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INNOVATION IN THE CEREAL SUPPLY CHAIN FOR THE DEVELOPMENT OF HEALTHY AND SUSTAINABLE FOODS

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Summary

Cereal grains are a reservoir of non-nutrient minor constituents with biological activity, such as micronutrients (minerals and vitamins), dietary fiber, and many secondary metabolites, which are mainly accumulated in the outer layers of the kernels, i.e., bran and germ. The consumption of whole grains, which have been associated with numerous beneficial effects and a healthy aging, remains below the minimum dietary intakes recommended by food agencies and national guidelines around the world. The growing demand for foods offering healthy features beyond basic nutrition has encouraged the development and marketing of food commodities, such as cereals, as specialty for the production of functional foods.

This PhD thesis has adopted a comprehensive “farm-to-fork” approach to assess the potential of minor cereal species, old varieties, and newly bred biofortified genotypes for the development of functional food products to increase consumer attractiveness to whole grains and improve the adherence to healthy dietary patterns.

The research highlighted substantial variability in the content of several bioactive compounds across species and genotypes, and a strong influence of the growing environment. Pigmented wheat and rice genotypes showed significantly higher levels of anthocyanins and phenolic acids, while bread wheat with yellow endosperm, tritordeum, and novel maize hybrids represented a relevant source of carotenoids, when compared to standard varieties. Intra-group variability allows the selection of the most promising varieties for cultivation and end-use applications, highlighting the fundamental role of breeding to enhance both agronomic and nutritional traits. The agronomic traits, grain composition, and bread quality of rye landraces cultivated in marginal environments open up opportunities for niche market value chains. Conversely, hulled wheats and old varieties of bread wheat showed limited adaptability to non-marginal cropping systems, emphasizing the need for genetic improvement.

The research has also highlighted the key role of milling strategies to optimize phytochemical retention while minimizing contaminant risk. Combining innovative genotypes with tailored milling strategies resulted in high nutritional quality and improved food safety. Using phytochemical-rich varieties for food production proved to be the best way of maintaining the highest levels of bioactive compounds and antioxidant capacity of the end products, although alternative strategies and bioavailability studies should be explored to further support their health advantages. The findings contribute to the ongoing research efforts aimed at identifying effective strategies that preserve bioactive compounds in cereal-based products. They offer practical insights for varietal selection, agricultural management, and processing strategies. Future research directions include wider genotype

screenings, *in vitro* or *in vivo* digestion and bioavailability studies, and closer collaboration between breeders, farmers, and the food industry. Indeed, biofortified cereal genotypes, especially those rich in pigment compounds, are promising alternatives to stimulate whole grain consumption and promote a diverse and healthy diet, but the generation of market and consumer awareness will be crucial for their widespread adoption.

Riassunto

I cereali rappresentano una componente essenziale delle diete umane a livello globale, non solo per il loro contributo calorico, ma anche per il potenziale ruolo salutistico, grazie al contenuto di fibre, vitamine, minerali e composti bioattivi, accumulati principalmente negli strati corticali delle cariossidi. Nonostante il consumo di cereali integrali sia stato associato a numerosi effetti benefici per la salute, i livelli di assunzione rimangono al di sotto delle quantità minime raccomandate dalle linee guida internazionali. La crescente domanda di alimenti in grado di offrire benefici salutistici ha stimolato lo sviluppo e la commercializzazione di *food commodity*, tra cui i cereali, come *specialty* per la produzione di alimenti ad alto valore aggiunto salutistico.

La presente tesi di dottorato ha valutato l'adozione di innovazioni di prodotto, ovvero specie cerealicole minori, varietà antiche e nuovi genotipi pigmentati, e innovazioni di processo, ovvero strategie di molitura e di trasformazione, con l'obiettivo di sviluppare filiere cerealicole innovative ad alto valore aggiunto.

L'attività di ricerca ha evidenziato un'elevata variabilità inter e intraspecifica nel contenuto di numerosi composti bioattivi, nonché una forte influenza delle condizioni ambientali. I genotipi pigmentati di frumento e riso hanno mostrato livelli significativamente più elevati di antociani e acidi fenolici, mentre frumenti teneri ad endosperma giallo, varietà di tritordeum e nuovi ibridi di mais si sono distinti per il maggiore contenuto in carotenoidi rispetto alle varietà convenzionali. La variabilità intraspecifica ha consentito l'identificazione delle varietà più promettenti per la coltivazione e per usi alimentari, sottolineando il ruolo fondamentale del miglioramento genetico per il raggiungimento di specifici obiettivi agronomicie nutrizionali. Le caratteristiche agronomiche e la composizione nutrizionale e salutistica di ecotipi locali di segale coltivati in ambienti marginali hanno messo in luce opportunità di valorizzazione in filiere di nicchia. Al contrario, farri e varietà antiche di frumento tenero hanno mostrato una limitata adattabilità a contesti colturali non marginali, evidenziando la necessità di miglioramenti genetici.

La ricerca ha inoltre confermato il ruolo cruciale delle strategie di molitura nel preservare i composti bioattivi e ridurre i rischi sanitari, in particolare quelli derivati dalla presenza di micotossine. Inoltre, l'utilizzo di varietà ricche in composti bioattivi si è dimostrato il metodo più efficace per mantenere le proprietà antiossidanti nei prodotti trasformati, sebbene ulteriori studi su metodi di processamento alternativi e sulla biodisponibilità di tali composti siano necessari per confermarne il vantaggio salutistico.

I risultati ottenuti contribuiscono in modo significativo alla definizione di strategie efficaci per la valorizzazione in filiere ad alto valore aggiunto di prodotti cerealicoli, offrendo indicazioni sul ruolo della selezione varietale, della gestione agronomica e delle tecnologie di prima e

seconda trasformazione nel fornire competitivà ad innovative varietà ad alto contenuti di composti salutistici. Tra le prospettive future si delineano l'estensione di *screening* varietali, sutdi sulla biodisponibilità *in vitro* o *in vivo* dei composti di interesse e di alimenti derivati, e una più stretta collaborazione tra genetica, mondo agricolo e industria alimentare. In particolare, varietà ad alto contenuto di composti bioattivi, soprattutto pigmentate, rappresentano una promettente alternativa per stimolare il consumo di cereali integrali e favorire una dieta più varia e salutare.

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Preface

This Ph.D. thesis is the result of the experimental work carried out at the Unit of Agronomy, Department of Agricultural, Forest and Food Sciences (DISAFA) of the University of Torino (Grugliasco, Italy) under the supervision of Prof. Massimo Blandino.

During the Ph.D. period, part of the experimental activities contributing to the topics discussed in the present thesis was carried out in cooperation with other Italian departments and universities, such as the Department of Life Sciences and Systems Biology (University of Turin), the Department of Pharmaceutical Science (University of Eastern Piedmon, Novara), the Department of Agriculture, Environmental and Food Sciences (University of Moline, Campobasso), the Department of Food and Drug (University of Parma), the Department of Food, Environmental and Nutritional Sciences (Università of Milan), the Department of Food Science (University of Napoli Federico II). Other research institutes and industrial partners were also involved, especially as far as the breeding and the milling and processing operations are concerned. In addition, a research period abroad was carried out at the Department of Chemistry of the Faculty of Agrobiology, Food and Natural Resources in Prague, Czech Republic, from September to December 2022, and from February to March 2023. A second visiting period was carried out in Thessaloniki, Greece, from July to October 2024, at the School of Agriculture, Department of Food Science and Technology, Aristotle University of Thessaloniki.

The topic of the Ph.D. activity has been related to improving the nutritional and health-related properties of cereal-based products through an integrated supply chain approach. The focus has been on the raw materials, including old and newly developed cereal species and varieties, but the investigation has ecompassed the role of growing conditions, agronomic management, milling and processing strategies on the fate of biologically active compounds. The thesis consists of nine chapters as described below.

Chapter I considers the role of cereals in the diet and the contributions of phytochemical compounds to the health-promoting effects of cereal consumption. Subsequently, this chapter focuses on the possibility of further enhancing the nutritional and health benefits of foods through the cultivation and processing of biofortified cereal genotypes characterized by enhanced phytochemical accumulation. The objectives of the experimental activities carried out during the Ph.D. period are presented at the end of this chapter.

Chapters II to **VII** present the original papers published, submitted, or in preparation for submission to international peer-reviewed journals:

- **Chapter II** – "The cultivation of rye in marginal Alpine environments: a comparison of the agronomic, technological, health and sanitary traits of local landraces and commercial cultivars" by Claudia Sardella, Luca Capo, Martino Adamo, Matteo Donna, Simone Ravetto Enri, Francesca Vanara, Michele Lonati, Marco Mucciarelli, Massimo Blandino. Published in Frontiers in Plant Science in May 2023.
- **Chapter III** – "Growing hulled Triticum species and old bread wheat genotypes in non-marginal production situations: an agronomic, qualitative, phytochemical, and sanitary comparison with modern varieties" by Claudia Sardella, Francesca Vanara, Mattia Scapino, Valentina Scarpino, Clara Pedrazzani, Chiara Dall'asta, Massimo Blandino. Submitted to Journal of Food Composition and Analysis in March 2025.
- **Chapter IV** – "Influence of agronomic practices on the antioxidant compounds of pigmented wheat (*Triticum aestivum* spp. *aestivum* L.) and tritordeum (× *Tritordeum martinii* A. Pujadas, nothosp. Nov.) genotypes" by Claudia Sardella, Barbora Burešová, Zora Kotíková, Petr Martinek, Raffaele Meloni, Luboš Paznocht, Francesca Vanara, and Massimo Blandino. Published in Journal of Agricultural and Food Chemistry in August 2023.
- **Chapter V** – "From paddy to polished rice: investigating antioxidant activity and phenolic compounds in Italian conventional and pigmented rice varieties and their by-products" by Yassine Jaouhari, Debora Giordano, Fabiano Travaglia, Claudia Sardella, Francesca Vanara, Jean Daniel Coisson, Monica Locatelli, Massimo Blandino. Submitted to Food Chemistry in April 2025.
- **Chapter VI** – "Comparing large-scale dry milling processes of conventional and innovative maize hybrids for the production of value-added cornmeal semolina" by Claudia Sardella, Alessandra Fratianni, Valentina Scarpino, Francesca Vanara, Gianfranco Panfili, Massimo Blandino. Submitted to Food Chemistry in April 2025.
- **Chapter VII** – "The potential of cereals as key sources of bioactive compounds: the role of milling and processing in wheat and tritordeum" by Claudia Sardella, Barbora Burešová, Zora Kotíková, Luboš Paznocht, Francesca Vanara, Matyáš Orsák, Massimo Blandino. Submitted to Journal of Agriculture and Food Research in April 2025.

Chapter VIII summarizes and discusses the results achieved within the Ph.D. thesis, highlighting limitations and suggesting future perspectives.

Chapter IX consists of all the publications produced during the Ph.D. period, including those not discussed in the thesis chapters.

Chapter I

Global relevance of cereals as staples

Cereal grains have served as a fundamental source of nourishment for humans since their domestication around 10,000 years ago, serving as staple foods across various cultures and regions. As the edible seeds of grasses from the *Poaceae* family, cereals include a wide array of species, such as bread and durum wheat, oat, barley, rice, sorghum, millet, maize, and rye. Among these, wheat, maize, and rice now dominate world agricultural production and harvested area (FAO, 2023a). Their historical prominence stems from their adaptability to diverse climates, ease of cultivation and storage, and versatility in culinary application. Today, cereal grains are globally traded commodities that significantly contribute to food security and, as processed foods, provide a substantial share of daily caloric intake. Between 2010 and 2021, approximately 46-49% of dietary energy in Asia and Africa derived directly from cereals, with an average supply of 1,447 and 1,175 kcal/cap/day, respectively. By comparison, direct cereal consumption in American and European countries provided almost 30% of calories, with stable patterns and an average of around 950 kcal/cap/day (FAO, 2023b). Wheat and rice account for the majority of this intake globally, providing 42% and 40% of the cereal-based food supply, respectively, followed by maize at 11%, which also plays a crucial role in animal feed and industrial uses (Awika, 2011). Within the European Union, wheat and its derivatives account for the largest share of the cereals available for domestic consumption (115.66 million tons), followed by maize (66.11 million tons) and barley (46.26 million tons; FAO, 2023a). Future consumption patterns are expected to remain relatively stable (EC, 2024), with an average daily intake of 219 grams of cereals per adult.

The nutritional relevance of cereals as the basis of the world's population diet relies on the grain energy-providing macronutrients (WHO, 2023; Poutanen et al., 2022), being cereal grains consisting of three major components: 65–75% carbohydrates, mainly starches, 6–15% proteins, 2–6.5% lipids, with the rest 10-15% being composed of dietary fiber (Ficco et al., 2024; Poutanen et al., 2012). The long-standing demand for cereals has driven selective breeding for yield-related traits, including non-brittle rachis, hulled kernels, reduced plant size, increased grain weight and starch accumulation, especially in wheat (Boukid et al., 2018). Indeed, the endosperm has gathered most of the scientific and technological interest for food processing purposes until the end of the previous century. This grain anatomical part mainly accumulates starch and sulfur amino acids proteins, such

as gluten-forming proteins, so that it primarily affects the milling and the processing properties (Poutanen et al., 2012).

Health-promoting phytochemicals in cereals

Today, the demand for ever-increasing grain production has not diminished, and it has perhaps become even more necessary given the future growth of the world's population. However, the nutritional relevance of cereals is not only related to its macronutrient composition. Cereal grains are also a reservoir of non-nutrient minor constituents with biological activity, such as micronutrients (minerals and vitamins), dietary fiber, and many secondary metabolites naturally produced by plants under physiological development as well as in response to abiotic and biotic stresses. Among these, the most significant besides fiber are *n*-3 fatty acids, sulfur amino acids, oligosaccharides (stachyose, raffinose, and fructans), lignin, minerals, trace elements, vitamins B and E, carotenoids, polyphenols (especially phenolic acids such as ferulic acid and smaller amounts of flavonoids and lignans), alkylresorcinols, phytic acid, betaine, total choline-containing compounds, inositols, phytosterols, policosanol, and melatonin (Poutanen et al., 2012). However, these compounds are unevenly distributed within the grain botanical parts (Figure 1, 2, 3), and this distribution also varies according to the type of cereal considered. Some (mainly soluble fiber, selenium, some B vitamins, carotenoids, and flavonoids) are present in significant quantities in the endosperm, but most are in the bran (especially the aleurone layer) and germ fractions, which are usually removed during milling operations (Farcas et al., 2022). Wheat bran and germ, for instance, contain about 45 and 18% of dietary fiber, and at least 7% and 6% of bioactive compounds, respectively, which represent about 52% and 24% of these fractions (Fardet, 2010).

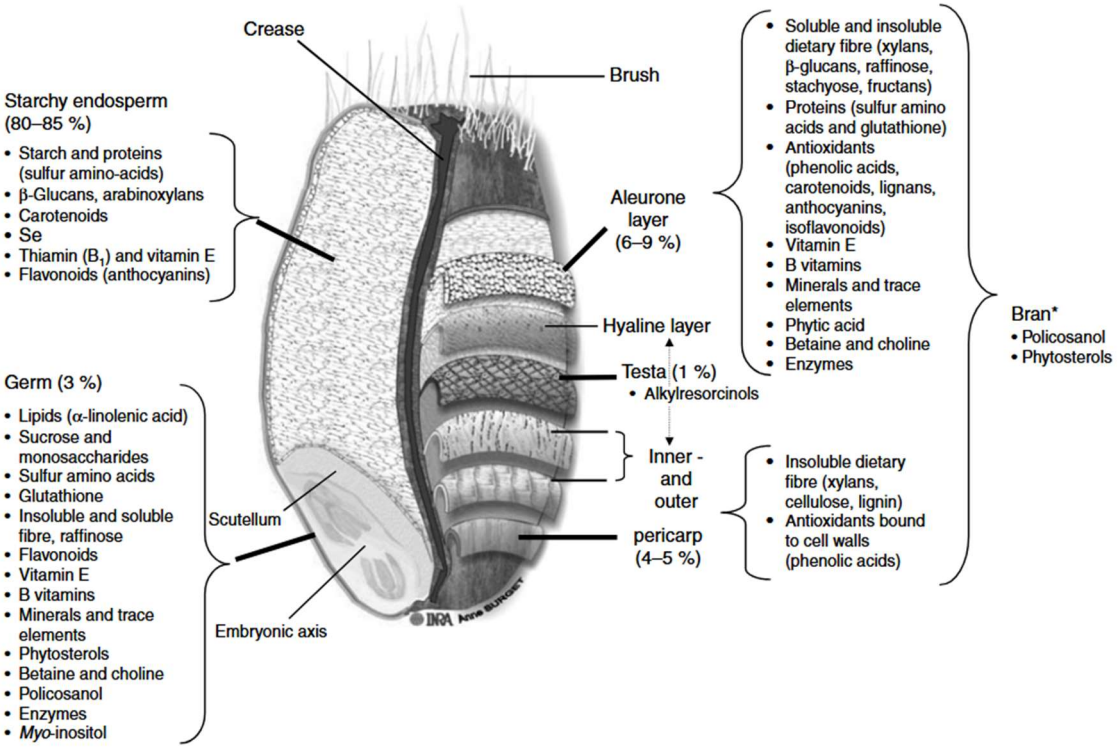

Figure 1. The three fractions (bran, germ, and endosperm) of wheat kernels with their main biologically active compounds (Fardet, 2010).

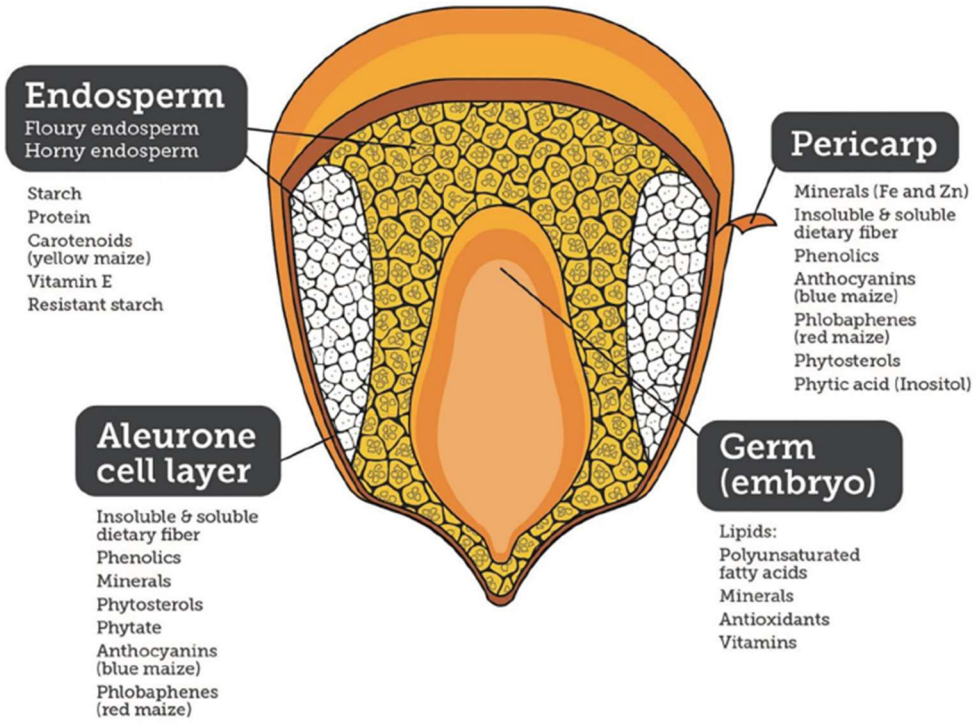

Figure 2. The kernel fractions of maize with the different components (Prasanna et al., 2020).

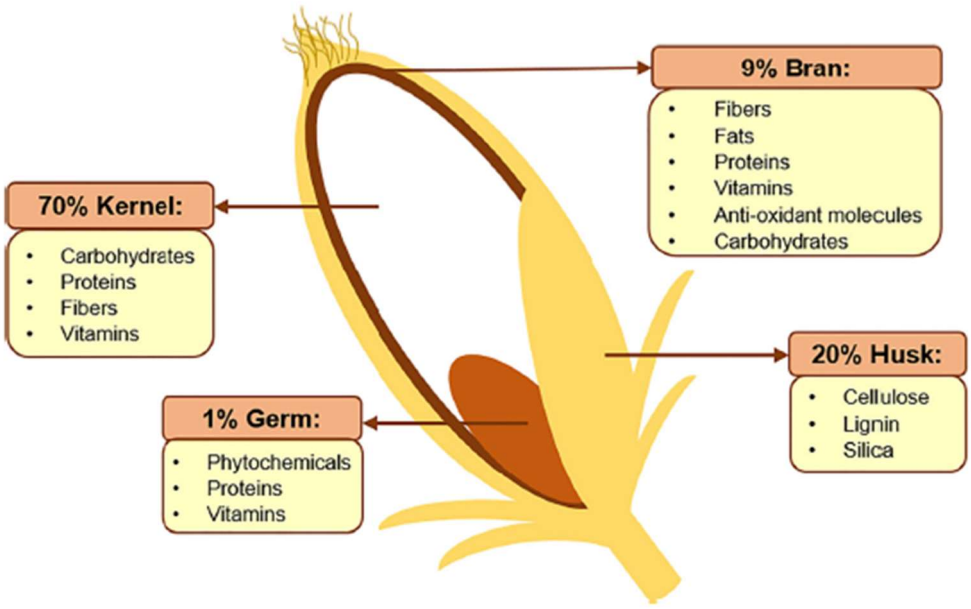

Figure 3. Schematic representation of rice kernel constituents (Fraterrigo Garofalo et al., 2020).

Significance of whole grain consumption for a healthy aging

The recognition of the importance of the outer grain layers for health maintenance has risen in the last decades, as epidemiological evidence and prospective studies strongly support a link between whole grain consumption and a reduced risk of non-communicable diseases, including coronary heart disease, cardiovascular disease, type 2 diabetes, and cancer, along with a reduction in all-cause mortality (Aune et al., 2016; Gil et al., 2011). Meta-analyses further demonstrate improvements in biomarkers such as LDL cholesterol, HbA1c, and C-reactive protein with whole grains consumption as opposed to refined grains or placebo consumption (Marshall et al., 2020). Although further interventional research is needed to better understand the preventive and treatment potential of whole-grain and whole pseudo-grain dietary intake for cardiovascular disease, a regular intake of whole grain derivatives together with the optimization of the protein/carbohydrate ratio in the diet and a regular physical activity has been recognized to significantly reduce the risk of developing aging-related diseases and increases healthy life expectancy (Capurso et al., 2021). This is particularly important in today's world, which is characterized by a rapidly aging population. It is expected that by 2050, the global population of people over 65 will surpass 2.1 billion people, more than double compared to 2015. Specifically, the elderly population will grow more rapidly in emerging-economy countries, such as Latin America and the Caribbean (expected increase of 71%), Asia (66%), and Africa (64%), followed by Oceania (47%), North America (41%), and Europe (23%) (United Nations, 2015). This

means that while European countries have had more than 150 years to adjust to an increase of up to 20% in the proportion of the population over 65, countries like Brazil, China, and India will have less than 20 years to adapt to a similar one (EUROSTAT, 2025). Longevity is the result of several factors among which nutrition plays a pivotal role, so that dietary strategies to promote healthy aging become critical. A higher adherence to diets characterized by a high consumption of fruits, vegetables, whole grain cereals, legumes, nuts, unsaturated fats, and low-fat dairy products is associated with a reduced risk of chronic degenerative diseases and total mortality, and greater odds of healthy aging (Bizzozero-Peroni et al., 2022; Tessier et al., 2025).

Many studies have attempted to elucidate the physiological mechanisms involved in the protective role of regular whole grain consumption. It is now agreed that the beneficial physiological effects of whole grain cereal products are not only due to the higher fiber content of the bran, but also to the synergetic action of the numerous bioactive compounds which are predominantly found in the bran and germ fractions of cereals (Fardet, 2010). Each of them has individually linked to certain biological actions and recognized health benefits, mainly contributing to antioxidant protection and diminished oxidant stress, so that whole grain flour possesses higher antioxidant activity than refined flour (Adom & Liu, 2002).

Challenges for whole grain cereal consumption

In view of this evidence, research interest has been moved towards the enhancement of the nutritional and health-related quality of cereals and cereal-based products. Given the uneven distribution of bioactive compounds within the kernel layers, the best way to preserve a high nutritional density in bioactive compounds is the preservation of the bran fraction during the milling process. However, the maintenance of the outer layers of the kernels has some well-known drawbacks. Firstly, they are the portions most subjected to contamination by natural toxins, such as mycotoxins, e.g. deoxynivalenol and zearalenone in wheat or aflatoxins and fumonisins in maize, or synthetic contaminants, e.g. heavy metals cadmium, lead, and arsenic, especially in rice, and pesticides (Cheli et al., 2010; Scarpino et al., 2021; Sovrani et al., 2012). Given the negative impact of exposure to contaminants on human and animal health (El-Sayed et al., 2022; Mostafalu & Abdollahi, 2016; Rizwan et al., 2018), it is particularly relevant that recommendations for the consumption of more whole grain cereal products should be accompanied by the production of less contaminated cereals, or the adoption of milling strategies alternative to the use of whole grain flour.

In this regard, innovative milling strategies has been recently proposed to carefully select specific kernel fractions that are lower in contaminants yet rich in phytochemicals (Liyana-

Pathirana & Shahidi, 2007; Sovrani et al., 2012). Recent works have demonstrated that grain fractionation processes such as pearling or air-classification of phytochemical-rich varieties of wheat (Ficco et al., 2018), barley (Bellido & Beta, 2009), tritordeum (Giordano et al., 2019), and maize (Chen et al., 2017) can effectively be applied to obtain milling co-products with relatively higher content of health-beneficial compounds. These enriched fractions can subsequently be used either for bioactive compounds extraction or as functional food ingredients to be directly incorporated into pasta, snacks, and bakery products (Zanoletti et al., 2017).

Other than food safety concerns, it is well known that the use of whole grain flour and bran in food applications poses challenges related to dough workability, technological properties and consumers' acceptance when compared to refined flour. Indeed, the high fiber content of whole grains and bran fractions causes disruption of the gluten network and thus affects dough stability and rheology, with negative outcomes on the sensory and technological properties of the end-use products. The higher ban content in whole grain flours usually reduces the loaf volume and causes a darker crust color and higher crumb firmness in bread (Zhang et al., 1999); lower strength and cooked firmness and greater cooking loss are typically reported in whole grain or bran-enriched pasta (Bresciani et al., 2022).

Consumer perception and market trends

Despite technological limitations, consumer perception of whole grains and derived products has evolved over time (Laureati et al., 2016; Nielsen, 2015). In 2015, a Nielsen study of 30,000 consumers in 60 countries found that 3 in 10 consumers, on average, are seeking foods that contain whole grains, with some variation in that percentage based on region (Nielsen, 2015). A predominant trend toward more healthy eating for improved well-being and longevity has emerged, leading consumers to choose a food product over another with a view of obtaining some desirable health end-state (Pappalardo & Lusk, 2017; Annunziata & Vecchio, 2011). In 2018, Euromonitor International named "healthy living" as the most impactful of eight megatrends influencing the food industry and one of the main reasons for determining consumer food choices (Hosafci, 2018). However, despite supportive policies, an increasing number of whole-grain alternatives on the market, and an upward trend in the overall consumption, whole grain intakes in many developed countries remain below the minimum dietary intakes recommended by food agencies and national guidelines around the world. This is particularly evident in many Mediterranean countries, including Italy, which have witnessed a shift away from the Mediterranean diet, of which whole grain consumption is one of the main pillars, over the past decades (Obeid et al.,

2022). In a recent survey conducted in Italy, for instance, only the 23.79% of participants met the recommended consumption frequency of 1-2 servings/main meal for cereals such as bread, breakfast cereals, rice, and pasta (Cardamone et al., 2024).

Research has primary suggested the necessity to implement tailored intervention strategies such as communication and education interventions at national level. However, the growing demand for foods offering healthy features beyond basic nutrition has encouraged the development and marketing of food commodities, such as cereals, as specialty for the production of functional foods. Innovative biofortified ingredients to be used in whole-grain formulations can add functionality, thus increasing consumer attractiveness to whole grain products and improving the adherence to healthy dietary patterns.

Nevertheless, the consumer acceptance of novel and innovative food alternatives is a complex and challenging issue. In Italy, for instance, biofortified products (with selenium, iodine, etc.) are increasingly widespread, confirming consumer demand for this category of functional foods. An analysis pointed out that the informed consumer of biofortified products is extremely determined to purchase them and is willing to pay high prices. However, in Italy today, aware and informed consumers are a very limited segment of the market dynamics (Timpanaro et al., 2020). As the analysis shows, consumers also make their purchasing decisions on the basis of information on labels or communicated in advertising campaigns, and considers foods bearing health-related claims slightly healthier than others without any message (Domínguez Díaz et al., 2020).

Emphasis should be given to the role of market information and campaigns to decrease the information asymmetry between consumers and products and increase the credibility and effectiveness of the various claims. However, it should be taken into account that in Europe functional foods and nutraceuticals do not have neither a specific regulatory framework, nor a statutory definition. The claims that suggest or imply that a food has particular characteristics, including nutrition, health claims, and reduction of disease risk claims, are clearly regulated in the European market by Regulation (EC) No 1924/2006 and depend on the specific nutrients or compounds contained in the products (EC, 2006). Up to date, a total of 239 health-related claims has been approved: 10 claims for fat, 5 for carbohydrates, 14 for fiber, 3 for protein, 98 for minerals, 68 for vitamins, 24 for other substances, 12 for food and finally 5 related to food categories (EC, 2025). A comprehensive review highlighted that health-related claims included in the labeling and approved by official government agencies positively affect consumers food choices and lead to healthier consumption patterns (Domínguez Díaz et al., 2020). However, globally only a few countries, such as the United States, United Kingdom, Sweden, and Singapore, have authorized whole grain health claims for the reduced risk of heart disease and certain cancers, while others, such

the European regulation, have concluded that there is currently insufficient scientific evidence to support a whole grain health claim (Mathews & Chu, 2020).

Genotype innovation for value-added supply chains

Minor and ancient species

Within this framework, interest in foods based on minor and undervalued cereals, such as rye, spelt, emmer, einkorn, and Khorasan, is growing, driven by consumer perceptions of these varieties as healthier, tastier, and more sustainable (Arzani & Ashraf, 2017; Shewry, 2018). The exploitation of secondary crops such as ancient wheat, local and old varieties can satisfy emotionally-driven trends, increase crop and food diversity, and provide farmers, millers, and manufacturers with new market positions (Longin & Würschum, 2016).

