)
PIL	45.30 ± 0.69^{b} (+ 293%)	0.87 ± 0.02^{b} (- 42%)	8.90 ± 0.16^{a}	6.61 ± 0.50^{cd} (+ 8%)
SV99	62.29 ± 0.43^{a} (+ 441%)	1.41 ± 0.07^{a}	8.83 ± 0.16^{a}	7.06 ± 0.45^{bc} (+ 16%)
SV89	62.47 ± 1.37^{a} (+ 442%)	1.42 ± 0.10^{a}	8.95 ± 0.09^{a}	7.52 ± 0.19^{ab} (+ 23%)

Concerning the ashes values, no statistically significant differences were found between raw rice and RIS, SV99 and SV89 samples, while a relevant decrease was observed after pilaf cooking (- 42%). Concerning PIL, it is possible that the inorganic substances dissolved in the cooking-water, following its evaporation, remained on the surfaces of the pan, thus obtaining a reduction of their concentration in the rice.

Regarding proteins, no significant differences ($p > 0.05$) were found between raw and cooked rice, with an average value of 8.93% (d.w.), suggesting that cooking does not affect the total proteins content of Artemide black rice.

The average content of total dietary fibre in raw rice was 6.10%, a lower value that than obtained in a previous work (10.8%) **[16]**; this discordant result could be given by factors related to cultivation such as the climate conditions, the use of fertilizers and the degree of ripeness of the grain at the time of harvest **[36]** and/or by major impact of the rice dehusking. Following cooking there was an increase in the values, in particular for RIS, SV99 and SV89, which increased their fibre content by 28.52%, 15.76% and 23.28%, respectively. This result could be linked to a degradation of the grain structure, which facilitated the fibre extraction.

3.2. Protein profiling

Considering that the total protein content not varied after cooking, a qualitative electrophoretic separation (SDS-PAGE) of the main protein fractions (albumins and globulins, prolamins and glutelins) was performed, in order to evaluate the eventual impact of the cooking methods on the protein profile.

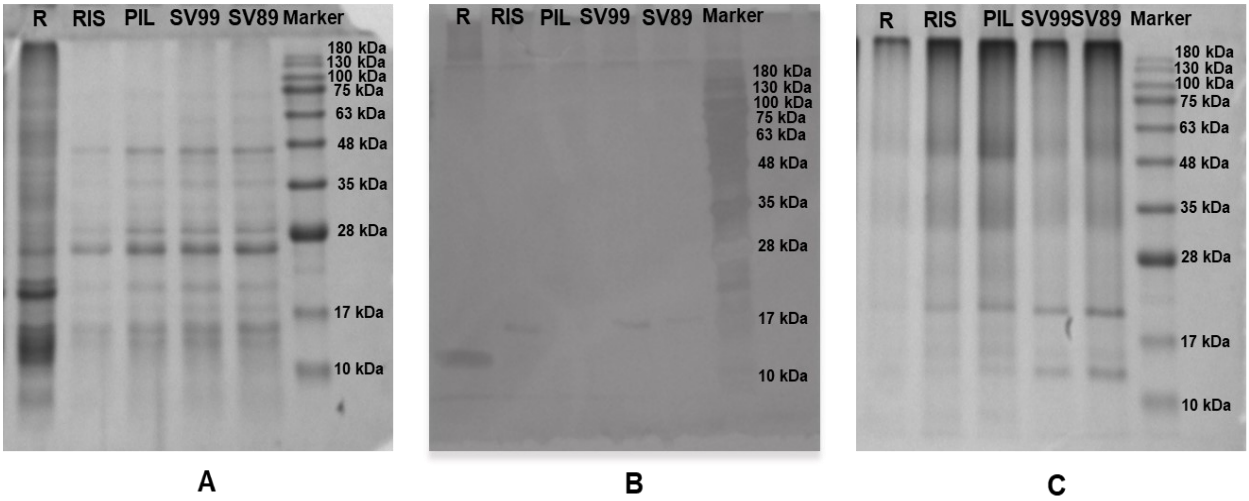

Figure 1. *Electrophoretic separation (SDS-PAGE) of the protein fractions containing albumins and globulins (A), prolamins (B) and glutelins (C).*

Regarding albumins and globulins (**Figure 1**, panel A), raw rice presented higher-intensity bands in respect to the cooked samples. This could be due to a partial protein denaturation during cooking; in addition, after cooking, the intensity of bands at 28 and 26 kDa slightly increased, suggesting hydrolysis phenomena involving higher molecular weight proteins.

As reported by Krishnan *et al.* **[37]** the main prolamins in rice are characterized by a molecular weight between 12 and 17 kDa. In **Figure 1**, panel B, it is possible to note in raw rice a broad band at about 13 kDa, which was not detected after cooking. A band at 17 kDa was instead detected in cooked samples, but not in the raw one. It can be hypothesized that prolamins present in uncooked rice, following the heat treatment, may have undergone structural changes, or may have linked to other molecules, thus increasing their size and molecular weight. A particular case is the PIL sample, in which it was not possible to detect prolamins: this could be related to problems in the extraction process, which is particularly delicate, since the prolamins in rice are present in very low concentrations, but also to the effect of high cooking temperatures (175 °C, the highest temperature considering all cooking methods), which could have induced a degradation of these proteins.

The main glutelins in rice are three: pro-glutelin (57 kDa), an acidic subunit (37-39 kDa) and a basic subunit (22-23 kDa) **[38]**. Observing **Figure 1**, panel C, all these bands can be identified, and, in general, cooking does not significantly impact on them. However, more pronounced bands can be seen in the cooked samples, compared to uncooked rice, at the 17 kDa molecular weight marker and below the 10 kDa molecular weight marker. This could be due to degradation phenomena of proteins with higher molecular weight, due to the thermal treatments.

3.3. Phenolic composition

3.3.1. Phenolic, anthocyanins, proanthocyanidins content and antioxidant activity

The phenolic content of raw and cooked rice, expressed as milligrams of catechin equivalents (CE) per gram of rice (dry weight, d.w.), is represented in **Figure 2** (panel A). A significant decrease of PC in all cooked sampled was observed respect to the raw rice. The average decrease of PC was 34%, that is a lower reduction compared to that observed in our previous work (average decrease: -83%) **[16]**. The greatest loss of polyphenols occurred in PIL rice (-44%) and SV99 rice (-42%), while RIS and SV89 cooking allowed to preserve a greater quantity of polyphenols (-29% and -22%, respectively). The PIL cooking led to a great loss of polyphenols, probably caused by the high temperatures applied (175 °C). In addition, as observed for ashes, water-soluble polyphenols could have been deposited on the pan after water evaporation; in fact, a coloured layer on the edges of the pan appeared at the end of the cooking, thus suggesting a further loss of phenolic compounds. The lower impact of cooking in the SV89 sample seems to be linked to the reduced temperature used; in fact, by increasing the temperature to 99°C, a relative greater loss of polyphenols was observed (-42%). These results, together with other previous literature evidences **[16,17,19]** suggest a significant impact of temperature values on the polyphenolic component of rice. However, even if temperatures around 100 °C were reached, in the RIS sample the highest PC values, similar to that obtained for the SV88, were observed. In this case, it could be hypothesized a role of the toasting process (not foreseen for the other cooking methods); in this cooking phase, following structural changes of the grain, a protective barrier could be formed, permitting to retain polyphenolic compounds inside the kernel. Furthermore, the optimization of cooking conditions for the risotto preparation (toasting phase: 3 minutes; total cooking time: 35 minutes; ratio rice/water (g/mL): 100/500) allowed to limit the loss of PC (-29%) respect to our previous work (-79%), where the toasting phase lasted 5 minutes, the total cooking time was 40 minutes and the ratio rice/water (g/mL) was 200/500 **[16]**.

The antioxidant activity was determined by DPPH• assay and expressed as milligrams of Trolox equivalents (mg TE/g d.w.) (**Figure 2**, panel B). The AA of raw rice (14.41 mg TE/g d.w.) is in line with that reported by Bordiga *et al.* (13.79 mg TE /g d.w.) **[2]**, but lower than that found in our previous studies (21.4 mg TE /g d.w.) **[16]**. This difference could be related to the different polyphenols' concentration, which in turn varies depending on pedo-climatic conditions, agronomic treatments and/or attack by parasites, but also on the degree of dehusking process. After cooking a decrement of the antioxidant activity was observed. The most significant loss was observed in PIL sample (-47%), while RIS and SV89 allowed to preserve greater antioxidant activity with a loss of 33% and 37%, respectively.

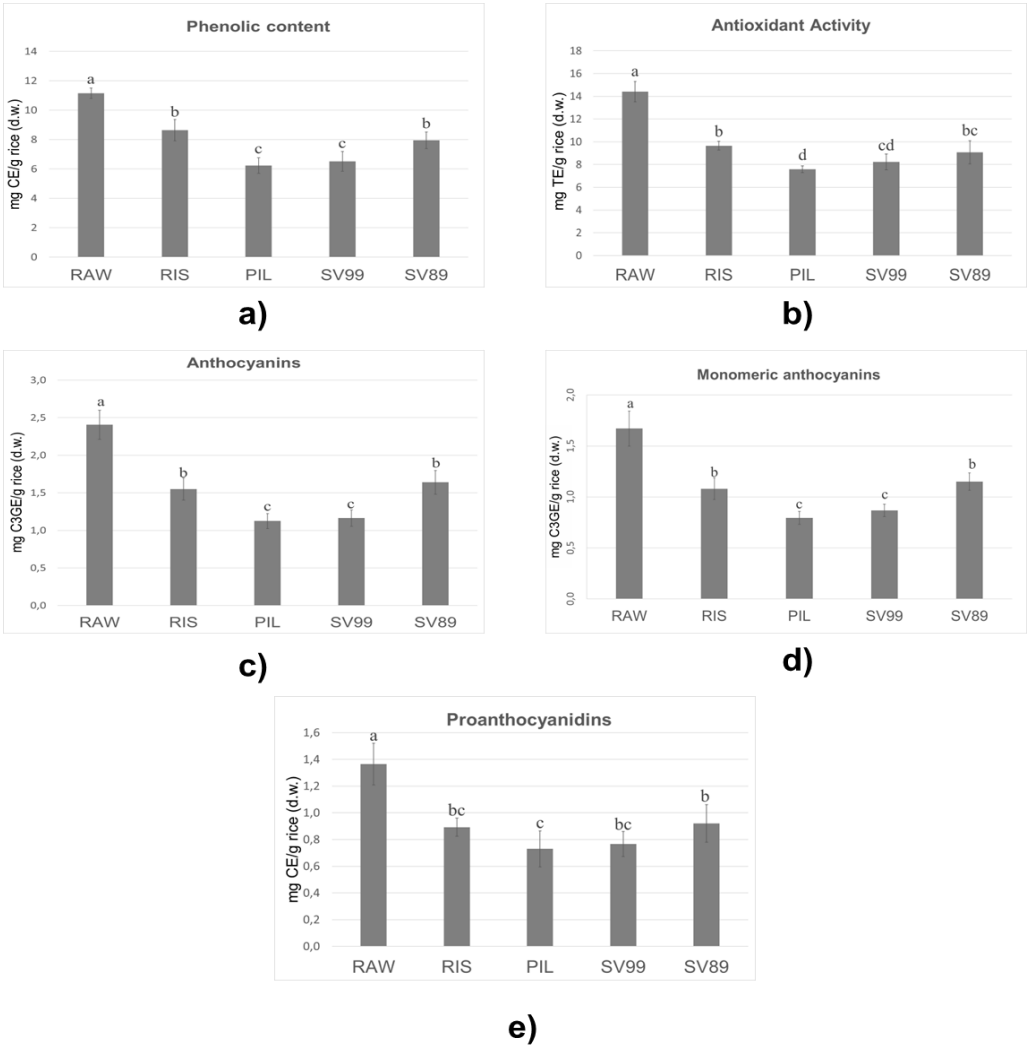

Figure 2. ***(a)*** *Free phenolic content (mg CE/ g d.w.),* ***(b)*** *antioxidant activity (mg TE/ g d.w.),* ***(c)*** *anthocyanins (mg CnE/ g d.w.),* ***(d)*** *monomeric anthocyanins (mg CnE/ g d.w.), and* ***(e)*** *proanthocyanidins (mg CE/ g d.w.) quantified in raw and cooked black rice, expressed as mean ± standard deviation. For each parameter, values with different letters are significantly different (p<0.05). CE: catechin equivalents; CnE: cyanidin-3-Oglucoside equivalents; TE: Trolox equivalents.*

The anthocyanins content, expressed as cyanidin-3-O-glucoside equivalents (mg CnE/g d.w.), was determined as both anthocyanins (AC) and monomeric anthocyanins (MAC) content (**Figure 2**, panel C and D). The AC value in Artemide raw rice (2.41 mg CnE/g d.w.) is slightly lower than that reported by Fracassetti *et al.* (3.71 mg CnE/g d.w.) **[17]**. Concerning the monomeric anthocyanins (1.67 mg CnE/g d.w.), they accounted for about 70% of the total value. After cooking a significant decrease of AC and MAC was observed, evidencing in both cases a similar behaviour. PIL and SV99 samples were the mostly affected by the cooking, which determined a reduction of 53% and 52% for the AC, and 52% and 48% for the MAC, respectively. RIS and SV89 samples allowed to maintain a good concentration of anthocyanins, with a more contained loss of 35% and 31% for MAC and 36% and 32% for AC, respectively. These reductions in the anthocyanin content after cooking were observed also by other authors. The anthocyanin content determined by Melini et al. in the raw Artemide black rice (1.99 mg/g d.w.) decreased of about 50% after “risotto” cooking (1.00 mg/g d.w.) **[39]**. Similarly, Fracassetti *et al.* observed a decrease in Artemide black rice anthocyanin content of about 48% after cooking in a rice cooker with different additions of water (from 3.41 mg/g d.w. of raw rice to 1.76 mg/g d.w. of cooked one) **[17]**.

The proanthocyanidins content (PAC), expressed as milligrams of catechin equivalents (CE) per gram of rice (dry weight, d.w.), was determined through the BL-DMAC assay (**Figure 2**, panel E). All the cooking methods examined led to a reduction in the concentration of proanthocyanidins. The greatest loss was found in PIL sample (-46%), while SV89 sample gave the best results with a loss of 32%. The RIS and SV99 cooking, on the other hand, gave an intermediate percentage of loss compared to the previous cooking (respectively 35% and 43%). Several factors could have led to the loss of proanthocyanidins, such as the degradation in the presence of oxygen **[40]**. In addition, proanthocyanidins are able to bind in a non-specific way proteins; during cooking, the formation of protein-proanthocyanidins complexes could made them insoluble in the extraction solvent, avoiding their detection and quantification. Furthermore, oxidative condensation reactions involving other tannins could occur, giving rise to high molecular weight insoluble proanthocyanidins; other chemical reactions could finally cause their cleavage in different molecules not detectable by this assay **[41]**.

3.3.2. Characterization of individual phenolics through RP-HPLC-DAD analysis

3.3.2.1. Anthocyanins

Individual monomeric anthocyanins were mainly detected in hydroalcoholic extracts (free form), with the only exception of cyanidin-3-O-rutinoside and peonidin-3-O-rutinoside, which were identified in the bound phenolic fraction, but at concentration under the limit of detection. The anthocyanin content in raw and cooked rice, expressed as µg/g (d.w.), is reported in **Table 3**.

Table 3. *Anthocyanins (µg/g d.w.) identified in both uncooked and cooked black rice samples; the results are expressed as mean ± standard deviation. Values with different letters in the same column are significantly different ($p < 0.05$). Values between brackets indicate significant percentage variations in respect to raw rice. Cn-3-glc: Cyanidin-3-O-glucoside; Pn-3-glc: Peonidin-3-O-glucoside; Cn-3-rut: Cyanidin-3-O-rutinoside; Pn-3-rut: Peonidin-3-O-rutinoside; Cn-3-gent: Cyanidin-3-O-gentiobioside.*

	Cn-3-glc (µg/g d.w.)	**Pn-3-glc (µg/g d.w.)**	**Cn-3-rut (µg/g d.w.)**	**Pn-3-rut (µg/g d.w.)**	**Cn-3-gent (µg/g d.w.)**
RAW	1258 ± 89^{a}	120.0 ± 7.9^{a}	68.60 ± 4.1^{a}	6.065 ± 0.42^{a}	10.91 ± 0.66^{a}
RIS	691.7 ± 61^{c} (- 45%)	62.54 ± 5.2^{c} (- 48%)	37.40 ± 4.7^{c} (- 46%)	3.145 ± 0.37^{c} (- 48%)	7.277 ± 1.1^{b} (- 33%)
PIL	564.1 ± 22^{d} (- 55%)	51.22 ± 2.4^{d} (- 57%)	29.85 ± 1.5^{d} (- 56%)	3.284 ± 0.38^{c} (- 46%)	5.314 ± 0.72^{c} (- 51%)
SV99	554.0 ± 34^{d} (- 56%)	50.50 ± 3.3^{d} (- 58%)	27.36 ± 2.0^{d} (- 60%)	3.711 ± 0.43^{bc} (- 39%)	6.934 ± 0.58^{b} (- 36%)
SV89	797.1 ± 11^{b} (- 37%)	72.00 ± 0.9^{b} (- 40%)	44.83 ± 1.6^{b} (- 35%)	4.088 ± 0.66^{b} (- 33%)	10.12 ± 0.68^{a} (- 7%)

Cyanidin-3-O-glucoside was the most abundant anthocyanin in Artemide black rice, representing about 86% of total anthocyanins content in raw rice. This data agrees with the results obtained by Ito & Lacerda **[42]**, which reported for other black rice varieties a contribution of this anthocyanins to the total anthocyanin corresponding to about 88%. Furthermore, the cyanidin-3-O-glucoside content quantified in Artemide black rice in this work (1258 µg/g) is greater than that determined by Bordiga *et al.* **[2]** (1004 µg/g), while cianidin-3-O-gentiobioside and peonidin-3-O-rutinoside content (10.9 and 6.7 µg/g, respectively) were found in lower concentrations (42.0 and 36.9 µg/g).

Differences in anthocyanins composition regarding different lots of Artemide rice probably depend on year of cultivation, environmental climatic conditions and processing conditions (especially the husk removal).

For all the anthocyanins identified, a reduction in their content after cooking was recorded if compared to the raw sample. Regarding cyanidin-3-O-glucoside, the most abundant

compound, SV89 cooking was found to be the best cooking method, which has resulted in a reduction of its content of 37%, followed by RIS (-45%). Significantly lower values were recorded for SV99 (554 µg/g) and for PIL (564 µg/g), with a loss compared to raw rice of 56 and 55%, respectively. A similar trend was observed also for the peonidin-3-O-glucoside, for which SV89 determined a reduction of 40% respect to the raw rice, against the 48, 57 and 58% of RIS, PIL and SV99 cooking, respectively. In a similar way, SV89 was the cooking method that best preserved cyanidin-3-O-rutinoside and peonidin-3-O-rutinoside, with a loss of only 35 and 33%, respectively, compared to the raw rice.

Finally, cyanidin-3-O-gentiobioside, even though present at low concentrations, is particularly interesting because seems more stable to the thermal treatment than to the other identified anthocyanins. Particularly, in the SV89 sample the reduction of this anthocyanin compared to raw rice is not statistically significant. A possible explanation for this result could lie in the chemical structure of this anthocyanin, as the presence of the disaccharide could have conferred greater stability to the molecule **[43]**.

From these results it is evident that the temperature has a strong impact on the total anthocyanin content. It's evident that the use of a lower temperature in *sous vide* cooking (SV89) better preserved anthocyanin content, compared to the same cooking method carried out at a higher temperature (SV99). Also RIS cooking has allowed to obtain good results in terms of preservation of the anthocyanin content, even if a maximum temperature of 100 °C was reached. This could be explained by the toasting phase typical of this cooking mode, which could have created a sort of "barrier" effect on the surface of the rice, able to protect the anthocyanins from their possible degradation, as demonstrated in Colasanto *et al.* **[16]**. This effect could justify the limited loss of anthocyanins, even if higher temperatures are reached in RIS compared to SV89. Considering PIL cooking, in which higher temperature are reached (175 °C), it resulted in a type of cooking which, like SV99, in almost all cases (except for peonidin-3-O-rutinoside) determines a considerable reduction of the total content of anthocyanins. Our results agree with Catena *et al.* **[19]**, in which it was seen that PIL cooking does not allow preserving good quantities of anthocyanins in the sample after cooking.

3.3.2.2. Free phenolic acids and flavonoids

In the free polyphenolic fraction, phenolic acids belonging to two different classes were found: 1) benzoic acid derivatives (gallic acid, protocatechuic acid and vanillic acid) and 2) cinnamic acid derivatives (ferulic acid and coumaric acid). In addition, a flavan-3-ol

(catechin) and a flavonol (myricetin) were also found. The results of quantification obtained from the analysis of these compounds are shown in **Table 4**; all the results are expressed in µg/g of rice (dry weight) and presented as mean ± standard deviation.

Table 4. *Free phenolic acids (μg/g d.w.) in both uncooked and cooked black rice samples; the results are expressed as mean ± standard deviation. Values with different letters in the same column are significantly different ($p < 0.05$). Values between brackets indicate significant percentage variations in respect to raw rice. Gal: gallic acid; Proto: protocatechuic acid; Van: vanillic acid; Fer: ferulic acid; Coum: coumaric acid; Cat: catechin; Myr: myricetin.*

	Gal	**Proto**	**Van**	**Fer**	**Coum**	**Cat**	**Myr**
RAW	5.31 ± 0.48^{c}	86.0 ± 3.6^{e}	54.5 ± 1.7^{c}	9.34 ± 0.26^{c}	16.7 ± 0.7^{a}	17.0 ± 2.9^{d}	2.98 ± 0.29^{c}
RIS	6.06 ± 0.32^{b} (+ 14%)	208 ± 10^{b} (+ 142%)	51.2 ± 3.1^{c}	11.4 ± 1.3^{ab} (+ 22%)	13.1 ± 0.8^{c} (- 22%)	61.0 ± 4.2^{b} (+ 259%)	5.81 ± 0.68^{a} (+ 95%)
PIL	5.04 ± 0.28^{c}	185 ± 5.0^{d} (+ 115%)	51.8 ± 10.6^{c}	10.3 ± 0.6^{bc}	13.4 ± 1.3^{c} (- 20%)	50.0 ± 3.8^{c} (+ 194%)	4.58 ± 0.19^{b} (+ 54%)
SV99	7.32 ± 0.18^{a} (+ 38%)	228 ± 5.0^{a} (+ 165%)	84.6 ± 8.2^{a} (+ 55%)	12.5 ± 0.3^{a} (+ 34%)	15.2 ± 1.2^{ab}	78.5 ± 2.8^{a} (+ 362%)	4.68 ± 0.48^{b} (+ 57%)
SV89	6.09 ± 0.31^{b} (+ 15%)	197 ± 3.0^{c} (+ 129%)	67.2 ± 3.5^{b} (+ 23%)	11.7 ± 0.6^{a} (+ 25%)	13.6 ± 0.7^{bc} (- 19%)	51.8 ± 1.1^{c} (+ 205%)	4.32 ± 0.71^{b} (+ 45%)

Protocatechuic acid is the most abundant phenolic acid in the free phenolic fraction, both before and after cooking, with values of 86 µg/g in RAW and 208 µg/g, 185 µg/g, 228 µg/g and 197 µg/g in RIS, PIL, SV99 and SV89, respectively. All the cooking methods determined an increase of this phenolic acid compared to the levels detected in raw rice. In particular, SV99 is the cooking method that caused the greatest increase in the levels of protocatechuic acid (+ 165%), while PIL caused the smallest one (+ 115%).

The increase in the quantities of protocatechuic acid in black rice samples after cooking agrees with what observed by Hiemori *et al.* **[44]**, who demonstrated that the increase in protocatechuic acid derives from the degradation of cyanidin-3-O-glucoside.

High concentrations in free form were obtained also for vanillic acid, with a value of 54.5 µg/g in uncooked rice. RIS and PIL cooking did not cause significant variations in its content, while SV cooking determined an increase in its concentrations compared to the RAW sample, evidencing a positive relation with the temperature employed: the concentration of vanillic acid in SV89 (67.2 µg/g) was lower than that registered in SV99 (84.6 µg/g).

Regarding gallic acid in free form, its contribution is not very relevant (5.31 µg/g in uncooked rice); the concentration values were rather low both before and after cooking, also for the methods that caused a statistically significant increase (RIS, SV89 and SV99).

Considering the hydroxycinnamic acids in the free form, coumaric acid showed the highest average content (16.7 µg/g in RAW sample), while ferulic acid showed slightly lower values (9.34 µg/g in RAW sample). However, their trend after cooking is different: for coumaric acid a general decrease is observed after cooking, while for ferulic acid an increase is noted.

Finally, discrete levels of catechin and smaller quantities of myricetin were also found. About catechin all cooking types determined an increase in its concentration, compared to the raw rice (from 17 µg/g in RAW sample to 78.5 µg/g in SV99).

Summarizing, a general increase of free phenolic acids concentrations after cooking was observed, in contrast with our previous work in which all phenolic acids, except protocatechuic acid, decreased after cooking. These differences could be related to the different cooking conditions adopted for the risotto preparation and to the different cooking methods used in this work **[16]**. However, in agreement with what observed in this work, an increase in free phenolic acids concentrations after cooking was observed also by Ryu et al. **[20]**.

3.3.3. Characterization of bound polyphenolic fraction

Phenolic acids present in this fraction are those bound to fiber components such as cellulose, hemicellulose, lignin, pectin and structural proteins. Five benzoic acid derivates (gallic acid, protocatechuic acid, vanillic acid, *p*-hydroxybenzoic acid and syringic acid), two cinnamic acid derivates (ferulic and coumaric acids) and three other flavonoids (catechin, myricetin and epicatechin) were found in this form. Their content in the samples is reported in **Table 5**.

Analysing this table, it is clear that the cinnamic acid derivatives are more present than benzoic acid derivatives. Among the compounds that are present in both free and bound form there are gallic acid, protocatechuic acid, vanillic acid, ferulic acid, coumaric acid, catechin and myricetin, while *p*-hydroxybenzoic acid, syringic acid and epicatechin were identified in the only bound fraction.

Ferulic acid and coumaric acid are the most abundant compounds. In particular, ferulic acid (308 µg/g in the RAW sample) is present for 97% in the bound form and for only 3% in the free form. It is interesting to note that no type of cooking significantly affected the ferulic acid content of Artemide rice in bound form. A possible explanation of this phenomenon lies in the fact that in the structure of ferulic acid there is a methyl group linked to oxygen in position R1, which confers stability to the molecule **[45]**.

The coumaric acid is present for 90% in the bound form and only for 10% in the free form (the values refer to the RAW sample). Following cooking, no significant changes were found in the content of bound coumaric acid compared to the values recorded in the RAW rice.

Vanillic acid is most abundant in the bound form (in the RAW sample it is present for the 62% in this form) and after cooking its concentrations increased (+ 41% in RIS and PIL, + 38% in SV99 and + 35% in SV89). The other phenolic acids belonging to the same chemical group have been registered in bound form in very low quantities; in fact, the percentage of gallic acid and protocatechuic acid are only 19% and 4%, respectively.

The quantities of protocatechuic acid, *p*-hydroxybenzoic acid and syringic acid did not change after cooking, except for protocatechuic acid, that increased in SV99 sample in a significant manner (more than twice), and syringic acid, that slightly decreased after RIS cooking.

In general, the relative ratios between the free fraction and the bound fraction of phenolic acids after cooking do not change and remain almost similar to those recorded for the RAW

sample. The only exception is the gallic acid: although its content in the bound form tends to increase after cooking, the free form increases more than the corresponding bound form, therefore the relative percentage of the bound form tends to decrease.

In addition to the various phenolic acids, some flavonoids in bound form (catechin, myricetin and epicatechin) were also quantified in the Artemide black rice samples. Catechin and myricetin were found in both free and bound forms, while epicatechin was found only in the bound form. No variation in the content of myricetin and epicatechin were observed after cooking. Catechin is the only one of the three where significant increases in concentrations are recorded after cooking, compared to the RAW sample. In addition, although the catechin content in the bound form tends to increase after cooking, the free catechin increases more than in the corresponding bound form, therefore the relative percentage of the bound form tends to decrease. The most significant increase of catechin in the free fraction was recorded for SV99 (from 72% in RAW to 87% in SV99).

Finally, regarding anthocyanin component, it was observed that it is mainly present in free form, but two anthocyanins, cyanidin-3-O-rutinoside and peonidin-3-O-rutinoside, were identified in trace amounts (unquantifiable values) in the extracts of the bound fraction.