Biofortification for phytochemicals-rich varieties

Furthermore, staple food crops like cereals can be the subject of biofortification, the process of breeding crops for higher levels of micronutrients or any health-related substance and/or for increased bioavailability after digestion (Timpanaro et al., 2020). While in poor countries at risk of hunger, biofortified food crops are a type of functional food that serve to compensate for deficiencies in microelements and to reduce malnutrition (Birol et al., 2015), in developed countries enhanced commodities can represent a form of a contribution to the diet for oxidative stress mitigation and increased life expectancy purposes as well as for product innovation and differentiation (Domínguez Díaz et al., 2020).

Pigmented varieties of rice, maize, wheat

Recent breeding efforts have been aimed to further enhance the grain accumulation of pigmented compounds that can be naturally synthesized in plant cells via secondary metabolisms. These compounds, mainly belonging to the group of anthocyanins and carotenoids, have been extensively studied in fruits and vegetables for their human health-related benefits against oxidative stress and chronic diseases, thus they can potentially provide functional attributes apart from color (Bassolino et al., 2022). The anthocyanins-rich varieties possess the ability to synthetize and store anthocyanins in the pericarp and/or aleurone layers of the grain, resulting in recognizable purple, blue, or black pigmentation, depending on the types and position of the anthocyanins in the kernel layers (Garg et al., 2022). Being anthocyanin accumulated in the outer layers, the refining process do not maintain these compounds and coloration in the final refined flour (Giordano et al., 2017).

Thus, the health-related properties of anthocyanin-rich varieties are further enhanced by the fact that the grains are typically exploited as whole grain flour or as mechanically separated bran fractions, thereby increasing the grain content and dietary intake of fiber and other associated phytochemical components. On the other hand, carotenoid-rich varieties typically accumulate in the endosperm a much higher concentration of carotenoids than conventional varieties (Giordano et al., 2017, 2019; Saenz et al., 2021), so that refining processes mainly recovering the starchy endosperm can result in flour with a strong yellow coloration and improved levels of antioxidant compounds.

Pigmented rice and maize genotypes displaying anthocyanin coloration are already cultivated worldwide, being the derived food and beverages part of the diet as typical products in many parts of the world, from Asia to Central and South America. Pigmented rice, represented by black, purple and red rice varieties, is considered a delicacy in Europe and United States (Ito & Lacerda, 2019), but an expansion of the niche market has been recently observed. In Italy, the main rice producer in Europe, a large collection of black and red rice is registered, and a great culinary appreciation has been shown by the Italian population (Bordiga et al., 2014). Additionally, food companies have shown interest in creating all-Italian value chain, such as in the case of the Italian brand “Riso Scotti” with the black rice Venere.

Maize with pigmented pericarp is less common in Europe, although the majority of maize varieties including those with white or yellow grains have the genetic information for the anthocyanin biosynthetic pathway (Lago et al., 2014). In northern regions of Italy and Spain, open-pollinated maize varieties traditionally cultivated in mountainous areas for human feeding, such as “Nero Spinoso”, “Millo Corvo”, “Rostrato Rosso di Rovetta” are often characterized by the presence of pigments, such as phlobaphenes, anthocyanins, flavonols, and phenolic acids (Cassani et al., 2017; Lago et al., 2015; Sangiorgio et al., 2021). Interest has been shown in restoring landraces cultivation for the development of niche supply chains and the promotion of diversity preservation (Domínguez-Hernández et al., 2022). Furthermore, pigmented landraces or south American germoplasm can be used as recurrent parents in cycles of backcrossing with modern lines to obtain novel improved genotypes adapted to the cultivation in temperate climate with enhanced nutritional and commercial value (Lago et al., 2014; Colombo et al., 2021).

On the other hand, bread wheat commonly does not exist in colored forms. Introgression processes introduced the purple pericarp or blue aleurone trait into wheat (*T. aestivum*) from the tetraploid Abyssinian wheat (*T. aethiopicum*) or various wild relatives, respectively (Garg et al., 2022). Wheat of black grains, the color resulting from the presence of both purple pericarp and blue aleurone, was developed by hybridizing purple pericarp and blue

aleurone wheat by Chinese and Japan researchers, and later transferred to Indian and European germoplasm (Martinek et al., 2014). Genotypes of bread wheat with increased carotenoid accumulation in the endosperm have also been bred and are available on the market (Burešová et al., 2021). Moreover, a combination of anthocyanins and carotenoid accumulation is possible and can result in both black bran and yellow endosperm (Dhua et al 2021).

New cereal species: the case of tritordeum

Other than commonly cultivated cereal species, also newly developed species can hold promises for health-related value-addition purposes in the food sector. Tritordeum (x *tritordeum martinii* A. Pujadas, nothosp. nov.) is a novel cereal species (Martín et al., 1999) developed with the aim of combining in its genome the properties of a wild relatives of *Hordeum vulgare* (*H. chilense* Roem. et Schultz.) with those of the parental durum wheat (*T. durum*). Despite the first hexaploid hybrids derived from backcrossing date back to the 70s, only in the last 15 years have promising varieties of tritordeum been genetically selected (Shewry et al., 2023). Interest in this species has raised for its grain carotenoid content, significantly higher than that of durum wheat as inherited from the *Hordeum* parent (Paznocht et al., 2018).

Constraints and challenges for specialty cereal chains

The (re)introduction of a yet not cultivated species or variety only has a sustainable and profitable future if a comprehensive assessment is carried out throughout the whole product chain (Longin & Würschum, 2016). Breeding is a central component, but a careful selection of the most appropriate genetic pool should be carried out based on the input from the players along the entire product chain. Firstly, agronomic properties such as adaptation, disease tolerance, yield potential, and sanitary risks should be assessed and compared with standard species or varieties. Secondly, the product quality should be tested in order to identify and address potential milling applications and technological uses. Notably, scientific evidence is needed when health-promoting features are claimed, and the nutritional advantages should be assessed encompassing all the steps of the supply chain, to help justify potentially higher prices. Overall, the amount of specific bioactive compounds and their profile of cereal species and varieties may depend on the plant genetic background, the environmental growth conditions, including the possible occurrence of abiotic and biotic stresses, as well as the agronomic practices applied (Marone et al., 2022). Milling processes may strongly influence the preservation of these compounds in the final

flour and semolina, due to their specific localization within kernel anatomical parts (Liyana-Pathirana & Shahidi, 2007). Storage conditions and commonly used methods for cereal secondary processing such as baking and cooking can cause various degradation rates of phytochemicals, depending on their stability to oxidation and high temperatures, on their solubility, and on the type of food matrix, ultimately affecting their bioavailability, digestibility, and health-related potential (Burešová et al., 2023).

Objectives of the thesis

The present work has attempted to evaluate the (re)introduction of minor cereal species and innovative, newly-developed varieties for the production of foods characterized by improved health-promoting properties. This investigation has been carried out using an integrated "farm-to-fork" approach, with the objective of identifying both opportunities and limitations for the long-term success of sustainable cereal-based value chains. The potential health-related benefits of these minor or innovative cereals were assessed in comparison with standard reference varieties suited to the same growing environments or market applications. The research was specifically designed to identify the possible field, milling, and processing strategies capable of delivering flour and processed products with enhanced phytochemical concentration. However, the study also considered other key aspects influencing the competitiveness and adoption of these cereals, including agronomic and yield-related traits, food safety, milling performances, and technological functionality, to fully support acceptance from all the involved parties of the product chain.

The feasibility of reintroducing local landraces of rye in marginal Alpine environments was explored for the development of specialty food chains, by comparing genetically diverse landraces with modern rye and wheat cultivars. In parallel, in response to the renewed interest for ancient species, the cultivation of hulled wheats (einkorn, emmer, and spelt) and old varieties of bread wheat was investigated in non-marginal growing environments. This work evaluated their agronomic and qualitative responses under standard cultivation practices to determine their suitability for modern industrial supply chains.

Focusing on newly bred varieties, anthocyanins- and carotenoids-rich genotypes were cultivated in different experiments under standard agronomic practices, with the aim of ascertaining the main factors contributing to the variation in the levels of both agronomic and qualitative traits and thus supporting an expansion of their cultivation.

The impact of milling on phytochemical retention and their distribution across milling co-products was studied first in rice and subsequently in maize, using both conventional and innovative varieties processed through different milling strategies.

Finally, the combined effects of milling and processing on phytochemical retention were investigated in wheat and tritordeum.

Overall, the findings contribute to the ongoing research efforts aimed at identifying effective strategies that preserve bioactive compounds in cereal-based products. The broadening of such knowledge can inform and guide farmers and manufacturers' decisions and ultimately support the development of safe foods which could represent a relevant source of health-promoting substances for consumers.

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Chapter II – "The cultivation of rye in marginal Alpine environments: a comparison of the agronomic, technological, health and sanitary traits of local landraces and commercial cultivars"

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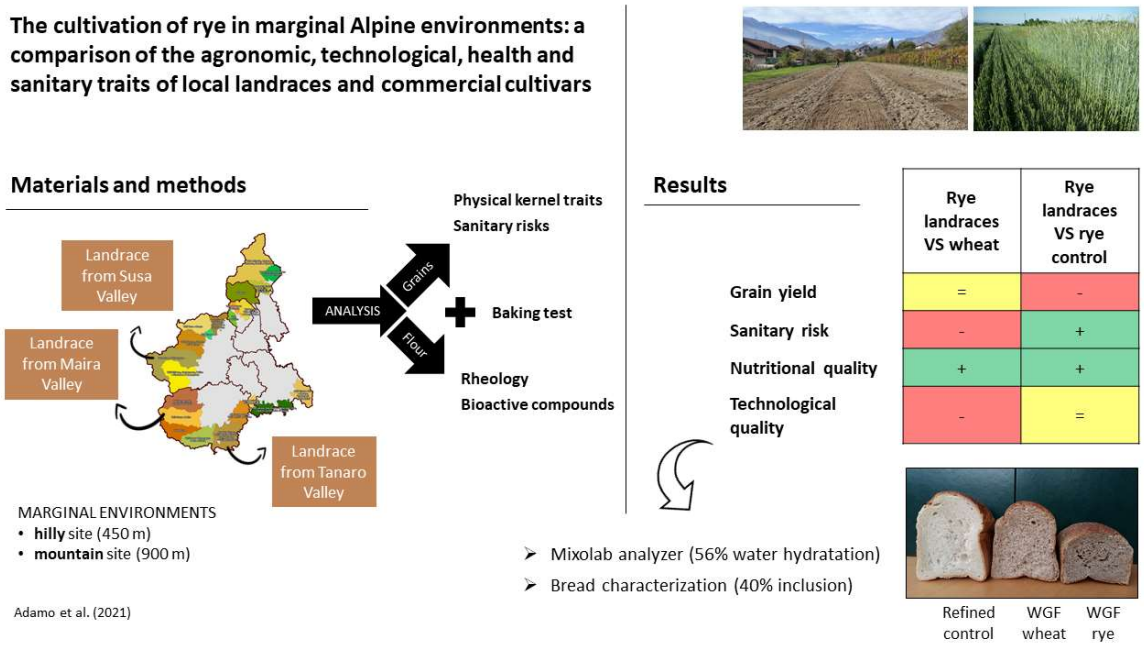
Graphical abstract



Abstract

Rye is a secondary crop that is characterized by a higher tolerance to climatically less favorable conditions than other cereal species. For this reason, rye was historically used as a fundamental raw material for bread production and as a supply of straw in northern parts of Europe as well as in mountain environments, such as Alpine valleys, where locally adapted landraces have continued to be cultivated over the years. In this study, rye landraces, collected in different valleys in the Northwest Italian Alps, have been selected as the most genetically isolated within their geographical contexts and cultivated in two different marginal Alpine environments. The traits concerning their agronomy, mycotoxin contamination, bioactive content, as well their technological and baking quality were assessed to characterize and compare rye landraces with commercial wheat and rye cultivars. Rye cultivars showed the same grain yield level as wheat in both environments. Only the genotype selected from the Maira Valley was characterized by tall and thin culms and a proneness to lodging, thereby resulting in a lower yield capacity. Among the rye cultivars, the hybrid one presented the highest yield potential, but also the highest susceptibility to the occurrence of ergot sclerotia. However, the rye cultivars, especially the landraces, were characterized by higher concentrations of minerals, soluble fibers, and soluble phenolic acids, and thus both their flours and breads had superior antioxidant properties. A 40% substitution of refined wheat flour with whole-grain rye flour led to a higher dough water absorption and a lower stability, thereby resulting in lower loaf volumes and darker products. Agronomically and qualitatively speaking, the rye landraces diverged significantly from the conventional rye cultivars, thus reflecting their genetic distinctiveness. The landrace from the Maira Valley shared a high content in phenolic acids and good antioxidant properties with the one from the Susa Valley, and, when combined with wheat flour, turned out to be the most suitable for bread making. Overall, the results have highlighted the suitability of reintroducing historic rye supply chains, based on the cultivation of local landraces in marginal environments and the production of value-added bakery goods.

Keywords: landraces, marginal environments, rye, Italian Alps, agronomic parameters, bioactive compounds, technological quality, ergot sclerotia.

Abbreviations: ABTS, 2-2′-Azino-bis(3-ethylbeenzothiazoline-6-sulfonic acid) diammonium salt; AC, antioxidant capacity; ANOVA, analysis of variance; A/X, arabinose/xylose; BF, bread formulation; CA, caffeic acid; CWBPAs, cell wall- bound phenolic acids; DDT, dough development time; DON, deoxynivalenol; DPPH, 2,2-diphenyl-1-picrylhydrazyl-hydrate; DS, dough stability; DW, dry weight; FA, ferulic acid; FRAP, ferric reducing antioxidant power; G, genotype; GA, gallic acid; GDDs, growing degree days; GPC, grain protein content; GS, growth stage; GY, grain yield; HBA, hydroxybenzoic acid; HI, harvest index; HPLC/RID, high-performance liquid chromatography with reflectance index detection; LOQ, limit of quantification; *p*-CA*, p*-coumaric acid; PCA, protocatechuic acid; REGW-F test, Ryan/Einot and Gabriel/Welsch test; RP- HPLC/DAD, reversed-phase high-performance liquid chromatography with diode array detection; SA, sinapic acid; SPAs, soluble phenolic acids; SRA, syringic acid; S-SRC, sucrose-solvent retention capacity; TAX, total arabinoxylans; TDF, total dietary fiber; TE, Trolox equivalents; TFA, trifluoroacetic acid; TKW, thousand kernel weight; TW, test weight; VA, vanillic acid; WA, water absorption; WEAXs, water-extractable arabinoxylans; WGF, whole-grain flour; W-SRC, water-solvent retention capacity; Y, harvest year.

Introduction

Rye (*Secale cereale* L.) is a minor cereal crop that has been cultivated for centuries in mountainous (Fracasso et al., 2022) and northern areas of Europe, where it has traditionally been used to provide flour, fodder and straw material for roof construction and basket making (Gaetano et al., 2012). The greater adoption of rye in unfavorable environments than other cereal species is due to its better adaptation to infertile and sandy soils, low temperatures, and drought conditions, which leads to a higher yield potential than that of wheat under similar conditions (Bushuk, 2001; Schittenhelm et al., 2014). Therefore, rye has spread as a crop in marginal areas, and autochthonous landraces have developed over time. Landraces are genetically heterogeneous populations generated in subsistence agriculture that have adapted to specific environmental conditions of the region where they have evolved (Camacho Villa et al., 2005). As a result of being isolated from other populations of the species, and because of the selective pressure of the local environmental factors over time, landraces are characterized by a distinct identity and good capacity to tolerate biotic and abiotic stress, and they often result in an enhanced yielding stability and intermediate yield level in low input agricultural systems (Zeven, 1998). Landraces have played a key role in the history of crops worldwide, as the practice of farmers of saving seeds from one harvest for the following sowing has promoted intra-specific diversity and enhanced genetic variation (Camacho Villa et al., 2005). The combined phenomenon of the exodus of the young workforce from mountain and rural areas, where landraces have been cultivated and thus preserved the most, has posed serious concerns about their extinction and subsequent loss of genetic diversity (de Carvalho et al., 2013). Currently, only a few rye varieties are cultivated (Persson and von Bothmer, 2002) and these are genetically less diverse and differentiated than landraces (Targońska et al., 2016). Hybrid cultivars are widespread in the growing areas of northern Europe, due to a considerable increase in grain yield linked to the heterosis phenomenon, which has been verified in intensive cropping systems (Hansen et al., 2004; Miedaner and Hübner, 2011; Laidig et al., 2017). However, crop improvement practices often utilize landrace diversity to develop new cultivars (Camacho Villa et al., 2005), particularly when the aim is to develop cultivars for marginal environments. In this regard, rye landraces are considered a source of several disease resistance genes, to share, with their wild rye ancestors, much more variation than modern cultivars, and as a useful resource for the development of sustainable crops (Newton et al., 2010). As far as the qualitative traits are concerned, rye flour has been shown to possess a high nutritional value, as it is particularly rich, just like whole-grain, in dietary fiber, including arabinoxylans, fructans, and β-glucans (Andersson et al., 2009), as well as in a

wide range of bioactive compounds, such as phenolic acids, alkylresorcinols, lignans, sterols, vitamins, and minerals (Nyström et al., 2008; Andersson et al., 2014; Kulichováet al., 2019). Rye bread is typically made with different proportions of rye and wheat flour and, in the past, was considered a staple food in the marginal areas where rye was traditionally cultivated (Netting, 1981). Interest in this product has recently increased, as whole-grain rye flour can be incorporated into wheat flour in order to provide several bioactive compounds and to produce value-added baked goods (Aprodu and Banu, 2017) that meet the consumers' growing request for functional foods (Euromonitor, 2016). However, the rheological and technological aspects of rye flour differ greatly from those of wheat flour, as its bread-making quality is mainly controlled by the carbohydrate fraction, especially by starch and arabinoxylans, and by the amylase activity, and, albeit to a lesser extent, by proteins and by the proteolytic activity (Bushuk, 2001; Buksa et al., 2010; Buksa et al., 2013). Therefore, the rheological traits and bread-making aptitude of mixes made of wheat and rye flour should be carefully evaluated in order to successfully produce landrace-based products and develop high value supply chains for marginal environments. In addition to the qualitative traits, local rye landraces are regarded as key components, from a cultural and social perspective, as the resumption of historic and traditional rye supply chains in specific environments can contribute to the recovery of abandoned and marginal areas where rye was a typical agricultural product in the past (Leoni et al., 2021). The genetic variation and diversity of landraces originating from the northern and eastern parts of Europe, which are characterized by cold winters, as well as the genetic distances and relationships among landraces and between landraces and commonly cultivated improved varieties have already been investigated (Kühn and Hammer, 1979; Persson and von Bothmer, 2002; Larsson et al., 2019). A few studies (Peratoner et al., 2016; Leoni et al., 2021; Colombo et al., 2022) have recently agronomically and/or qualitatively characterized and compared modern populations of rye cultivars with landraces collected from farmers in the central Italian Alpine valleys, thereby highlighting the current interest in restoring the cultivation of traditional cereal crops, such as rye, and their supply chains. The reconversion of abandoned farmlands into productive areas through farming can in fact positively affect the access to and relaunching of marginal areas. Nevertheless, to the best of the authors' knowledge, no study that takes into account a wide multiplicity of agronomic and qualitative parameters has been published about the cultivation of rye landraces in marginal Alpine environments. A previous study (Adamo et al., 2021) examined the genetic diversity and structure of rye landraces sampled in different valleys in the Northwest Italian Alps to understand which ones deserved conservation efforts, due to their authentic adaptation to the environmental conditions at their site of origin. In the present work, we have selected three out of the 16

analyzed landraces that have shown the highest genetic distance and we have carried out field experiments in two different marginal Alpine environments over two growing seasons. The landraces were characterized, from an agronomic, technological, and qualitative point of view, according to the low-input cropping system in force in the growing areas. One wheat and two modern rye cultivars, which were used as controls, were cultivated side by side and investigated along with the rye landraces.

Materials and Methods

Experimental design

Field experiments were carried out in the 2019-20 and 2020-21 growing seasons in a hilly location (Bruzolo, TO; 45° 08' N, 7° 11' E; altitude 450 m a.s.l., in the Susa Valley) and in a mountain location (Entracque, CN; 44° 14' N, 7° 23' E; altitude 900 m a.s.l., in the Gesso Valley), both of which are located in Alpine valleys in Northwest Italy, where rye was traditionally cultivated for the production of flour. The position of the Susa Valley, that is, at the center of the Alps, determines less rainfall than other Northwest Italian valleys; it has mild winters and generally dry springs and summers, and a high frequency of windy days. The experiment was carried out on the south-facing side of the central sector of the hilly valley in a shallow alluvial soil of medium fertility. Since the territory is surrounded by high mountains, the climate in the Gesso Valley mountains is characterized by cold winters and hot summers, although it is not far from the Mediterranean Sea. The trial was set up in deep and shallow soil, originating from a glacial deposit. The locations where the field experiments were located are remarkably different in terms of ecological conditions: the climate in Bruzolo is sub-Mediterranean, while Entracque is characterized by a hypomesoxeric climate, according to Gaussen-Bagnouls classification method (Cagnazzi and Marchisio, 1998), with an average annual air temperature of 12 °C and 10 °C and an average annual precipitation of 792 mm and 1107 mm in Bruzolo and Entracque, respectively, considering 19 years of observations (2002-2021; ARPA Piemonte agrometeorological service).

Five rye genotypes were sown in each location, together with an ordinary bread-making wheat (cv. Solehio, Agroalimentare Sud Spa, Melfi, Italy), which was used as a control. The three compared local rye landrace genotypes, called Susa, Maira and Tanaro, according to the respective Valleys of origin, were identified during a survey carried out in the alpine growing areas of Northwest Italy by Adamo et al. (2021) and selected as the genotypes with the highest genetic distances. The rye landraces originating from the Maira Valley and from the Susa Valley showed high values of nucleotide diversity and resulted to be the most genetically isolated within this geographical context. The rye landraces were compared with a commercial population rye cultivar (cv. Antoninskie, with Poznańska Hodowla Roślin Sp. z.o.o., Tulce, Poland, being responsible for its selection and conservation, and the Società Italiana Sementi, San Lazzaro di Savena, Italy for its supply), and with a hybrid rye cultivar (cv. Su Nasri, with Saaten-Union GmbH, Isernhagen, Germany, being responsible for its selection and conservation, and RV Venturoli, Pianoro, Italy for its supply), both of which

are widely cultivated in the growing areas of north Europe and available on the Italian market.

The cereal genotypes from each location were assigned to experimental units using a completely randomized block design, with three replicates. The plot was 12 m^2 (6 x 2 m) in size. The previous crop in each growing season had been bread wheat or a meadow in the hilly and mountain locations, respectively, and the latter was often also used for grazing. The plots were seeded after an autumn ploughing (30 cm) and disk harrowing to prepare a suitable seedbed. Mechanical sowing was conducted in 12 cm wide rows (on October 30 in both 2019 and 2020 at Bruzolo, and on October 18 and October 5 in 2019 and 2020, respectively, at Entracque) at a seeding rate of 350 seeds m^{-2}, except for the hybrid rye for which a seeding rate of 200 seeds m^{-2} was applied. According to the low- input cropping system in force in the growing areas, no mineral or organic fertilizations, or chemical and mechanical treatments were applied to control weeds, diseases or insects during the cultivation.

Agronomic and yield parameters

The full flowering date, that is, when 50% of the ears had reached the complete extrusion of anthers from the spikelets (growth stage, GS 65, according to the BBCH scale), was monitored during spring and expressed as days from April 1st, while the flowering duration was counted considering the days that had elapsed between the first release of the anthers and complete anthesis (GS 60-69). The number of ears per unit of grown area (ear density) was counted manually before the harvest in two sampling areas (625 cm^2) per plot. The number of kernels per ear was calculated in 10 randomly selected ears from each sampling area. The average plant height and the percentage of plot with severe lodging were recorded for each plot. The culm diameter was measured on the 1st internode length in 10 plants per plot using a digital gauge.

The plots were harvested (on July 14 and July 23 in Bruzolo 2020 and 2021, and on August 6 and August 12 in Entracque 2020 and 2021, respectively), by means of a Walter Wintersteiger cereal plot combine-harvester, and the straw was collected behind the combine-harvester and weighed immediately after harvesting. The grain yield (GY) and straw yield results were expressed on a dry weight (DW) basis, and the harvest index (HI) values were obtained by dividing the grain yield by the aboveground biomass (grain + straw) of each sample, expressed as a percentage. Aliquots of 2 kg of grain were taken from each plot, to determine the test weight (TW) and the grain moisture content using a GAC® 2000 Grain Analyzer (Dickens-John Auburn, IL, USA). The thousand-kernel weight (TKW) was determined on two 200-kernel sets for each sample using an electronic balance.

Sanitary risk

The number of ergot sclerotia were visually counted in 500 g grain samples randomly collected from each plot and expressed as g of sclerotia per kg of grain, according to the Commission Regulation (EU) 2021/1399 of 24 August 2021 (European Commission, 2021). The deoxynivalenol (DON) concentration was determined in whole-grain flour, using the ELISA method, by means of RIDASCREEN® DON direct competitive immunoassays (R-Biopharm, Darmstadt, Germany), according to the method reported by Nguyen et al. (2019).

Chemical analyses

Chemicals

2-2′-Azino-bis(3-ethylbeenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), ethanol (CHROMASOLV®, 99.8%), ethylacetate (CHROMASOLV®, 99.8%), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, 97%), hydrochloric acid (HCl, 37.0%), Iron (III) chloride hexahydrate ($FeCl_3$*$6H_2O$, ≥ 98.0%), methanol (CHROMASOLV®, 99.9%), potassium sulfate (K_2SO_4, ≥ 99.0%), sodium hydroxide (NaOH, ≥ 98.0%), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), trifluoroacetic acid (TFA), sodium carbonate (Na_2CO_3) and phenolic acid standards (caffeic acid ≥ 98%, *p*-coumaric acid ≥ 98%, *t*-ferulic acid ≥ 99%, gallic acid ≥ 99%, protocatechuic acid ≥ 99%, *p*-hydroxybenzoic acid ≥ 99%, sinapic acid ≥ 98%, syringic acid ≥ 95% and vanillic acid ≥ 97%) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Monosaccharide standards (L(+) Arabinose and D(+) Xylose) were purchased from Extrasynthese (Genay, France). Water (HPLC grade) was obtained from ELGA PURELAB Ultra system (M-medical, Cornaredo, Milan, Italy).

Proximate composition analyses

The harvested grains were mixed thoroughly, and a 2 kg grain sample was taken from each plot, while representative sub-samples (500 g) were ground to whole-meal using a laboratory centrifugal mill equipped with a 1-mm sieve (Model ZM-200, Retsch, Haan, Germany). The grain protein content (GPC) and the ash content were determined according to AACC method 39-10.01 (AACC International, 2008) on whole-grain flour, using an NIRsystems 6500 monochromator instrument (Foss-NIRsystems, Laurel, MD, USA). The samples were ground to a fine powder (particle size < 500 mm) with a Cyclotec 1093 sample mill (Foss, Padua, Italy), and stored at -25 °C, prior to the chemical analyses. The moisture content, which was determined to express the results on a DW basis, was obtained by oven-drying the < 500 mm samples at 105 °C for 24 h. The β-glucan determination was performed

using a Megazyme mixed-linkage β-glucan assay kit, according to the producer's instructions (Megazyme International Ireland Ltd, Wicklow, Ireland). The total dietary fiber (TDF) was determined according to the AOAC 985.29-1986 enzymatic-gravimetric method (AOAC International, 2003) on a mixed representative sample for each genotype. All the samples were analyzed in triplicate, except for the TDF, which was instead analyzed once for each genotype, and the results are presented as DW%.

Determination of the total arabinoxylans (TAX)

The monosaccharide composition of the TAX was determined by means of high-performance liquid chromatography with reflectance index detection (HPLC/RID) after acid hydrolysis, according to the procedure proposed by Buksa et al. (2012), albeit with some modifications. Aliquots of 0.5 mL of deionized water and 2 mL of 2 M TFA were added to 25 mg of each sample, and the mixtures were hydrolyzed for 3 h at 90 °C in a water bath. The samples were vortexed every 30 min during hydrolysis to aid dispersion. The samples were then cooled and subsequently centrifuged at 3300 rpm for 10 min. Aliquots (2 mL) of the clear supernatants were collected, to which 8 mL of bidistilled water was added. The samples were neutralized with dry Na_2CO_3 to pH 7, filtered using a syringe filter (pore diameter 0.20 µm) and then 10 µL of the sample was injected into an HPLC system, Agilent 1200 Series (Agilent Technologies, Santa Clara, CA, USA) coupled with an Agilent 1200 Series RI detector. Separations were carried out using SecurityGuard Cartridges, Carbo-Pb (4 x 3.0 mm, Phenomenex) and an SP08010 column (Shodex); the column temperature was set at 70 °C and the mobile phase was bidistillated water (flow rate of 0.6 mL min^{-1}). Arabinose and xylose were identified using the retention times and the reflectance indexes of their respective standards, and the TAX content was calculated as the sum of the two sugars. Solutions of individual monosaccharide standards (L(+) Arabinose and D(+) Xylose) were prepared and diluted to different concentrations to obtain calibration curves for quantification purposes. All the samples were analyzed in triplicate, and the results are presented as DW%. The degree of arabinose substitution to the xylose units (A/X ratio) was calculated by dividing the arabinose content by the xylose content.

Determination of the phenolic acid composition and antioxidant capacity (AC)

Soluble (free and conjugated, SPAs) and cell wall-bound phenolic acids (CWBPAs) were extracted and quantified according to the procedure described in detail by Giordano et al. (2019). Briefly, SPAs were extracted twice with an 80:20 (*v/v*) ethanol:water solution. The supernatants were collected and then evaporated to dryness under a nitrogen stream. Samples were hydrolyzed with 2 M NaOH (400 mL) for 2 h under continuous stirring at 4

°C. After acidification to pH 2 with HCl, the soluble phenolic acids were extracted three times with 500 mL of ethyl acetate. For the extraction of CWBPAs, the samples were extracted as described before in order to remove the soluble phenolic acids. The remaining pellet was hydrolyzed for 4 h under continuous stirring at 4 °C, by adding 2 M NaOH (400 mL). After acidification to pH 2 with HCl, the bound phenolic acids were extracted twice with 800 mL of ethyl acetate and then centrifuged. The phenolic extracts were filtered through a 0.2 mm filter and then analyzed by means of a reverse phase high- performance liquid chromatograph Agilent 1200 Series (Agilent Technologies, Santa Clara, CA, USA) coupled with an Agilent 1200 Series diode array detector (RP-HPLC/DAD). Separations were carried out using a 150 x 4.6 mm, 5 mm, Gemini RP-18 column (Phenomenex, Torrance, CA, USA); the column temperature was set at 35 °C. Phenolic acids were identified using the retention times and the UV/Vis spectra of their respective standards.