***Table 5**. Bound phenolic acids (µg/g d.w.) in both uncooked and cooked black rice samples; the results are expressed as mean ± standard deviation. Values with different letters in the same column are significantly different ($p < 0.05$). Values between brackets indicate significant percentage variations in respect to raw rice. Gal: gallic acid; Proto: protocatechuic acid; Van: vanillic acid; p-OH: p-hydroxybenzoic acid; Syr: syringic acid; Fer: ferulic acid; Coum: coumaric acid; Cat: catechin; Myr: myricetin; Epi: epicatechin.*

	Gal	**Proto**	**Van**	***p*-OH**	**Syr**	**Fer**	**Coum**	**Cat**	**Myr**	**Epi**
RAW	1.24 ± 0.30^{c}	3.93 ± 0.19^{b}	90.3 ± 4.7^{b}	3.46 ± 0.08^{ab}	1.72 ± 0.20^{a}	308 ± 10^{a}	147 ± 6.0^{a}	6.66 ± 0.76^{c}	88.3 ± 3.4^{a}	11.7 ± 1.3^{a}
RIS	4.39 ± 1.06^{ab} (+ 254%)	5.00 ± 1.15^{b}	127 ± 20^{a} (+ 41%)	4.47 ± 1.39^{a}	1.07 ± 0.42^{b} (-38%)	282 ± 39^{a}	143 ± 10^{a}	16.8 ± 2.1^{a} (+ 152%)	81.0 ± 13^{a}	18.4 ± 5.7^{a}
PIL	3.60 ± 1.06^{ab} (+ 190%)	6.11 ± 1.31^{ab}	127 ± 9.0^{a} (+ 41%)	3.53 ± 0.74^{ab}	1.52 ± 0.36^{ab}	288 ± 28^{a}	143 ± 14^{a}	13.1 ± 2.0^{b} (+ 97%)	77.7 ± 14^{a}	18.8 ± 4.7^{a}
SV99	5.23 ± 0.68^{a} (+ 322%)	8.24 ± 2.64^{a} (+ 110%)	125 ± 23^{a} (+ 38%)	3.78 ± 1.64^{ab}	1.24 ± 0.32^{ab}	301 ± 18^{a}	142 ± 10^{a}	12.1 ± 1.5^{b} (+ 82%)	69.2 ± 15^{a}	19.9 ± 9.9^{a}
SV89	2.76 ± 0.67^{bc}	6.64 ± 0.64^{ab}	122 ± 14^{a} (+ 35%)	2.47 ± 0.45^{b}	1.58 ± 0.16^{ab}	311 ± 18^{a}	143 ± 8.0^{a}	8.40 ± 1.02^{c}	76.0 ± 6.6^{a}	20.0 ± 2.2^{a}

4. Conclusions

Three different cooking methods, traditional Italian risotto and pilaf methods, and the more innovative (and less applied) *sous vide* technique (using a controlled temperature temperatures of 89 and 99 °C), were tested to evaluate their impact on the chemical and nutritional composition of Italian Artemide black rice.

The *sous vide* at 89 °C and the risotto were the best cooking methods to preserve anthocyanins, total polyphenols content and antioxidant capacity, while no particular differences between cooking methods were observed regarding their impact on the protein fraction. Concerning individual compounds, a general decrease of anthocyanins content was observed. Cyanidin-3-O-glucoside, the most abundant anthocyanin in black rice, decreased of 37 and 45% in *sous vide* at 89 °C and risotto samples, respectively, against the 55 and 56% of pilaf and *sous vide* at 99 °C samples. Free phenolic acids in general increased after cooking, probably for a better extraction due to modifications of food structure and/or following their release from the degradation of more complex phenolic compounds, while the fiber-bound fraction remained unchanged after all cooking methods.

Each cooking method was optimized in order to limit the loss of phenolics, thus allowing to reduce the average loss of polyphenols compared to our previous work **[16]**.

The possibility to improve the functional properties of cooked black rice, preserving at the best the antioxidant polyphenolic fraction simply modifying the cooking condition, seems particularly relevant: a correct information on good practices in food preparation could have a great impact on the health promoting habits of the population. In this regard, among the two best cooking methods identified, risotto, which is a traditional method, could be easily employed for home preparations, while the sous vide cooking (at 89 °C), requiring more professional skills and equipment, could be used in restaurants, canteens of schools, hospitals and companies.

Concluding, future research could be focused on rice digestive process, in particular exploiting *in vitro* simulated methods, in order to evaluate the fate of polyphenolic and anthocyanic compounds along the oro-gastrointestinal tract, and to estimate their bioaccessibility and bioavailability.

Funding: This research was funded by Università degli Studi del Piemonte Orientale, Dipartimento di Scienze del Farmaco, through the grant “Fondi di Ricerca Locale-FAR 2019”, and “NUTRAcore Project”. A.C. is the recipient of a fellowship grant within the PhD

program in “Food, Health and Longevity” (Università degli Studi del Piemonte Orientale) (MINDFUL project).

Data Availability Statement: Data sharing not applicable.

Acknowledgments: The authors would like to thank Claudio Santin for the technical support for the *sous vide* cooking, and Cristina Brizzolari (Azienda Agricola Luigi e Carlo Guidobono Cavalchini, tenuta La Mondina) for having provided rice samples.

Conflicts of Interest: The authors declare no conflicts of interest.

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Chapter 5

The impact of simulated digestion on the polyphenolic fraction of cooked Italian Artemide black rice

Abstract

Black rice can be defined a sort of natural functional food, due to its high content of antioxidant polyphenols, particularly anthocyanins and phenolic acids. However, to exert their beneficial effect to the human organism, these compounds must be bioaccessible and bioavailable. The aim of this work was to evaluate the impact of digestion process on the polyphenolic fraction of cooked Artemide black rice, through the application of *in vitro* INFOGEST simulated static digestion protocol. Anthocyanins, other flavonoids and phenolic acids content were determined by RP-HPLC-DAD following digestion. In general, anthocyanins were found to be stable up to the gastric level, while their concentrations decreased significantly in the intestine, with a consequent reduction also in the bioaccessibility values. The free fraction of phenolic acids showed a reduction in their concentration in the insoluble portion and a simultaneous increase in the soluble one during digestion. The bound phenolic acids, on the other hand, were detected almost unchanged, after digestion. The flavonoids free fraction showed an increase in the concentration, with a consequent increase in their bioaccessibility, during the digestion. These results allowed to highlight the main changes in the polyphenolic composition of one variety of black rice after cooking (“risotto” mode) and digestion.

1. Introduction

The Italian Artemide black rice (*Oryza sativa* L.) is a pigmented rice variety obtained from the breeding between Venere black rice and a white *indica* variety. It is particularly rich in healthy polyphenols, mainly anthocyanins and phenolic acids, which make it a sort of natural functional food **[1,2]**. Polyphenols, as described in scientific literature can trigger a lot of potential beneficial effects on the human health, due to their capacity to prevent the cellular oxidative damage implicated in various chronic diseases, such as cancer and cardiovascular diseases, also reducing the natural aging of the cells **[3]**. Cyanidin-3-O-glucoside represents the most abundant anthocyanin quantified in the Artemide black rice, as in other black rice varieties, representing on average about 83% of total anthocyanins. The phenolic acids mostly present in black rice in free form are cinnamic, protocatechuic and gallic acids, moreover the main represented bound (insoluble) forms of phenolic acids are ferulic, coumaric and caffeic acids **[1-7]**.

However, since rice must be consumed after cooking, it's important to study the impact of this treatment on rice bioactive compounds. In general, regarding anthocyanins, due to their high instability, all heat treatments determine a decrease **[8-10]**. In previous works we investigated the impact of different cooking methods (boiling, microwave, pressure cooker, risotto, pilaf and *sous vide*) on Artemide black rice composition, and "risotto" and *sous vide* at 89 °C were the methods following which the smallest anthocyanin decrease was observed **[4]**.

Furthermore, to better understand the destiny of rice phenolics after consumption, it's important to consider the digestive process, for example through *in vitro* simulated digestion tests, such as the standard INFOGEST protocol. In this protocol SSF (Simulated salivary fluid) and salivary amylase, SGF (Simulated gastric fluid) and pepsin, SIF (Simulated intestinal fluid) and pancreatin with bile salt are used to simulate oral, gastric and intestinal digestion **[11]**.

Anthocyanins are unstable at basic pH and they can be degraded to other metabolites. For this reason, it has been observed in various foods that their absorption in the intestine is somewhat limited (about 1%) **[12]**. However, in order to explain their biological effects, probably anthocyanins are absorbed in the intestine and therefore are bioavailable **[13]**.

Only few studies were conducted on the bioavailability of anthocyanins and phenolic acids in different food matrices, such as purple potatoes and pigmented rice. The main results are about the anthocyanins. They seem to be stable up to the gastric level, while a significant

decrease in their concentration was observed in the intestinal tract **[12-15]**. Furthermore, it has been observed that also the food matrix effect is important: in purple sweet potatoes the high amount of starch hinders the adsorption of anthocyanins through the gastro-intestinal barrier **[12]**. In the same manner, the dietary fiber contained in black rice decreases the availability (and bioaccessibility) of flavonoids **[13]**.

Moreover, most of the literature concerning the bioavailability of phenolics during the digestion is referred to the analysis of raw samples, or to anthocyanin/phenolics extracts. There is a gap in knowledge about the impact of the digestion on the phenolic fraction in cooked foods. So, the aim of this work is to investigate the behaviour of phenolic compounds in cooked Artemide black rice during the different digestion phases. Based on our previous works, the “risotto” cooking method (widely used in Italian cuisine) was chosen, because its capacity to preserve the polyphenols when compared to other cooking methods, applying the international standardised INFOGEST protocol to simulate in vitro the human digestion **[11]**.

2. Materials and methods

2.1. Chemicals

α-Amylase from *Bacillus spp.* (A6380, CAS 9000-90-2), pepsin from porcine gastric mucose (V001221, CAS 9001-75-6), fungal lipase from *Candida rugosa* (L1754, CAS 9001-62-1), pancreatin from porcine pancreas (P3292, CAS 8049-47-6), porcine bile extract (B8631, CAS 8008-63-7), acetonitrile, methanol (all HPLC grade), and formic acid (50%, LC–MS grade) were obtained from Sigma–Aldrich (Milan, Italy). Anthocyanins (cyanidin-3-O-glucosid, peonidin-3-O-glucoside, cyanidin-3-O-rutinoside and peonidin-3-O-rutinoside) were purchased from LGC Standard srl (Sesto San Giovanni, Milan, Italy). All the other polyphenol reference compounds (protocatechuic acid, ferulic acid, *p*-hydroxybenzoic acid, gallic acid, vanillic acid, coumaric acid, myricetin, catechin), chemicals, and reagents were of analytical grade from Merck KGaA (Darmstadt, Germany). Ultrapure water (18.2 MΩ cm at 25 °C) was produced by ELGA PURELAB Ultra system (M-medical, Cornaredo, Milan, Italy).

2.2. Rice samples and cooking procedure

The local company “Azienda Agricola Luigi e Carlo Guidobono Cavalchini, tenuta La Mondina”, located in Casalbeltrame, Novara (Italy), supplied the “Artemide” black rice samples (harvest year: 2018) in under-vacuum commercial packages, kept at room temperature.

Prior to the application of the digestion protocol, the rice was cooked in the “risotto” mode. The choice of this cooking method lies in the fact that in our previous work it was the most performing in preserving the polyphenolic fraction of “Artemide” black rice **[5]**, being this cooking approach as optimal in order to preserve the antioxidant/antiinflammatory property.

100 g of black rice was toasted for 3 minutes in a cooking pan, then it was cooked adding in several times a total of 500 mL of hot distilled water. The total cooking time was 35 minutes and at the end of the cooking the water was completely absorbed by the rice or evaporated. All the other cooking specifics are described in the previous chapter of this thesis. After cooking the rice was kept left to cool at room temperature (20 °C), and grinded with a meat grinder to simulate the chewing.

2.3. Static *in vitro* digestion

For the static *in vitro* digestion, the standardized INFOGEST protocol was followed **[11]**. This protocol involves the succession of three phases: oral, gastric and intestinal. Simulated Salivar Fluid (SSF), Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF) were prepared 1.25 x concentrated; their composition is resumed in **Table 1**.

Table 1. *Composition of simulated digestive fluids.*

Salt solution	**SSF**	**SGF**	**SIF**
KCl (mM)	15.1	6.9	6.8
KH_2PO_4 (mM)	3.7	0.9	0.8
$NaHCO_3$ (mM)	6.8	12.5	42.5
$MgCl_2*6H_2O$ (mM)	0.5	0.4	1.1
$(NH_4)_2CO_3$ (mM)	0.06	0.5	-
HCl (mM)	0.09	1.3	0.7
NaCl (mM)	-	11.8	9.6

5 g of cooked rice was added with 4 mL of SSF, 0.75 mL of bacterial α-amylase from *Bacillus spp.* to obtain an activity of 75 U/mL, 0.225 mL of deionized water and 0.025 mL of 0.3M $CaCl_2*2H_2O$. So, the ratio of sample to SSF was 50:50. This mixture was incubated for 2 minutes at 37 °C on a swing plate in a stove.

Then, 8 mL of SGF, 0.667 mL of porcine pepsin to obtain an activity of 2000 U/mL, 0.48 mL of fungal lipase to obtain an activity of 60 U/mL, 0.448 mL of deionized water and 0.005 mL of 0.3M $CaCl_2*2H_2O$ were added. The pH was adjusted to 3.0 using HCl 6M and the mixture was incubated for 2 hours at 37 °C on a swing plate in a stove.

Subsequently, 8 mL of SIF, 5 mL of porcine pancreatin to obtain an activity of 100 U/mL, 3 mL of a 10 mM porcine bile salts aqueous solution, 3.16 mL of deionized water and 0.04 mL of 0.3M $CaCl_2*2H_2O$ were added, and the pH was adjusted to 7.0 with NaOH 6M. This mixture was incubated for 2 hours at 37 °C on a swing plate in a stove.

At the end of each digestive step, all digested samples were immediately centrifuged, and the supernatants and pellets collected, freeze dried and stored until the extraction of phenolics. For the gastric digestion, before the centrifugation, the sample was neutralized to pH 7.0 with NaOH 6M, to stop the enzymatic activity.

For each sample a blank was prepared in which the food matrix was omitted, to eliminate any possible interference due to enzymes and other components added to conduct the digestive process (for example, the bile extract).

2.4. Extraction procedure of phenolics

2.4.1. Extraction of free phenolics

An opportune amount of grinded sample (50 mg for the insoluble intestinal digests and 100 mg for all other insoluble fraction of digests and for the undigested risotto) was extracted in ultrasonic bath for 2 minutes, three times with 1.5 mL of a 50% *v/v* hydroalcoholic mixture, for a total of 4.5 mL. After each step, the test tube was centrifugated (Eppendorf Centrifuge 5417 R, Hamburg, Germany) at 9,200 x g for 2 minutes and the upper phases were collected in a new 15 mL test tube, while the solid lower phase was used for the extraction of bound phenolics. The extracts were stored at -20 °C until use. For each rice sample the extraction was performed in triplicate.

For the blank and the soluble fraction of each digest, 50 mg of freeze-dried powder was solubilized in 1 mL of a 50% *v/v* hydroalcoholic mixture. These solutions were stored at -20 °C until use.

2.4.2. Extraction of bound phenolics

The solid residues from the extraction of free phenolics were extracted with 10 mL of NaOH 4M for 210 minutes, placed on a stirring plate with a magnetic stir bar. Then, the pH was adjusted to 2.30 with HCl 6M and transferred into a separating funnel, where three extractions with ethyl acetate (30, 20 and 10 mL, respectively) were carried out. The organic phases were collected in a 100 mL flask and finally the solvent was removed by a rotary evaporator (Rotavapor® BUCHI R-210, Flawil, Switzerland). The dry extract obtained was solubilized in 2 mL of HPLC grade methanol, filtered on a 0.45 µm filter (Spartan™ 30/0.45 RC) and transferred in a 2 mL test tube, dividing in aliquots for the analyses and stored at -20 °C until use. For each sample the extraction was performed in triplicate.