The AC of the whole-grain flour and bread samples was determined by means of both ABTS (AC_{ABTS}) and ferric reducing antioxidant power (AC_{FRAP}) assays, following the QUENCHER approach (direct measurement of AC on solid samples) previously described by Serpen et al. (2012). Three individual extractions were carried out for each sample (n = 3), and the results are expressed as mg kg^{-1} (DW) for SPAs and CWBPAs, and as mmol Trolox equivalents (TE) kg^{-1} of sample (DW) for AC.

Technological quality analyses

The technological characterization was carried out by employing whole-grain flour blends of both bread wheat and the rye varieties with refined wheat flour in a proportion of 40:60, according to the conventional commercial use of rye flour for baking in the growing areas. The refined wheat flour was used on its own as a control. The whole-grain wheat and rye flours were obtained by completely grinding the harvested grains, using a Retsch ZM 200 (Retsch GmbH, Haan, Germany) fitted with a 1-mm aperture sieve. The refined wheat flour was supplied by a milling company (0.76% ash content) and the main rheological properties are reported in Table S1.

Rheological properties of the flour

The mixing and pasting behaviors of the refined control and of the whole-grain wheat and rye flour blends were studied using a Mixolab® analyzer (Chopin Technologies, Villeneuve la Garenne, France), according to the ICC Standard Method 173 (ICC, 2011). This instrument allows specific information to be obtained about the behavior of dough constituents (starch, protein, water) by continuously measuring the torque (Nm) produced

by the passage of the dough between two mixing blades submitted to both shear stress and temperature changes. The resulting Mixolab® curve is separated into five different phases. A detailed description of the physical changes that occur during a Mixolab® measurement was reported in Rosell et al. (2007). Analyses were carried out through two different approaches. According to the standard 'Chopin+' protocol, an adapted water supply was used to quantify the water absorption capacity (WA, %) and the dough development time (DDT, min) of each of the flour blends, which represent the amount of water that flour can absorb and the time necessary to reach a pre-set reference dough consistency and to produce a torque (peak C1) of 1.1 Nm ± 0.05 at 30 °C. A faster Mixolab® mixing protocol, called FAST protocol, was then applied at a constant WA of 56%, as described by Dubat and Lebrun (2019), albeit with a few modifications of the assay conditions. The starting temperature was set at 45 °C and the heating cycle, which was initiated after 4 min, was set between 45 and 90 °C, while the cooling cycle, which was initiated after 5 min, was set between 90 and 50 °C. The heating and cooling rates were set at 8 °C min^{-1}. The following parameters, which were derived from the curve, were obtained: DDT, or the time necessary to reach the maximum torque (C1) at 45 °C; dough stability (DS, min), or the elapsed time at which the produced torque is kept at the C1 torque value (± 11%); thermal weakening, i.e. decrease in dough consistency due to mechanical shear stress and a temperature increase from 45 to 60 °C (C1-C2, Nm), which provides information about the protein quality; starch gelatinization, i.e. an increase in viscosity due to the absorption of water by the starch granules and an increase in temperature from 60 to 90 °C (C3-C2, Nm); cooking stability, i.e. the stability of the hot gel at a constant temperature of 90 °C (C3-C4, Nm); and cooling setback, i.e. the retrogradation of the starch in the cooling phase from 90 to 50 °C (C5-C4, Nm).

The solvent retention capacity (SRC) test was adopted to provide information on the water adsorption and on the content of pentosans, according to AACC method 56-11.02 (AACC International, 2008). The following solvents were tested separately: water (W-SRC) and 50% sucrose (S-SRC). Briefly, deionized water or a 50% sucrose solution was added to 5 g of a flour sample, in different centrifuge tubes. After 20 min of hydration and a periodic vigorous shaking (every 5 min for 5 s), the samples were centrifuged at 1000×g for 15 min. The supernatant was decanted, and the pellet was weighed. The quantity of the absorbed solutions is expressed as % SRC.

Bread-making procedure and bread characterization

The twelve mixes (one with whole-grain wheat and five with whole-grain rye flours blended with refined flour in the proportion 40:60, from each experiment) and the refined flour on its own were employed to prepare bread in a bread-making machine. The whole- grain flours of the wheat and rye genotypes were both collected from field experiments carried out in the 2019-20 growing season and milled as previously reported. Flour (1000 g) was added to water (670 g), as were 25 g of powder yeast and 20 g of salt. After 13 min of kneading, the dough was manually divided into three loaves of 550 g each and left to rest for 15 min at room temperature. Leaving was then carried out for 50 min at 30 °C and at 70% humidity. The loaves were baked for 40 min at 235 °C in a bakery oven. The specific volume, color, moisture content and antioxidant capacity of each loaf of bread were determined. The loaf volume was determined 1 h after baking, by means of rapeseed displacement, according to AACC method 10-05.01 (AACC International, 2008). The chromatic characteristics of the bread crust and crumbs were determined using a Minolta Chroma Meter reflectance spectrophotometer (Model CR-400, Minolta Co., Osaka, Japan). Standard illuminant C was used as the reference. The analysis was performed in triplicate at three different points for each loaf. The lightness (L^*), redness (a^*) and yellowness (b^*) color values were determined directly by the instrument, in accordance with Commission Internationale de l'Éclairage (CIE, 1986). The total color difference, that is, the ΔE value of the bread slices from the refined control, was calculated according to Mohd Jusoh et al. (2009).

Bread samples were freeze-dried using a Lyovapor L-200 Freeze Dryer (Buchi, Flawil, Switzerland) and the lyophilized samples were ground to a fine powder using a Cyclotec 1093 sample mill. The moisture content was obtained by oven-drying the samples at 105 °C for 24 h and the AC was determined by conducting the same assays that had been performed on the whole-grain samples (AC_{ABTS} and AC_{FRAP}). The AC results are expressed as mmol TE kg^{-1} (DW).

Statistical analyses

The data were compared separately for the two different Alpine environments, by means of an analysis of variance (ANOVA), to evaluate the effect of genotype (G), harvest year (Y) and their interaction (G × Y) on the agronomic and sanitary traits, the qualitative parameters, and the phytochemical content and profile of the whole-grain flours obtained from the wheat and rye varieties. The effect of bread formulation (BF) and Y and their interaction (BF × Y) on the rheological properties of the twelve blends and the refined control flour was also investigated separately for the two environments. One-way ANOVA was performed on samples from 2020 harvest to evaluate the effect of BF on the bread-making aptitude and

the antioxidant properties of the composite breads. Means were identified as significantly different ($p \leq 0.05$) based on the Ryan/Einot and Gabriel/Welsch (REGW-F) statistical test. Prior to the analysis, data were tested for normality and homoscedasticity. Pearson correlation analysis was performed to investigate the relationships between the agronomic, nutritional and phytochemical, qualitative and rheological traits. Statistical analyses were carried out by means of SPSS for Windows statistical package, version 28.0 (SPSS, Inc., Chicago, IL, USA).

Genetic data were obtained using a ddRAD (double digest Restriction Associated DNA) sequencing approach. Libraries were produced according to the IGATech (Udine, Italy) custom protocol, with minor modifications with respect to Peterson et al. (2012). The adopted raw data clean-up and preliminary analysis procedures are described in Adamo et al. (2021). Nucleotide diversity (p) was calculated in the Stacks 2.0 environment (Catchen et al., 2011). The genetic distances between the rye cultivars were calculated as Prevosti's distances between the populations (Prevosti et al., 1975) in the R environment with the *prevost.dist* function of the R package, 'poppr' v 2.9.3 (Kamvar et al., 2015) and visualized by means of a neighbor joining tree (nj). The tree topologies were sampled with a 1000 bootstrap, and a minimum bootstrap value of 75 was considered as significant.

Agronomic and qualitative parameters were used to cluster the rye cultivars into the main groups using a similarity approach. The most representative variables were selected by experts: culm diameter, plant height, HI, GY and TKW were selected to describe the variability of the agronomic parameters, while TW, GPC, β-glucans, TAX, SPAs, CWBPAs, DS, thermal weakening (C1-C2) and ergot sclerotia were selected to describe the variability of the quality parameters. A cluster analysis was performed, in the R environment, using the R package 'factoextra' v1.0.7 (Kassambara and Mundt, 2020). A preliminary principal component analysis (PCA) was performed to determine the influence of the variables on the cultivar ordination. The most probable number of clusters was predicted, using both the silhouette and the wss (or elbow) methods, and hierarchical clustering dendrograms were then prepared, using Ward's minimum variance method (Ward, 1963), to describe the Euclidean distance of the cultivars.

Results

Meteorological trends

The two growing seasons showed different meteorological trends throughout the cereal crop cycles (Table S2). The precipitations in the 2019-20 growing season were 298 and 494 mm greater than in the 2020-21 season in the hilly and mountain environment, respectively. The difference in rainfall was mainly concentrated in the tillering and stem elongation stages (March to May). The growing degree days (GDDs) were higher (on average +8 °C day^{-1}) from April to June in 2019-20 than in 2020-21. As expected, the rainfall and temperatures were higher and lower, respectively, in the mountain environment than in the hilly one.

Agronomic parameters

On average, the GY was higher in the hilly environment (4.4 t ha^{-1}) than in the mountain one (3.3 t ha^{-1}), due to an overall higher number of kernels per ear in 2020, and ear density and TKW in 2021 (Table 1). ANOVA did not show any significant differences between the GY of the rye genotypes and bread wheat in either site, except for Maira. Overall, the rye genotypes had the same ear density as wheat, except for the hybrid sown at a lower seeding rate in the mountain environment, but they resulted in a higher number of kernels per ear and in a lower TKW, although TKW only led to significant differences in the hilly site. As expected, the rye genotypes showed a higher straw yield (hilly site +52%) and plant height (+94% and +89% in the hilly and mountain environments, respectively, Table S3) than wheat, which resulted in a higher HI (+26%) in the hilly site. Moreover, wheat had the same precocity in the mountain site as most of the rye genotypes, but a shorter duration of flowering (Table S3). As far as the rye genotype comparison is concerned, the hybrid resulted in the highest GY, although this difference was not significant. The heterosis effect of this cultivar resulted in a good tillering ability, thus leading to a complete recovery of the ear density in the hilly site, and to the highest culm diameter in both environments, compared to the other genotypes. Moreover, the hybrid had a lower plant height and a higher HI than the other rye genotypes.

Maira showed the greatest differences in the agronomical and yield traits in the landrace ryes: this genotype resulted in a lower GY (-73%) in both environments, as a consequence of the lower TKW; moreover, it had the longest flowering duration, the lowest HI, the greatest plant height and the thinnest culm diameter. These morphological traits led to an extensive lodging phenomenon in both years, especially in the hilly environment, which was not

observed for the other genotypes. Susa and Tanaro reported very similar agronomical traits to the commercial population cultivar.

Overall, the GY was higher in 2021 than in 2020 in the hilly site, while an opposite trend was observed for the straw yield in both locations, although the G × Y interaction was never significant.

Table 1. Effect of the genotype, the harvest year and their interaction on the agronomic traits[a] of the investigated wheat and rye cultivars, cultivated in two different marginal Alpine environments.

Environment	Factors	Source of variation	GY (t ha^{-1})	Ear density (n° m^{-2})	Kernels ear^{-1} (n°)	TKW (g)	Straw yield (t ha^{-1})	HI (%)
Hilly (450 m)	**Genotype (G)**	Bread wheat	4.7 a	436 a	43 b	47.5 a	5.7 b	45.4 a
		Susa rye	4.8 a	388 a	57 ab	32.7 c	9.6 a	34.6 c
		Maira rye	2.6 b	377 a	59 a	27.7 d	7.9 a	24.5 d
		Tanaro rye	4.8 a	352 a	60 a	34.3 bc	9.6 a	34.1 c
		Commercial rye	4.0 a	323 a	69 a	37.1 b	8.2 a	33.9 c
		Hybrid rye	5.2 a	320 a	67 a	36.2 b	8.3 a	39.8 b
		p (F)	***	ns	***	***	***	***
	Year (Y)	2020	4.0 b	361 a	56 a	37.4 a	10.1 a	28.5 b
		2021	4.7 a	371 a	62 a	34.4 b	6.4 b	42.3 a
		p (F)	**	ns	ns	***	***	***
	G × Y	*p* (F)	ns	ns	*	*	ns	ns
Mountain (900 m)	**Genotype (G)**	Bread wheat	3.0 ab	473 a	34 b	40.5 a	6.3 a	33.3 ab
		Susa rye	3.4 a	387 ab	55 a	36.4 a	6.9 a	33.6 ab
		Maira rye	2.2 b	412 ab	53 a	31.2 a	6.6 a	26.2 c
		Tanaro rye	3.7 a	347 ab	56 a	33.8 a	6.9 a	37.3 ab
		Commercial rye	3.2 ab	323 ab	53 a	34.9 a	7.5 a	31.7 b
		Hybrid rye	4.0 a	301 b	59 a	34.1 a	7.5 a	38.1 a
		p (F)	**	**	***	ns	ns	***
	Year (Y)	2020	3.2 a	416 a	44 b	37.6 a	9.0 a	26.3 b
		2021	3.3 a	332 b	60 a	32.6 b	4.9 b	40.5 a
		p (F)	ns	*	***	**	***	***
	G × Y	*p* (F)	ns	ns	ns	ns	ns	ns

[a]GY, grain yield; TKW, thousand kernel weight; HI, harvest index. The results are expressed on a DW basis. Means followed by different letters are significantly different, according to the REGW-F test [(*) p (F) ≤ 0.05, (**) p (F) ≤ 0.01, and (***) p (F) ≤ 0.001].

Ergot sclerotia contamination and DON contamination

Sclerotia of *Claviceps purpurea* (Fr.) Tul. were found in both sites and in both years among the collected samples of the rye genotypes, but they were never detected among those of the bread wheat (Figure 1). On average, the occurrence of ergot sclerotia was higher in the hilly environment in 2020 than in 2021, while no difference was reported between the years at the mountain site, which reported a lower risk than the hilly one. A significantly higher ergot sclerotia content was observed for the hybrid rye in the hilly environment than all the other genotypes, although Maira did not differ significantly from the hybrid rye. Furthermore, the sclerotia per grain in the hybrid cultivar and in Maira exceeded the legal limit (EU No. 2021/1399) at both sites, as well as in the commercial population cultivar in the hilly site. The DON content was under the limit of detection in all of the wheat and rye samples.

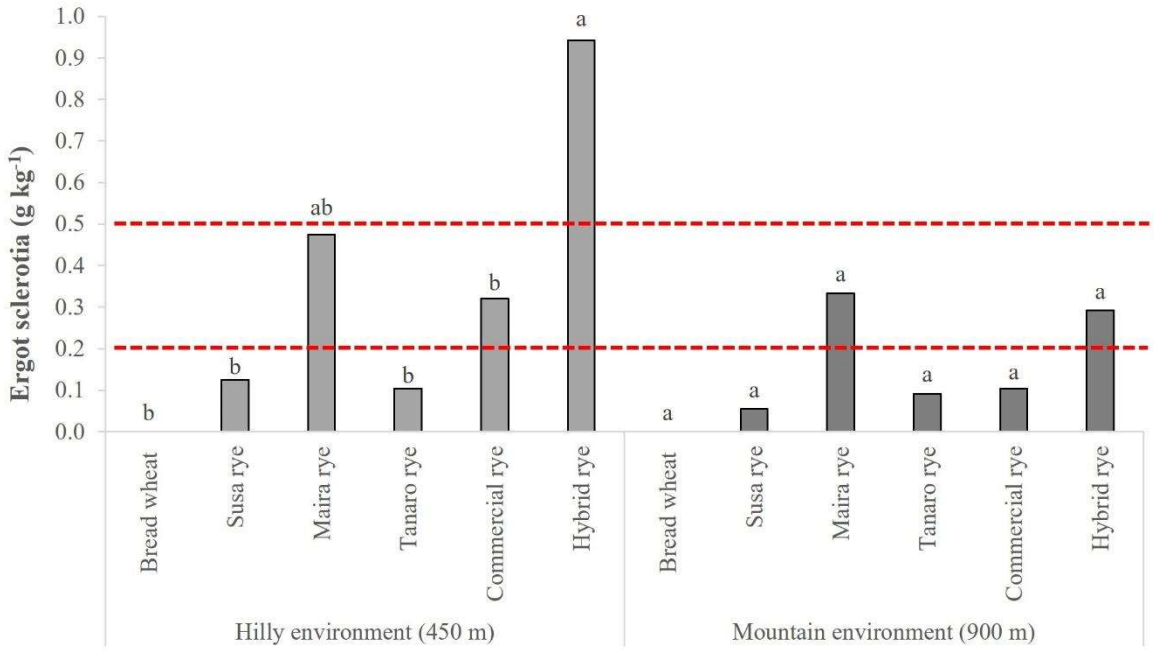


Figure 1. Comparison of ergot sclerotia contamination of the investigated wheat and rye genotypes. The data are averaged over 2 years and for 3 replications. Different letters on bars indicate significant differences [p (F) ≤ 0.05]. The 0.5 g kg^{-1} limit represents the maximum level until 30th June 2024, according to Reg. (EC) No. 1881/2006; the 0.2 g kg^{-1} limit represents the maximum level from 1st July 2024, according to Reg. (EU) No. 2021/1399.

Qualitative kernel traits and compositional analyses of the whole-grain flour

Two-way ANOVA showed significant effects of the genotype factor on all the investigated qualitative traits of the grain and of the whole-grain flour in both growing environments, except for the calculated A/X ratio, which was in the same range for wheat and rye (Table 2). The average values of TW for the rye samples were -1.2 and -3.8% lower than for the wheat control, in the hilly and mountain sites, respectively. However, a great variation of the TW trait was observed among the rye varieties, with Susa showing the highest value in both environments, followed by Tanaro, the hybrid rye, the commercial rye and Maira. As a consequence of the lower TW, Maira recorded the highest ash content in both environments, which was significantly higher than those of the hybrid rye and the wheat control (+8 and +7% in the hilly and mountain sites, respectively), although it did not differ significantly from those of the other two landraces and the commercial rye. On average, the highest ash content was observed in the rye varieties (+4 and +3%, compared to wheat, in the hilly and mountain sites, respectively) and in the mountain environment (+2% with respect to the hilly site), which was also characterized by higher GPCs than the hilly one (on average +4%). Moreover, Maira registered the highest GPC of all the investigated rye cultivars in both sites, but did not differ significantly from the wheat control (Table 2).

The TAX content was found to be significantly higher in all the ryes than in the wheat control (+37%), without any differences being observed among the rye genotypes in either location. Instead, there was no difference between rye and the wheat control as far as the calculated A/X ratio is concerned. The rye varieties had a higher β-glucans content than the wheat variety, that is, 1.2-fold greater at both sites. No significant differences among the rye varieties were found in the hilly site, whereas in the mountain site the highest value of β-glucans and the lowest were registered for Susa and for the commercial rye, respectively. The analysis of TDF was performed without any replicate, thus two-way ANOVA was not applied to this parameter. The rye varieties recorded higher values of TDF than the wheat control (on average +4% in the hilly site, and +5% in the mountain one), with the highest values observed for the hybrid rye in the hilly environment (16.7%) and for Susa in the mountain environment (16.9%).

The year had a significant effect on some of the analyzed parameters, and differences were detected between the two environments. TW was significantly higher in 2020 in the hilly site (+3%), whereas no significant differences between the two years were observed for the mountain site. The ash content was influenced to a great extent by the year in both environments, with higher values observed in 2021 than in 2020 in the hilly site (+9%), while an opposite trend was observed in the mountain site (+4% in plots grown during 2020 compared to 2021). Two-way ANOVA showed a significant effect of the year ($p < 0.05$) on

both the A/X ratio and β-glucans in the mountain environment, with higher values observed in 2020 than in 2021 (+4 and +8%, respectively). The G × Y interaction was only significant for β-glucans in the mountain environment, with more marked differences among the rye genotypes being observed in 2020 (data not shown). In the first year, the commercial population cultivar presented a β-glucans content that was significantly lower than all the other rye genotypes (-20%), whereas this difference was not recorded in the second harvest.

Table 2. Effect of the genotype, the harvest year and their interaction on the qualitative kernel parameters and compositional traits[a] of whole-grain flour of the investigated wheat and rye cultivars, cultivated in two different marginal Alpine environments.

Environment	Factors	Source of variation	TW (kg hL^{-1})	Ash (%)	GPC (%)	TAX (%)	A/X	β-glucans (%)	TDF (%)
Hilly (450 m)	**Genotype (G)**	Bread wheat	75.1 ab	1.89 c	10.1 ab	6.1 b	0.78 a	0.9 b	15.2
		Susa rye	76.0 a	1.94 abc	9.1 c	8.9 a	0.74 a	2.2 a	15.7
		Maira rye	71.8 c	2.04 a	10.6 a	8.7 a	0.74 a	2.0 a	15.7
		Tanaro rye	74.4 ab	1.99 abc	9.2 c	8.6 a	0.72 a	2.1 a	15.4
		Commercial rye	73.6 bc	1.99 ab	9.5 bc	8.5 a	0.78 a	2.1 a	15.5
		Hybrid rye	75.0 ab	1.91 bc	9.1 c	8.5 a	0.82 a	2.1 a	16.7
		p (F)	***	***	***	***	ns	***	
	Year (Y)	2020	75.4 a	1.87 b	9.7 a	8.2 a	0.80 a	1.9 a	14.6
		2021	73.3 b	2.05 a	9.5 a	8.3 a	0.73 a	1.9 a	15.8
		p (F)	***	***	ns	ns	ns	ns	
	G × Y	*p* (F)	ns	*	ns	ns	ns	ns	
Mountain (900 m)	**Genotype (G)**	Bread wheat	76.6 a	1.95 bc	11.3 a	6.4 b	0.86 a	0.8 c	15.0
		Susa rye	75.2 ab	1.99 ab	9.3 b	8.8 a	0.75 a	2.1 a	16.9
		Maira rye	72.8 c	2.08 a	11.1 a	8.4 a	0.74 a	2.0 ab	16.1
		Tanaro rye	73.9 bc	2.00 ab	9.4 b	8.7 a	0.81 a	2.1 ab	15.3
		Commercial rye	73.0 c	1.97 ab	9.6 b	8.5 a	0.75 a	1.9 b	14.4
		Hybrid rye	73.5 bc	1.96 b	9.3 b	8.6 a	0.77 a	2.0 ab	16.3
		p (F)	***	*	***	***	ns	***	
	Year (Y)	2020	74.2 a	2.03 a	10.1 a	8.3 a	0.81 a	1.9 a	16.8
		2021	74.1 a	1.96 b	9.9 a	8.1 a	0.75 b	1.8 b	15.6
		p (F)	ns	**	ns	ns	*	*	
	G × Y	*p* (F)	ns	ns	ns	ns	ns	**	

[a]TW, test weight; GPC, grain protein content; TAX, total arabinoxylans; A/X, arabinose to xylose ratio; TDF, total dietary fiber. The results are expressed on a DW basis. Means followed by different letters are significantly different, according to the REGW-F test [(*) p (F) ≤ 0.05, (**) p (F) ≤ 0.01, and (***) p (F) ≤ 0.001].

Phenolic acids and antioxidant capacity of the whole-grain flour

The effect of genotype and year on the content and composition of the SPAs and CWBPAs of the whole-grain wheat and rye flours is shown in Tables 3, 4, respectively. Two-way ANOVA showed a significant effect of the genotype on the total SPA content in the hilly and mountain environments, whereas the CWBPA content was not influenced by the genotype in either site. The SPA content was observed to be higher (+60%) in all the rye varieties than in the wheat control in both environments. Maira registered the greatest concentration of SPAs, followed by Susa, in the hilly site, while the highest SPA content in the mountain site was reached by both the Maira and Susa ryes, although Tanaro and the hybrid rye did not differ significantly from either. Sinapic acid was the most abundant SPA and was detected in all the analyzed genotypes, where it was found to represent 55-61% of the total SPAs, followed by ferulic acid (23-33%), *p*-coumaric and vanillic acid, which only accounted for 3- 4% and 2-6% of the total share, respectively. A characteristic profile, which depended upon the considered genotype, was observed. Overall, the rye varieties presented a significantly higher concentration of sinapic, ferulic and *p*-coumaric acids in both sites than the wheat control, and significant differences were detected among the varieties and between the two growing environments. Conversely, the vanillic and syringic acid contents were significantly higher in the wheat variety.

CWBPAs constituted most of the phenolic acid concentration, and on average represented 85-91% of the total phenolic acids. Ferulic acid was the predominant phenolic acid detected in the bound form, accounting for 76-81% of total bound phenolics. The other abundant CWBPAs were: sinapic acid (12-17%) and *p*-coumaric acid (4-6%). Gallic acid showed the lowest values, ranging from 0.9 to 3.5 mg kg^{-1} in the bound form, but it was only detected in traces in the soluble form. The genotype did not influence the total content of CWBPAs, although the effect was significant for most of the individual compounds. The wheat variety registered the highest ferulic acid value in the hilly site, although Maira and the commercial rye did not differ significantly from the control. As far as sinapic acid is concerned, the rye varieties showed higher values than the control, although, in some cases, the differences were not significant. As previously observed for the SPAs, the concentrations of bound vanillic and syringic acids were higher in the wheat variety than in the ryes, but they only accounted for a small share of the total content of the bound phenolic acids (0.2-0.7%). Additionally, the variation patterns in the phenolic acid compositions differed among the rye genotypes.

Apart from the genotype, the year factor also affected the SPA and CWBPA concentrations. The highest content was observed in 2020 in both sites and for both parameters, but not for the SPAs in the mountain site, whose content did not differ significantly over the two years.

The 2020 harvest was in fact characterized by higher SPA and CWBPA contents in the hilly environment (+42 and +53%, respectively), while the CWBPAs increased by about 22% in the mountain environment in 2020, compared to 2021. A similar trend was observed for the individual phenolic acids, although some of them were not influenced by the year.

The G × Y effect was significant for both SPAs and CWBPAs in the hilly site, with marked differences among the rye genotypes being observed in 2020. Maira had a significantly higher content of both forms of phenolic acids in the 2020 harvest, while the differences were less distinct (SPAs) or were not detected (CWBPAs) among the genotypes in 2021 (data not shown). The interaction was only significant for SPAs in the mountain environment, with more marked differences among the rye genotypes being observed in 2021, with Maira presenting a higher content than all the other genotypes (data not shown).

The AC of the wheat and rye whole-grain flours was measured by means of two different assays, ABTS and FRAP. As shown in Figure 2, the AC values of the rye varieties were significantly higher than in the wheat control in both sites, and this result was confirmed by both assays. Furthermore, no significant differences were found between the five examined rye genotypes. In the hilly site, the AC of the rye varieties was on average 35 and 18% higher than in the wheat control, while the increase was recorded to be about 40 and 28% (ABTS and FRAP assays, respectively) in the mountain site. Even though the AC of the rye landraces did not differ significantly from those of the commercial and the hybrid ryes, they were characterized by higher AC values in the mountain site (on average +7%). The growing year only had a significant effect on the AC_{ABTS} of the plots cultivated in the hilly environment (data not shown), where it resulted in higher levels in 2020 (+10%) than in 2021, in accordance with the higher phenolic acid concentration. Significant relationships were found between the phytochemicals and AC of the analyzed whole-grain flours (Table S4). Significant correlations were observed between the AC, measured with both assays, and the SPAs ($r = 0.507$ and $r = 0.361$, according to the ABTS and FRAP assays, respectively), whereas no correlation was observed between the AC and CWBPAs. The three most abundant phenolic acids, that is, sinapic, ferulic and *p*-coumaric, were highly correlated to AC in the soluble form, with the exception of *p*-coumaric acid and $AC_{FRAP,}$ whose correlations was not significant (data not shown). Less marked, but still significant correlations were registered for sinapic and *p*-coumaric acids in the insoluble form and AC (data not shown).

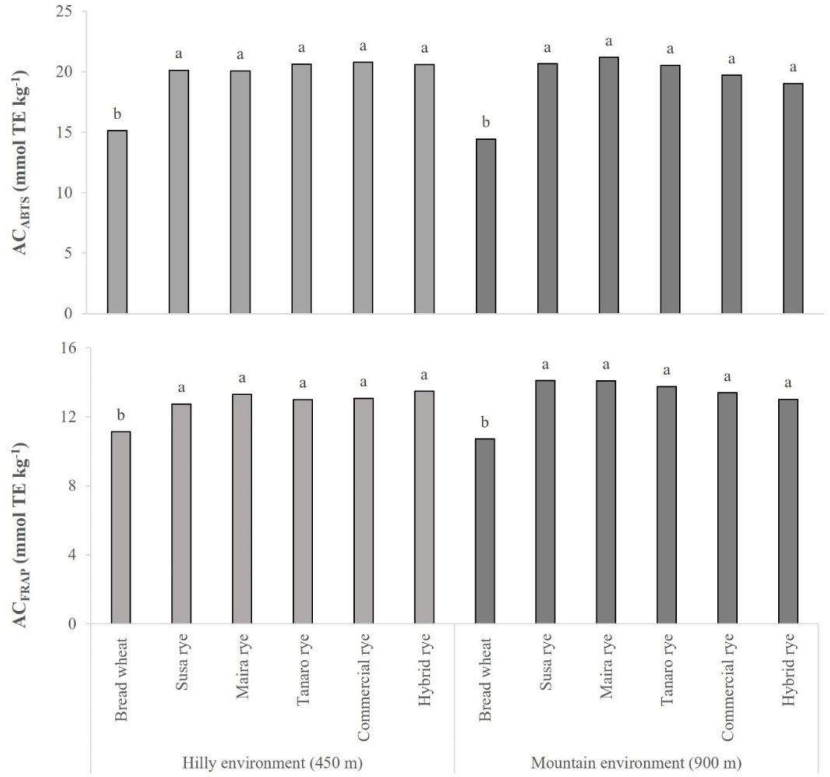


Figure 2. Comparison of the antioxidant capacity (AC) of the wheat and rye whole-grain flours, measured by means of two different methods (ABTS and FRAP assays). The data are averaged over 2 years and for 3 replications. The results are expressed on a DW basis. Different letters on bars indicate significant differences [p (F) ≤ 0.05].

Table 3. Effect of the genotype, the harvest year and their interaction on the total soluble phenolic acid content and on the content of the individual soluble compounds[a] of the whole-grain flour of the investigated wheat and rye cultivars, cultivated in two different marginal Alpine environments.

Environment	Factors	Source of variation	SPAs (mg kg^{-1})	GA (mg kg^{-1})	PCA (mg kg^{-1})	HBA (mg kg^{-1})	VA (mg kg^{-1})	CA (mg kg^{-1})	SRA (mg kg^{-1})	*p*-CA (mg kg^{-1})	FA (mg kg^{-1})	SA (mg kg^{-1})
Hilly (450 m)	**Genotype (G)**	Bread wheat	107 d	< LOQ	0.6 c	2.6 ab	6.3 a	0.3 a	6.2 a	3.4 c	22.9 e	65.0 c
		Susa rye	188 b	< LOQ	3.5 b	2.5 ab	5.6 ab	1.6 a	2.8 b	9.0 a	60.6 b	101.9 a
		Maira rye	202 a	< LOQ	4.8 a	3.0 a	4.9 b	2.1 a	2.2 b	9.5 a	69.6 a	105.6 a
		Tanaro rye	159 c	< LOQ	4.7 a	2.1 bc	5.0 b	0.6 a	2.4 b	8.5 a	46.9 c	89.0 b
		Commercial rye	160 c	< LOQ	3.3 b	2.3 bc	4.6 b	0.7 a	2.5 b	7.0 b	47.7 c	91.9 b
		Hybrid rye	148 c	< LOQ	3.9 ab	1.8 c	3.5 c	0.3 a	2.7 b	5.6 b	39.0 d	91.0 b
		p (F)	***	-	***	***	***	ns	***	***	***	***
	Year (Y)	2020	187 a	< LOQ	4.4 a	2.6 a	6.1 a	0.8 a	3.4 a	8.6 a	57.2 a	103.6 a
		2021	132 b	< LOQ	2.3 b	2.1 b	3.8 b	1.2 a	2.9 b	5.5 b	37.8 b	76.3 b
		p (F)	***	-	***	***	***	ns	**	***	***	***
	G × Y	*p* (F)	***	-	**	ns	ns	ns	***	***	***	**
Mountain (900 m)	**Genotype (G)**	Bread wheat	81 c	0.3 a	1.4 d	2.1 a	4.8 a	0.1 b	4.6 a	2.1 b	20.1 c	45.1 c
		Susa rye	136 a	< LOQ a	3.6 bc	1.7 bc	3.6 bc	1.0 a	1.9 bc	4.8 a	42.4 a	77.2 a
		Maira rye	134 a	0.4 a	3.0 c	2.0 ab	3.2 bc	0.8 a	1.4 c	4.4 a	41.5 ab	77.5 a
		Tanaro rye	131 ab	< LOQ a	4.2 b	1.6 c	3.6 b	0.6 ab	2.0 bc	4.0 a	36.0 ab	78.5 a
		Commercial rye	110 b	< LOQ a	3.0 bc	1.5 c	2.7 c	0.9 a	1.5 c	3.9 a	32.1 b	64.1 b
		Hybrid rye	129 ab	< LOQ a	5.4 a	1.7 c	3.2 bc	1.1 a	2.5 b	3.8 a	32.4 b	79.3 a
		p (F)	***	ns	***	***	***	***	***	***	***	***
	Year (Y)	2020	120 a	0.2 a	4.1 a	1.6 b	3.7 a	0.9 a	2.2 a	4.2 a	33.9 a	69.4 a
		2021	122 a	0.0 a	2.9 b	1.9 a	3.2 b	0.7 a	2.3 a	3.5 b	35.1 a	72.7 a
		p (F)	ns	ns	***	***	*	ns	ns	**	ns	ns
	G × Y	*p* (F)	***	ns	***	***	**	ns	ns	ns	**	***

[a]SPAs, soluble phenolic acids; GA, gallic acid; PCA, protocatechuic acid; HBA, hydroxybenzoic acid; VA, vanillic acid; CA, caffeic acid; SRA, syringic acid; *p*-CA, *p*-coumaric acid; FA, ferulic acid; SA, sinapic acid; LOQ, limit of quantification. The results are expressed on a DW basis. Means followed by different letters are significantly different, according to the REGW-F test [(*) p (F) ≤ 0.05, (**) p (F) ≤ 0.01, and (***) p (F) ≤ 0.001].

Table 4. Effect of the genotype, the harvest year and their interaction on the total cell wall-bound phenolic acid content and on the content of the individual insoluble compounds[a] of the whole-grain flour of the investigated wheat and rye cultivars, cultivated in two different marginal Alpine environments.

Environment	Factors	Source of variation	CWBPAs (mg kg^{-1})	GA (mg kg^{-1})	PCA (mg kg^{-1})	HBA (mg kg^{-1})	VA (mg kg^{-1})	CA (mg kg^{-1})	SRA (mg kg^{-1})	*p*-CA (mg kg^{-1})	FA (mg kg^{-1})	SA (mg kg^{-1})
Hilly (450 m)	**Genotype (G)**	Bread wheat	1004 a	1.3 a	1.0 c	3.6 a	6.2 a	9.8 a	7.7 a	36.9 c	820.8 a	116.7 b
		Susa rye	919 a	3.2 a	4.2 b	2.4 bc	4.8 bc	9.1 a	2.7 bc	50.5 b	696.2 bc	146.0 ab
		Maira rye	1052 a	2.3 a	3.3 bc	3.2 ab	5.2 b	7.8 a	2.9 bc	61.3 a	815.6 ab	150.7 ab
		Tanaro rye	962 a	1.7 a	7.4 a	1.5 d	3.9 c	8.1 a	1.8 c	53.5 ab	719.7 bc	164.5 a
		Commercial rye	959 a	2.8 a	2.0 bc	2.5 bc	4.7 bc	3.6 b	3.5 b	49.9 b	742.5 abc	147.2 ab
		Hybrid rye	917 a	2.4 a	3.9 bc	1.8 cd	4.6 bc	6.8 a	2.2 bc	40.3 c	695.6 c	159.2 ab
		p (F)	ns	ns	***	***	***	***	***	***	***	*
	Year (Y)	2020	1176 a	3.2 a	4.4 a	2.8 a	6.3 a	8.8 a	4.2 a	58.6 a	919.9 a	167.1 a
		2021	769 b	1.4 b	2.9 b	2.2 b	3.5 b	6.3 b	2.8 b	38.6 b	582.7 b	128.6 b
		p (F)	***	**	*	***	***	***	***	***	***	***
	G × Y	*p* (F)	***	ns	*	ns	*	**	**	***	***	**
Mountain (900 m)	**Genotype (G)**	Bread wheat	998 a	2.0 a	2.8 a	3.5 a	5.6 a	11.7 a	7.0 a	42.2 bc	809.1 a	114.4 b
		Susa rye	974 a	1.2 a	4.3 a	2.1 b	4.2 b	10.2 ab	2.8 ab	48.6 abc	740.0 a	160.4 a
		Maira rye	885 a	2.3 a	3.0 a	2.6 b	4.1 b	9.2 ab	2.3 b	55.3 a	677.1 a	129.4 ab
		Tanaro rye	994 a	3.5 a	3.3 a	2.2 b	4.5 b	7.8 b	5.9 ab	50.0 ab	767.7 a	149.3 a
		Commercial rye	931 a	1.5 a	3.7 a	2.3 b	4.1 b	8.4 ab	2.5 b	49.7 ab	713.3 a	145.1 ab
		Hybrid rye	935 a	0.9 a	4.3 a	2.2 b	3.7 b	10.1 ab	2.3 b	39.7 c	718.0 a	153.4 a
		p (F)	ns	ns	ns	***	**	*	**	***	*	**
	Year (Y)	2020	1047 a	2.6 a	5.1 a	2.4 a	5.0 a	10.5 a	3.3 a	56.3 a	811.0 a	150.5 a
		2021	859 b	1.2 b	2.0 b	2.5 a	3.8 b	8.6 b	4.3 a	38.9 b	664.1 b	133.5 b
		p (F)	***	**	***	ns	***	**	ns	***	***	*
	G × Y	*p* (F)	ns	ns	**	ns	ns	ns	ns	ns	ns	ns

[a]CWBPAs, cell wall-bound phenolic acids; GA, gallic acid; PCA, protocatechuic acid; HBA, hydroxybenzoic acid; VA, vanillic acid; CA, caffeic acid; SRA, syringic acid; *p*-CA, *p*-coumaric acid; FA, ferulic acid; SA, sinapic acid. The results are expressed on a DW basis. Means followed by different letters are significantly different, according to the REGW-F test [(*) p (F) ≤ 0.05, (**) p (F) ≤ 0.01, and (***) p (F) ≤ 0.001].

Rheological parameters of the flour blends

Twelve blends of refined flour were formulated for bread making, with a 40% replacement of the whole-grain flour obtained from the wheat and rye genotypes cultivated in the hilly and mountain environments, and characterized for their rheological properties; the refined commercial wheat flour (no replacement) was considered as the control.

The refined flour control in the Mixolab test required a water supply of 55.8% (14% basis) to reach an adequate dough consistency (data not shown). The replacement with whole-grain wheat flour increased the amount of water (WA) required for the hydration process of the flour to 56.9% (average of the two sites), while the inclusion of whole-grain rye flour led to a further increase in the WA (59.0%, average of the two sites). The highest WA values were observed for the Tanaro blend in the hilly site and for the Maira blend in the mountain site (59.5%). The functionality of the whole- grain wheat and rye blends was estimated by determining the SRC profile and by comparing it with the SRC profile of the refined control (data not shown). SRC tests were carried out using two different solvents, namely water (W-SRC) and sucrose solutions (S- SRC), to provide information on some of the chemical and physical properties of the samples, and particularly on the ability of specific constituents to absorb and retain a given solvent. The water holding capacity (W-SRC) was similar for the refined control and the blend with whole-grain wheat flour substitution (76.5 and 75.3%, respectively, data averaged over two sites), while increasing values were observed for the rye mixes (+24% in both sites, compared to the refined flour). Maira registered the highest W-SRC value in both the hilly and the mountain environments (100.1 and 98.3%, respectively), while the lowest value was recorded for the commercial rye (90.8%). The rye blends were characterized by higher S-SRC values than the refined control (about +7% and +13% in the hilly and mountain sites, respectively).

Increasing DDT values were observed, for the adapted hydration protocol, for the whole-grain wheat and rye blends (9 min), compared to the refined flour (3 min), while DS progressively decreased (data not shown), thus confirming the role of increasing levels of dietary fiber on the rheological behavior of the whole-grain blends. However, a similar behavior was recorded for all the rye blends when the standard protocol was adopted. On the other hand, significant differences were found among the rye genotypes using the FAST protocol at constant WA, as a result of the higher mechanical shear stress (Table 5). The DDT parameter was similar for the refined control and the whole-grain wheat flour substitution, whereas it decreased by about 1 min for the mixes containing whole-grain rye flour in both sites. The DDT of the rye blends were homogeneous, with a few exceptions (the commercial and the hybrid rye showed lower DDT values in the hilly and the mountain sites, respectively). On the other hand, the inclusion of whole-grain wheat negatively

affected the DS, which was significantly lower than the refined control (-38% in both sites). A further decrease in DS was registered for the inclusion of whole- grain rye (-50 and -54% in the hilly and mountain environments, respectively), although to a different extent, depending on the rye variety. C1, the maximum torque, was significantly higher for the rye varieties than for the refined control and the whole-grain wheat blend. Significant differences were also detected for the (C1-C2) parameter (thermal weakening) in both sites. Maira showed a significantly high value, in the hilly site in particular, which was similar to the refined control and the whole-grain wheat blend, whereas the other rye blends presented lower values, albeit to different extents. The samples from the mountain site were more homogenous, except for the Susa blend. Gelatinization (C3-C2) was significantly higher for the whole-grain wheat blend than for the rye blends in both sites. Among the rye varieties, Tanaro was the one that was characterized by the highest gelatinization, while Maira and the hybrid rye were characterized by the lowest. The Maira blend was characterized by the highest cooking stability range (C3- C4), which, overall, was higher for all the rye blends than for the wheat substitution and the refined control, and it was also characterized by the lowest cooling setback (C5-C4).

Table 5. Effect of the bread formulation, the harvest year and their interaction on the rheological parameters[a] of a refined wheat flour (used as a control) and twelve blends containing refined wheat flour and wheat or rye whole-grain flour (WGF) at a 40% substitution level. The source genotypes of WGF were cultivated in two different marginal Alpine environments. The considered rheological parameters were obtained by applying the Mixolab® FAST protocol, using 56% of constant WA.

Environment	Factors	Source of variation	DDT (min)	DS (min)	C1 (Nm)	Thermal weakening, C1-C2 (Nm)	Gelatinization, C3-C2 (Nm)	Cooking stability, C3-C4 (Nm)	Cooling setback, C5-C4 (Nm)
Hilly (450 m)	**Bread formulation**	Refined control	3.4 a	4.3 a	1.42 b	0.50 ab	1.84 e	0.15 c	0.74 c
		40% WGF bread wheat	3.4 a	2.7 b	1.35 c	0.51 ab	2.16 a	0.23 c	0.98 b
		40% WGF Susa rye	2.7 b	2.4 c	1.53 a	0.45 de	2.00 c	0.51 b	1.10 a
		40% WGF Maira rye	2.6 bc	2.1 d	1.44 b	0.53 a	1.84 e	1.00 a	0.62 d
		40% WGF Tanaro rye	2.7 bc	2.1 cd	1.54 a	0.47 cd	2.08 b	0.51 b	1.03 ab
		40% WGF commercial rye	2.6 c	2.0 d	1.54 a	0.50 bc	2.01 c	0.54 b	0.96 b
		40% WGF hybrid rye	2.7 b	2.3 cd	1.46 b	0.43 e	1.97 d	0.49 b	0.98 b
		p (F)	***	***	***	***	***	***	***
	Year (Y)	2020	2.9 a	2.5 a	1.4 b	0.45 b	1.92 b	0.55 a	0.83 b
		2021	2.8 a	2.5 a	1.5 a	0.52 a	2.05 a	0.43 b	1.00 a
		p (F)	ns	ns	***	***	***	***	***
	Bread × Y	*p* (F)	***	ns	***	***	***	***	***
Mountain (900 m)	**Bread formulation**	Refined control	3.4 a	4.3 a	1.42 c	0.50 a	1.84 f	0.15 e	0.74 c
		40% WGF bread wheat	3.5 a	2.7 b	1.39 c	0.51 a	2.11 a	0.23 d	1.01 a
		40% WGF Susa rye	2.7 b	2.1 c	1.48 b	0.46 b	1.96 cd	0.60 c	0.94 ab
		40% WGF Maira rye	2.5 bc	2.1 cd	1.50 ab	0.51 a	1.93 de	0.75 a	0.85 b
		40% WGF Tanaro rye	2.6 bc	2.1 cd	1.54 a	0.52 a	2.00 b	0.67 b	0.95 ab
		40% WGF commercial rye	2.5 bc	1.9 de	1.51 ab	0.52 a	1.96 c	0.67 b	0.91 ab
		40% WGF hybrid rye	2.4 c	1.8 e	1.51 ab	0.52 a	1.91 e	0.59 c	0.89 ab
		p (F)	***	***	***	***	***	***	***
	Year (Y)	2020	2.8 a	2.4 a	1.4 b	0.50 a	1.91 b	0.65 a	0.81 b
		2021	2.8 a	2.4 a	1.5 a	0.51 a	2.01 a	0.39 b	0.99 a
		p (F)	ns	ns	***	ns	***	***	***
	Bread × Y	*p* (F)	***	***	***	***	***	***	***

[a]DDT, dough development time; DS, dough stability; C1, peak of 1.1 Nm ± 0.05 at 30 °C. The results are expressed on a DW basis. Means followed by different letters are significantly different, according to the REGW-F test [(*) p (F) ≤ 0.05, (**) p (F) ≤ 0.01, and (***) p (F) ≤ 0.001].

Bread characterization

Fourteen breads (one refined flour used as a control, one bread wheat variety, five rye varieties for each site) were produced with the previously described flours and were analyzed to establish some technological and antioxidant properties.

One-way ANOVA showed a significant effect of the bread formulation on the bread volume, with differences emerging between the two growing locations (Table 6). As far as the breads produced with flour obtained from the hilly site are concerned, a decrease in the volume was already observed in the bread produced with the whole- grain wheat flour replacement (-5%). Breads made with rye flour showed a further reduction (-22% on average), with differences emerging among the varieties. The highest value was observed for the bread made with the Maira blend. As far as the mountain site is concerned, the volume of the whole-grain wheat bread was statistically similar to that of the refined control, while the rye breads presented significantly lower volumes (-25% on average), although these values were slightly more homogenous than those of the hilly site.

The color of the crust and crumbs of each bread was analyzed, and the Δ*E* parameter was assessed considering the refined bread as the control (Table 6). The replacement with whole-grain flour resulted in a reduction in the lightness (*L**) value and an increase in the redness (*a**) value of the crust, while the blue-yellow component (*b**) was similar to that of the refined control, with the exception of Maira and Tanaro in the hilly site, while the values in the mountain site were more heterogeneous. Rye inclusion also affected the color of the crumbs, with differences among the varieties and between the environments. The rye breads from the hilly site appeared redder and yellower than the wheat bread and the refined control, but the lightness component was higher than that of the wheat bread and lower than that of the refined control. A similar trend was observed for the mountain site, but no significant differences were detected among the rye and wheat breads for the lightness component, which presented lower values than the refined control.

The AC of the breads increased significantly when the refined flour was replaced with whole-grain flour (Figure 3). A significant increase was recorded for the bread with whole-grain wheat, compared to the control, in both sites and for both assays, although the AC_{ABTS} value for the hilly site did not differ significantly from that of the control. The AC of the breads made with rye flour was significantly higher than the refined control, but similar or higher than the bread with whole-grain wheat flour. Large variations were observed among the rye varieties, contrary to what was observed for the whole-grain flours. The maximum observed increase was recorded for Maira, when employing both the ABTS (+69 and +65% in the hilly and mountain sites, respectively, compared to the refined control), and the FRAP (+91and +116%) assays.

Table 6. Effect of the bread formulation on the bread-making aptitude[a] of a refined wheat flour (used as a control) and twelve blends containing refined wheat flour and wheat or rye whole-grain flour (WGF) at a 40% substitution level. The source genotypes of WGF were cultivated in two different marginal Alpine environments during 2019-20 growing season.

Environment	Bread formulation	Bread volume (mL)	Color: lightness		Color: redness		Color: yellowness		Color: Δ*E*	
			Crust	Crumb	Crust	Crumb	Crust	Crumb	Crust	Crumb
Hilly (450 m)	Refined control	1573 a	68.0 a	67.1 a	8.2 b	1.1 c	28.3 a	14.3 d	-	-
	40% WGF bread wheat	1495 b	56.1 b	53.1 d	11.6 a	4.0 b	26.8 abc	16.0 c	8.2 b	16.2 a
	40% WGF Susa rye	1197 d	49.9 c	55.8 cd	14.2 a	4.4 ab	27.6 ab	17.4 abc	14.2 a	13.9 ab
	40% WGF Maira rye	1315 c	45.7 c	58.4 bc	13.6 a	5.1 a	24.3 c	18.8 a	18.9 a	12.1 bc
	40% WGF Tanaro rye	1280 c	49.2 c	60.4 b	13.3 a	4.6 ab	25.2 bc	18.6 a	15.3 a	10.2 c
	40% WGF commercial rye	1197 d	46.0 c	54.9 cd	14.2 a	4.3 ab	26.2 abc	16.8 bc	18.2 a	14.7 a
	40% WGF hybrid rye	1146 d	48.3 c	60.5 b	14.0 a	4.2 ab	27.1 ab	17.8 ab	15.7 a	9.7 c
	p (F)	***	***	***	***	***	**	***	***	***
Mountain (900 m)	Refined control	1573 a	68.0 a	67.1 a	8.2 c	1.1 d	28.3 a	14.3 c	-	-
	40% WGF bread wheat	1514 a	54.3 b	55.2 b	13.5 ab	3.4 c	27.9 b	13.8 c	9.7 b	14.0 a
	40% WGF Susa rye	1170 bc	45.9 c	57.0 b	13.7 ab	4.2 bc	25.2 c	16.8 ab	18.4 a	12.6 a
	40% WGF Maira rye	1139 c	47.4 c	57.6 b	13.9 ab	5.2 a	26.0 bc	18.3 a	16.9 a	12.7 a
	40% WGF Tanaro rye	1149 bc	48.4 c	58.0 b	14.9 a	4.4 ab	27.7 ab	18.8 a	15.7 a	12.3 a
	40% WGF commercial rye	1240 b	48.0 c	56.2 b	13.4 ab	4.7 ab	25.8 bc	17.8 ab	16.2 a	13.8 a
	40% WGF hybrid rye	1197 bc	54.2 b	56.4 b	12.1 b	3.9 bc	26.4 abc	16.3 b	10.1 b	13.1 a
	p (F)	***	***	***	***	***	***	***	***	ns

[a]Δ*E*: the total color difference from the refined control. The results are expressed on a DW basis. Means followed by different letters are significantly different, according to the REGW-F test [(*) *p* (F) ≤ 0.05, (**) *p* (F) ≤ 0.01, and (***) *p* (F) ≤ 0.001].

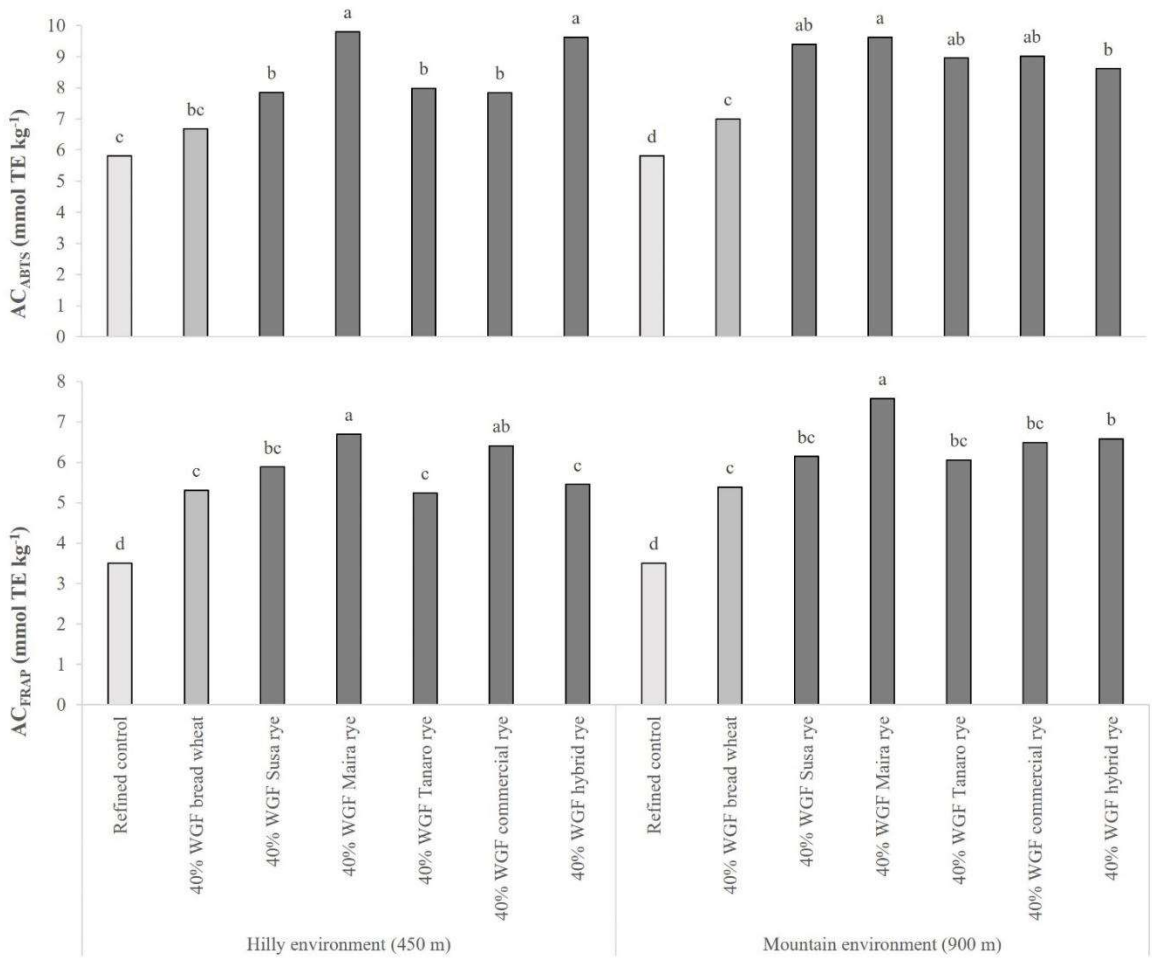


Figure 3. Comparison of the antioxidant capacity (AC) of the bread made of refined wheat flour (used as a control) and breads made of refined wheat flour and wheat or rye whole-grain flour (WGF) at a 40% substitution level, measured by means of two different methods (ABTS and FRAP assays). The source genotypes of WGF were cultivated in two different marginal Alpine environments during 2019-20 growing season. The data are the average of 3 replications. The results are expressed on a DW basis. Different letters on bars indicate significant differences [p (F) ≤ 0.05].

Discussion

The field experiments carried out in the present work offer evidence of the agronomical and qualitative differences between rye and bread wheat cultivated in marginal growing areas, such as in the Alpine valleys of Northwest Italy. These differences have already been pointed out for other growing areas, and in particular in northern, central and eastern Europe countries (Aprodu and Banu, 2017; Laidig et al., 2017; Johansson et al., 2021). No differences in yield potential were found between rye and wheat when comparing hilly and mountain environments. Indeed, although rye was found to be characterized by a lower TKW than wheat, this was compensated for with a greater number of kernels per ear. Rye was also characterized by higher plant heights and straw yields as, in the past, it was also grown to provide material for roofs and baskets, other than for flour (Gaetano et al., 2012). The rye kernels showed a lower TW and GPC than bread wheat, but presented, in both environments, higher concentrations of minerals, soluble fibers, and the soluble fraction of phenolic acids, which resulted in a superior AC of both the flours and the final baked products. Other studies have already pointed out the high AC of rye grains, due to their high content of compounds which have been shown to possess strong bioactivity *in vitro* (Bondia-Pons et al., 2009; Andersson et al., 2014). Phenolic compounds are regarded as potent antioxidants and have been suggested to play a role in the prevention of many degenerative diseases, mainly due to their antioxidative, anti- inflammatory and anti-carcinogenic effects (Li et al., 2008). The greater content of phenolic acids in the rye samples than in the wheat control should be ascribed to a higher contribution of the soluble fraction to the total amount. This hypothesis is in agreement with prior works (Nyström et al., 2008) and should be considered of great interest, as the non-bound fraction of phenolic acids is believed to be readily available for absorption in the small and large intestines of humans (Manach et al., 2004) and it has been indicated to be the most flavor-active, thereby contributing to the perceived flavor of rye products (Heiniö et al., 2008). Aprodu and Banu (2016), by means of the DPPH (2,2-diphenyl-1- picrylhydrazyl-hydrate) free radical scavenging method, observed a higher AC for rye than for wheat and triticale samples (1.4 and 1.3-times higher, respectively), while Michalska et al. (2007), who compared whole-grain rye breads with commercial wheat rolls, reported superior antioxidant properties for the rye baked samples. As far as the bread-making properties are concerned, the inclusion of whole-grain rye in a bread formulation resulted in a higher WA and a lower DS than those of wheat, thus leading to reduced loaf volumes and darker baked products. A previous study investigated the influences of rye inclusion on dough behavior during leavening and on bread-related features (Colombo et al., 2022). The authors observed a positive effect of rye

inclusion on dough development, as the dough rose faster and higher when rye was added to wheat. Furthermore, the bread features (volume, crumb firmness and crumb moisture) were similar to those of the control. These different outcomes can be ascribed to the different levels of rye inclusion (30% vs the 40% of the present study) and to the different protein contents of the rye flour. GPC is an important indicator of the bread-making aptitude of a flour, and it is usually correlated with the DDT of the dough and with the volume of the final bread. Nevertheless, no gluten network forms in rye flour during the dough-making step. Rye storage proteins are not able to form a strong viscoelastic network with good gas-retaining properties, as wheat gluten proteins are instead capable of forming, due to different composition and lower content (Meeus et al., 2020). The lack of the formation of gluten in a rye dough makes the swelling substances highly important for the dough and bread crumb structure. The carbohydrate fraction and the amylase activity were observed to play major roles in influencing the dough quality and baking performances of rye flour. Among the non- starch polysaccharides, pentosans, mainly arabinoxylans, are the main contributors to the high viscosity of rye dough, due to their high water-holding capacity (Deleu et al., 2020), which, in particular, characterizes the water-extractable fraction (WEAXs). The positive effect of WEAXs on bread quality has been related to an increased viscosity and elasticity of the dough and to an improved stabilization of the liquid films around the gas cells and, consequently, of the foam structure, thus resulting in improved gas retention and in an increased loaf volume and crumb softness (Buksa et al., 2013). Furthermore, arabinoxylans are the most abundant fibers in rye, ranging from 6.5 to 12.2% of DW, with 1.5-4% being WEAXs (Vinkx and Delcour, 1996; Nilsson et al., 1997; Hansen et al., 2003), and therefore contribute to a great extent to the beneficial effects related to gut health, bowel movement and increased satiety that are well-known for high- fiber cereal foods (Jonsson et al., 2018). In the present study, the total arabinoxylan contents of the rye varieties, which ranged between 8.4 and 8.9% (DW), were significantly higher than those of the wheat variety, as was the β-glucans component (1.9-2.2% DW). Furthermore, as far as bread making is concerned, the overall higher level of TDF that characterized the whole-grain rye flour resulted in higher SRC values, both S-SRC, which is associated with pentosan contents, and W-SRC, which provides information on all the compounds that are able to interact with water and which are characterized by a good water holding capacity (Aprodu and Banu, 2017). The competition of fibers with proteins for water resulted in rye doughs that were not sufficiently hydrated at 56% of WA and required shorter times to reach the maximum C1 torque, thereby leading to lower DDT and DS values than those of the refined wheat control. Indeed, the DDT and DS parameters were found to be negatively correlated with both TAX and the β-glucans content. The different dough resistances to

mechanical stress, due to a lack of adequate hydration, between the wheat and rye doughs were confirmed by the higher C1 values of the rye doughs, which were hence characterized by insufficient plasticity and were difficult to process. A positive effect of the TAX and β-glucans component was registered for the cooking stability range (C3-C4), as the doughs containing rye developed a more stable starch gel under a heating constraint and were characterized by a higher amylase activity than the doughs of the whole-grain wheat and the refined control.