2.5. RP-HPLC-DAD analysis

For the RP-HPLC-DAD analysis the protocol described by Colasanto *et al.* **[4]** was applied. Briefly, using a Shimadzu LC-20A Prominence chromatographic system equipped with a diode array detector (DAD detector SPD-M20A), the separation was performed on a reversed-phase Synergi TM 4 µm Max-RP 80 Å LC Column (250 x 4.6 mm i.d.)

(Phenomenex, Torrance, CA, USA), protected by a guard column containing the same phase, at 30 °C. Eluent A of the mobile phase consisted of water/ formic acid/ acetonitrile (87:10:3, *v/v*), while eluent B consisted of water/ formic acid/ acetonitrile (40:10:50, *v/v*). The applied program gradient was the following: from 6 to 20% B (20 min), from 20 to 40% B (15 min), from 40 to 60% B (5 min), from 60 to 90% B (5 min), isocratic 90% B (5 min), from 90 to 6% B (1 min), isocratic 6% B (29 min), with a total run time of 80 minutes. The flow rate and the injection volume were 400 µL/min and 5 µL, respectively. Polyphenolic rice extracts were centrifuged (14,000 rpm for 20 min, microcentrifuge 5417R, Eppendorf, Milan, Italy) prior to the injection in the chromatographic system. Cyanidin-3-O-glucoside, peonidin-3-O-glucoside, cyanidin-3-O-rutinoside and peonidin-3-O-rutinoside were tentatively identified by comparison with retention times of individual authentic standard molecules and their UV–Vis spectra; the quantification was performed on the basis of calibration curves obtained with the corresponding standard compounds.

2.6. Calculation of bioaccessibility and digestibility indexes

In agreement with Ortega et al. **[16]**, two different indexes were applied to evaluate the digestion impact on the polyphenolic fraction: the percentage of bioaccessibility and the percentage of digestibility.

The bioaccessibility index indicates in percentage terms the amount of substance released in the soluble fraction of the digest respect to the total content in the food, and in this work it is calculated in two different ways: 1) by comparing the quantification of the soluble fraction to the total content obtained after digestion (the sum of supernatant and pellet); 2) by comparing the soluble fraction to the total quantity of compound quantified in the sample before the digestion. The formulas used for the calculation are the following:

$$\text{Bioaccessibility index 1 (\%)} = [CC_{SF} / (CC_{IF} + CC_{SF})] \times 100 \quad (1)$$

$$\text{Bioaccessibility index 2 (\%)} = (CC_{SF} / CC_{BD}) \times 100 \quad (2)$$

where CC_{SF} is the compound content in the soluble fraction of digest; CC_{IF} is the compound content in the insoluble fraction of the digest and CC_{BD} is the compound content in the sample before the digestion.

The digestibility index (or total recovery %) indicates the ratio between the total portion of compound quantified after digestion (the sum of supernatant and pellet) and the compound quantification obtained in the sample before the digestion. This index represents the

"recoverable" quantity after the digestive process and was calculated with the following formula:

$$\text{Digestibility index (\%)} = [(CC_{IF} + CC_{SF}) / CC_{BD}] \times 100 \qquad (3)$$

Both indices were calculated for each of the three digestive phases (oral, gastric and intestinal).

2.7. Statistical analysis

All statistical analyses were performed using the free statistical software R 4.1.1 version **[17]** and results were expressed as mean ± standard deviation (SD) of at least three independent experiments. Differences were estimated by analysis of variance (ANOVA) followed by Tukey's honest significant difference test. The statistical significance level was set to 0.05.

3. Results and discussion

All samples were characterized for the content of phenolics (anthocyanins, phenolic acids and other flavonoids). Regarding the analysis of phenolic acids and other flavonoids, the undigested risotto and the insoluble portions of each digestive phase were quantified both in the free and bound form, while anthocyanins were found in bound form only in some cases and in unquantifiable amounts. **Figure 1** shows the scheme of the conducted work.

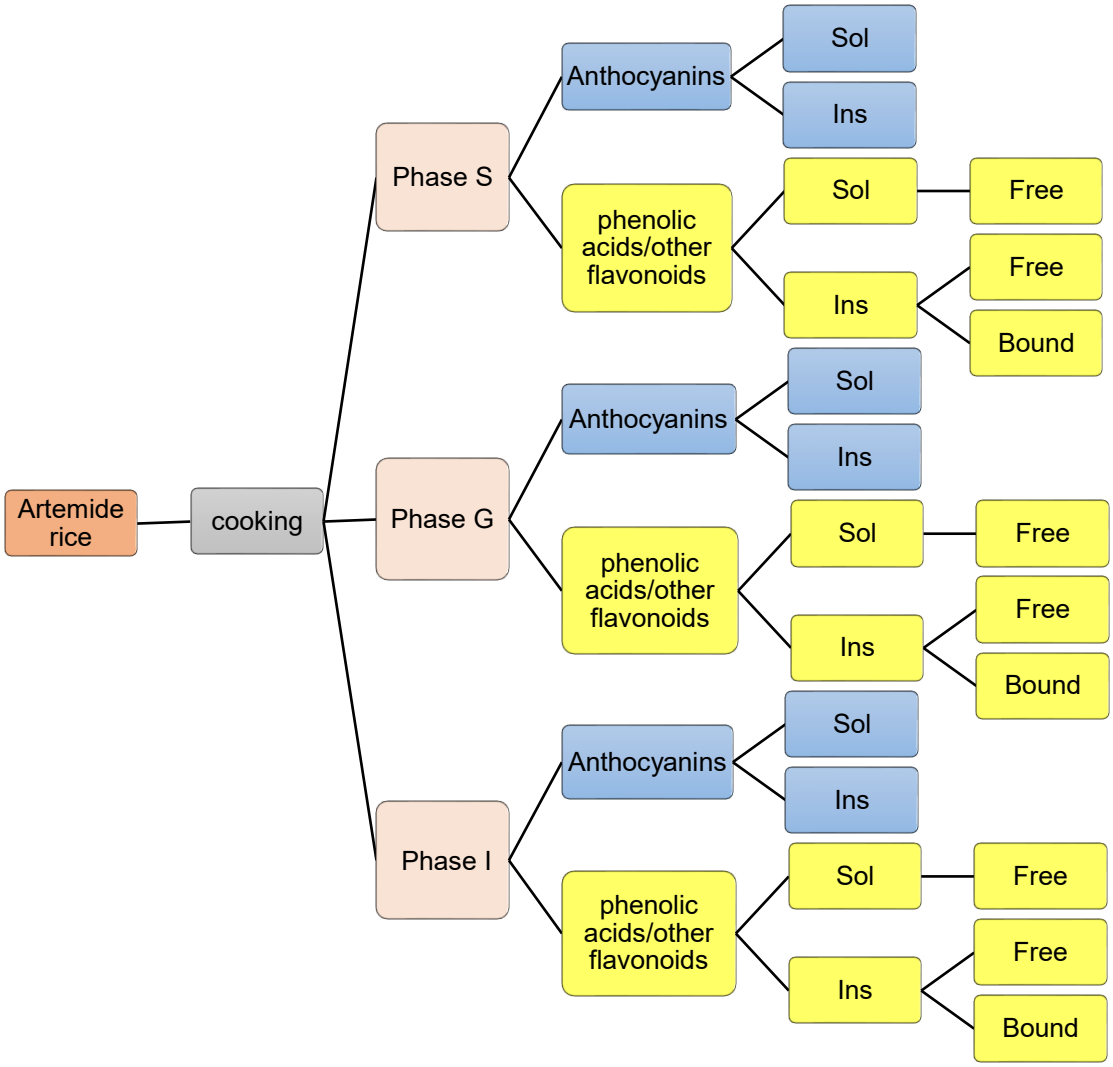

Figure 1. *Scheme of work and analyses carried out in the simulated digestion tests*

3.1. Quantification of anthocyanins

The first part of the activities was carried out for the evaluation of the digestive process impact on the rice polyphenolic fraction and concerned the quantification of individual anthocyanins (**Table 2**). Anthocyanins were identified using standard compounds and, in the case of cyanidin-3-O-gentiobioside, on the basis of previous information published in the literature **[1]**. The values related to the amount of cyanidin-3-O-gentiobioside were expressed as equivalents of cyanidin-3-O-glucoside.

Table 2 *Anthocyanin content in both undigested and digested rice samples, expressed as mean ± standard deviation and quantified in µg/g (d.w.) of rice.*

Anthocyanin	***Sample***	***Soluble portion (µg/g d.w.)***	***Insoluble portion (µg/g d.w.)***	***Digestibility index %***
Cn-3-glc	undigested		622 ± 12[a]	
	S	106 ± 8[c]	495 ± 12[b]	96,5
	G	315 ± 19[a]	301 ± 5[c]	98,9
	I	205 ± 1[b]	121 ± 10[d]	52,4
Cn-3-rut	undigested		27.6 ± 1.1[a]	
	S	4.45 ± 0.33[c]	22.7 ± 1.8[b]	98,2
	G	14.6 ± 1.5[a]	14.5 ± 0.6[c]	105
	I	11.2 ± 1.0[b]	6.99 ± 0.55[d]	65,8
Pn-3-glc	undigested		48.0 ± 0.48[a]	
	S	8.96 ± 0.59[c]	38.2 ± 0.5[b]	98,3
	G	25.7 ± 2.2[a]	21.8 ± 0.6[c]	99,1
	I	20.8 ± 0.3[b]	9.34 ± 1.18[d]	62,8
Pn-3-rut	undigested		2.86 ± 0.22[a]	
	S	0.777 ± 0.065[c]	2.62 ± 0.31[a]	119
	G	2.09 ± 0.16[a]	1.46 ± 0.12[b]	124
	I	1.31 ± 0.13[b]	0.972 ± 0.233[b]	79,8
Cn-3-gent	undigested		5.79 ± 0.38[a]	
	S	1.48 ± 0.11[c]	4.91 ± 0.24[b]	110
	G	4.10 ± 0.36[a]	2.61 ± 0.05[c]	116
	I	3.27 ± 0.14[b]	1.57 ± 0.21[d]	83,7

Cn-3-glc: cyanidin-3-O-glucoside; Pn-3-glc: peonidin-3-O-glucoside; Cn-3-rut: cyanidin-3-O-rutinoside; Pn-3-rut: peonidin-3-O-rutinoside; Cn-3-gent: cyanidin-3-O-gentiobioside.

Cyanidin-3-O-glucoside is the most abundant anthocyanin in Artemide black rice cooked as risotto (622 μg/g d.w.), representing about 88% of the total content of quantified anthocyanins. This data is in line with that reported in the previous chapter of this thesis. The cyanidin-3-O-glucoside values determined in the digested samples indicate a certain stability at the salivary and gastric level, while following intestinal digestion the value (326 μg/g, given by the sum of the soluble and the insoluble portion) is considerably reduced, if compared to the initial one (622 μg/g). The digestibility index values (96.5, 98.9 and 52.4% for the salivary, gastric and intestinal phases, respectively) confirm that the recovered amount of cyanidin-3-O-glucoside at the end of the digestive process is clearly reduced, suggesting a degradation of this anthocyanin at the intestinal level. The same trend was confirmed for the other detected anthocyanins.

Considering the ratio between the anthocyanins contained in the soluble fraction and those in the insoluble one (in each digestive phase), a decrease of the anthocyanins content was observed in the insoluble portion during the digestion, and a following increase in the soluble one, but only up to the gastric level. This increase in the soluble fraction is desirable, since the soluble portion represents the bioaccessible and potentially bioavailable one. This result indicates a gradual release of the substance in solution. However, the concentration in the intestinal soluble portion, while remaining higher than in the salivary phase, decreases respect to gastric phase. This reduction may be due to degradation processes of anthocyanins due to the change of pH values from the gastric to the intestinal phase, as previously described **[18,19]**. By way of example, **Figure 2** shows the graph relating to the change in the soluble/insoluble ratio during the digestion of cyanidin-3-O-glucoside. A similar trend was observed for all the considered anthocyanins (data not shown).

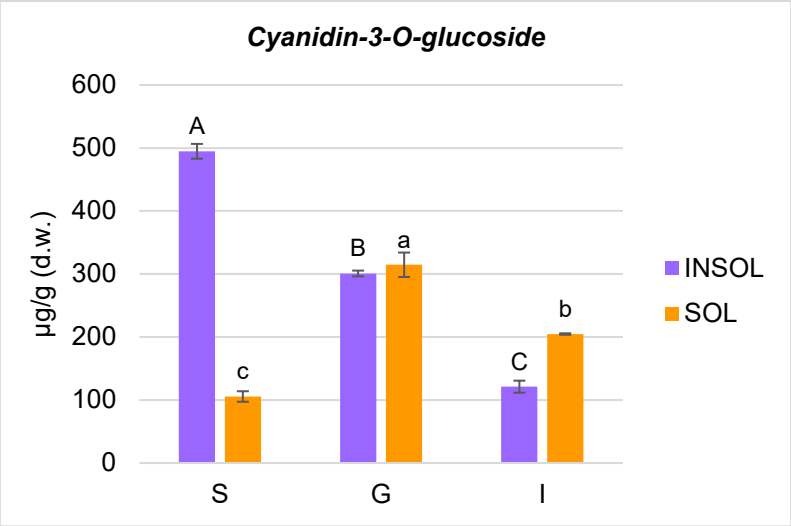

Figure 2. *Total cyanidin-3-O-glucoside content quantified in the soluble and insoluble fractions of each digestive phase. Lowercase letters indicate the comparison between digested samples of the soluble portion, uppercase letters indicate the comparison between digested samples of the insoluble portion. Different letters indicate significant differences between samples ($p < 0.05$).*

Since anthocyanins must be bioaccessible to exert their beneficial effects on the human organism **[18,19]**, their bioaccessibility index was calculated. This index was calculated in two different ways: by comparing the percentage of substance in solution, both to its total content after digestion (sum of soluble portion + insoluble portion) (Bioaccessibility index 1), and to total quantified content in the undigested risotto sample (Bioaccessibility index 2). In **Table 3** the bioaccessibility indexes of the various anthocyanins after each individual digestion phase are reported.

Table 3. *Bioaccessibility indexes (%) of anthocyanins expressed for the different phases of the in vitro simulated digestive process.*

Anthocyanin	***Sample***	***Bioaccessibility index 1 (%)***	***Bioaccessibility index 2 (%)***
Cn-3-glc	S	17.6	17.0
	G	51.1	50.6
	I	62.8	32.9
Cn-3-rut	S	16.4	16.1
	G	50.1	52.7
	I	61.5	40.5
Pn-3-glc	S	19.0	18.7
	G	54.1	53.6
	I	69.0	43.4
Pn-3-rut	S	22.9	27.2
	G	58.9	72.9
	I	57.4	45.9
Cn-3-gent	S	23.1	25.5
	G	61.1	70.7
	I	67.5	56.5

Cn-3-glc: cyanidin-3-O-glucoside; Pn-3-glc: peonidin-3-O-glucoside; Cn-3-rut: cyanidin-3-O-rutinoside; Pn-3-rut: peonidin-3-O-rutinoside; Cn-3-gent: cyanidin-3-O-gentiobioside.

Observing the bioaccessibility indexes 2 of all quantified anthocyanins, we note quite high values at the gastric level, which tend to considerably decrease in the intestinal phase. A possible explanation for this significant reduction could be the high susceptibility of anthocyanins to pH. Their passage from an area with acidic pH (gastric environment) to an area with basic pH (intestinal environment) can strongly compromise their stability, leading to the lowering of the anthocyanin levels. This reduction is not evident observing the bioaccessibility indexes 1, because in this case the soluble portion is considered in comparison with the total residue after the digestive process.

Finally, the insoluble residue deriving from the three digestion steps was characterized to evaluate the possible presence of bound anthocyanins, but they are almost completely absent in bound form. The only anthocyanins found in trace amounts were cyanidin-3-O-

rutinoside and peonidin-3-O-rutinoside. Probably, the presence of the rutinose makes the anthocyanins less susceptible to enzymes and pH changes, as demonstrated also by Xu *et al.* **[20]**.

3.2. Quantification of phenolic acids

The main phenolic acids were quantified in both undigested and digested risotto (soluble and insoluble portions). The undigested risotto, the insoluble portion and the soluble portion of each digestion were characterized for the fraction of free phenolic acids; moreover, the undigested risotto and the insoluble portion of each digestion were also characterized for the bound fraction of phenolic acids. The bioaccessibility and digestibility indexes were calculated for each compound.

3.2.1. Free phenolic acids

In **Table 4** the phenolic acids content determined in both soluble and insoluble portions of undigested and digested risotto is summarised, including also the digestibility indexes.

Table 4. *Free phenolic acid content (μg/g d.w.), expressed as mean ± standard deviation, and digestibility index (%) of the free fraction. Different letters indicate significant differences between the samples deriving from the different digestive phases within the same soluble and/or insoluble fraction ($p < 0.05$).*

Phenolic acid	*Sample*	*Soluble portion (μg/g d.w.)*	*Insoluble portion (μg/g d.w.)*	*Digestibility index %*
***p*-hydroxybenzoic acid**	undigested		tr	
	S	0.471 ± 0.052^{b}	tr	nd
	G	1.21 ± 0.27^{b}	tr	nd
	I	20.6 ± 2.3^{a}	2.34 ± 0.26	nd
Protocatechuic acid	undigested		201 ± 3^{a}	
	S	58.3 ± 3.6^{c}	143 ± 4^{b}	100
	G	124 ± 11^{b}	78.8 ± 1.2^{c}	101
	I	145 ± 10^{a}	25.1 ± 2.1^{d}	84.3
Vanillic acid	undigested		57.4 ± 1.3^{a}	
	S	23.2 ± 1.9^{b}	43.8 ± 2.9^{b}	117
	G	31.8 ± 4.0^{a}	21.2 ± 0.6^{c}	92,4
	I	28.6 ± 2.3^{ab}	3.27 ± 0.28^{d}	55.6
Gallic acid	undigested		5.59 ± 0.26^{a}	
	S	1.39 ± 0.11^{c}	4.32 ± 0.08^{c}	102
	G	7.28 ± 1.18^{b}	4.78 ± 0.33^{bc}	216
	I	13.3 ± 1.1^{a}	5.11 ± 0.32^{ab}	329
Coumaric acid	undigested		14.8 ± 1.1^{a}	
	S	1.92 ± 0.14^{c}	12.1 ± 2.4^{a}	94.6
	G	5.52 ± 0.47^{b}	7.99 ± 0.43^{b}	91.4
	I	11.8 ± 0.6^{a}	3.01 ± 0.22^{c}	100
Ferulic acid	undigested		11.1 ± 0.3^{a}	
	S	1.86 ± 0.16^{c}	8.35 ± 0.06^{b}	91.8
	G	6.31 ± 1.20^{b}	5.47 ± 0.09^{c}	106
	I	9.91 ± 0.71^{a}	1.74 ± 0.15^{d}	105

Protocatechuic acid is the most abundant phenolic acid, with a value of 201 µg/g (d.w.) in the undigested risotto. It is possible to note that, during the digestive process, the insoluble portion tends to decrease, while the soluble portion increases significantly; this behaviour can be explained by the fact that the prolonged contact with the digestive fluids and the action of the various enzymes favoured the passage of this phenolic acid into solution.

Considering the digestibility index, protocatechuic acid seems stable up to the gastric level, where there is complete recovery of the substance (digestibility index: 101%), while it decreases slightly in the intestine (84.3%).

The concentration of vanillic and gallic acid are 57.4 and 5.59 µg/g (d.w.), respectively, while the *p*-hydroxybenzoic acid is present only in traces in the undigested risotto. These values are consistent with those reported in a previous Chapter of this Thesis (54.5 µg/g (d.w.) for vanillic acid and 5.31 µg/g (d.w.) for gallic acid). It is interesting to observe how the *p*-hydroxybenzoic acid was found only in traces in the undigested risotto and in the insoluble portion (except for the intestinal phase, where 2.34 µg/g (d.w.) were quantified), while it was quantified in the soluble portion with values up to 20.6 µg/g (d.w.) in samples deriving from the intestinal phase.

The digestibility indexes of vanillic acid shown a decreasing trend along the various phases of the simulated digestion, in particular at the intestinal level (total recovery: 55.6%), where it is possible to hypothesize a structural degradation, with the possible formation of its metabolite 2-methoxyhydroquinone **[21]**. The concentration of gallic acid, on the other hand, tends to increase during the digestive phases, and in the intestinal one values are more than three times higher than those quantified in undigested risotto.

Considering ferulic and coumaric acids (belonging to the hydroxycinnamic series), it can be noted that the recorded quantities are significantly lower than those recorded for the phenolic acids deriving from benzoic acid (*p*-hydroxybenzoic, protocatechuic, vanillic and gallic acids). Both ferulic and coumaric acids shown an increase in the levels of the soluble portion and a decrease in the levels of the insoluble portion; the first one promotes the bioaccessibility of these compounds, thus also increasing their potential bioavailability, a necessary condition to be able to carry out their bioactivity (antioxidant, anti-inflammatory, antidiabetic effects, *etc*.) **[18,19]**.

Observing the bioaccessibility indexes, reported in **Table 5**, seem that the bioaccessibility of these phenolic acids tends to increase during the digestion. The only exception was the

vanillic acid, for which a slight decrease was observed in the intestinal phase (evaluating the bioaccessibility index 2). This outcome is related to the fact that a reduction of the vanillic acid concentration was observed (due to probable degradation) during the digestive process. As regards *p*-hydroxybenzoic acid, it was not possible to calculate the bioaccessibility indexes in all conditions, as it was quantified only in the soluble portions of the digested samples and in the insoluble residue deriving from the intestinal phase.

Table 5. *Bioaccessibility indexes (%) of phenolic acids expressed for the different phases of the in vitro simulated digestive process.*

Phenolic acid	***Sample***	***Bioaccessibility index 1 (%)***	***Bioaccessibility index 2 (%)***
***p*-hydroxybenzoic acid**	S	nd	nd
	G	nd	nd
	I	89.8	nd
Protocatechuic acid	S	28.9	29.0
	G	61.2	61.9
	I	85.2	71.8
Vanillic acid	S	34.6	40.4
	G	60.1	55.5
	I	89.7	49.8
Gallic acid	S	24.4	25.0
	G	60.4	130
	I	72.2	237
Coumaric acid	S	13.7	13.0
	G	40.8	37.3
	I	79.7	79.8
Ferulic acid	S	18.2	16.7
	G	53.6	56.7
	I	85.1	89.1

3.2.2. Bound phenolic acids

The fiber-bound phenolic acids were quantified in the insoluble residues deriving from the polyphenols extraction from the undigested risotto and from the samples obtained after each digestive step, in order to evaluate if the digestive conditions (pH, enzymes, etc.) could release part of the fraction bound to the components of the matrix.

The values were expressed as mean ± standard deviation and quantified as μg/g (d.w.) of rice (**Table 6**).

Table 6. *Content of bound phenolic acids (μg/g d.w.) in samples of undigested risotto and insoluble digest, expressed as mean ± standard deviation, in the different digestive phases. For each phenolic acid, different letters indicate significant differences between the different digestive phases ($p < 0.05$). In the event of significant differences with respect to risotto, the percentage variations have been reported in brackets.*

Phenolic acid	***Sample***	***Bound insoluble portion (μg/g d.w.)***
***p*-hydroxybenzoic acid**	undigested	2.55 ± 0.80^{a}
	S	4.43 ± 0.91^{a}
	G	3.30 ± 0.64^{a}
	I	4.40 ± 0.75^{a}
Protocatechuic acid	undigested	7.88 ± 1.24^{b}
	S	9.59 ± 0.36^{a} (*+ 22%*)
	G	5.77 ± 0.37^{c} (*- 27%*)
	I	8.05 ± 0.36^{ab}
Vanillic acid	undigested	99.7 ± 5.1^{a}
	S	108 ± 10^{a}
	G	96.1 ± 8.7^{a}
	I	105 ± 8^{a}
Gallic acid	undigested	0.955 ± 0.039^{a}
	S	0.769 ± 0.103^{ab}
	G	0.509 ± 0.176^{b}
	I	0.631 ± 0.224^{ab}
Syringic acid	undigested	0.989 ± 0.031^{a}
	S	1.44 ± 0.45^{a}
	G	1.55 ± 0.50^{a}
	I	0.831 ± 0.337^{a}

Coumaric acid	undigested	122 ± 7[a]
	S	129 ± 3[a]
	G	115 ± 9[a]
	I	128 ± 5[a]
Ferulic acid	undigested	270 ± 15[a]
	S	292 ± 6[a]
	G	271 ± 25[a]
	I	296 ± 12[a]

Ferulic and coumaric acids (cinnamic series) were the most abundant phenolic acids in bound form, with values of 270 µg/g and 122 µg/g, respectively, in the undigested risotto. Following, vanillic acid (benzoic series) shown values of 99.7 µg/g. Ferulic acid is present for 96% in bound form, while coumaric and vanillic acids appears to be in bound form for 90% and 62%, respectively (the values are referred to the undigested sample).

No significant differences were observed in ferulic acid concentration before and after digestion, with an average value of 282 µg/g. This is contrast with the work of Ti et al. **[22]**, in which an increase in the concentration of ferulic acid in bound form following the digestive process was observed in TianYou 998 rice, a white variety from Guangzhou (China). Authors hypothesized that since ferulic acid is mainly present in the bran layer of the rice caryopsis, cooking and subsequent digestion may have damaged the cell walls causing greater extractability of this compound. The differences observed in this work could depend on the different cooking conditions/methods used, which may have impacted differently on the determination of ferulic acid. Also the chewing (in this study simulated by grinding) strongly impact the release of free phenolics, allowing the food matrix more accessible to the digestive enzymes **[23,24]**.

The coumaric acid was found at the concentration of 122 µg/g in the undigested sample, and 129, 115 and 128 µg/g in the three digestive phases. No statistically significant differences were found between the undigested risotto and digested one. The same was obtained for the vanillic acid, present in 62% in the bound form and in 38% in the free form (values referred to undigested risotto).

The concentrations of the other bound phenolic acids (*p*-hydroxybenzoic, protocatechuic, gallic and syringic acids) are rather low and in any case scarcely modified following the digestion process.

Overall, the bound fraction would appear to be not bioaccessible, as it remains almost unchanged after digestion. However, studies reported in the literature have shown that the intestinal microbiota is able to release part of the bound component of phenolic acids, increasing their bioavailability. This release by the intestinal microflora of the bound polyphenolic component, which it is not possible to highlight with the INFOGEST simulated digestion protocol, would seem to be due to the action of proteolytic enzymes capable of splitting the bond between the phenolics and the fiber components, thus allowing their release and the subsequent absorption **[25,26]**.

3.3. Quantification of other flavonoids

In addition to anthocyanins and phenolic acids, two other important flavonoids, catechin and myricetin, have been identified in both the free and bound polyphenolic fraction of undigested and digested cooked rice. Furthermore, in the bound fraction also the epicatechin was found. Their identification was confirmed by comparison of the retention times and the DAD profiles of the relative standard compounds.

3.3.1. Free flavonoids

Catechin and myricetin were the two flavonoids found in the free fraction. Their concentrations in undigested and digested rice are reported in **Table 7**.

Table 7. *Catechin (μg/g d.w.) and myricetin (μg/g d.w.) content in undigested and digested black rice samples in the different digestive phases, expressed as mean ± standard deviation, and digestibility indexes. Different letters indicate significant differences between the samples deriving from the different digestive phases within the same soluble and/or insoluble fraction ($p < 0.05$).*

Flavonoid	***Sample***	***Soluble portion (μg/g d.w.)***	***Insoluble portion (μg/g d.w.)***	***Digestibility index %***
Catechin	undigested		51.7 ± 0.9^{a}	
	S	8.43 ± 0.54^{c}	46.4 ± 2.9^{b}	106
	G	25.6 ± 3.6^{b}	30.5 ± 1.4^{c}	109
	I	49.4 ± 4.5^{a}	14.4 ± 0.9^{d}	123
Myricetin	undigested		3.40 ± 0.45^{b}	
	S	0.558 ± 0.030^{b}	5.00 ± 0.51^{a}	163
	G	1.15 ± 0.07^{b}	3.28 ± 0.44^{b}	130
	I	4.74 ± 0.82^{a}	1.93 ± 0.32^{c}	196

For both flavonoids the concentrations in the insoluble portion decrease during the digestion, while at the same time the values of the soluble portion increase. Furthermore, a significant increase has been highlighted during the intestinal phase for catechin, while for myricetin it has been already observed starting from the salivary phase. At the end of the digestion, the digestibility index reached a value of 123% for catechin and 196% for myricetin. The hypothesis to explain this increase is that during the digestive process these substances can be released following the degradation of more complex polymeric structures (for example procyanidins, in the case of catechins) or can be partially released from the corresponding bound fraction.

The bioaccessibility indexes shown an increasing trend during the digestion steps for both the flavonoids (bioaccesibility index 2: 95.6 and 139% in the intestinal phase for catechin and myricetin, respectively).

3.3.2. Bound flavonoids

Catechin, epicatechin and myricetin also in bound form were detected (**Table 8**). Myricetin is predominant compared to the other two flavonoids and its content in bound form is greater than that present in free form (in the sample of undigested risotto, for example, there are 70.3 µg/g of myricetin in bound form and 3.40 µg/g in free form).