The differences between the rye genotypes and the wheat control were slightly greater in the hilly environment than in the mountain one, but only concerning a few agronomical and sanitary parameters, e.g. TKW, straw yield, HI and ergot sclerotia. On the other hand, the different behaviors observed between rye and wheat were similar for all the qualitative and phytochemical traits, and for the bread-making aptitude, in both of the considered marginal growing areas.

This paper also highlights the differences that exist in the qualitative traits between rye genotypes, when comparing commercial cultivars (one population and one hybrid) with local landraces, which were selected according to their genetic diversity, and were cultivated side by side in the same marginal environments. Overall, only very small differences were reported for the agronomical and yield traits. The higher potential yield of the hybrid cultivar is the most evident trait, even in marginal environments, and it benefits from the heterosis effect, which resulted in a greater plant functionality and stability and in a higher HI. The superior yield capacity of hybrids than that of population cultivars has already been reported for intensive cropping systems (Hansen et al., 2004; Miedaner and Hübner, 2011). However, the hybrid cultivar was also the most susceptible to sanitary risks, and for the occurrence of ergot sclerotia in particular, and thus potentially alkaloids that are able to cause severe mycotoxicosis in both humans and animals, with an always higher content than the maximum regulatory levels (EU No. 2021/ 1399). Recent experiments have observed a much higher degree of ergot severity in hybrid cultivars than in open-pollinated population cultivars (7.5 vs 2.2%, on average), due to the occurrence, in the former, of poorly restored plants that shed less pollen (Mirdita et al., 2008; Mirdita and Miedaner, 2009). As ergot is able to efficiently infect florets that have not yet pollinated or those that have just pollinated, even a reduced amount of pollen favors ergot infection. This occurs when a hybrid cultivar is not fully restored to fertility and therefore, on average, sheds less pollen than population cultivars or landraces, which are instead characterized by full pollen shedding (Miedaner and Geiger, 2015). The risk related to this disease was found to be higher in the hilly environment than in the mountain one, and in particular for rainy spring seasons during the flowering period. On the other hand, the risk of contamination of other

mycotoxins, such as DON, was observed to be very low in the considered marginal environments, and similar to what has been reported for other Alpine areas (Colombo et al., 2022). A previous study, carried out in an Alpine valley in Northeast Italy, investigated the differences between modern rye cultivars and South Tyrolean landraces collected from local farmers (Peratoner et al., 2016). The authors observed that traits related to grain yield and plant stability were significantly less favorable for landraces than for modern cultivars, as were the investigated parameters related to baking quality. On the other hand, they found that landraces showed a good adaptation to the altitude of the site of origin. In the present study, no significant differences were detected among the landraces and commercial rye cultivars for the proximate and dietary fiber composition. Conversely, Hansen et al. (2003) found higher TDF and TAX contents in population varieties than in hybrid cultivars, while the hybrid rye considered in the present work was characterized by higher TDF contents in the hilly site and retained a high content in the mountain site.

The rye landraces were characterized by higher levels of SPAs and CWBPAS, in both environments, than the conventional rye cultivars, although the AC of the whole-grain flour, as measured by means of two assays, was similar among the rye genotypes, in agreement with that of Leoni et al. (2021) for the whole-grain flour of rye landraces from a Northwest Italian valley. Instead, Kulichová et al. (2019) reported considerable differences in the antioxidant properties of 19 rye genotypes, as measured by four different methods. The authors ascribed the different profiles and amounts of antioxidants in the rye genotypes to the provenience of the genotypes from different parts of Europe, and to their different morphological and agronomical traits. A larger variation has been recorded in the present study for the AC of bread obtained from the rye varieties than for the AC of the source flours, with landraces on average being characterized by higher antioxidant properties than the conventional varieties. Many studies have reported that the antioxidant status of flour can be changed by the bread-making process. Changes in the antioxidant activity of bread can in particular occur because the baking step leads to the destruction of natural-labile antioxidants and to the formation of other thermally-induced compounds that have antioxidant properties, with differences that depend upon the chemical content of the flour and the technological conditions adopted during the process (Delgado-Andrade et al., 2010).

The differences among the rye genotypes were more evident for distinctive agronomic and qualitative parameters, when specific landraces were considered. In particular, the genotype from the Maira Valley showed the most notable morphological differences, as it had the tallest and thinnest culm and suffered from lodging phenomena, which reduced its yield potential and HI. Maira also showed the main differences as far as the qualitative traits

are concerned, compared to the other rye genotypes. The proximate composition analyses revealed the superiority of Maira over the rye landraces and commercial cultivars for bread-making purposes, as it was characterized by the highest protein and ash contents in both environments. On the other hand, the Susa and Tanaro landraces were characterized by low GPC values, like those of the hybrid rye, but higher TAX and β-glucans contents. Maira showed the highest SPA and CWBPA contents in the hilly site, while Susa registered the highest values in the mountain site. The dough formulation including the Maira flour showed the greatest ability to absorb and retain a highly concentrated sugar solution in both locations, and this was followed by Susa, which was in turn characterized by the highest level of soluble fibers. Differences in protein network weakening among the rye varieties were registered at constant hydration. In the hilly site, the blend including Maira was characterized by the highest thermal weakening (C1-C2), which can be explained by the high GPC of the source flour, which in turn resulted in bread characterized by the highest volume for that location. On the other hand, the blend containing the hybrid rye cultivated in the hilly site had a considerably low (C1-C2) value, mainly due to the low GPC and high TDF contents observed in the same site, which led to a final bread characterized by the lowest volume. Low bread volumes were registered for the blend with Susa in both environments, which was characterized by high β-glucans, high TDF and low GPC contents. Furthermore, this blend presented a low C2 value for the adapted hydration (data not shown), thus suggesting that the dough resisted deformation during kneading and heating, and this was confirmed by the DDT value, which was higher than those of all the other analyzed blends. The rye mixes presented a similar or lower starch gelatinization level (C3-C2) than the refined control, due to starch dilution of the DF compounds, except for the Maira blend, which registered much lower values and was also characterized by a low cooling setback (C5-C4). Furthermore, the Maira-based bread retained the highest AC, as measured by both assays, in both sites, thus suggesting that this landrace, in combination with wheat flour, was the most suitable for bread making, as it showed the best performances when the rheological and antioxidant properties were considered.

Our results highlighted that genetically diverse genotypes have distinctive agronomic features which have been observed in different cultivation environments. Individuals from different cultivars resulted to be separated into coherent clades, thus revealing that the rye genotypes were well differentiated among populations, and the clades were well supported, with bootstrap values ≥ 75 (Figure 4A). This genetic distinctiveness can be ascribed to the geographical isolation and historical background of rye, which was cultivated as a staple subsistence crop in many isolated valleys in the Northwest Italian Alps (Adamo et al., 2021). However, the Tanaro and Susa ryes were positioned close to the commercial population

cultivar and to the hybrid cultivar, respectively (Figure 4A), thus possibly indicating a lower genetic isolation for the former, and a less distinct origin for the second, with respect to the Maira rye. In fact, as also pointed out in Adamo et al. (2021), many local rye varieties were shown to share a common genetic background with the hybrid and the commercial ryes. Overall, the genetic distance from conventional rye cultivars was found to influence specific agronomic and qualitative traits, even in the marginal Alpine areas, with differences being observed among the individual landraces. Hierarchical clustering of the agronomic parameters indicated that Maira was part of a separated cluster, that showed a high genetic distance from all the other rye cultivars (Figure 4B). On the other hand, from the qualitative perspective, Susa was found to be part of a cluster that was separated from the other rye cultivars, and this was followed by Maira (Figure 4C).

In summary, to the best of the authors' knowledge, this study is the first to have investigated a wide multiplicity of agronomic, nutritional, and qualitative parameters of locally selected rye landraces grown in marginal environments, along with wheat and commercial rye cultivars. The genotype selected from Tanaro Valley was found to be close to the commercial population cultivar, from the genetic, agronomic, and qualitative points of view. Maira, according to the genetic investigation, was found to be qualitatively divergent from all the other analyzed commercial and landrace rye genotypes, and to have less favorable morphologic and agronomic traits. Nevertheless, it was also characterized by the highest content of health-related substances and a better bread-making aptitude. A good bioactive compound content was also registered for Susa, together with a relevant number of soluble fibers, which, however, proved to be detrimental for its rheological properties. The present work has demonstrated the feasibility of cultivating local landraces in marginal Alpine environments, that is, in both hilly and mountain areas, to produce landrace-based foods with high nutritional value. The revival of historic rye supply chains could prove to be beneficial for the recovery process of marginal and mountain environments as well as for the preservation of the genetic heritage of rye.

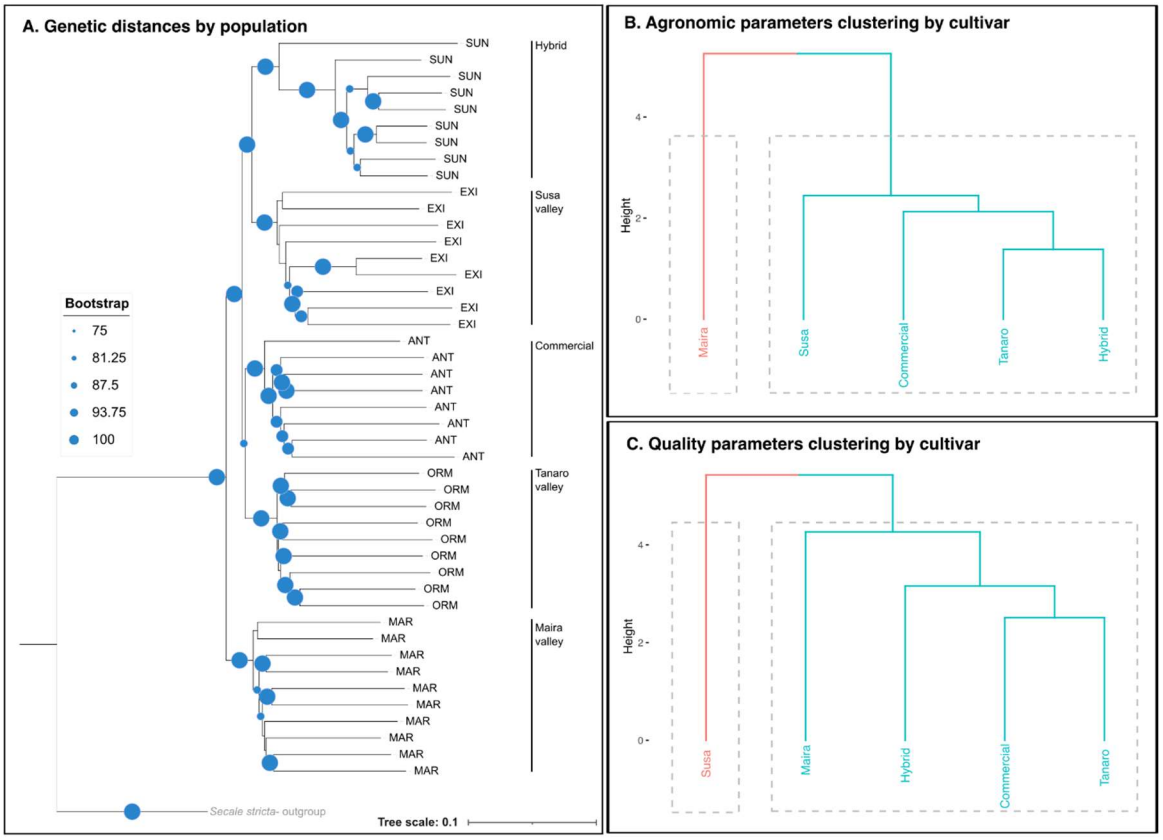


Figure 4. Genetic distances between the rye genotypes[a] visualized by means of a neighbor joining tree (A); hierarchical clustering of the rye genotypes based on agronomic (B) and qualitative parameters (C).

[a]SUN, EXI, ANT, ORM, MAR refer to the accession codes adopted in Adamo et al. (2021).

Supplementary material

Table S1. Rheological and mixing properties[a] of the refined flour used as a control.

Alveograph test	P (mm H_2O)	109
	L (mm)	96
	P/L	1.14
	W (10E-4J)	393
	Ie (%)	65.6
Farinograph test	WA (%)	61.1
	DDT (min)	2.3
	DS (min)	22.5
	Degree of softening, after 10 min (FU)	26
	Degree of softening, at 12 min after maximum (FU)	16

[a]P, maximum over pressure; L, average abscissa at rupture; P/L, curve configuration ratio; W, alveographic strength; Ie, elasticity index; WA, water absorption; DDT, dough development time; DS, dough stability; FU, farinograph units.

Table S2. Monthly cumulative rainfall, rainy days, and growing degree days (GDDs)[a] measured in the experimental areas during the growth cycle in both experimental sites in the 2019-2021 period.

Environment	Month	Rainfall (mm)		Rainy days (n°)		GDDs (Σ °C day^{-1})	
		2019-20	2020-21	2019-20	2020-21	2019-20	2020-21
Hilly (450 m)	October	180	114	12	9	422	337
	November	225	1	20	2	227	241
	December	72	12	10	7	178	125
	January	4	47	2	8	157	129
	February	4	16	1	4	234	188
	March	28	7	12	3	247	252
	April	48	54	6	12	355	305
	May	130	77	14	10	509	430
	June	106	113	14	16	557	643
	July	62	54	8	12	660	647
	August	32	14	11	6	687	663
	Nov-July	**679**	**381**	**87**	**74**	**3124**	**2960**
Mountain (900 m)	October	186	257	12	9	378	304
	November	332	16	22	2	142	228
	December	127	106	8	13	138	66
	January	20	88	3	8	125	62
	February	0	23	0	3	207	128
	March	77	9	10	5	179	210
	April	112	86	7	16	312	225
	May	225	137	14	13	426	374
	June	86	51	14	8	481	561
	July	75	44	16	10	603	591
	August	42	19	13	9	630	611
	Nov-July	**1054**	**560**	**94**	**78**	**2613**	**2445**

[a]Accumulated growing degree days for each experiment using a 0 °C base value. Data obtained from the ARPA Piemonte agrometeorological service.

Table S3. Effect of the genotype, the harvest year and their interaction on the agronomic traits of the investigated wheat and rye cultivars, cultivated in two different marginal Alpine environments.

Environment	Factors	Source of variation	Full flowering (days from the 1st of April)	Flowering duration (days)	Plant height (cm)	Culm diameter (mm)
Hilly (450 m)	**Genotype (G)**	Bread wheat	41 b	7 c	76 d	3.7 d
		Susa rye	46 a	13 b	145 bc	4.1 bc
		Maira rye	45 a	17 a	161 a	3.9 cd
		Tanaro rye	45 a	13 b	144 bc	4.5 ab
		Commercial rye	46 a	12 b	149 b	4.4 ab
		Hybrid rye	44 a	12 b	138 c	4.7 a
		p (F)	***	***	***	***
	Year (Y)	2020	42 b	11 b	132 b	4.1 b
		2021	47 a	13 a	139 a	4.4 a
		p (F)	***	***	***	**
	G × Y	*p* (F)	***	ns	**	ns
Mountain (900 m)	**Genotype (G)**	Bread wheat	60 a	8 c	81 d	3.4 c
		Susa rye	60 a	10 b	149 b	3.8 ab
		Maira rye	61 a	12 a	177 a	3.5 bc
		Tanaro rye	61 a	11 b	148 b	3.9 ab
		Commercial rye	61 a	10 b	154 b	3.8 ab
		Hybrid rye	57 b	11 b	139 c	4.0 a
		p (F)	***	***	***	***
	Year (Y)	2020	60 a	10 b	135 b	3.3 b
		2021	60 a	11 a	148 a	4.2 a
		p (F)	ns	***	***	***
	G × Y	*p* (F)	*	***	ns	ns

Means followed by different letters are significantly different, according to the REGW-F test [(*) p (F) ≤ 0.05, (**) p (F) ≤ 0.01, (***) p (F) ≤ 0.001, and ns, non-significant].

Table S4. Pearson correlation coefficients of the agronomic (A), nutritional and phytochemical (B), and qualitative and rheological (C) traits[a].

(A)

Traits	Ear density	Kernels ear^{-1}	TKW	Straw yield	HI	Full flowering	Flowering duration	Plant height	Culm diameter
GY	-0.011	0.250 *	0.206	0.169	0.591 **	-0.027	-0.536 **	-0.197	-0.433 **
Ear density		-0.410 **	0.267 *	0.118	-0.168	-0.175	0.073	-0.315 **	-0.399 **
Kernels ear^{-1}			-0.381 **	-0.198	-0.328 **	0.459 **	-0.362 **	0.565 **	0.637 **
TKW				0.129	0.078	-0.582 **	0.012	-0.568 **	-0.256 *
Straw yield					-0.670 **	0.064	-0.066	0.053	-0.203
HI						-0.117	-0.310 **	-0.213	-0.510 **
FFD							-0.394 **	0.659 **	0.382 **
FD								0.089	-0.543 **
Plant height									0.260 *

(B)

Traits	TAX	β-glucans	SPAs	CWBPAs	AC_{ABTS}	AC_{FRAP}
TKW	-0.510 **	-0.519 **	-0.389 **	0.163	-0.446 **	-0.442 **
TAX		0.847 **	0.444 **	-0.026	0.704 **	0.676 **
β-glucans			0.513 **	-0.045	0.821 **	0.705 **
SPAs				0.466 **	0.507 **	0.361 **
CWBPAs					0.061	0.016
AC_{ABTS}						0.699 **

(C)

Traits	GPC	Ash	TAX	β-glucans	DDT	DS	C1	C1-C2	C3-C2	C3-C4	C5-C4
TKW	0.149	-0.319 **	-0.510 **	-0.519 **	0.560 **	0.509 **	-0.514 **	-0.196	0.249 *	-0.328 **	-0.022
GPC		0.298 *	-0.342 **	-0.489 **	0.342 **	0.232	-0.290 *	0.283 *	0.005	0.011	-0.253 *
Ash			0.267 *	0.186	-0.274 *	-0.250 *	0.375 **	0.433 **	0.047 **	0.281 *	0.029
TAX				0.847 **	-0.759 **	-0.566 **	0.414 **	-0.111	-0.450 **	0.548 **	-0.112
β-glucans					-0.817 **	-0.601 **	0.392 **	-0.814	-0.574 **	0.617 **	-0.815
DDT						0.758 **	-0.521 **	-0.041	0.126	-0.690 **	-0.106
DS							-0.345 **	-0.060	-0.285 **	-0.646 **	-0.302 **
C1								0.385 **	0.362 **	-0.004	0.548 **
C1-C2									0.075	-0.004	-0.090
C3-C2										-0.377 **	0.827 **
C3-C4											-0.367 **
C5-C4											

[a]GY, grain yield; TKW, thousand kernel weight; HI, harvest index; TAX, total arabinoxylans; SPAs, soluble phenolic acids; CWBPAs, cell wall-bound phenolic acids; AC, antioxidant capacity (ABTS and FRAP assays); GPC, grain protein content; DDT, dough development time; DS, dough stability; C1, peak of 1.1 Nm ± 0.05 at 30 °C; C1-C2, thermal weakening; C3-C2, gelatinization; C3-C4, cooking stability range; C5-C4, cooling setback. Marked factors are statistically significant at (*) $p < 0.05$, and (**) $p < 0.01$.

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Chapter III – "Growing hulled *Triticum* species and old bread wheat genotypes in non-marginal production situations: an agronomic, qualitative, phytochemical, and sanitary comparison with modern varieties"

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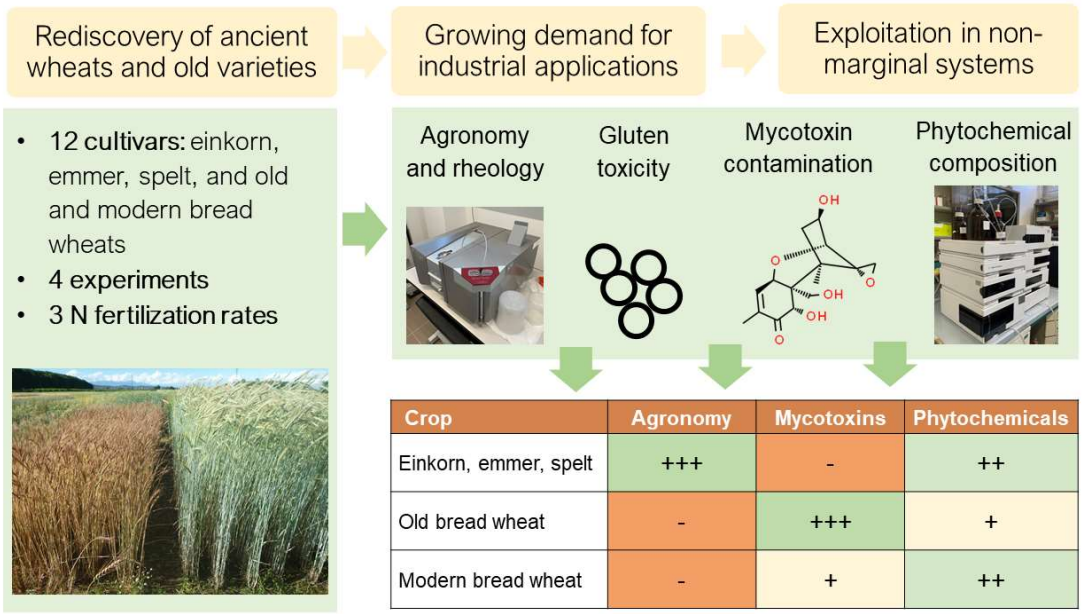
Graphical abstract



Abstract

Ancient *Triticum* species and old varieties of bread wheat (*Triticum aestivum* spp.) have been attracting attention in plant breeding and in the food industry for food diversification and value addition purposes. This study investigated the agronomic behavior and the grain quality of thirteen genotypes belonging to einkorn, emmer, spelt, and bread wheat, including old and modern bread wheat varieties, cultivated under standard agronomic practices. The modern bread wheats displayed better agronomic performances, while the old genotypes had higher protein contents but were characterized by higher dough weakening, even with increasing levels of nitrogen fertilization. The old genotypes did not offer advantages concerning toxic celiac disease epitopes but exhibited an overall reduced susceptibility to mycotoxin accumulation, although a large variability was observed within the hulled species and modern bread wheats. The bioactive compound content of the genotypes varied extensively and was closely related to the growing conditions. The results highlighted considerable differences among genotypes and a broader variability in the modern varieties than the old ones for many of the studied traits. Additionally, substantial agronomic and qualitative differences were observed within the hulled wheats, thus suggesting opportunities for genetic selection and development of improved hulled or bread wheat cultivars.

Keywords: einkorn; emmer; spelt; grain quality; rheology; deoxynivalenol, bioactive compounds.

Abbreviations: AC, antioxidant capacity; ARs, 5-n-alkylresorcinols; AUCGC, area under the canopy greenness curve; BW, bread wheat; CWBPAs, cell wall-bound phenolic acids; DON, deoxynivalenol; E, experiment; FB: wheat for biscuits (frumento biscottiero); FF: improver high protein wheat (frumento di forza); FP: ordinary bread-making wheat (frumento panificabile); G, genotype; GDDs, growing degree days; GPC, grain protein content; GS, growth stage; GY, grain yield; HI, harvest index; N, nitrogen; NDVI, normalized difference vegetation index; SPAs, soluble phenolic acids; TKW, thousand kernel weight; TW, test weight; Y, harvest year.

Introduction

Bread wheat (*T. aestivum* ssp. *aestivum,* BW) has been a primary component of human diets since its evolution and first cultivation, about 9000 years ago. This hexaploid species (genome AABBDD) has benefited from its free-threshing character, which allows the naked grains to be easily released from the glumes upon threshing, and sets it apart from the earliest cultivated species, which were characterized by hulled grains. Einkorn (*T. monococcum* ssp. *monococcum*, genome AA), emmer (*T. turgidum* ssp. *dicoccum*, genome AABB) and spelt (*T. aestivum* ssp. *spelta*, genome AABBDD) are some of the first domesticated crops and represent a bridge between wild and cultivated wheats, hence they are often designated as ancient wheats (Arzani & Ashraf, 2017). Nowadays, bread wheat is the most frequently grown wheat species, although the so-called ancient wheats continue to be cultivated in small amounts, mainly in marginal lands and for specialty foods. Ancient wheat species have never been the subject of modern plant breeding programs and they became obsolete because of adverse agronomical attributes (Boukid et al., 2018). However, recent breeding efforts have been made in response to a market interest, and modern genotypes of hulled wheat, i.e., einkorn, emmer, and spelt, which were released after 2000, are currently available on the market (Longin et al., 2016). Autochthonous landraces and old BW genotypes were also replaced in the late 1960s by modern semi-dwarf cultivars, which, being cultivated in high-input cropping systems, made it possible to obtain the higher grain yields and better technological properties required for the industrial processing of wheat (Boukid et al., 2018). However, over the last few decades, there has been a renewed interest in marginal cereals for a multitude of reasons. Ancient species and old varieties are often perceived as safer, healthier, and more sustainable than modern cultivars (Shewry, 2018), and derived products have thus been proposed as alternatives in response to the consumers' demands for sustainable and high value-added foods. Despite their relatively low yield potential, such traits as their relatively low nutrient requirements and adaptability to marginal conditions have made them a highly attractive crop option, especially for organic farming (Migliorini et al., 2016). Extensive research has been carried out on both ancient species and old wheat genotypes to explore the perceived benefits that promoted their reintroduction, emphasizing their good phytochemical compound contents under limited input conditions and their potential to enhance the nutritional value of bread formulations (Dinelli et al., 2011; Ziegler et al., 2016). However, direct comparisons with modern BW are limited, especially for non-marginal production systems (Shewry & Hey, 2015). Several authors have also highlighted that the selection over time for starch accumulation and increased grain weight purposes has led to lower mineral (Simsek et al.,

2019) and protein contents of modern cultivars (Shewry & Hey, 2015). However, this lower grain protein concentration has been matched by a quality improvement in the protein composition over the last few decades, with greater strength values of the flour, which have better suited the technological needs of the market (Ormoli et al., 2015). The selection of high gluten strength, which has acted more on glutenin genes, thus decreasing the gliadin/glutenin ratio, supports the conclusions of some authors (Ribeiro et al., 2016) who have observed a reduction in the number of toxic gliadin epitopes for celiac disease patients in modern BW varieties compared to old ones. Breeding efforts over the last century were also aimed at increasing disease resistance to prevent yield and quality losses and to limit the health risks posed by contamination of the grains by toxic fungal metabolites, such as the mycotoxins produced by Fusarium head blight (FHB) pathogens. The knowledge on genetic variation pertaining to FHB resistance has been enlarged in the past few decades, and landraces, wild relatives, and ancient lines of wheat have actively been explored (Buerstmayr et al., 2020).

Notwithstanding, the successful reintegration of ancient wheats and old BW genotypes requires knowledge about the available genetic material and comparative studies with modern varieties that include different agronomic practices and field conditions that are favorable for each group. Ancient and old wheat genotypes have usually been compared with reference material under subsistence and low-input conditions, while modern varieties are usually bred for high-input production systems. The ongoing demand of the market and, in particular, of the industrial supply chain, points out the need for an expansion of the cultivation of these genotypes, even in non-marginal cereal-growing areas. This motivated us to investigate the quality of some hulled wheat species and old Italian BW genotypes in a series of field trials under state-of-the-art wheat production and conventional agronomic practices, and to compare them with modern, reference BW varieties for the Italian market, characterized by diverse technological properties and commercial uses. Several key agronomic, qualitative, phytochemical, and sanitary traits were investigated to fully evaluate the behavior of the genotypes in the considered production situations and to address the advantage of their use in the food supply chain. Finally, we collected and analyzed a representative subset of old and modern BW varieties to establish the responses of their agronomic and technological properties to three varying input rates of nitrogen (N) fertilization, which is currently the most widespread agronomic practice in the area, and allows both high yields and high grain quality for bread-making to be achieved simultaneously. The information was used to complete the comparison between ancient and modern species and varieties of *Triticum*, and can be used to more appropriately guide the agronomic choices of farmers and food supply chain operators. It is hypothesized that

modern varieties would be able to overcome the agronomic and rheological deficiencies of old genotypes in non-marginal cropping systems, while providing a similar amount of health-related compounds and ensuring low sanitary risks.

Materials and methods

Experimental design

Thirteen genotypes, including diploid, tetraploid, and hexaploid species (Table S1), were considered for the experiment. The einkorn, emmer, and spelt genotypes were selected as hulled varieties from among improved varieties released after 2000. The old bread wheat genotypes were chosen from among the landraces cultivated in the XIX° century and from old varieties developed in the XX° century from varietal crosses, all released before 1953 (Migliorini et al., 2016). The selected modern cultivars were released after 2000 and were chosen as references, in the Italian context, of three different quality categories: wheat for biscuits (FB, frumento biscottiero), for ordinary bread-making (FP, frumento panificabile) and improver high protein (FF, frumento di forza), on the basis of their potential technological use, according to the Synthetic Index of Quality (Foca et al., 2007). The studied genotypes were cultivated over two growing seasons (2016-2017 and 2017-2018) in two different locations in north-west Italy: a field in Carmagnola (44° 50' N, 7° 40' E, 245 m a.s.l.), in a deep fertile loam-silty soil and a field in Cigliano (45° 18' N, 8° 01' E, 237 m a.s.l.), in a shallow sandy-loam soil (Table S2). The soils were sampled as described by Sardella, Burešová, et al. (2023) at tillering, i.e. growth stage (GS) 23. Precipitations and daily temperatures were measured at meteorological stations near the experimental fields (Table S3). Given the marked differences in the soil characteristics and weather conditions, four different experiments were set up: SL17 (Cigliano, 2016-17, sandy-loam soil), LS17 (Carmagnola, 2016-17, loam-silty soil), SL18 (Cigliano, 2017-18, sandy-loam soil), LS17 (Carmagnola, 2017-18, loam-silty soil). The standard agronomical management practices for the area were adopted in all the plots (Table S2). Briefly, planting was performed at the end of October in 12 cm wide rows at a seeding rate of 300 seeds·m^{-2} for einkorn, emmer, and spelt, and of 400 seeds·m^{-2} for bread wheat. The N fertilizer (80 kg of N·ha^{-1}) was manually provided to all the plots as ammonium nitrate (granular, N 27%), and split between the tillering stage (GS 23) and the beginning of stem elongation (GS 32). The weed control was conducted chemically at GS 23, while no fungicides or insecticides were used. A completely randomized block design with 3 replications (n = 156) was set up, with plot dimension of 7 × 1.5 m. Only in the Cigliano experimental field were three N fertilization treatments applied, as ammonium nitrate (N_0, N_{80} and N_{160}, at fertilization rates of 0, 80, and 160 kg of N·ha^{-1}, respectively), to the old and modern BW genotypes, and were split equally between GS 23 and GS 32.