Table 8. *Catechin (µg/g d.w.), myricetin (µg/g d.w.) and epicatechin (µg/g d.w.) content in bound form in undigested and digested black rice samples in the different digestive phases, expressed as mean ± standard deviation. Different letters indicate significant differences between the samples deriving from the different digestive ($p < 0.05$).*

Flavonoid	***Sample***	***Insoluble portion (µg/g d.w.)***
Catechin	undigested	5.81 ± 0.66^{a}
	S	4.26 ± 1.06^{ab} (- *27%*)
	G	4.50 ± 1.64^{ab} (- *23%*)
	I	3.05 ± 0.17^{b} (- *48%*)
Myricetin	undigested	70.3 ± 3.3^{a}
	S	60.5 ± 9.5^{a}
	G	74.6 ± 3.7^{a}
	I	73.9 ± 5.9^{a}
Epicatechin	undigested	21.7 ± 5.9^{a}
	S	19.5 ± 4.8^{a}
	G	17.3 ± 5.2^{a}
	I	22.0 ± 2.6^{a}

Both myricetin and epicatechin values obtained in the digested samples are unchanged if compared to values of the undigested risotto. Catechin concentrations tend to decrease during the digestive process, until obtaining at the end of the digestion a percentage reduction of 48%. The data obtained therefore suggest that during the digestive process the catechins can be partially released from the bound component; this hypothesis is also confirmed by the concomitant increase in catechin values quantified in free form, and is in agreement with that reported in Om *et al*, who states how enzymes and pH changes could release free polyphenols from the bound fraction **[27]**.

4. Conclusions

The aim of this work was to evaluate the impact of the digestive process on the polyphenolic composition of Italian Artemide black rice cooked in risotto mode, with particular attention to anthocyanins, other flavonoids, and phenolic acids. To simulate the human digestion the international standardised INFOGEST protocol was applied, subjecting the rice to salivary, gastric and intestinal phases, respectively.

In general, anthocyanins were found to be stable up to the gastric level, while their concentrations decreased significantly in the intestine, with a consequent reduction also in the bioaccessibility values. The free fraction of phenolic acids showed a reduction in their concentration in the insoluble portion and a simultaneous increase in the soluble portion during the digestion. The prolonged contact with the digestive fluids and the action of the enzymes have evidently favoured their release from the matrix. Furthermore, a progressive increase in the bioaccessibility index of all the considered phenolic acids was observed, except for vanillic acid, which may have been partially degraded during the intestinal phase. The bound phenolic acids, on the other hand, remain almost unchanged after digestion. This could suggest that the bound polyphenol fraction is not bioaccessible. However, the simulated digestion protocol used in this work does not allow the evaluation of some important aspects of the digestive process, such as the absorption and the impact of the intestinal microbiota. Indeed, studies reported in the literature have shown that the intestinal microbiota is able to release part of the bound component of phenolic acids, thus increasing its bioavailability. Finally, also the flavonoids fraction showed an increase in the concentration in free form, with a consequent increase in their bioaccessibility, during the digestion.

In conclusion, the results obtained allowed to highlight the main changes in the polyphenolic composition of one variety of black rice after cooking and digestion. In future, the research activities would be focused to continue the detailed study of the cooking conditions (for example, the evaluation of the ranges temperature-pressure, the toasting phase, the addition of other ingredients), to identify the cooking conditions more useful to preserve the polyphenolic component. Furthermore, it will be interesting to investigate the evaluation of the impact of the microbiota, to understand which further transformations the rice polyphenolic fraction can undergo during digestion, particularly concerning this mode of cooking able to preserve the bioactive properties of pigmented rice.

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Chapter 6

Different types of rice and cooking methods: impact on glycaemic trend in children with type 1 diabetes

This chapter is based on:

Colasanto, A., Savastio, S., Pozzi, E., Gorla, C., Coïsson, J. D., Arlorio, M., & Rabbone, I. (2023). Different types of rice and cooking: impact on glycaemic trend in children with type 1 diabetes. In preparation for the submission to Nutrients.

Abstract

(1) Background: some types of food as rice are difficult to manage in terms of glycaemic response in type 1 diabetes (T1D) subjects. Our aim was to evaluate the chemical and nutritional composition of rice before and after cooking, studying i) the postprandial glycaemic trend due to two different types of rice ("Gigante Vercelli" white rice, and "Artemide" black rice) and ii) cooking modes (risotto vs boiled) in T1D children and adolescents, using an advanced hybrid closed loop (AHCL) system (Tandem ControlIQ^{TM}). (2) Methods: general composition and spectrophotometric analyses on raw and cooked rice were performed. Eight T1D subjects (M4/F4, aged 11±1.4 ys) using an AHCL system were enrolled. "Gigante Vercelli" cooked as risotto or in boiling mode or "Artemide" rice in boiled mode were prepared by the same cook in two evenings. Continuous glucose monitoring metrics were evaluated for 12 hours after meal consumption. (3) Results: both rice varieties showed a higher total dietary fiber after cooking, when compared to raw ones. Cooking as "risotto" determines an increase in phenolic and antioxidant content ($p<0.05$) in both varieties, while, after boiling, a decrease in total starch was found ($p< 0.05$) for "Gigante Vercelli". Analyzing clinical data, a significantly different change in glycaemic trend after risotto and boiled white rice ($p<0.05$) was found. Instead, Artemide boiled rice never overcomed a mean glycaemic peak over 180 mg/dL. (4) Conclusion: the findings here showed suggest a different chemical and nutritional profile of rice impact on the glycaemic trend, however, well controlled by the use of AHCL systems.

1. Introduction

Rice (*Oryza sativa* L.) is one of the most consumed cereals worldwide; it is present on the market in different types (white, brown, or pigmented) **[1]**, color (white, red, black, depending on the pigment contents), caryopsis shape, and texture.

White rice (milled rice were husk, bran, and germ are removed) is primarily composed of starch, hypoallergenic proteins and bioactive components (including fiber and tocopherols) and offers high levels of energy and glycaemic index.

The consumption of unmilled rice (brown or pigmented) is preferable because of its lower amount of starch and a higher content of nutritional components, in particular proteins, fibers, vitamins and minerals. Thereby, it has a lower glycaemic index. The glycaemic index (GI; 0-100) is a recognized rating system based on the measure of the increase in the level of blood glucose caused by eating a specific carbohydrate (food/ingredient that contains sugar) compared with eating a standard amount of glucose. The rate of glycaemia increase is generally expressed by curves that clearly report and describe the behavior of a specific food **[2]**.

A food (or ingredient) with a high glycaemic index release glucose quickly, causing a rapid rise in blood glycaemia, when compared with foods/ingredients with a low glycaemic index, contrary leading to a slow release of glucose into the blood. Furthermore, the pigmented varieties such as Artemide black rice are generally richer in polyphenols, antioxidants, and anthocyanins respect to the white varieties **[3,4]**.

Diet and insulin therapy are critically important in the management of type 1 diabetes (T1D) to improve glycaemic control and to reduce the risk of complications.

Some types of food are very difficult to manage in terms of glycaemic response, like pizza or rice. Since rice presents a high glycaemic index (GI) (58-93%), T1D children often struggle to control their postprandial glycaemic levels after its intake. While many experiments about pizza and glycaemic control in T1D have been performed **[5,6]**, no studies have evaluated postprandial glucose values after eating rice, especially considering different types of cooking. The glycaemic index is influenced by various factors such as the intrinsic characteristics of the starch (amylose/ amylopectin ratio), the type of post-harvest processing, and the consumer processing (cooking, storage and heating) **[7,8]**.

In recent years, the development of new technologies has been of great help in glycaemic control and management of complex meals, thanks to continuous glycaemic monitoring systems and to Advanced Hybrid Closed Loop (AHCL) systems **[9,10]**.

The primary aim of this study was to evaluate the chemical and nutritional composition of white rice before and after cooking ("risotto" mode or boiling). The secondary aim was to study the glycaemic impact of two different types of rice ("Gigante Vercelli" white rice **[11]** and Artemide black pigmented rice **[4]**) as well as two different cooking modes in children and adolescents with T1D using an AHCL system (Tandem ControlIQTM).

2. Materials and methods

2.1. Chemical characterizations: techniques

Moisture, protein, and total dietary fiber were quantified following the protocols applied in the previous work on Artemide black rice **[4]**. Except for the moisture content, data were expressed on a dry weight (d.w.) basis.

The amounts of the total, non-resistant (NR), and resistant (R) starch were determined using the respective assay procedures of Megazyme (K-DSTRS) kits which are based on a modified method of Englyst *et al.* **[12]** developed by McCleary *et al.* **[13]**.

Total polyphenols and antiradical activity were obtained using methods previously validated, applying the extraction procedure described in Colasanto *et. al.* **[4]**.

The cooking conditions of rice are detailed in **Table 1**.

Table 1. *Cooking conditions applied in the experiment.*

	Cooking method	***Ratio rice/water (g/mL)***	***Cooking time (min)***	***Notes***
Gigante Vercelli	Boiling (BOI)	100/500	15	Rice added when the water started boiling
	Risotto (RIS)	100/350	15	Time including 2 minutes toasting
Artemide	Boiling (BOI)	100/500	35	Rice added when the water started boiling

2.2 Subjects

Eight subjects (M 4/F 4) with T1D were enrolled at the Division of Pediatrics of Maggiore della Carità University Hospital in Novara, Italy in 2022.

All subjects attended two evenings to eat rice all together within a balanced meal established by our dietician (rice with zucchini, chicken, and fruit salad). Written informed consent was obtained from all participants and their parents before the enrolment.

During the first evening, children ate 80 grams of white “Gigante Vercelli” rice (79,3 g of carbohydrates in 100 g of rice). Four children ate rice cooked as “risotto” and four subjects ate boiled one.

Instead, during the second evening, all subjects received 80 grams of Artemide black rice (62 g CHO in 100 g rice) in the only boiled version, selected on the basis of the results of

the first evening. The dinner was served at 7.30 pm, prepared by the same cook, and consumed after a standard bolus delivered 10-20 minutes earlier according to glycaemic values.

To uniform experimental conditions on each day some instructions were given: on the morning before each study day, to change the infusion set and sensor to ensure positioning of the cannula and optimal sensor accuracy; in the afternoon before dinner, not to play sports; before and after the meal, to correct any hypoglycemia or hyperglycemia according to the same standardized protocol.

All patients used the same short-acting insulin analog (insulin Aspart, Novorapid, Novo Nordisk A/S, Bagsværd, Denmark).

Continuous glucose sensor data were collected from the Diasend platform for 12 hours (7:00 pm to 7:00 am) of the following day. Glucose values and times in target were evaluated every 5 minutes for 12 hours after dinner. Total automatic basal insulin (U/kg) and total auto-bolus insulin (U/kg) infused during the entire observation period were evaluated.

The primary inclusion criteria were therapy with AHCL (Tandem ControlIQTM) **[14,15]** for at least 3 months and an age between 10 and 16 years. Patients with HbA1c >8.5% (69 mmol/mol), food allergies, treated with drugs that could affect immunity or glucose metabolism (corticosteroids, ciclosporin, and tacrolimus), concurrent illness or psychiatric disease/eating disorders were excluded.

The diagnosis of T1D was performed according to the American Diabetes Association criteria. Children's height, weight, and BMI were evaluated using the Italian growth charts **[16]**.

Clinical data, HbA1c%, disease duration, and insulin requirement were collected.

2.3 Statistical analysis.

Data were expressed as mean ± SD. Differences in rice composition were estimated by analysis of variance (ANOVA) followed by Tukey's honest significant difference test. The differences between subjects were evaluated for the continuous variables through U Mann-Whitney test. The Chi-square test was used for comparison of nominal variables between groups. Trend evaluation across glycaemic values was performed through multinomial regression analyses. Significant p values were less than 0.05. The statistical analyses were performed using SPSS 22.0 (SPSS Inc., Chicago, IL, USA) and R Software 4.2.2 version.

3. Results

General composition of raw and cooked “Gigante Vercelli” white rice was determined, while data related to Artemide black rice derived from a previous work **[4]**.

3.1. White Gigante Vercelli rice: Chemical characterization

Proximate composition, total phenolic content (TPC), and total antioxidant activity (TAA) of raw and cooked “Gigante Vercelli” white rice were determined (**Table 2**).

Table 2. *Proximate composition, total phenolic content (mg CE/g d.w.), and total antioxidant activity (mg TE/g d.w.) determined in raw and cooked “Gigante Vercelli” white rice.*

Sample	***Moisture (%)***	***Proteins (% d.w.)***	***Total Dietary Fiber (% d.w.)***	***Total Polyphenols (mg CE/g d.w.)***	***Antiradical activity (mg TE/g d.w.)***
RAW	10.8 ± 0.4^{b}	7.02 ± 0.3^{a}	1.14 ± 0.1^{b}	0.21 ± 0.02^{b}	0.20 ± 0.01^{b}
RISOTTO	65.5 ± 0.2^{a}	7.49 ± 0.3^{a}	2.01 ± 0.2^{a}	0.24 ± 0.01^{a}	0.25 ± 0.01^{a}
BOILED	65.4 ± 0.7^{a}	7.39 ± 0.1^{a}	1.79 ± 0.04^{a}	0.09 ± 0.01^{c}	0.10 ± 0.01^{c}

The results are expressed as mean ± standard deviation. Values with different letters in the same column are significantly different ($p < 0.05$).

A higher moisture content in cooked rice was found, when compared to raw samples, as expected, without significant differences between risotto and boiled cooking methods.

No statistically significant variations occurred in protein content after cooking, while an interesting increase in total dietary fiber was observed after both cooking protocols ($p<0.05$).

A reduction in TPC and TAA was observed after boiling mode ($p<0.01$). After “risotto” cooking an increase of TPC and TAA was instead observed, compared to the uncooked rice ($p<0.05$).

Moreover, the starch composition of white rice was investigated (**Table 3**). After boiling, a decrease of non-resistant (NR) starch and consequently total starch was found, while no statistical differences in both NR and total starch were observed in risotto cooking, comparing with raw sample ($p < 0.05$). Furthermore, in both risotto and boiled rice an increase of resistant starch was observed compared to the raw analysed samples, reaching values statistically equivalent (**Table 3**).

Table 3. *Non-resistant (NR), Resistant (R) and Total starch in raw and cooked "Gigante Vercelli" white rice.*

Sample	*NR starch*	*R starch*	*Total starch*
RAW	84.6 ± 1.2[a]	0.22 ± 0.1[b]	84.8 ± 1.3[a]
RISOTTO	84.8 ± 1.1[a]	0.80 ± 0.1[a]	85.6 ± 1.2[a]
BOILED	79.1 ± 2.3[b]	0.77 ± 0.03[a]	79.9 ± 2.3[b]

The results are expressed as mean ± standard deviation. Values with different letters in the same column are significantly different (p < 0.05).

3.2. Postprandial glucose trend after risotto or boiled rice

We enrolled 8 T1D children, mean age 11±1.4 ys, diseases duration of 4.1±3 ys>; HbA1c 6,5±0.4% (48 ±0.4% mmoli/moli). All children were normal weight (BMIz score -0.08±0.6).

We found a significant change in glycaemic trend ($p<0.0001$) after the consumption of risotto and boiled white rice ($p=0.02$) during the following 12 hours. Instead, boiled black rice does not induce a significant alteration in glycaemic trend. In particular, Artemide boiled rice never determined a mean glycaemic peak over 180 mg/dL at each time point.