Agronomic and physical parameters of the grains

A few crop indexes were determined during the growth cycle. The area under the canopy greenness curve (AUCGC), which expresses both the vigor and the chlorophyll content of a plant, was calculated throughout the growing seasons as previously described (Sardella, Burešová, et al., 2023). The ear density (number of ears per unit of grown area), the average plant height, and the percentage of plot with severe lodging were recorded for each plot (Sardella, Capo, et al., 2023). At harvest (early July; Table S2), the straw was collected behind the combine-harvester and weighed immediately. The grain yield (GY) and the straw yield were calculated on a plot basis and adjusted to a 13% moisture content, and the harvest index (HI) values were expressed as a percentage (Sardella, Capo, et al., 2023). After harvesting, the einkorn, emmer, and spelt husks were removed using a laboratory dehusking machine (FC2K Otake, Dellavalle S.r.l., Mezzomerico, Italy). The harvested grains were thoroughly mixed and 2 kg samples were taken from each plot for determination of moisture content, test weight (TW), and thousand-kernel weight (TKW), as described by Sardella, Burešová, et al. (2023).

Qualitative parameters

Representative sub-samples (500 g) were ground to whole-meal (Sardella, Burešová, et al., 2023) to determine the grain protein content (GPC) and the ash content according to the AACC method 39-10.01. For the chemical analyses, samples were ground to fine powder (particle size < 500 µm) and stored at -25 °C.

Rheological parameters

Only the grains (2 kg) of the old and modern bread wheat genotypes from the Cigliano experimental field, treated with different N fertilization rates, were milled using the Bona 4RB mill (Bona, Monza, Italy) to study the mixing and pasting behaviors of the refined flours. The analyses were performed using a fast Mixolab® (Chopin Technologies, Villeneuve-la-Garenne, France) mixing protocol, according to the ICC Standard Method 173, and a constant water absorption (WA) of 55%, as described by Sardella, Capo, et al. (2023).

Gliadin quantification by means of R5 ELISA

The refined flour of the considered genotypes was analyzed by means of RIDASCREEN® Gliadin (Art. No. R7001, R-Biopharm AG, Darmstadt, Germany) to determine the gliadin content ($mg \cdot kg^{-1}$). The samples were extracted with Cocktail Solution (Art. No.

R7006/R7016, patent WO 02/092633, R-Biopharm AG), which was used according to the manufacturer's instructions.

Deoxynivalenol (DON) analysis

The harvested grains were thoroughly mixed and 4 kg samples were ground completely as described in Sardella, Capo, et al. (2023) to determine the DON concentration using RIDASCREEN® DON direct competitive immunoassays (R-Biopharm AG).

Phytochemical compound analyses

Chemicals

The used 2,2-diphenyl-1-picrylhsydrazyl (DPPH), ethanol (CHROMASOLV®, 99.8%), ethyl acetate (CHROMASOLV®, 99.8%), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, 97%), hydrochloric acid (37%), iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$, ≥ 98%), methanol (CHROMASOLV®, 99.9%), sodium hydroxide (≥ 98%), 2,4,6-Tris(2-pyridyl)-*s*-triazine, butylated hydroxytoluene (≥ 99%), phenolic acid standards (caffeic acid ≥ 98%, *p*-coumaric acid ≥ 98%, ferulic acid ≥ 99%, gallic acid ≥ 99%, protocatechuic acid ≥ 99%, *p*-hydroxybenzoic acid ≥ 99%, sinapic acid ≥ 98%, syringic acid ≥ 95% and vanillic acid ≥ 97%), and alkyl resorcinol standards (5-nonadecyl-resorcinol, 5-heneicosylresorcinol, 5-tricosyl-resorcinol, 5-heptadecylresorcinol, all 10 mg powder) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Water (HPLC grade) was obtained from ELGA PURELAB Ultra system (M-medical, Cornaredo, Milan, Italy). LC-MS grade methanol, ethyl acetate, and 2-propanol were purchased from Scharlab Italia S.r.l. (Milan, Italy). MS-grade ammonium formate and formic acid were obtained from Fisher Chemical (Thermo Fisher Scientific, Inc., San Jose, CA, USA).

Extraction and quantification of the phenolic acids and total alkylresorcinol (AR) content

The soluble (free and conjugated, SPAs) and cell wall-bound phenolic acids (CWBPAs) were extracted and quantified by means of high-performance liquid chromatography with diode array detection (Giordano et al., 2019). The ARs were extracted and quantified using high-performance liquid chromatography coupled with tandem mass spectrometry, as reported in Pedrazzani et al. (2021). The results were expressed as $mg \cdot kg^{-1}$ of dry weight (DW).

Antioxidant capacity (AC) determination

The total AC was measured directly, using the QUENCHER procedure, on the whole-meal flour samples by means of two assays and expressed as mmol Trolox equivalents (TE)·kg^{-1} of DW. Both the DPPH scavenging capacity (AC_{DPPH}) and the ferric reducing antioxidant power (AC_{FRAP}) were determined according to Serpen et al. (2012).

Statistical analyses

The data obtained from the plots supplied with 80 kg of N·ha^{-1} allowed a comparison to be made of all the genotypes across species (n = 13). An analysis of variance (ANOVA) was performed using the SPSS for Windows statistical package, version 29.0.1.0 (SPSS Inc., Chicago, IL, USA), to evaluate the effect of the genotype (G), the experiment (E), and their interactions on the agronomic and qualitative parameters, on the R5 gliadin contents, DON, and bioactive compounds, as well as on the antioxidant capacity. The data were tested for normality and homoscedasticity, and means were identified as significantly different (p < 0.05) on the basis of the Ryan-Einot-Gabriel-Welsch F (REGW-F) statistical test. The effect of the N fertilization on the agronomic and rheological parameters was tested by means of ANOVA, considering only the BW genotypes cultivated in the SL soil at Cigliano over the 2 years. The considered treatments were factorial combinations of 8 genotypes (G), 3 N fertilization rates (N), and 2 harvest years (Y).

Results and discussion

Meteorological data

The meteorological data showed remarkable differences in the monthly cumulative rainfall and the growing degree days (GDD) across years, particularly in April and May (Table S3). An excess rainfall level in the 2017-18 period from booting (April) to flowering (May) favored a higher FHB infection, especially in the Carmagnola site (LS18), with a strong impact on the agronomic, qualitative, and sanitary parameters.

Agronomic and qualitative parameters

Two-way ANOVA, carried out on the agronomic and qualitative data, revealed highly significant differences for the genotype and the experiment factors (Table 1). The grain yield (GY) differentiated the genotypes according to the species, with the modern cvs. (cultivars) presenting the highest values (mean value of 5.5 $t \cdot ha^{-1}$), and significantly lower yields for spelt (-23%), emmer (-42%), the old wheats (-51%), and einkorn (-68%). These results corroborate previous findings on the mean grain yields of spelt, emmer, and einkorn that were 40, 55, and 62%, respectively, lower than that of bread wheat under conventional management conditions (Longin et al., 2016; Rachoń et al., 2020). Significant differences were found between experiments, as the plots grown in LS17 performed better (4.7 $t \cdot ha^{-1}$) than those in SL17 and SL18 (-21%), whilst LS18 showed the lowest average yield (-36%, compared to the highest yielding experiment) despite the good fertility of the soil. The seasonal heavy rains, which facilitated N-soil leaching, plant lodging, and fungal infection, were largely responsible for the resulting low yield of SL18. The genotypes responded differently to the improved yield conditions (Figure S1), and the modern BW genotypes in particular, which showed a higher yield potential and an improved responsiveness to better growing conditions. On the other hand, the old BW genotypes showed high yield stability across the experiments, but this did not imply a better performance under low-yielding conditions, as previously reported by other authors (Ferrante et al., 2017), since they were the least productive in all the considered production systems. In addition, the spelt cvs. performed better for a low nutrient availability of the soils (+20% in both SL17 and SL18, compared to LS17). Lodging was a severe problem for the old BW genotypes (mean value of 80%) and hulled wheats, although the response of the latter differed according to the specific cv., mainly related to the plant height. The old BW genotypes were on average 60 cm taller than the modern wheats, while the hulled wheats, such as the spelt cv. Rossella and the emmer cv. Giovanni Paolo, were characterized by lower heights, and were also less prone to lodging, similarly to the modern BWs. As expected, large differences in HI

were observed for the modern BW (around 50%), compared to the hulled and old BW genotypes (HI < 40%, with the emmer cv. Monlis presenting an extremely low value of around 15%), except for the spelt cv. Rossella (around 50%). Lodging was affected significantly by the experiment factor, with higher values observed in the experiment characterized by high fertility of the soil (LS17, in particular for the old BW genotypes), while the G × E effect was not significant.

The einkorn cv. presented the highest ear density, which led to the lowest TKW of all the genotypes (Table 1). Nevertheless, its TW was found to be the highest among the hulled wheats (71.7 vs 63.3 $kg \cdot hL^{-1}$). The TKW discriminated the hulled and old wheats from the modern cvs., as the values were on average higher for spelt (+32%), emmer (+23%) and the old BW genotypes (+20%) than for the modern cvs., while, as expected, spelt and emmer presented significantly lower TW values than the modern cvs. (-13 and -16%), among which the high protein cv. Bologna emerged (77.2 $kg \cdot hL^{-1}$). The old BW genotypes were characterized by good TW values, similar to that of the ordinary bread-making cv. Solehio (74.0 $kg \cdot hL^{-1}$). The experiment factor had a significant effect on the ear density, and all the studied genotypes showed a similar response between experiments, with a higher number of spikes per square meter where the fertility of the soil was greater (LS17 > LS18 > SL17 > SL18, with 89%, 73%, 36% more than SL18). The TKW and TW traits showed differences among the experiments, with SL17 > SL18 > LS17 > LS18, although the genotypes behaved differently. The TW values of the old BW genotypes were always above the commercial threshold of 75 $kg \cdot hL^{-1}$ in the SL17 and SL18 experiments, while the modern genotypes gave better results in 2017 (data not shown), presumably due to their higher FHB infection and grain shriveling in 2018 than the old ones.

The grains of all the genotypes belonging to the hulled wheats and to the old BWs were characterized by a much higher GPC than the grains of the modern cvs. (+17% as average values for both types), except for the emmer cv. Luni, whose average content (13.7%) was similar to that of the high protein BW cv. Bologna (FF), and the spelt cv. Rossella (12.6%), which was similar to that of the bread-making (FP) and biscuit-making (FB) wheats. The highest values were observed for the einkorn cv. Monlis (16.1%), although the emmer cv. Giovanni Paolo, the spelt cv. BC Vigor, and the old BW cv. Andriolo did not differ significantly. The ash content was significantly high in the einkorn (2.17%), the old BW genotypes (mean value of 2.03%) and the spelt cv. BC Vigor (2.02%) grains, while low values were recorded for the modern BWs (mean value of 1.88%). The potential of producing high protein and ash contents under the same crop conditions is in line with previous reports for einkorn, emmer, and spelt (Longin et al., 2016; Golea et al., 2023), and for old BW genotypes (Migliorini et al., 2016; Simsek et al., 2019). The lower protein and

ash contents of modern cvs. have been attributed to dilution effects following a selection for starch accumulation in order to increase the grain size and productivity (Simsek et al., 2019; Shewry & Hey, 2015), although also no effect on the GPC of the old and modern BW cvs. was observed (Simsek et al., 2019). The GPC rose, albeit differently among the cvs., along with the fertility of the soils, with +20% in LS17 and LS18, compared to SL18 and SL17 (Figure 1). The highest ash content was observed in the LS18 experiment, probably due to the lower recorded TW values and consequent higher bran/endosperm ratio, and the rise occurred at different rates in relation to the genotype. GPC is one of the qualitative parameters that are used to commercially classify BW flour into quality categories for industrial processing (Foca et al., 2007), and the ash content is an indicator of the mineral content of the grain, which is essential for both human nutrition and for the leavening properties of the dough. However, bread-making quality hinges not only on the protein quantity, but also on its composition, which can be explored through empirical rheological analyses.

Table 1. Effect of the genotype, the experiment, and their interaction on the agronomic traits and qualitative parameters[a] of the investigated genotypes cultivated in the experimental trials and supplied with 80 kg of N·ha^{-1}.

Factor	Crop		Source of variation	GY (t·ha^{-1})	Lodging (%)	Ear density (n°·m^{-2})	TKW (g)	TW (kg·hL^{-1})	GPC (%)	Ash (%)
Genotype (G)	Einkorn		Monlis	1.5 h	37.0 cd	741 a	28.4 h	71.7 de	16.1 a	2.17 a
	Emmer		Giovanni Paolo	3.4 de	6.7 e	335 e	49.7 b	63.6 f	16.1 ab	1.92 d
			Luni	3.5 d	45.3 bc	474 bcd	46.1 c	61.2 g	13.7 e	1.90 def
	Spelt		BC Vigor	4.3 c	26.0 cde	459 bcd	48.9 b	64.2 f	15.6 abcd	2.02 bc
			Rossella	4.5 c	14.0 de	434 d	53.5 a	64.4 f	12.6 f	1.87 f
	BW	Old genotypes	Andriolo	2.5 fg	86.9 a	530 bcd	43.9 de	74.4 bc	15.7 abc	2.05 b
			Gentilrosso	2.7 fg	84.7 a	536 bc	50.1 b	75.0 b	15.0 cd	2.00 c
			Frassineto	2.4 g	80.3 a	493 bcd	49.8 b	74.5 bc	14.9 d	2.01 c
			Verna	2.9 ef	67.1 ab	535 bc	42.7 e	75.5 b	15.3 bcd	2.05 b
		Modern genotypes	Arabia (FB)	5.7 ab	6.7 e	443 cd	42.8 e	73.2 cd	12.7 f	1.88 def
			Solehio (FP)	5.8 a	14.0 de	492 bcd	45.0 cd	74.0 bc	12.1 f	1.91 de
			Aubusson (FP)	5.4 ab	14.4 de	530 bcd	36.8 f	70.3 e	12.6 f	1.86 f
			Bologna (FF)	5.3 b	7.0 de	538 b	32.9 g	77.2 a	13.5 e	1.88 ef
			p (F)	***	***	***	***	***	***	***
Experiment (E)			SL17	3.8 b	32.0 bc	442 c	48.3 a	73.8 a	12.6 b	1.87 c
			LS17	4.7 a	59.6 a	614 a	44.3 b	71.1 c	15.8 a	1.97 b
			SL18	3.7 b	21.0 c	325 d	44.9 b	72.0 b	12.8 b	1.98 b
			LS18	3.0 c	42.4 b	561 b	37.9 c	66.8 d	16.0 a	2.04 a
			p (F)	***	***	***	***	***	***	***
G × E			*p* (F)	***	ns	ns	***	***	***	***

[a]GY, grain yield; TKW, thousand kernel weight; TW, test weight; GPC, grain protein content; BW, bread wheat; FB, wheat for biscuits (frumento biscottiero); FP, ordinary bread-making wheat (frumento panificabile); FF, improver high protein wheat (frumento di forza); SL17, sandy-loam soil, harvested in 2017; LS17, loam-silty soil, harvested in 2017; SL18, sandy-loam soil, harvested in 2018; LS17, loam-silty soil, harvested in 2018. Means followed by different letters are significantly different, according to the REGW-F test [(*) *p* (F) < 0.05, (**) *p* (F) < 0.01, (***) *p* (F) < 0.001, and ns, not significant]

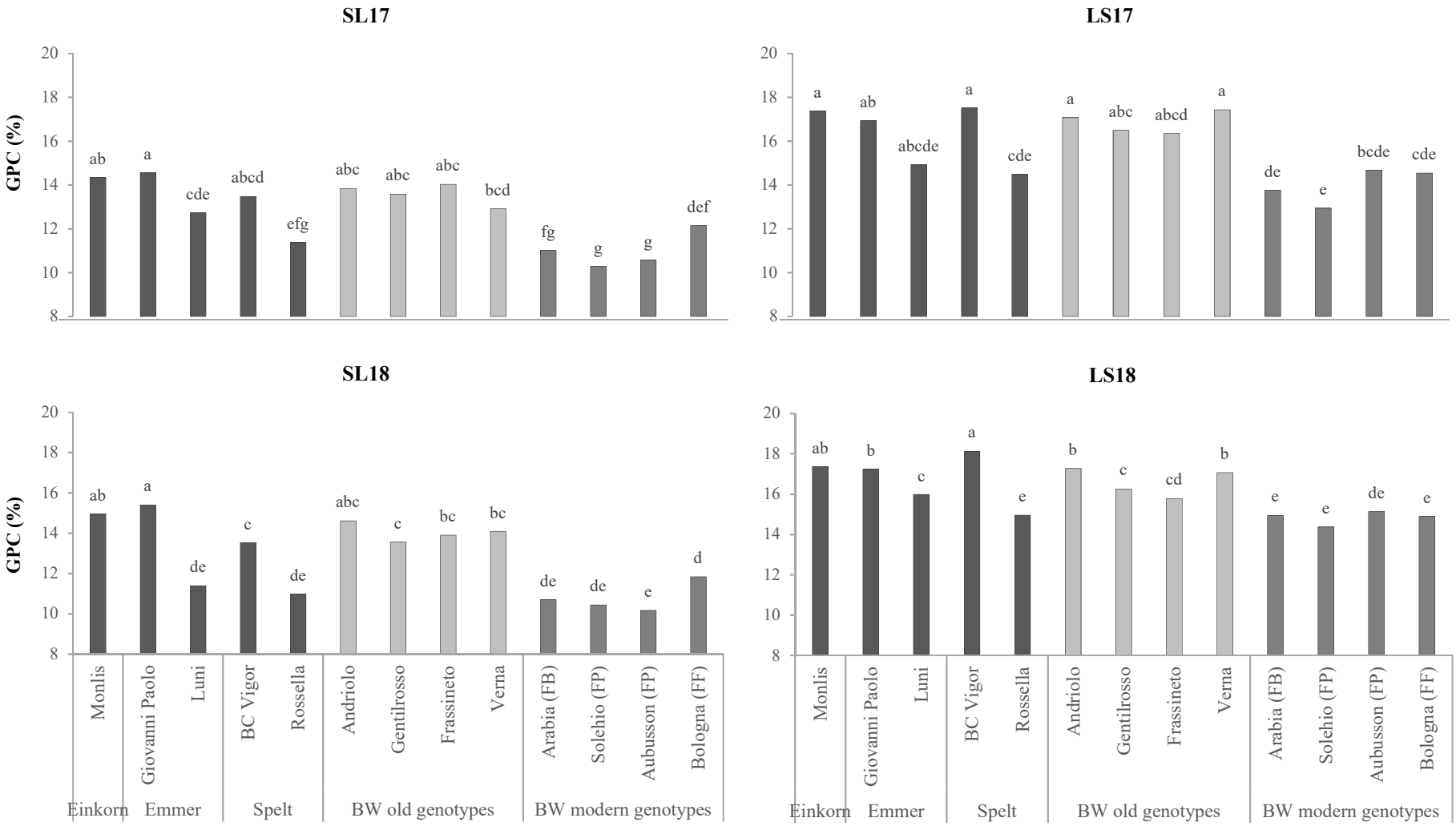


Figure 1. Comparison of the grain protein contents (GPC) of the investigated genotypes across the growing environments.

SL17, sandy-loam soil, harvested in 2017; LS17, loam-silty soil, harvested in 2017; SL18, sandy-loam soil, harvested in 2018; LS17, loam-silty soil, harvested in 2018; BW, bread wheat; FB, wheat for biscuits (frumento biscottiero); FP, ordinary bread-making wheat (frumento panificabile); FF, improver high protein wheat (frumento di forza). Different letters above the columns indicate significant differences between the groups for $p(F) < 0.05$, according to the REGW-F test.

Both the old and modern BW cvs. were grown under N-input regimes of 0, 80, and 160 kg of $N \cdot ha^{-1}$ in the same location (Cigliano) over the 2 growing seasons (*n* = 144). This approach was aimed at further elucidating the agronomic and bread-making qualities of these genotypes within the context of a high-input production system. The N effect was evident for all the investigated traits (Table 2), with significantly higher values of GY (+47%), AUCGC (+27%), lodging (mainly pertaining to the old varieties, with no significant difference in the N_{80} and N_{160} range), and GPC (+31%) from N_0 to N_{160}. Overall, better GY results were achieved in 2018 (+9%), which, however, was characterized by lower TKW, TW, and GPC values. The TKW rose for N_{80} and diminished for N_{160}, but a differing N effect on this trait was found in relation to the genotype. The significant decrease observed for the TW along with the scale-up of the N rate was mainly observed for the old genotypes (3-5% decline at N_{160} with respect to N_0), as it remained the same or even rose slightly (0-2%) in the modern ones (data not shown). The effect of the significant G × N interaction on GY and GPC is shown in Figure 2. The old varieties had the same GY at N_0, N_{80}, and N_{160}, and, in some cases, the increase in N input rates determined yield losses, although not in a significant way. The old varieties bred in the XX° century, that is, Frassineto and Verna, showed an optimized productivity for N_{80}, while the modern ones showed a maximum for N_{160}. Indeed, except for the biscuit-making cv. (FB), which showed a better yield, no significant differences were detected between the old and modern genotypes for N_0. The modern BW cvs., unlike the old ones, instead showed significant increases for N_{80} (+64%) and N_{160} (+84%). The GPC rose, albeit differently, among the varieties, along with the N supply. All the genotypes exhibited a maximum level for N_{160}, but the modern cvs. required the highest N application to reach similar values to those of the old genotypes with no N input, except for the modern high protein (FF) cv., which reached a similar level for N_{80} and further increased it for N_{160}. The responses of the old genotypes to increasing N inputs were higher: the GPC rose on average by 16 and 8% for the old and modern cvs., respectively, for N_{80}, and by 32 and 28% for N_{160}.

Table 2. Effect of the genotype, the N fertilization rate, the harvest year, and their interaction on the agronomic traits and qualitative parameters[a] of the investigated bread wheat genotypes cultivated in the Cigliano experimental area.

Factor	Bread wheat	Source of variation	GY ($t \cdot ha^{-1}$)	AUCGC (NDVI-Day)	Lodging (%)	TKW (g)	TW ($kg \cdot hL^{-1}$)	GPC (%)
Genotype (G)	Old genotypes	Andriolo	2.1 e	64.8 a	68.3 a	46.3 c	76.0 c	14.3 a
		Gentilrosso	2.8 cd	65.3 a	66.8 a	53.1 a	76.6 bc	13.7 b
		Frassineto	2.4 de	65.1 a	63.3 a	52.6 a	75.8 c	14.0 ab
		Verna	2.9 c	64.0 a	52.3 a	43.6 e	77.4 b	13.8 b
	Modern genotypes	Arabia (FB)	5.2 ab	64.6 a	0.2 b	45.0 d	74.3 d	11.2 d
		Solehio (FP)	5.6 a	64.6 a	0.0 b	49.5 b	76.1 c	10.8 e
		Aubusson (FP)	5.4 a	60.4 b	0.0 b	41.1 f	73.6 d	10.9 e
		Bologna (FF)	4.8 b	57.7 c	0.2 b	34.8 g	78.9 a	12.4 c
		p (F)	***	***	***	***	***	***
N fertilization (N) (kg of $N \cdot ha^{-1}$)		0	3.2 c	53.7 c	3.9 b	45.5 b	76.7 a	10.9 c
		80	4.0 b	64.4 b	38.3 a	46.4 a	76.1 b	12.5 b
		160	4.4 a	68.4 a	42.2 a	45.3 b	75.7 c	14.3 a
		p (F)	***	***	***	***	***	***
Harvest year (Y)		2017	3.9 a	62.0 b	40.1 a	47.3 a	76.7 a	12.8 a
		2018	3.9 b	64.3 a	24.9 a	44.6 b	75.6 b	12.5 b
		p (F)	***	***	ns	***	***	**
G × N		*p* (F)	***	**	***	***	***	***
G × Y		*p* (F)	ns	ns	ns	***	***	***
N × Y		*p* (F)	**	***	ns	**	**	***
G × N × Y		*p* (F)	ns	ns	ns	ns	***	ns

[a]GY, grain yield; AUCGC, area under the canopy greenness curve; TKW, thousand kernel weight; TW, test weight; GPC, grain protein content; FB, wheat for biscuits (frumento biscottiero); FP, ordinary bread-making wheat (frumento panificabile); FF, improver high protein wheat (frumento di forza). Means followed by different letters are significantly different, according to the REGW-F test [(*) *p* (F) < 0.05, (**) *p* (F) < 0.01, (***) *p* (F) < 0.001, and ns, non-significant].

Rheological properties

The old genotypes were subsequently characterized for their rheological behavior and compared with the modern ones. The Mixolab® parameters, obtained to estimate the bread making quality of the refined flour, were affected to a great extent by the genotype and the N fertilization (Table 3). The old varieties did not differ significantly from the modern ones, in terms of dough development time (DDT), and only the FF wheat (cv. Bologna) emerged. The dough stability (DS) discriminated the old from the modern BWs, with a net improvement of 189% on average observed in the latter ones, which reported differences according to the bread-making quality (a higher value in FF than in the FP and FB cultivars). The high protein content of the old genotype flours could have led to doughs that were not sufficiently hydrated at 55% of WA, thereby resulting in higher mechanical stress, as indicated by the higher values of C1 (1.05 vs 0.81 Nm for the old and modern cvs.), i.e. the maximum torque during mixing, which is an indication of insufficient dough plasticity due to a lack of hydration and, consequently, poor dough workability. The old genotypes were also characterized by lower values of C2 (data not shown), i.e. the minimum torque following kneading and temperature increase, and therefore by a significantly greater decrease in dough consistency (Bánfalvi et al., 2020), due to destabilization and unfolding of the proteins, which become hydrophobic, as reflected by the higher dough weakening (C1-C2) parameter (0.63 vs 0.35 Nm). Protein denaturation involves the release of large amounts of water, which can hinder dough processability, and can negatively affect gas retention and the final bread volume. The increase in dough consistency caused by the hydration of the starch granules with the water released by the thermally degraded proteins was indicated by the starch gelatinization (C3-C2) parameter, which was similar among the old genotypes and slightly higher than the modern ones (2.47 vs 2.42 Nm), since the FB and FF modern cvs. performed poorly. The hot gel was found to be more stable for the modern varieties than for the old ones, as indicated by the cooking stability (C3-C4) parameter (0.57 vs 0.48 Nm). C3-C4 gives indications about the decrease in dough consistency under a constant heating, before cooling and starch retrogradation, which depends on the rate of enzymatic hydrolysis (Bánfalvi et al., 2020) and affects the bread volume and its porosity. No significant differences were detected for the cooling setback (C5-C4) parameter for any of the analyzed factors. All the investigated traits progressively increased, as a function of the N-rate, from N_0 to N_{160}, except for the gelatinization and the cooking stability, which declined as the rate increased, due to a higher protein content. All the parameters showed higher values in 2017, except for dough weakening, for which no significant differences were observed. The significant G × N interaction for the DS trait exhibited an interesting response (Figure 2), as the old genotypes did not show different values for a rising N supply,

while most of the modern genotypes performed better than the old ones for N_0 and optimized this parameter for N_{160}, except for the FB cv., which showed a peak for N_{80}. Data reported in the literature point out that the decreasing trend in the protein concentration of grains over time is nevertheless accompanied by an enhanced quality of the composition (Ribeiro et al., 2016) and rheological traits (Ormoli et al., 2015; Mefleh et al., 2022), such as in the W and P/L indices of the alveograph as well as of the SDS sedimentation volume, although some other authors have not observed any trend as a function of the release year when evaluating the physicochemical properties of old and modern cultivar-based breads (Boukid et al., 2020). Our work, despite suffering from a lack of technological evaluation of the derived products, has the strength of comparing an equal number of old and modern cvs. under the same agronomic conditions. The achievement of the objective of improving both the yield and grain quality was confirmed by estimating the bread-making quality of the flour using a Mixolab® protocol at constant hydration, and was also evident for no N supply. A greater variability was found among the modern cvs., which could thus be selected for specific purposes. On the other hand, the dough network formed by the flours obtained from the old genotypes always resulted to be weaker and more difficult to process, even after the application of N fertilizer. Their suboptimal pasting and workability behavior required improvement, which could be achieved by adjusting the formulations and processing parameters (blends with stronger flours or the addition of appropriate enzymes) and/or by optimizing the technological processes (for instance, indirect fermentation).

Table 3. Effect of the genotype, the N fertilization rate, the harvest year, and their interaction on the rheological parameters[a] of the refined flour of the investigated bread wheat genotypes cultivated in the Cigliano experimental area.

Factor	Bread wheat	Source of variation	DDT (min)	DS (min)	C1 (Nm)	C1-C2 (Nm)	C3-C2 (Nm)	C3-C4 (Nm)	C5-C4 (Nm)
Genotype (G)	Old genotypes	Andriolo	1.1 b	0.9 d	1.1 ab	0.6 a	2.6 ab	0.4 de	0.9 a
		Gentilrosso	1.1 b	0.9 d	1.0 abc	0.6 a	2.4 abc	0.5 bcd	0.6 a
		Frassineto	1.1 b	0.9 d	1.0 ab	0.6 a	2.4 abc	0.6 b	0.6 a
		Verna	1.2 b	1.2 d	1.1 a	0.6 a	2.4 abc	0.4 e	0.8 a
	Modern genotypes	Arabia (FB)	1.2 b	1.9 c	0.9 c	0.5 b	2.3 c	0.5 bcde	0.8 a
		Solehio (FP)	1.2 b	2.9 b	0.7 d	0.3 b	2.6 a	0.8 a	0.8 a
		Aubusson (FP)	1.2 b	2.1 c	0.7 d	0.4 b	2.5 abc	0.6 bc	0.7 a
		Bologna (FF)	2.0 a	4.6 a	0.9 bc	0.3 b	2.3 bc	0.4 cde	0.9 a
		p (F)	***	***	***	***	*	***	ns
N fertilization (N) (kg of N·ha^{-1})		0	1.0 c	1.7 b	0.8 c	0.4 b	2.5 a	0.6 a	0.7 a
		80	1.2 b	2.1 a	0.9 b	0.5 a	2.5 a	0.5 ab	0.7 a
		160	1.5 a	2.1 a	1.1 a	0.5 a	2.3 b	0.5 b	0.8 a
		p (F)	***	*	***	*	*	***	ns
Harvest year (Y)		2017	1.5 a	2.1 a	1.0 a	0.5 a	2.5 a	0.8 a	0.8 a
		2018	1.1 b	1.9 b	0.9 b	0.5 a	2.4 b	0.4 b	0.7 a
		p (F)	**	*	***	ns	**	***	ns
G × N		*p* (F)	**	***	ns	ns	ns	ns	ns
G × Y		*p* (F)	***	ns	ns	ns	ns	***	ns
N × Y		*p* (F)	ns	ns	ns	ns	ns	*	ns
G × N × Y		*p* (F)	ns	*	ns	ns	ns	ns	ns

[a]DDT, dough development time; DS, dough stability; C1, maximum torque (peak of 1.1 Nm ± 0.05 at 45 °C); C1-C2, dough weakening; C3-C2, gelatinization; C3-C4, cooking stability; C5-C4, cooling setback; FB, wheat for biscuits (frumento biscottiero); FP, ordinary bread-making wheat (frumento panificabile); FF, improver high protein wheat (frumento di forza). Means followed by different letters are significantly different, according to the REGW-F test [(*) *p* (F) < 0.05, (**) *p* (F) < 0.01, (***) *p* (F) < 0.001, and ns, non-significant].

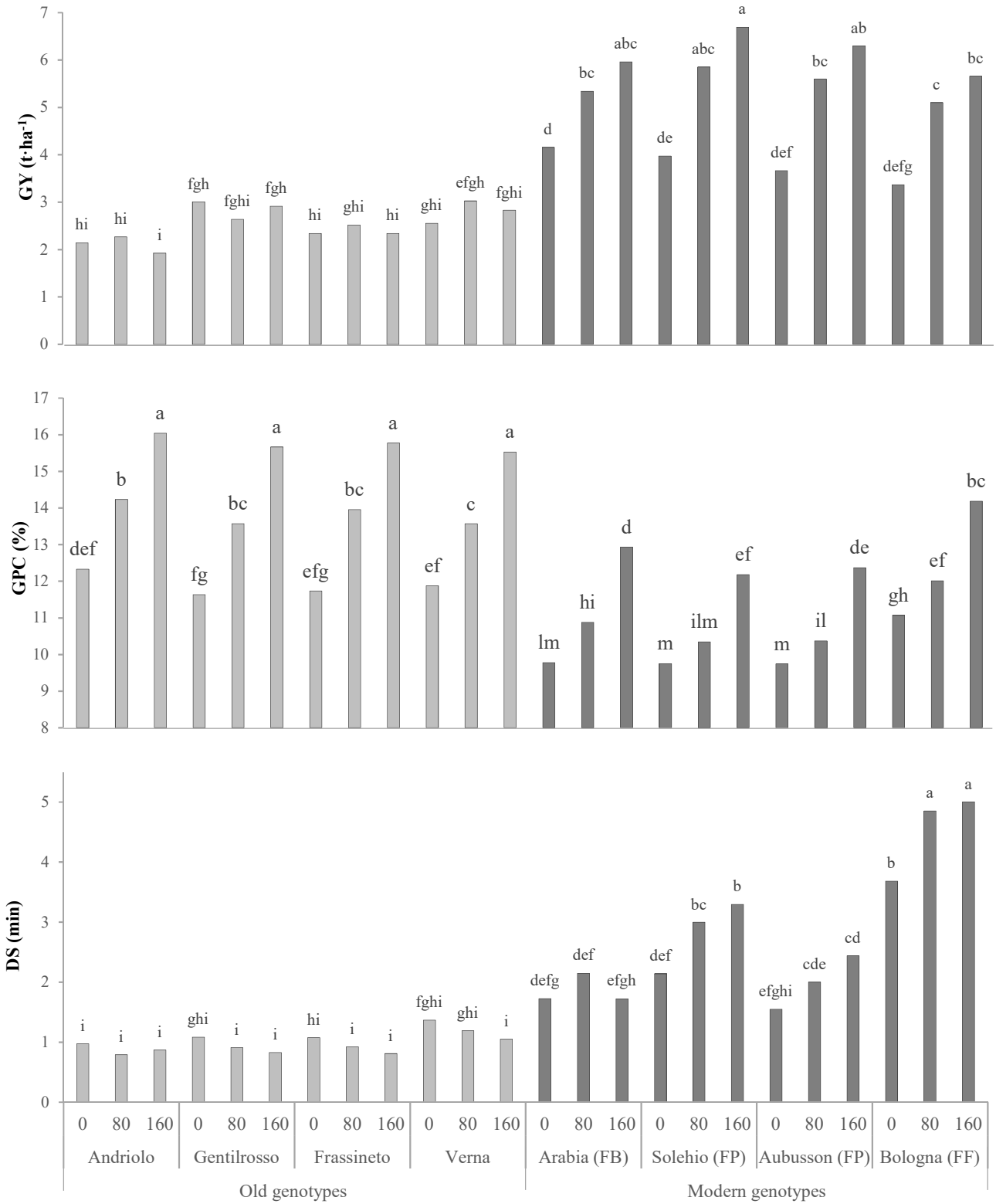


Figure 2. Comparison of the grain yields (GY), grain protein contents (GPC) and dough stability (DS) of the investigated bread wheat genotypes cultivated in the Cigliano experimental area and supplied at three different soil N rates (0, 80, and 160 kg of N·ha^{-1}).

FB, wheat for biscuits (frumento biscottiero); FP, ordinary bread-making wheat (frumento panificabile); FF, improver high protein wheat (frumento di forza). Data are the average of 2 years. Different letters above the columns indicate significant differences between the groups for p (F) < 0.05, according to the REGW-F test.

Gluten immune toxicity

The quality of the investigated genotypes was also evaluated in terms of gluten immune toxicity, by targeting potential toxic celiac-related epitopes with the R5 monoclonal antibody (Table 4). The R5 reactivity varied significantly among the genotypes, with no clear discrimination between the emmer, spelt or modern wheats. Overall, the highest concentrations were observed in the old BW genotypes, with cv. Andriolo presenting the maximum value (266 $mg \cdot kg^{-1}$). The modern cvs. showed more heterogenous values, and they did not differ significantly from the old genotypes, with the sole exception of the FP cv. Solehio, which had the lowest absolute concentrations of R5 gliadins (133 $mg \cdot kg^{-1}$). Despite their high protein content, the einkorn cv. Monlis and the emmer cv. Giovanni Paolo had a low R5 reactivity, while the emmer cv. Luni showed a similar concentration to those of some BW cvs., thereby supporting the idea that immunotoxicity might be genotype-dependent, even in wheat species that lack the D genome, which has been found to be related to celiac epitope expression (Sharma et al., 2020). The experiment led to 38% lower values in SL18 than in LS17, and intermediate values for SL17 and LS18, with an experiment-dependent response of the genotype. The data of the present study, obtained by means of R5 gliadin quantification, do not support the hypothesis of a higher immunotoxicity of the modern genotypes that had been postulated to be a consequence of wheat breeding oriented toward increasing gluten to achieve the desired rheological properties. Indeed, the genetic improvement of wheat through breeding has mainly been focused on glutenins, which are mainly responsible for dough strength and are less immunogenic than gliadins (Sharma et al., 2020). Recent studies on the *in vitro* digestibility of ancient and modern grains have found that, even after digestion, ancient and old genotypes exhibited a higher immunotoxicity and produced a larger number of peptides containing immunogenic and toxic sequences (Ficco et al., 2019; Prandi et al., 2017), except for *T. monococcum.*

Mycotoxin contamination

The sanitary quality was evaluated in terms of DON content, which was influenced significantly by both the genotype and the experiment factors (Table 4), with concentrations that were 37 and 28 times higher in LS18 and SL18 than in SL17. Although the modern BW cvs. were more contaminated overall, the DON content of the FP and FF cvs. did not differ significantly from that of the old genotypes, while only the FB cv. showed higher susceptibility (Figure S2). The hulled wheats resulted to be the most susceptible to DON accumulation in all the experiments, and especially in the growing environments characterized by favorable weather conditions for FHB infection. The emmer cv. Giovanni Paolo showed the greatest DON content in all the experiments and always exceeded the

maximum level of DON set for unprocessed cereals (1000 μg·kg^{-1}; European Commission, 2023). The limit was exceeded in all the genotypes in the LS18 experiment, and the high protein (FF) cv. Bologna was the only variety that presented a content under the maximum level in SL18. Overall, these results indicated a lower DON contamination susceptibility of the old BW genotypes, due to a greater physical difficulty of the *Fusarium* conidia reaching and infecting the ear of taller plants (Chrpová et al., 2013), and to possible biochemical mechanisms of resistance (Dong et al., 2023). Furthermore, these data, obtained under natural infection conditions, highlighted a higher variability in the occurrence of DON in the modern hulled and bread wheats, thereby allowing genotypes to be identified, which are less prone to the disease and have the same level of mycotoxin contamination as the old ones. These findings are in conflict with previous publications that observed a significantly higher DON accumulation in modern BW cvs. than in other *Triticum* species (Wiwart et al., 2016) and wheat landraces (Chrpová et al., 2013; Wiwart et al., 2016) analyzed under artificial inoculation.

Phytochemical compounds and antioxidant capacity

Table 4 shows the health-related compounds of the whole-meal flour of the analyzed genotypes across the experiments. All the parameters were affected significantly by the genotype and experiment factors, and by their interactions. Most of the differences among the genotypes emerged from the SPA fraction, although its contribution to the total content was the lowest, ranging from 10% in the spelt and modern BWs to 15% in the einkorn, emmer, and the old BW genotypes. The highest share (18%) and content (130 mg·kg^{-1}) of SPAs were observed in the old cv. Verna, and this was followed by the einkorn cv. Monlis and the emmer cv. Luni (average value of 111 mg·kg^{-1}). The modern cvs. presented the lowest SPA content (average value of 71 mg·kg^{-1}) and were not significantly different from the spelt, except for the cv. Solehio, whose content was lower but statistically equal to that of the old cvs. Andriolo and Gentilrosso (average value of 96 mg·kg^{-1}). The CWBPA concentrations of the investigated genotypes showed less marked differences. The modern cvs. showed the highest values, but neither the old genotypes nor the spelt and einkorn wheats were significantly different from them. As expected, the sinapic acid was the most abundant phenolic acid in the soluble form (average value of 52 mg·kg^{-1}, ranging from 51 to 71% of the total SPA content; Figure S3), while ferulic acid made up most of the bound fraction (543 mg·kg^{-1}, ranging from 79 to 88% of the total CWBPA content; data not shown). The second predominant compound in the soluble form was ferulic acid (20 mg·kg^{-1}, 18-26%), and this was followed by vanillic (6.4 mg·kg^{-1}, 5-9%), syringic (4.6 mg·kg^{-1}, 2-9%), hydroxybenzoic (2.7 mg·kg^{-1}, 2-5%), and *p*-coumaric (2.2 mg·kg^{-1}, 2-4%) acid (Figure S3),

while the CWBPA composition was less heterogenous (data not shown), with sinapic acid accounting for 4-9% of the total CWBPA content (average value of 43 mg·kg^{-1}), followed by *p*-coumaric acid (29 mg·kg^{-1}, 3-12%). The bound caffeic, vanillic, syringic and hydroxybenzoic acids accounted for less than 1% each. Qualitative differences in the SPA and CWBPA composition emerged across the genotypes. Einkorn showed the highest concentration of *p*-coumaric acid (both soluble and bound) and sinapic acid (only bound), similarly to what Barański et al. (2020) observed when comparing the phenolic compositions of einkorn, emmer, and spelt accessions. The SPA content and composition of the emmer cvs. were different from each other, with cv. Luni showing the highest total amount in all the environments and higher concentrations of the ferulic, sinapic, and vanillic acids. The old BW genotypes had above average soluble ferulic, synapic, syringic, and vanillic acid contents, while the soluble and bound phenolic acid compositions of the modern genotypes were more heterogenous. The SPAs and CWBPAs showed a similar response to the varying growing conditions, with larger concentrations recorded in the LS18 (+150 and +58%) and SL18 (+126 and +44%) experiments than in the LS17 and SL17 ones.

The average AR content (Table 4) varied significantly among the analyzed *Triticum* cvs., with higher levels being observed in the modern BWs than in the old genotypes. The modern cvs. Bologna and Aubusson presented the highest concentrations (average of 1258 mg·kg^{-1}), while the spelt cv. Rossella showed the lowest (673 mg·kg^{-1}). The old BW genotypes displayed similar values to that of the aforementioned spelt cv. (average of 756 mg·kg^{-1}). The variation in this parameter can be attributed to the localization of the ARs in the testa and pericarp of wheat kernels (Pedrazzani et al., 2021), which makes the total content susceptible to differences in the kernel size among genotypes and negatively correlated with the TKW. The experiments conducted in 2018 resulted in a significantly higher AR accumulation than LS17 (-11%) and SL17 (-18%). The observed inter-experiment fluctuations were primarily influenced by the modern cvs., which appeared to generally be less stable across experiments, due to their improved responsiveness to the varying production conditions, with consequent changes in such grain traits as kernel size and grain composition.

The DPPH and FRAP assays were used to evaluate the *in vitro* AC of the flour samples (Table 4), by measuring, respectively, the scavenging of the DPPH radicals and the reduction of Fe^{3+} to Fe^{2+}, which lead to the concentration of electron-donating antioxidants (Serpen et al., 2012). The old cv. Gentilrosso exhibited superior AC in both assays, while the other old genotypes and the modern bread-making (FP) cv. were characterized by lower but statistically similar values. The significantly high AC_{DPPH} registered for the einkorn (cv. Monlis) and the FF modern wheat (cv. Bologna) was not consistent with the poor AC_{FRAP}

values. As far as the environment is concerned, the AC generally showed a similar response to that found for the investigated phenolic compounds (LS18 > SL18 > LS17 > SL17).

An analysis of recent literature has not revealed any major differences at the nutritional and phytochemical level between the ancient species (einkorn, emmer, and spelt) and the modern BW cultivars (Shewry & Hey, 2015; Spisni et al., 2019), with only einkorn, emmer, and khorasan showing high concentrations of the carotenoid lutein (Ziegler et al., 2016). On the other hand, old BW genotypes have shown greater numbers of total phenolic compounds and total isomers than modern cultivars (Dinelli et al., 2011) and higher concentrations free soluble phenolic compounds (Montevecchi et al., 2019), in accordance with the data of the present study. However, a higher content of ferulic acid has also been reported for modern cultivars (Qayyum et al., 2024). Our collected data revealed a great variability among the modern BW cvs., with some of them characterized by similar phenolic acid contents to those of the old genotypes, or even higher amounts for AR phenolic lipids.

Table 4. Effect of the genotype, the experiment, and their interaction on the R5 gliadin content of the refined flour, and on the DON content, the bioactive compounds, and the antioxidant capacity[a] of the whole-meal flour of the investigated genotypes cultivated in the experimental trials and supplied with 80 kg of N·ha^{-1}.

Factor	Species		Source of variation	R5 gliadins (mg·kg^{-1})	DON (µg·kg^{-1})	SPAs (mg·kg^{-1})	CWBPAs (mg·kg^{-1})	ARs (mg·kg^{-1})	AC_{DPPH} (mmol TE·kg^{-1})	AC_{FRAP} (mmol TE·kg^{-1})
Genotype (G)	Einkorn		Monlis	150 de	7798 bc	110 bc	668 ab	770.3 de	3.9 abc	8.8 bcde
	Emmer		Giovanni Paolo	178 cde	9891 a	79 fg	551 cd	809.0 cde	3.6 ef	8.1 ef
			Luni	251 ab	5851 cd	112 b	536 d	878.1 bcd	3.7 bcdef	7.6 fg
	Spelt		BC Vigor	244 abc	5377 d	71 gh	666 ab	939.0 bc	3.8 bcde	8.1 def
			Rossella	179 bcde	9485 ab	72 gh	604 bcd	672.5 e	3.6 def	9.2 abc
	BW	Old genotypes	Andriolo	266 a	2326 ef	94 de	635 abc	772.4 de	3.8 abcd	9.6 ab
			Gentilrosso	227 abc	1504 ef	98 de	654 ab	719.0 de	3.9 ab	9.7 a
			Frassineto	234 abc	1179 f	99 cd	639 ab	774.7 de	3.9 abc	9.0 abc
			Verna	260 ab	1323 f	130 a	609 bc	758.0 de	3.7 bcdef	9.0 abcd
		Modern genotypes	Arabia (FB)	239 abc	6663 cd	66 h	610 bc	946.0 b	3.6 f	8.5 cde
			Solehio (FP)	133 e	2415 ef	87 ef	704 a	758.1 de	3.8 abcde	9.5 ab
			Aubusson (FP)	217 abcd	3472 e	63 h	647 ab	1307.7 a	3.8 bcdef	9.6 ab
			Bologna (FF)	263 ab	1039 f	68 gh	682 ab	1408.9 a	4.0 a	7.3 g
			p (F)	***	***	***	***	***	***	***
Experiment (E)			SL17	230 b	244 c	52 c	502 c	797.3 c	3.6 c	8.0 c
			LS17	275 a	769 c	57 c	514 c	859.4 b	3.7 b	8.3 c
			SL18	170 c	6957 b	117 b	722 b	950.6 a	4.0 a	9.1 b
			LS18	201 b	9045 a	129 a	793 a	970.3 a	4.0 a	9.6 a
			p (F)	***	***	***	***	***	***	***
G × E			*p* (F)	***	***	***	***	***	***	***

[a]DON, deoxynivalenol; SPAs, soluble phenolic acids; CWBPAs, cell wall-bound phenolic acids; ARs, 5-*n*-alkylresorcinols; AC, antioxidant capacity (DPPH and FRAP assays); BW, bread wheat; FB, wheat for biscuits (frumento biscottiero); FP, ordinary bread-making wheat (frumento panificabile); FF, improver high protein wheat (frumento di forza); SL17, sandy-loam soil, harvested in 2017; LS17, loam-silty soil, harvested in 2017; SL18, sandy-loam soil, harvested in 2018; LS17, loam-silty soil, harvested in 2018. The results are expressed on a DW basis. Means followed by different letters are significantly different, according to the REGW-F test [(*) *p* (F) < 0.05, (**) *p* (F) < 0.01, (***) *p* (F) < 0.001, and ns, not significant].

Conclusions

The comparative study we conducted underscores the challenges inherent to the agronomic management of both hulled species and old BW genotypes when grown in non-marginal cropping systems. The selected cvs. demonstrated diminished adaptability to diverse environmental conditions and limited responsiveness to improved production conditions. The grains of the old cvs had a significantly higher protein content than the modern cvs., although the dough of the old BWs presented greater processing difficulties, even after N fertilizer. The old cvs. did not confer advantages, in terms of toxic celiac disease epitopes, as their concentration of R5 gliadin sequences was comparable or even higher than those of the modern cvs. in all the experiments. Nonetheless, they exhibited reduced susceptibility to mycotoxins, although the genotypes affected less by the contamination were also found within the hulled wheats and the modern BWs. According to the assessed phytochemical profile, a variable composition was observed for the hulled species, while the old BWs appeared to have slightly higher phenolic acid contents than the modern cvs. However, these differences were quite modest and highly variable among the genotypes and depended on the growing conditions, so that, overall, they might not be considered relevant in the context of human diets. On the other hand, the content and variability of the phenolic lipids were lower in the old genotypes than in the modern cvs. The large set of samples analyzed in the present study, albeit limited to a selection of representative cvs. for each category, confirmed the genetic determination of compositional differences between hulled, old, and modern BW varieties, even within the same species, as described in previous studies, as well as the important effect of the environment. In addition, a higher level of biodiversity is generally pointed out as a strength of landraces and old BW genotypes; on the basis of the limited, although representative number of cvs. that we analyzed in different production situations, a clearly greater variability of many of the studied traits was observed for the modern BW cvs. than for the old ones. Moreover, the recently released hulled wheat cvs., despite suffering from agronomic deficiencies, have exhibited a wide range for all the agronomic and qualitative traits. This points out the great importance of targeted genetic improvement efforts and varietal selection, to allow the progressive identification of new modern genotypes, as they are capable of ensuring high yields and technological performances, even with a low N supply, and can be further improved, in terms of sanitary risk tolerance and phytochemical quality, in accordance with the requirements of the food supply chain.

Supplementary material

Table S1. The einkorn, emmer, spelt, and bread wheat (BW, old and modern) genotypes compared in this study.

Crop	Taxonomic classification	Ploidy level[a]	Type[b]	Accession[c]	Plant height (cm)[d]	Origin	Year of release
Einkorn	*T. monococcum* spp. *monococcum*	Diploid (AA)	Hulled genotypes	Monlis	122 - 172	CREA-IT-RM, Rome (RM), Italy	2006
Emmer	*T. turgidum* spp. *dicoccum*	Tetraploid (AABB)		Giovanni Paolo	78 - 141	CREA-CI-FG, Foggia (FG), Italy	2008
Spelt	*T. aestivum* spp. *spelta*	Hexaploid (AABBDD)		Luni	103 - 175	S.I.S. S.p.A., San Lazzaro di Savena (BO), Italy	2002
				BC Vigor	128 - 184	Bc Institut, d.d., Zagreb, Republika Hrvatska	2012
				Rossella	103 - 151	CREA-CI-FG, Foggia (FG), Italy	2016
BW	*T. aestivum* spp. *aestivum*	Hexaploid (AABBDD)	Old genotypes	Andriolo	119 - 173	Tuscany, Italy	From the XIX° century
				Gentilrosso	128 - 174	Tuscany, Italy	From the XIX° century
				Frassineto	113 - 164	Tuscany, Italy	1922
				Verna	124 - 182	Tuscany, Italy	1953
			Modern genotypes	Arabia (FB)	78 - 105	Apsovsementi S.p.A., Voghera (PV), Italy	2009
				Solehio (FP)	75 - 106	Agroalimentare Sud S.p.A., Melfi (PZ), Italy	2008
				Aubusson (FP)	49 - 97	Limagrain Italia S.p.A., Fidenza (PR), Italy	2003
				Bologna (FF)	70 - 91	S.I.S. S.p.A., San Lazzaro di Savena (BO), Italy	2002

[a]Number of the chromosome sets. [b]Andriolo, Gentilrosso, Frassineto, and Verna are historically cultivated bread wheat cultivars (from before 1985); Andriolo and Gentilrosso are landraces, that is, genetically heterogenous populations that have been cultivated from the XIX° century, while Frassineto and Verna are old genotypes that were developed from varietal crosses; all the other bread wheats, the einkorn, emmer, and spelt cultivars are recent genotypes that were released after 2000. [c]FB: wheat for biscuits (frumento biscottiero); FP: ordinary bread-making wheat (frumento panificabile); FF: improver high protein wheat (frumento di forza). [d]The minimum and maximum plant heights measured in the experimental trials.

Table S2. Main information on the field experiments conducted in the 2016-18 period in north-west Italy: the physical and chemical characteristics of the soils and the timing of the main agronomic management practices applied to the plots.

		2016-17		2017-18	
		Cigliano	Carmagnola	Cigliano	Carmagnola
Parameter	**Measuring unit**	**SL17**	**LS17**	**SL18**	**LS18**
Geographic coordinates		45° 18' N, 8° 01' E	44° 50' N, 7° 40' E	45° 18' N, 8° 01' E	44° 50' N, 7° 40' E
Altitude	m	236	245	236	245
Soil (USDA classification)		Typic Hapludalfs	Typic Udifluvents	Typic Hapludalfs	Typic Udifluvents
Sand (2 -0.05 mm)	%	41.7	27.7	44.8	28.6
Silt (0.05 - 0.002 mm)	%	47.5	65.4	45.9	64.8
Clay (< 0.002 mm)	%	10.8	6.9	9.3	6.5
pH		5.9	7.9	5.7	8.1
Organic matter	%	1.51	1.62	1.69	1.44
C/N		9.4	8.3	12.0	8.0
Cation Exchange Capacity (C.E.C.)	$Cmol(+)\cdot kg^{-1}$	10.2	10.7	11.2	11.3
Exchangeable Potassium	$mg\cdot kg^{-1}$	69	30	18	58
Available Phosphorus	$mg\cdot kg^{-1}$	42	12	89	7
Total Nitrogen	$g\cdot kg^{-1}$	0.94	1.14	0.82	1.05
Crop tecniques	**Growth stage**				
Sowing date	-	4 November 2016	27 October 2016	31 October 2017	31 October 2017
N fertilization	Tillering (GS 23)	7 March 2017	9 March 2017	12 March 2018	14 March 2018
	Stem elongation (GS 32)	3 April 2017	07 April 2017	18 April 2018	19 April 2018
Harvest date	-	05 July 2017	14 July 2017	04 July 2018	16 July 2018

SL17, sandy-loam soil, harvested in 2017; LS17, loam-silty soil, harvested in 2017; SL18, sandy-loam soil, harvested in 2018; LS17, loam-silty soil, harvested in 2018. The soils were sampled at a depth of 0-30 cm, using Eijkelkamp cylindrical augers, at the beginning of March, just before the N fertilization at tillering (growth stage, GS 23).

Table S3. Monthly cumulative rainfall and growing degree days (GDDs)[a] from sowing (November) to the end of the ripening stage (June) measured in the experimental areas.

Year	Month	Rainfall (mm)		GDDs (Σ °C·d^{-1})	
		Cigliano (SL)	Carmagnola (LS)	Cigliano (SL)	Carmagnola (LS)
2016-17	November	158	257	238	250
	December	45	77	144	159
	January	4	12	97	111
	February	45	62	152	175
	March	69	69	349	356
	April	34	51	415	412
	May	79	77	554	558
	June	149	103	673	698
	November – June	583	708	2622	2719
	April – May	113	128	969	970
2017-18	November	48	66	224	220
	December	33	27	113	82
	January	107	117	178	141
	February	60	86	113	89
	March	109	103	223	209
	April	93	116	456	444
	May	138	310	583	565
	June	35	14	665	672
	November – June	623	839	2555	2422
	April – May	231	426	1039	1009

[a]GDDs: accumulated growing degree days for each month using a 0 °C base; SL, sandy-loam soil; LS, loam-silty soil. Data obtained from the Regione Piemonte agrometeorological service.

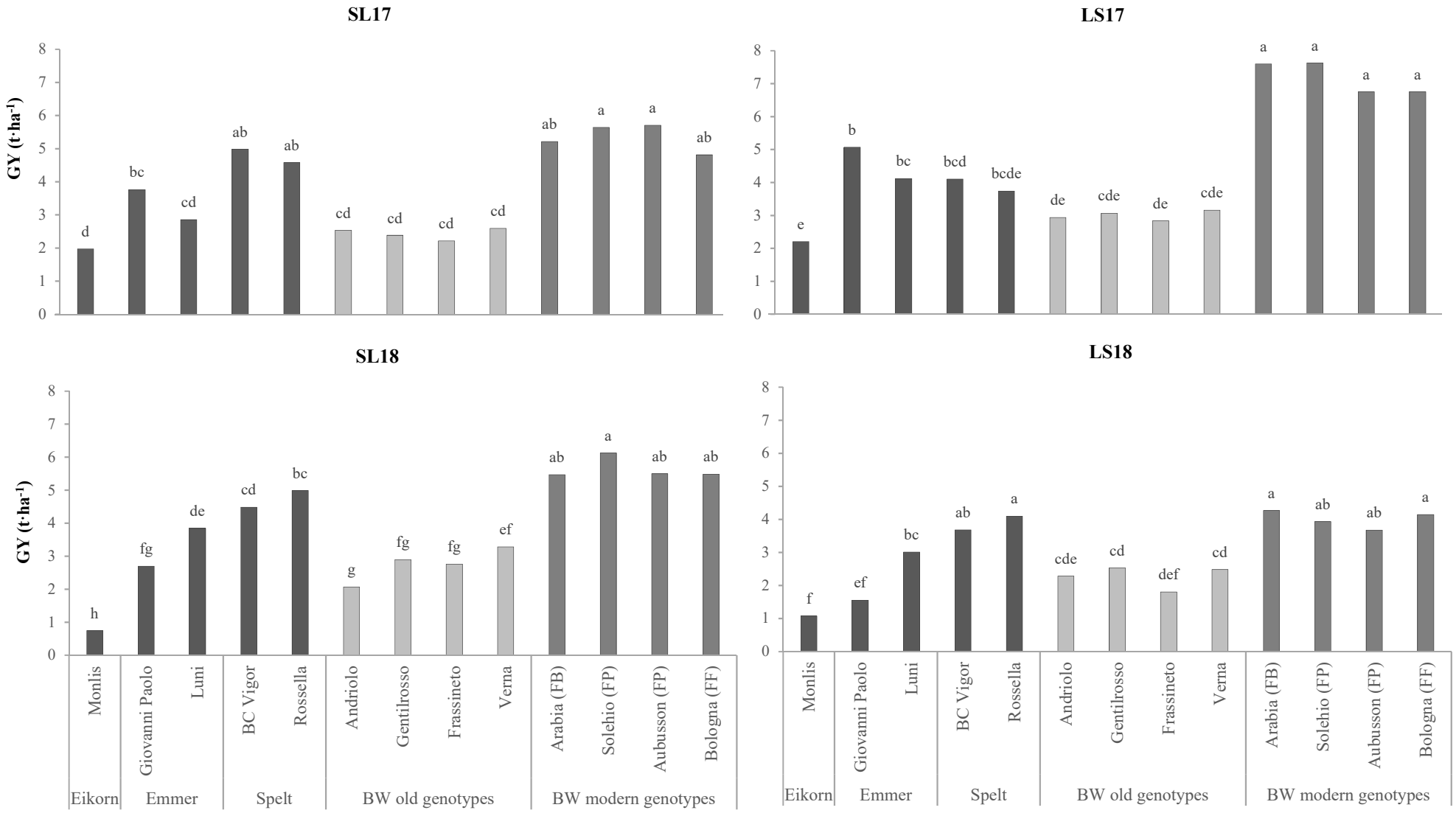


Figure S1. Comparison of the grain yields (GY) of the investigated genotypes across the growing environments.

SL17, sandy-loam soil, harvested in 2017; LS17, loam-silty soil, harvested in 2017; SL18, sandy-loam soil, harvested in 2018; LS17, loam-silty soil, harvested in 2018; BW, bread wheat; FB, wheat for biscuits (frumento biscottiero); FP, ordinary bread-making wheat (frumento panificabile); FF, improver high protein wheat (frumento di forza). Different letters above the columns indicate significant differences between the six groups for p (F) < 0.05, according to the REGW-F test.