Analyzing data by a different type of cooking, a lower mean glucose value at 2 (T2) and 4 hours (T4) was found after eating boiled than risotto Gigante Vercelli white rice ($p< 0.05$).

Moreover, subjects who ate boiled Artemide rice showed better glucose levels at 2, 3, 4, 5 hours than white risotto ($p<0.05$) (**Figure 1**). No differences in glycaemic values between white and black boiled rice were found (**Figure 1**).

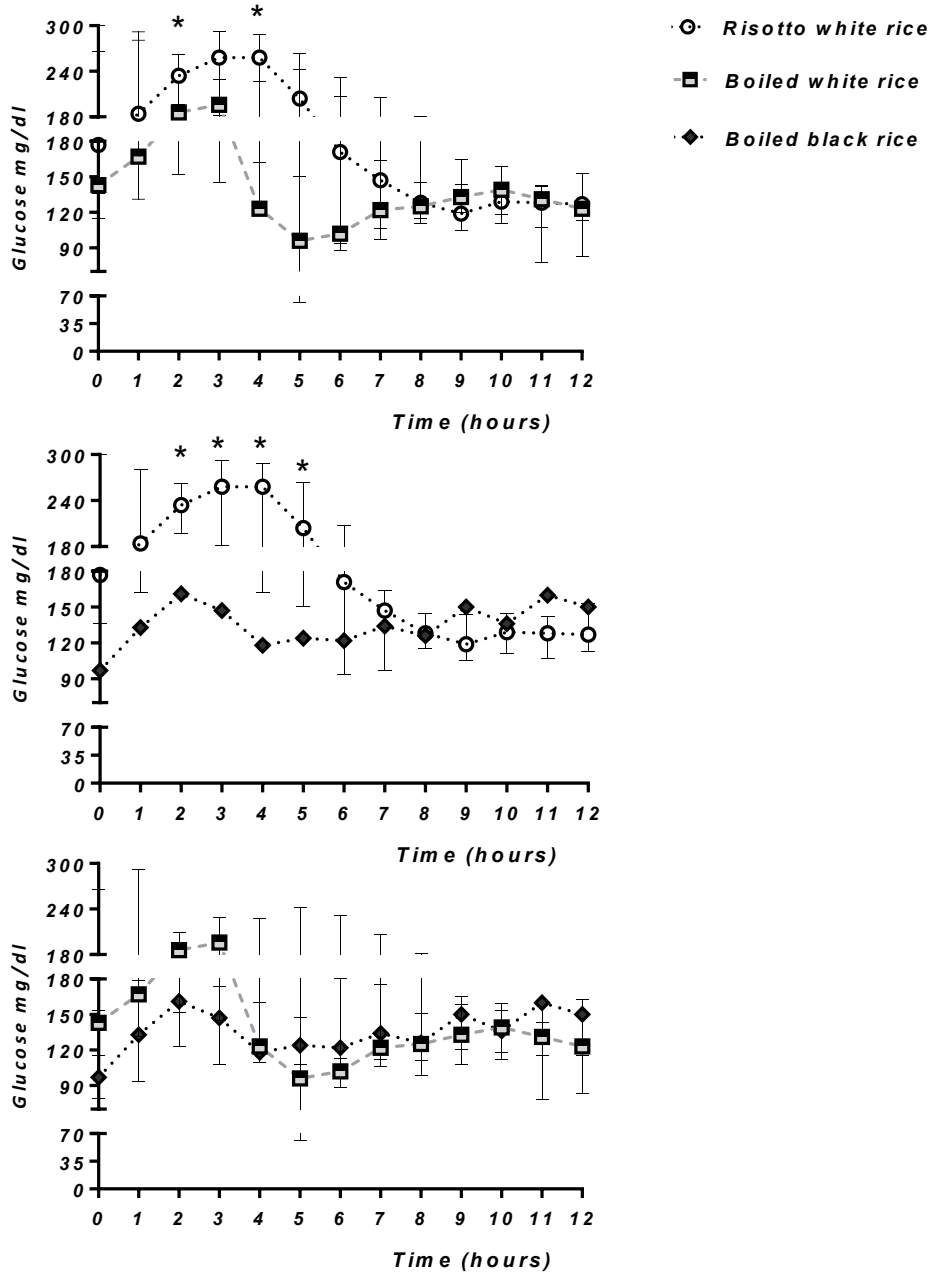

Figure 1. *Glycaemic trend after the assumption of risotto white rice, boiled white rice and boiled black rice during the following 12 hours. *= p<0.05*

Not significant differences in automatic basal or auto bolus insulin delivered by the AHCL were found, according to different types of rice or cooking.

4. Discussion

Rice is a common food for humans, widely consumed around the world; its intake in diabetes diet is not easy to manage depending on several factors, such as glycaemic index and type of cooking. Moreover, brown and white rice have different GI and, consequently, a different impact on the glycaemic trend on diabetic people **[3]**. As reported in literature, some Authors found how the different cooking techniques can influence the composition, digestibility, and GI of the rice **[1,17]**. For example, raw or partially cooked starch could be considered a low-GI food. The ingestion of cooked white rice, cooled to 4 °C for 24 hours and then heated before eating, determines a lower glycaemic response than the ingestion of freshly cooked white rice. So, this reflects the impact of the rice preparation by the consumer on glycaemic variability **[18]**. As well known, starch structure, gelling or retrogradation status in foods (as well as the ratio between amylose and amylopectin) lead to different digestive profiles, so modifying the GI.

In our paper, analyzing the composition of white rice, no significant variations in protein contents were found after cooking, while an interesting increase in total dietary fiber occurred. Considering TPC and TAA, they increased after cooking in “risotto” mode, while a decreasing trend in boiling mode, compared to the uncooked rice, was highlgithed.

In a previous work, the data related to the chemical composition of Artemide rice caryopsis were shown **[4]**. Moreover, protein content in Artemide black rice, showed no significant differences after both types of cooking (average values of 10.4 vs 10.5% of raw rice). Contrary, a significant decrease of total dietary fiber content, compared to the raw rice (7.9 vs 10.8%) was observed, probably depending on the partial degradation and release of some fiber components during cooking.

The polyphenols content and antiradical activity in Artemide black rice were better preserved in risotto mode (21% of the total polyphenols content), while boiling cooking preserved only 10%, making the risotto mode the most suitable for the healthy properties of Artemide black rice **[4]**.

However, Artemide black rice is very difficult to prepare in “risotto” mode, depending on its typical brown-related character, its high fiber content and its rheology, leading to long time of cooking.

Similar results were observed in white “Gigante Vercelli” rice, even if the polyphenols concentration was sharply lower than Artemide’s one (51.8 mg/g of dry weight raw rice for

Artemide vs 0.205 mg/g of dry weight raw rice for “Gigante Vercelli”) with the difference that risotto white rice is easy to prepare and very palatable.

Analyzing glucose sensor values, a good postprandial glycaemic control thanks to the help of technology was found. In relation to the type of cooking, we showed better postprandial glycemic control after eating white boiled rice vs risotto, and an important change in glycaemic trend after eating white rice, in particular, cooked as risotto. These facts suggest a great glycaemic impact determined by its GI depending on the type of cooking. These differences could be explained, as demonstrated, by the loss of some soluble starch during boiling into the cooking water. On the other hand, in the risotto mode, in which all the cooking water is typically absorbed by the rice, no starch loss was observed, according with the higher glycaemic values found in the risotto group.

Instead, a significant reduction in total starch, as expected, was found in Gigante Vercelli boiled rice, which facilitates the lower glycaemic impact if cooking in boiled mode. Moreover, an increase of resistant starch (RS) was observed in both cooking methods; this could be explained by a structural rearrangement, after cooking, of starch granules, that from digestible (non-resistant) turned into non digestible (resistant) ones. This could be interesting because the use of food rich in RS is a potential strategy suggested in literature for reducing starch digestion rate and blood glucose levels **[7,8]**.

Instead, boiled Artemide black rice determined a better glycaemic control at each time point than white rice with mean values always in a target range under 180 mg/dL. This is probably due to the different composition of Artemide black rice which, being an unmilled rice, has a higher fiber content and, consequently, a lower starch concentration with respect to the “Gigante Vercelli” white rice.

The main limitation of this study is the small number of children enrolled. However, this is only a pilot study in which we wanted to consider the chemical and nutritional characteristics of meal-based of rice and its management with different cooking technologies. The second limitation is not to have utilized all functions of the monitoring devices used by diabetic children to cover a particular food or meal.

5. Conclusions

The rice cooked as “risotto” is an important dish in Italian cuisine, and it is more palatable than boiled one, as well known. However, the risotto cooking mode permits to better preserve the total polyphenol content and antioxidant activity in both Artemide and “Gigante Vercelli” rice, making this type of cooking more suitable to enhance healthy properties.

The recent technology applied to diabetes represented by the Advanced Hybrid Closed Loop (AHCL) **[9,19]** has an important role in postprandial glycaemic control and management of complex meals.

Our findings suggest that AHCL systems can help children with T1D manage the coverage of complex meals based on rice. However, knowing the different types of rice and cooking can improve metabolic outcomes. Black Artemide rice gives the best post-prandial glycaemic values; white Gigante Vercelli rice gives a lower glycaemic peak if cooked by boiling than risotto.

Further studies and probably different types of extended boluses able to cover the glycaemic peaks thanks to advanced technology applied to diabetes care, might help people with diabetes to obtain better postprandial glycaemic control when eating meals with high glycaemic index and a less favorable cooking type.

Author Contributions: Conceptualization, A.C. and I.R.; methodology, A.C., S.S., J.D.C. and E.P.; formal analysis, S.S.; investigation, C.G.; data curation, A.C. and S.S.; writing—original draft preparation, S.S.; writing—review and editing, S.S., J.D.C, M.A. and I.R.; visualization, A.C. and S.S.; supervision, S.S.; project administration, S.S. and I.R. All authors have read and agreed to the published version of the manuscript.

Funding: *This research received no external funding.*

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of Ethics Committee of Novara (protocol code 108/22).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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Chapter 7

In vitro batch fermentation of cooked white and black rice to evaluate the impact on gut microbiota functionality: preliminary results

Abstract

Due to the importance of the interaction between food and microbiota, two different rice varieties (black and white, pigmented and refined respectively) were subjected to a simulated *in-vitro* gastric and small intestinal digestion. The residue was subjected to a subsequent microbiota fermentation through *in-batch* fermentation with human faecal microbiota from a healthy volunteer. SCFAs (Short Chain Fatty Acids) production was monitored via GC-FID. Important differences between the two rice varieties were found. In particular, the black variety determined higher production of acetate and propionate, positive metabolites of a functional microbiome.

Introduction

The metabolism and bioavailability of polyphenols compounds strictly depends on the gut microbiota composition, which also determine their functionality **[1]**. On the other hand, the microbiota composition depends on the action of polyphenols, but also other bioactive and functional compounds provided by the diet, such as dietary fiber. This double interaction contributes significantly to the human health **[2]**. The gut microbiota, more particularly, metabolizes the non-digestible fraction of the dietary fiber, often acting as prebiotic ingredient, yielding metabolites with a low molecular weight, such as SCFAs, positive and desired indicators of the microbiota metabolism **[3]**. Different static and dynamic *in vitro* methods are used to replicate the human digestion. Completing the information coming from these methods (e.g. INFOGEST protocol) **[4]**, the batch fermentation using human microbiota is a challenging (and simple) *in vitro* way to obtain a first indication about the impact of the food on that microbiota, as well as the effect of microbiota on the fermentation of the selected food. The aim of this work was to evaluate the impact of black rice (polyphenols- and fiber-rich food) on a healthy human gut microbiota, compared to white polished rice, through the determination of SCFAs production.

Materials and methods

Rice samples and cooking methods

“Artemide” black rice and “Gigante Vercelli” white rice were subjected to cook. The white variety was cooked in two different ways (risotto preparation and boiling), in order to observe differences between the cooking methods; while black rice was used in the only boiled version (the most commonly used for the unmilled pigmented varieties), to evaluate the

different impact of an unmilled pigmented variety against a polished white one. The cooking specifications are reported in Chapters 4 and 6 of this Thesis.

Simulated in vitro digestion

After cooking, the samples were subjected to the *in vitro* INFOGEST simulated digestion protocol **[4]**. This protocol involves the succession of three phases: oral, gastric and intestinal. More details about the protocol application are reported in Chapter 5 (*2.3. Static in vitro digestion*) of this thesis. At the end of digestion, the samples were centrifuged, and stabilized by freeze drying.

In vitro batch fermentation

Faecal sample for the batch fermentation was collected from a healthy adult volunteer (28 y.o.), who declared no smoking, no antibiotic, no alcohol, and no pre- and pro-biotics consumption for 6 months before the beginning of the study. For the fermentation the protocol described by Pérez-Burillo *et al.* **[5]**, with some modifications, was followed. In brief: the faecal inoculum was prepared by weighting 5 g of fresh faeces and adding a sterile phosphate buffer solution (K_2HPO_4 8.8 g/L; KH_2PO_4 6.8 g/L) to a final volume of 25 mL. This solution was mixed and immediately centrifugated at 4,000 x g (Centrifuge 5804 R, Eppendorf, Milan, Italy) and the supernatant was considered as the faecal microbiota inoculum. An adequate amount (10% of the final volume) of the solid residue obtained after the sample digestion was weighted in a 20 mL sterile vial, then added with 7.5 mL of sterile basal medium (K_2HPO_4 5.22 g/L; KH_2PO_4 16.32 g/L; $NaHCO_3$ 2 g/L; Yeast extract 2 g/L; Peptone 2 g/L; Mucin 1 g/L; *L*-Cysteine HCl 0.5 g/L; TWEEN-80 1 mL/L). The vial was sealed with a rubber cup and flushed with nitrogen for 10 minutes to create anaerobic conditions. Then 2 mL of faecal microbiota inoculum was added. The fermentation started in a stove at 37 °C, protected from light under continuous gentle agitation (60 rpm). Each incubation was performed in duplicate with a negative control; sampling was obtained at 4, 6, 24 and 48 h, respectively. Immediately after sampling, the reaction was stopped in ice and the samples were centrifuged at 9,200 x g (Centrifuge 5417 R, Eppendorf, Milan, Italy) for 5 minutes. The supernatant (needed for the SCFAs quantification) and the solid residue (possibly useful to carry out the sequencing of the microbiota) were recovered and stored at -20 °C, until their use.

SCFAs quantification

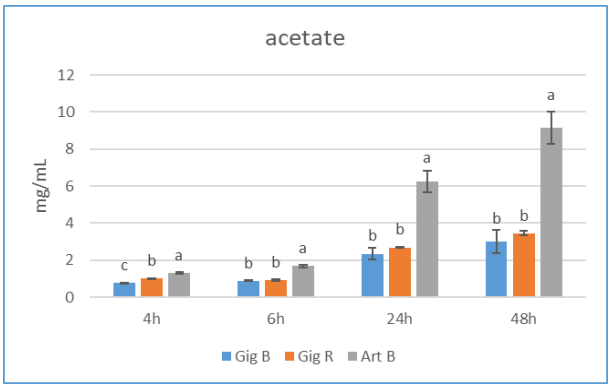
The detection and quantification of short chain fatty acids (SCFAs) was obtained via GC-FID, following the protocol described by Huang *et al.* **[6]**, with some modifications. 1 µL of the supernatant obtained after centrifugation of fermented samples was injected to gas chromatography (Thermo TRACE 1300, Thermo Fisher) equipped with a flame-ionization detector (FID) and a capillary column (NUKOL™ FUSED SILICA Capillary Column, 15m x 0.25mm i.d. x 0.25 µm film thickness). The injection was performed in splitless mode (split flow: 75.0 mL/minute; split ratio: 50) and the temperature of the injector was 250 °C. The oven temperature was initially set to 100 °C, and programmed to reach 200 °C in 10 minutes (rate: 10 °C/minute). This temperature was maintained for other 10 minutes, for a total running time of 20 minutes. The gas carrier was hydrogen at a flow rate of 1.5 mL/min. The detector temperature was maintained at 350 °C. Standard solutions of acetic, propionic and butyric acids (from 0.1 to 12 mg/mL) were used for the preparation of the standard curves and the identification and the quantification of the analytes.

Statistical analysis

Results were expressed as mean ± standard deviation (SD) and differences were estimated by analysis of variance (ANOVA) followed by Tukey's honest significant difference test. The statistical significance level was set to 0.05. All statistical analyses were performed using the free statistical software R 4.0.0 version.

Results

In all the considered samples, acetate and propionate were detected, while no presence of butyrate was found. SCFAs concentrations at each time of the fermentation process are reported in **Figure 1**.



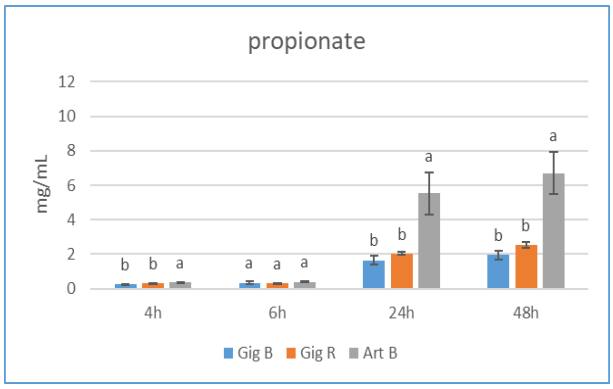


Figure 1. *Acetate (left) and propionate (right) concentrations (mg/mL of solutions obtained after the fermentation), expressed as media ± standard deviation. Different letters indicate significant differences between samples ($p < 0.05$).*

"Gig B" = boiled white "Gigante Vercelli" rice; "Gig R" = white "Gigante Vercelli" rice cooked as risotto; "Art B" = boiled black "Artemide" rice.

The differences in the SCFAs production between black and white rice were evident since the first sampling time (4h), becoming more evident at 24 and 48 h, where the maximum concentration was reached. Acetate concentrations of 9.17, 3.45 and 3.00 mg/mL were detected at 48 h in Art B, Gig R and Gig B samples, respectively. 6.70, 2.53 and 1.94 mg/mL were detected for propionate in the same samples, at 48 h. No statistically differences were found between the two white rice cooked in different ways.

Discussion

Significant differences in the SCFAs production between black and white rice fermentations were found. The elevated amounts of acetate and propionate detected in the Artemide black rice fermented sample, compared to the white rice samples, could be related to its higher content in total dietary fiber and polyphenols, that are well known for their fermentable properties, thus increasing the production of SCFAs **[7,8].** These data suggest a possible positive modulation of the gut microbiota by this black rice variety, leading to a significant level of "secondary modulators" of the microbiota effect, like SFCAs. However, no statistically significant differences were observed between the two cooking methods of white rice. We assume that the small differences observed between rice samples in the total dietary fiber content (2.01% d.w. for the risotto preparation and 1.79% d.w. for the boiled sample) are not significant to modulate and to improve the production of SFCAs.

Further studies are needed to investigate the potential changes in the microbiota composition and profile, caused by the two rice varieties. Furthermore, it would be

interesting to understand which is the most determinant component (fiber or polyphenols) in black rice correlated with the production of SCFAs by the human microbiota. About that, it will be useful to test other cooking methods of black rice, basing on the fact that, as previously reported by Colasanto and collaborators **[9]**, different cooking techniques impact differently on the polyphenolic content, but not particularly on the total fiber content in cooked rice. It must be considered that the risotto preparation, or the *sous vide* cooking at 89 °C (as reported in the Chapters 3 and 4 of this Thesis) were demonstrate to be the best performing methods to preserve polyphenols in rice matrix, when compared to the rice boiling, resulting this last the worst one in previous investigations.

Conclusion

This preliminary work confirmed that the *in vitro* batch fermentation using human microbiota isolated from faecal material is a powerful approach to study either the interplays between food and microbiota. The results here reported are correlated to a single analysis (single sample of microbiota) and must be replicated in order to substantiate the outcomes. Anyway, these preliminary results confirm a different trend of SCFAs production using different rice varieties, supporting the idea that pigmented rice must be considered an example of healthy foods and ingredients for humans. Moreover, the relative and more relevant impacts of dietary undigestible fiber and polyphenols at mechanistic level must be clarified in further studies.

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Chapter 8

Conclusions and future perspectives

The relationships and the interplays between food and health were suggested (and substantiated) by a lot of studies during the last decades. The necessity to deeply explore the impact of technology (and particularly the cooking methods) and the impact of human digestion is now a challenging topic in order to better understand the bioaccessibility and the consequent bioavailability of nutrients (and non-nutrients with a physiological role, like polyphenols).

The main results obtained in this Thesis work concern the impact of cooking and digestive processes on the chemical and nutritional composition of black rice, which can be considered as a sort of natural functional food, due to its high content of antioxidant polyphenols, in particular anthocyanins. Furthermore, important evidence about the impact of black and white rice on the glycaemic trend in type 1 diabetic children and adolescents was obtained. Finally, the basis to study the interactions between cooked rice and gut microbiota in healthy individuals have been laid.

The results obtained and described in **Chapter 3** demonstrated the importance to study the cooking methods, evaluating their impact on rice chemical composition, in order to preserve its nutritional (and beneficial) properties. Different cooking methods (boiling, microwaves oven, under pressure pot and risotto preparation) had significant and different impact on rice composition. The risotto mode, with a preliminary toasting phase, was established to be the best cooking method to preserve antioxidant capacity, polyphenols, and anthocyanin content, while boiling turned out to be the worst, due to the fact that part of polyphenolic compounds remained in the exceeding cooking water, as expected. The data obtained were not limited to polyphenols variations, but have also improved the information about the cooking impact on the main nutrients. Proteins and ashes content didn't significantly change after cooking, while total dietary fiber, following the exposure to all cooking methods, decreased, probably because cooking led to the partial degradation of some fiber components, depending on temperature, percentage of water as solvent and interaction with other compounds during thermal exposure.

As described in **Chapter 4,** the evaluation of the cooking impact was then extended to other methods, such as *sous vide* and pilaf preparations, in comparison with the risotto mode. The optimization of the cooking conditions, probably influenced by my personal participation in the TV show "Masterchef Italia 10", allowed to reduce the average loss of polyphenols compared to the previous work (-34% reduction, against the -83% as described in Chapter 3). The *sous vide* mode performed at 89 °C and the risotto mode were the best cooking

methods to preserve anthocyanins, total polyphenols content and antioxidant capacity, while no particular differences between cooking methods were observed regarding their impact on the protein fraction. The *sous vide* mode at 99 °C and the pilaf mode resulted less efficient, highlighting the importance of the temperature of cooking conditions. It is interesting to note that one of the best cooking methods (risotto) is a traditional one, easily reproducible at home, while the second one (*sous vide* at 89 °C), even though not so easy at home, could be used in the canteens of schools, hospitals and Companies.

Since polyphenols, to exert their beneficial effect to the human organism, must be bioaccessible and bioavailable, the evaluation of the impact of digestion process on the polyphenolic fraction of cooked Artemide black rice, through the application of *in vitro* INFOGEST simulated digestion protocol, was reported and discussed in **Chapter 5**. Interesting findings about the behaviour of anthocyanins, other flavonoids and phenolic acids were done. Anthocyanins were found to be stable up to the gastric level, while their concentrations decreased significantly in the intestine, with a consequent reduction also in the bioaccessibility values. For example, cyanidin-3-O-glucoside, the most abundant anthocyanin recovered in black rice, decreased during the digestion, degrading in protocatechuic acid, which increased in its concentration in the soluble portion. The free fraction of phenolic acids showed a reduction in their concentration in the insoluble portion and a simultaneous increase in the soluble portion during the digestion, while the bound phenolic acids remain almost unchanged after digestion. This could suggest that the bound polyphenol fraction is not bioaccessible, but this protocol not considers the role of gut microbiota, which could release part of the bound component of phenolic acids, increasing their bioavailability. The flavonoids fraction showed an increase in the concentration in free form, with a consequent increase in their bioaccessibility, during the digestion, confirming other studies. In general, our results allowed to highlight the main changes in the polyphenolic composition of one variety of black rice after cooking and digestion.

In future, the research activities would be focused to continue the detailed study of the cooking conditions (for example, the evaluation of the ranges temperature-pressure, the toasting phase, the addition of other ingredients, applying some specific project designs), to identify the cooking protocol more useful to preserve the polyphenolic component, particularly concering the binomial time-temperature.

Chapter 6 reports and discuss the studies about the glycaemic impact of two different types of rice (“Gigante Vercelli” white rice and Artemide black rice) and two different cooking

modes (boiling and risotto) in children and adolescent patients with type 1 diabetes (T1D). The glycaemic trend of each patient was monitored through an advanced hybrid closed loop (AHCL) system (Tandem ControlIQ™). Our findings suggest that AHCL systems can help children with T1D manage the coverage of complex meals based of rice. The different rice varieties and cooking methods impacted differently on the glycaemic trend. In particular, Artemide black rice gave the best post-prandial glycaemic values, while “Gigante Vercelli” white rice gave a lower glycaemic peak if cooked by boiling than risotto. This agrees with the minor content of soluble starch and dietary fiber determined in the boiled sample of white rice.

However, the risotto preparation of “Gigante Vercelli” white rice was the most appreciated by children. So, further studies focused to obtain better postprandial glycaemic control when eating meals with high glycaemic index, could be useful. Probably, different types of extended boluses could be tested to find the better conditions to cover the glycaemic peaks. About that, we recently conducted a second study: eighteen T1D subjects (aged 11.8 ± 2.6 years old) ate the “Gigante Vercelli” rice cooked in the risotto mode, and a half of the patients received insulin as a single bolus, while the other half as an extended bolus (70% immediately, and the remaining 30% in the next 2 hours). The preliminary results are reported in **Figure 1**. We found an optimal postprandial glycemic control for both types of boluses with mean glucose values in target (<180 mg/dl) within 2 hours after eating risotto. At 3 hours after dinner, subjects using extended bolus had lower median glucose values than the ones using single bolus. So, the extended bolus might determine better postprandial glycemic excursions in the case of a meal with high glycemic index, but further elaborations of data must be considered.

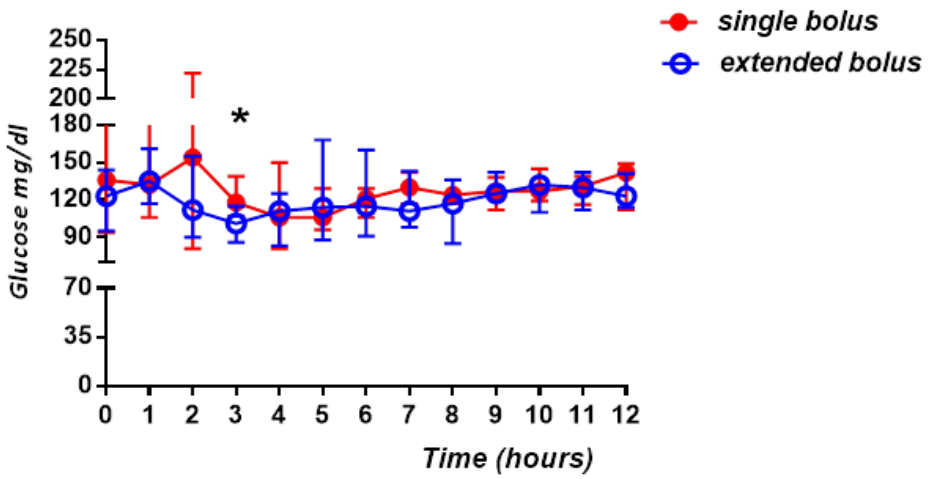

Figure 1. *Glycaemic trend after the assumption of risotto white rice, in subjects used single or extended bolus, during the following 12 hours. *= p<0.05*

Due to the importance of the interaction between food and gut microbiota, as described in **Chapter 7**, the impact of cooked rice, subjected to an *in vitro* simulated digestive process, on a faecal microbiota from a healthy person was investigated. Boiled Artemide black rice and "Gigante Vercelli" white rice cooked in both boiling and risotto mode, after the simulated digestion process, were chosen to an *in vitro* batch fermentation with a faecal microbiota of a healthy donator. In this thesis the production of short chain fatty acids (SCFAs), positive and desired indicators of the microbiota metabolism, was investigated. Significant differences in the SCFAs production between black and white rice fermentations were found. The elevated amounts of acetate and propionate detected in the Artemide black rice fermented sample, compared to the white rice samples, could be related to its higher content in total dietary fiber and polyphenols that are well known for their fermentable properties and thus to increase the production of SCFAs. These data suggest a possible positive modulation of the gut microbiota by this black rice variety. However, no statistically significant differences were observed comparing the two cooking methods of white rice, in which polyphenols concentrations were very low and minimal differences in the other parameters, such as fiber content, were observed.

In future, it would be interesting to understand which is the most determinant component (fiber or polyphenols) in black rice for the production of SCFAs by the human microbiota. To do this, the black rice cooked in a way that can best preserve the polyphenols (for example the risotto or the *sous vide* method at 89 °C), or directly the raw rice, could be subjected to

digestion and fermentation, and compared with boiled rice, which contains less polyphenols than the previous ones, but the same fiber content. Considering the raw material, the drying methods and the preserving methods of rice should be investigated, in order to evaluate the natural fate of the polyphenols during caryopsis storage. Literature needs to be implemented on this direction.

Furthermore, other studies are needed to investigate eventually changes in the microbiota composition caused by the two rice varieties. Actually, the next-generation sequencing (NGS) of faecal microbiota of the digested samples is in progress. This could reveal interesting information on the relative changes of the main families of bacteria present in the faecal microbiota, confirming the real impact of rice digested on the microbiota profile.

Future perspectives could also include the application of the protocols described in this thesis to other functional foods or ingredients, to better understand their interaction with the human microbiota and consequently their impact on human health.