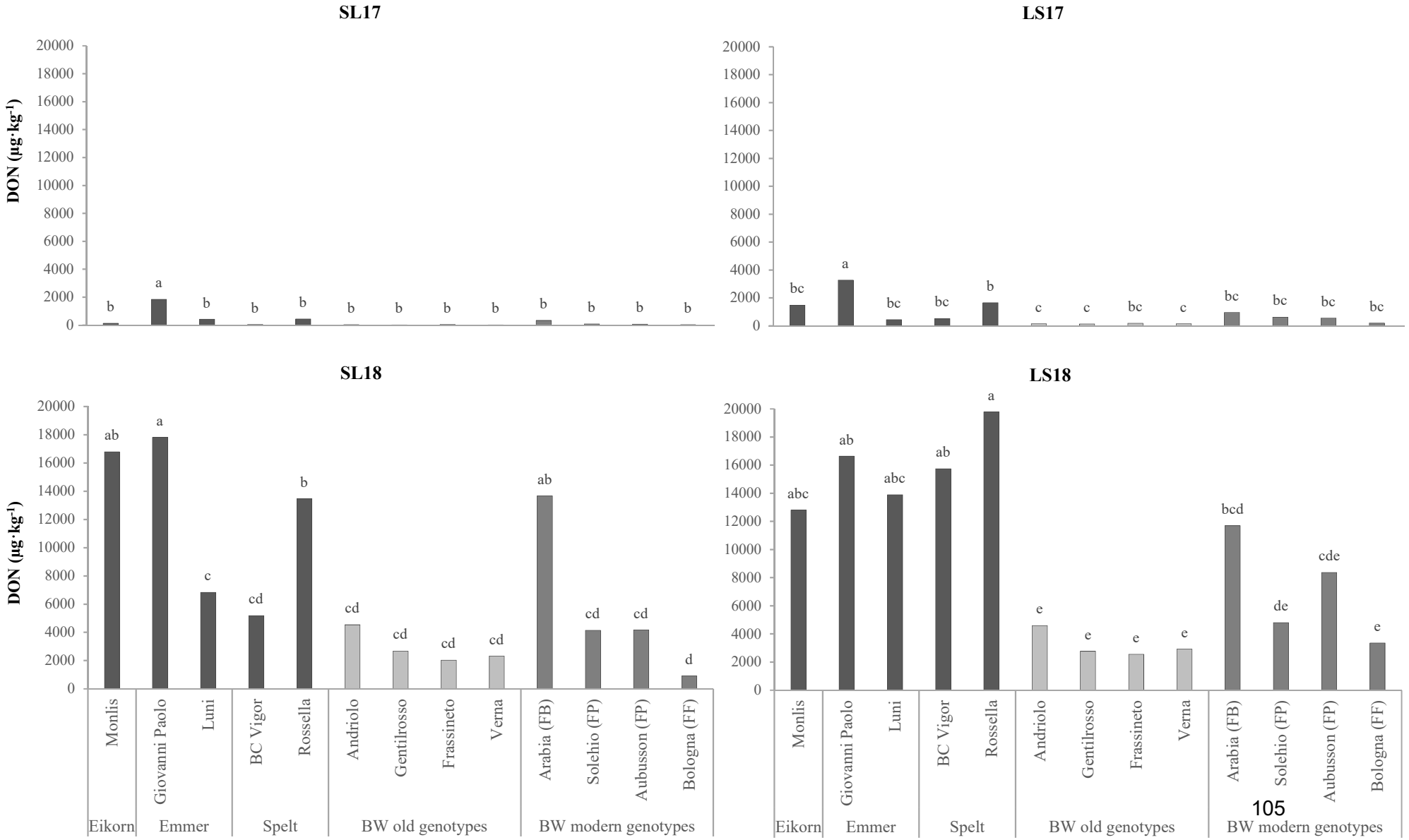

SL17
LS17
SL18
LS18
DON (µg·kg-1)
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
b a b b b b b b b b b b
bc a bc bc b c c bc c bc bc bc bc
ab a c cd b cd cd cd cd ab cd cd d
abc ab abc ab a e e e e bcd de cde e
Monlis
Giovanni Paolo
Luni
BC Vigor
Rossella
Andriolo
Gentilrosso
Frassineto
Verna
Arabia (FB)
Solehio (FP)
Aubusson (FP)
Bologna (FF)
Eikorn
Emmer
Spelt
BW old genotypes
BW modern genotypes

Figure S2. Comparison of the deoxynivalenol (DON) contents of the investigated genotypes across the growing environments.

SL17, sandy-loam soil, harvested in 2017; LS17, loam-silty soil, harvested in 2017; SL18, sandy-loam soil, harvested in 2018; LS17, loam-silty soil, harvested in 2018; BW, bread wheat; FB, wheat for biscuits (frumento biscottiero); FP, ordinary bread-making wheat (frumento panificabile); FF, improver high protein wheat (frumento di forza). The results are expressed on a DW basis. Different letters above the columns indicate significant differences between the groups for p (F) < 0.05, according to the REGW-F test.

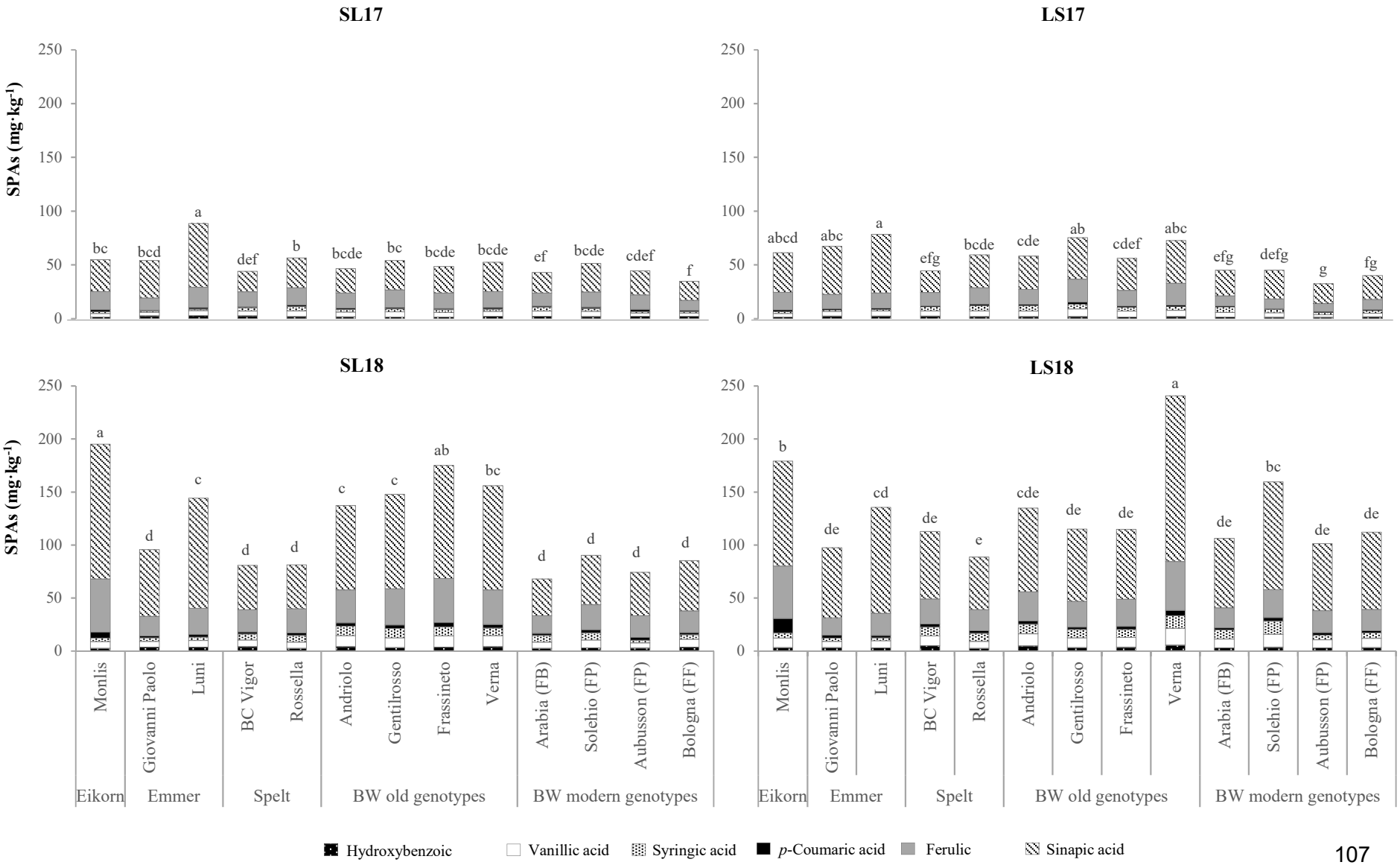

SL17
LS17
SL18
LS18
SPAs (mg·kg-1)
250
200
150
100
50
0
bc bcd a def b bcde bc bcde bcde ef bcde cdef f
abcd abc a efg bcde cde ab cdef abc efg defg g fg
a d c d d c c ab bc d d d d
b de cd de e cde de de a de bc de
Monlis
Giovanni Paolo
Luni
BC Vigor
Rossella
Andriolo
Gentilrosso
Frassineto
Verna
Arabia (FB)
Solehio (FP)
Aubusson (FP)
Bologna (FF)
Eikorn
Emmer
Spelt
BW old genotypes
BW modern genotypes
Hydroxybenzoic
Vanillic acid
Syringic acid
p-Coumaric acid
Ferulic
Sinapic acid

Figure S3. Comparison of the soluble phenolic acid (SPA) profiles of the investigated genotypes across the growing environments.

SL17, sandy-loam soil, harvested in 2017; LS17, loam-silty soil, harvested in 2017; SL18, sandy-loam soil, harvested in 2018; LS17, loam-silty soil, harvested in 2018; BW, bread wheat; FB, wheat for biscuits (frumento biscottiero); FP, ordinary bread-making wheat (frumento panificabile); FF, improver high protein wheat (frumento di forza). The results are expressed on a DW basis. Different letters above the columns indicate significant differences between the groups for p (F) < 0.05, according to the REGW-F test.

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Chapter IV – "Influence of agronomic practices on the antioxidant compounds of pigmented wheat (*Triticum aestivum* spp. *aestivum* L.) and tritordeum (× *Tritordeum martinii* A. Pujadas, nothosp. nov.) genotypes"

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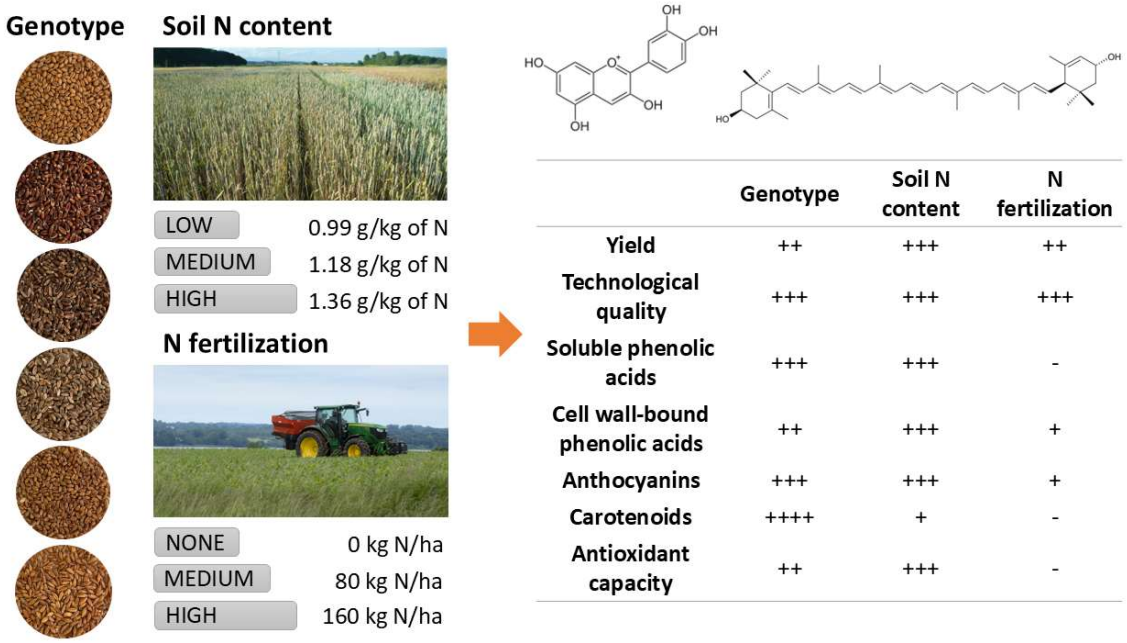
Graphical abstract



Abstract

Twelve pigmented wheat genotypes, one tritordeum, and one common wheat were grown in three field experiments under varying nitrogen (N) fertilization rates to investigate the contributions of genotype, environment, and fertilization on the levels of phenolic acids, anthocyanins, carotenoids and antioxidant capacity of the grains. Soluble phenolic acids increased significantly (+16%) in the environment with high soil N content, while bound phenolic acids and anthocyanins decreased (−16 and −57%). N fertilization affected the agronomic and qualitative traits but had limited effects on some bioactive compounds (bound phenolic acids and anthocyanins). The greatest differences appeared among the color groups and within the same color types, with the black group showing the most anthocyanins and phenolic acids (34.4 and 1207 $mg \cdot kg^{-1}$) and the highest antioxidant capacity. Some of the cultivars could be promising for the development of innovative supply chains and the production of functional foods, as they showed good yield and quality performances, and good antioxidant features.

Keywords: *Triticum aestivum*, × *Tritordeum martinii*, pigmented cereals, nitrogen, phenolic acids, anthocyanins, carotenoids, antioxidant capacity, technological quality.

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbeenzothiazoline-6-sulfonic acid) diammonium salt; AC, antioxidant capacity; ANOVA, analysis of variance; AUCGC, area under the canopy greenness curve; BHT, butylated hydroxytoluene; CWBPAs, cell wall-bound phenolic acids; cyd-3-glu, cyanidin-3-glucoside; cyd-3-rut, cyanidin-3-rutinoside; dpd-3-glu, delphinidin-3-glucoside; dpd-3-rut, delphinidin-3-rutinoside; DW, dry weight; E, environment; FRAP, ferric reducing antioxidant power; G, genotype; GDDs, growing degree days; GPC, grain protein content; GS, growth stage; GY, grain yield; HPLC/DAD, high- performance liquid chromatography with diode array detection; LOD, limit of detection; N, nitrogen; NDVI, normalized difference vegetation index; PCA, principal component analysis; pnd-3-glu, peonidin-3-glucoside; REGW-F test, Ryan−Einot−Gabriel−Welsch F test; SPAs, soluble phenolic acids; TAC, total anthocyanin content; TCC, total carotenoid content; TE, Trolox equivalents; TKW, thousand kernel weight; TPTZ, 2,4,6-tris(2-pyridyl)-*s*-triazine; TW, test weight.

Introduction

Bread wheat (*Triticum aestivum* spp. *aestivum* L.) products are the basis of most human diets across the globe, and they contribute to a great extent to the daily intake of antioxidant compounds and the prevention of aging processes caused by oxidative stress. Indeed, the consumption of whole-meal wheat has been associated with multiple health benefits, which are related to the high dietary fiber content and to the synergetic action of several bioactive compounds, such as micronutrients and phytochemicals.[1] The most consumed wheat types are the white- and red-grained ones.[2] However, some newly developed pigmented wheat varieties have been receiving a great deal of attention from breeders and scientists around the world as they appear to be a valuable source of protective phytochemicals, particularly those responsible for conferring the color to the kernels.[3] Anthocyanins and carotenoids, apart from giving attractive blue-purple and yellow-orange colors to grains, respectively, have been associated with numerous biological activities[4] and are regarded as potent antioxidant compounds that can add functionality to the energy-rich cereal matrix. Pigmented wheat exists in different forms, which depend on the type and location of the pigments within the kernel layers. Other works have confirmed that purple wheat contains anthocyanins in the pericarp layer (purple pericarp - Pp) and blue wheat in the aleurone layer (blue aleurone - Ba),[3] whereas black wheat accumulates anthocyanins in both layers, combining the genetics of both purple and blue wheat (Pp + Ba).[5] On the other hand, wheat genotypes concentrations of carotenoids in the starchy endosperm are characterized by a bright yellow color of the kernels (yellow endosperm - Ye).[3] Apart from innovative Ye wheat varieties, the novel cereal species tritordeum (× *Tritordeum martinii* A. Pujadas, nothosp. nov.) has shown promise, because of its high carotenoid content in the grain, which has been found to be more than five times higher than that of durum wheat.6 Furthermore, some tritordeum cultivars exhibit similar technological properties to those of bread wheat and therefore show a good bread-making aptitude.[7] Thus, these novel and unconventional genotypes might be valuable resources to increase the nutritional value of wheat-derived food products. Nevertheless, the main challenge for their exploitation on a large scale is their lower yield than commonly cultivated non- pigmented varieties.[2,7] However, different agricultural practices can be adopted to grow wheat and tritordeum with the aim of improving both the grain yield and quality. Since these techniques can have an effect on the concentration of health-promoting substances,[8−11] more knowledge on the impact of different agronomic conditions on the phytochemical concentrations of these grains is needed to understand whether the agricultural management practices usually applied to common wheat could be helpful in enhancing the yield and agronomic performances of innovative pigmented genotypes, without having any detrimental effect on their high nutritional value. Nitrogen (N) is a fundamental nutrient for plant growth, and the supply of N is a relevant agronomic practice for crop production, as it influences the yield, the protein content, and the technological quality of the grains.[12] The effect of N fertilization on the phytochemical

concentrations of grains has been reported for different cereal crops.[13–15] However, few data are available on pigmented cereals, and, at present, there is little information about the effect of the N fertility of the soil on the phytochemical accumulation of the grains of wheat and tritordeum genotypes. Therefore, the aim of the present study has been to evaluate the relative contribution of the genotype, the environment (defined as a combination of location and year), and the N fertilization to the variation in the levels of bioactive compounds, such as soluble and bound phenolic acids, anthocyanins, and carotenoids, and in the antioxidant capacity of a selected set of non-traditional genotypes with pigmented grains, including anthocyanin- and carotenoid-rich registered varieties and breeding lines, and considering one known commercial variety as a control.

Materials and Methods

Experimental design

Thirteen bread wheat genotypes and one tritordeum genotype were used in this study. Among the considered wheat genotypes, there were six with Pp, three with Ba, two with Pp and Ba, one with Ye, and one common (red) bread wheat, which was chosen as a widely cultivated variety and used as a control (Figures 1 and S1; Table 1). The studied genotypes were grown during the 2018−19 growing season in two different locations in north-west Italy (Cigliano, Piedmont, 45° 18′ N, 8° 01′ E; Carmagnola, Piedmont, 44° 50′ N, 7° 40′ E). The field trial was repeated during the 2019−20 growing season in Cigliano. The field in Cigliano was characterized by a shallow sandy-loam soil (Typic Hapludalfs), whereas the one in Carmagnola was characterized by a deep silty-loam soil (Typic Udifluvents). The N content of the soil in both locations was tested at the end of the winter period (Table 2). The soils were sampled at a depth of 0−30 cm, using Eijkelkamp cylindrical augers, at the beginning of March, just before the N fertilization at the tillering growth stage (GS 23, according to Zadoks et al.).[16] The daily precipitations and temperatures were measured at meteorological stations located near the two experimental areas (Table 3). Due to the remarkably different growing conditions observed in the field trials, both in terms of soil characteristics, mainly as N content, and weather conditions, three different environments were defined as a combination of location and year and were set up as follows: A (Cigliano, 2019−20 growing season), B (Cigliano, 2018−19), and C (Carmagnola, 2018−19). The genotypes were cultivated under N- input regimes of 0 kg of $N \cdot ha^{-1}$ (unfertilized), 80 kg of $N \cdot ha^{-1}$ (medium N fertilization), and 160 kg of $N \cdot ha^{-1}$ (high N fertilization). The total amount of administered N was split into two dosages to avoid leaching and to improve N availability, according to the ordinary N fertilization practices in the growing areas. The N fertilizer was provided manually and was evenly distributed as ammonium nitrate (granular, N 27%) at the tillering stage (GS 23) and at the beginning of stem elongation (GS 32).

Thus, the treatments compared at each location were factorial combinations of 14 genotypes (G), 3 environments (E), and 3 N fertilization rates (N). The treatments were assigned to experimental units using a completely randomized block design, with a 10.5 m^2 plot (7 × 1.5 m) and three replications (n = 378). The agronomic techniques commonly adopted in the area were applied to all plots. Briefly, the previous crop in all the experiments was maize, the field was plowed each year, incorporating the debris into the soil, and this was followed by disk harrowing to prepare a suitable seedbed. Planting was conducted in 12 cm wide rows at a seeding rate of 450 $seeds \cdot m^{-2}$ in October or November (Table S1). The weed control was conducted with mesosulfuron-methyl and iodosulfuron-methyl- sodium at stem elongation (GS 31). A fungicide (prothioconazole + tebuconazole) and an insecticide (deltamethrin) were applied to all the plots at anthesis (GS 65) to minimize the negative impact of fungal diseases and insects. Harvesting was carried out using a Walter Wintersteiger cereal plot combine harvester.

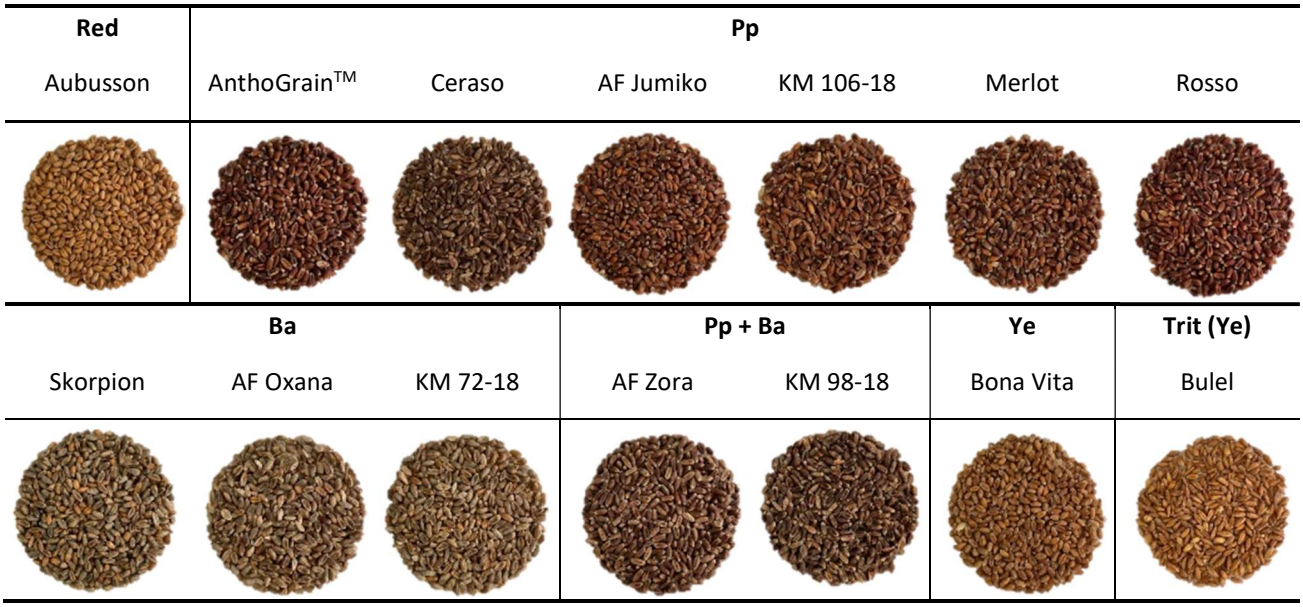


Figure 1. Wheat and tritordeum genotypes grouped according to the grain color, which depends on the type and position of the pigments within the kernel layers.

Pp, purple pericarp; Ba, blue aleurone; Pp + Ba, black grain; Ye, yellow endosperm; Trit, tritordeum.

Table 1. The wheat and tritordeum genotypes compared in the study and their origin.

Type	Genotype	In charge of breeding	Year of release
Red wheat (control)	Aubusson	Limagrain Italia S.p.A., Fidenza (PR), Italy	2002
Pp wheat	AnthoGrain™	Hetland Seeds Ltd, Naicam, SK, Canada	2013
	Ceraso	Saatzucht Donau GesmbH. & CoKG, Austria	2014
	AF Jumiko	Agrotest Fyto, Ltd., Kroměříž, Czech Republic	2018
	KM 106-18	Agrotest Fyto, Ltd., Kroměříž, Czech Republic	Breeding line
	Merlot	Saatzucht Donau GesmbH. & CoKG, Austria	2015
	Rosso	Saatzucht Donau GesmbH. & CoKG, Austria	2010
Ba wheat	Skorpion	Agrotest Fyto, Ltd., Kroměříž, Czech Republic	2011 (2012 - European Catalogue of Varieties)
	AF Oxana	Agrotest Fyto, Ltd., Kroměříž, Czech Republic	2019
	KM 72-18	Agrotest Fyto, Ltd., Kroměříž, Czech Republic	Breeding line
Pp + Ba wheat	AF Zora	Agrotest Fyto, Ltd., Kroměříž, Czech Republic	2021
	KM 98-18	Agrotest Fyto, Ltd., Kroměříž, Czech Republic	Breeding line
Ye wheat	Bona Vita	Istropol Solary a.s., Horné Mýto, Slovakia	2011
Tritordeum (Ye)	Bulel	Arcadia S.p.A., Pamplona, Spain	2015

Pp, purple pericarp; Ba, blue aleurone; Pp + Ba, black grain; Ye, yellow endosperm.

Table 2. Main information on the trial and on the physical and chemical characteristics of the soil for the field experiments conducted in the 2018-20 period in north-west Italy.

Parameters	Measuring unit	Environments		
		A	B	C
Growing season		2019-20	2018-19	2018-19
Location		Cigliano (VC)	Cigliano (VC)	Carmagnola (TO)
Geographic coordinates		45° 18' N, 8° 01' E	45° 18' N, 8° 01' E	44° 50' N, 7° 40' E;
Altitude	m	236	236	245
Soil (USDA classification)		Typic Hapludalfs	Typic Hapludalfs	Typic Udifluvents
Sand (2 - 0.05 mm)	%	41.7	39.4	32.1
Silt (0.05 - 0.002 mm)	%	47.5	52.3	65.5
Clay (< 0.002 mm)	%	10.8	8.3	5.3
pH		5.9	6.6	8.1
Organic matter	%	1.51	1.59	2.01
C/N		9.4	8.8	8.6
Cation Exchange Capacity (C.E.C.)	$Cmol(+)\cdot kg^{-1}$	10.2	10.6	9.8
Exchangeable potassium	$mg\cdot kg^{-1}$	69	135	72
Available phosphorus	$mg\cdot kg^{-1}$	42	25	15
Total N	$g\cdot kg^{-1}$	0.99	1.18	1.36
Estimated mineralized N[a]	$kg\cdot ha^{-1}\cdot year^{-1}$	134	159	201
AUCGC with different N rates (kg of $N\cdot ha^{-1}$)				
0	NDVI-Day	38.6	57.1	68.4
80	NDVI-Day	49.0	61.6	68.4
160	NDVI-Day	57.0	66.5	70.2
GY with different N rates (kg of $N\cdot ha^{-1}$)				
0	$t\cdot ha^{-1}$	3.1	3.9	6.5
80	$t\cdot ha^{-1}$	4.9	4.7	6.7
160	$t\cdot ha^{-1}$	5.8	5.8	6.4
GPC with different N rate s (kg of $N\cdot ha^{-1}$)				
0	%	10.9	10.6	12.9
80	%	10.7	10.9	13.8
160	%	11.5	12.5	14.6

The data reported for the Area Under the Canopy Greenness Curve (AUCGC), grain yield (GY) and grain protein content (GPC) refer to the averages of all the compared genotypes and replications.
[a]Calculated on the basis of total N and the physical parameters of the soil, according to Grignani et al. (2003).

Grain Yield and Physical Parameters

The area under the canopy greenness curve (AUCGC) was calculated from normalized difference vegetation index (NDVI) measurements taken throughout the growing seasons, according to De Santis et al.[17] The grain yield (GY) was calculated on a plot basis and adjusted to a 13% moisture content. The harvested grains were mixed thoroughly, and 2 kg grain samples were taken from each plot to determine the grain moisture means of a GAC 2000 Grain Analyzer (Dickey-John Corp., Auburn, IL, USA). The thousand-kernel weight (TKW) was determined on two 200 kernel sets of each sample, using an electronic balance.

Qualitative Parameters

Representative subsamples (500 g) were ground to whole-meal using a laboratory centrifugal mill equipped with a 1 mm sieve (Model ZM-200, Retsch, Haan, Germany). The grain protein content (GPC) was determined according to the AACC method 39-10.01[18] by means of an NIR System Model 6500 (FOSS-NIRSystems, Laurel, MD, USA), as was the ash content. All the samples were ground to a fine powder (particle size <500 μm) with a Cyclotec 1093 sample mill (Foss, Padua, Italy) and stored at −25 °C, prior to the chemical analyses. The moisture content, which was determined to express the concentrations of phenolic acids, anthocyanins, and carotenoids and the antioxidant capacity results on a dry weight (DW) basis, was obtained by oven-drying the <500 μm samples at 105 °C to a constant weight.

Chemical Analyses

Chemicals

2,2′-Azino-bis(3-ethylbeenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS), ethanol (CHROMASOLV, 99.8%), ethyl acetate (CHROMASOLV, 99.8%),(±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Tro- lox, 97%), hydrochloric acid (HCl, 37%), iron(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$, ≥98%), methanol (CHROMASOLV, 99.9%), potassium sulfate (≥99%), sodium hydroxide (≥98%), 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ), butylated hydroxytoluene (BHT, ≥99%), *tert*-butyl methyl ether (HPLC grade), and phenolic acid standards (caffeic acid ≥98%, *p*-coumaric acid ≥98%, ferulic acid ≥99%, gallic acid ≥99%, protocatechuic acid ≥99%, *p*-hydroxyben zoic acid ≥99%, sinapic acid ≥98%, syringic acid ≥95%, and vanillic acid ≥97%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anthocyanin standards (cyd-3-glu, cyanidin-3-glucoside; cyd- 3-rut, cyanidin-3-rutinoside; dpd-3-glu, delphinidin-3-glucoside; dpd- 3-rut, delphinidin-3-rutinoside; pnd-3-glu, peonidin-3-glucoside), all with a purity ≥97%, were purchased from Polyphenols (Sandnes, Norway). Lutein (≥95%) and zeaxanthin (≥98%) standards were obtained from Extrasynthese (Genay, France). Methanol, ethanol, acetone, and hexane, all p.a. grade, were obtained from Lachner (Neratovice, Czech Republic). Formic acid (99−100%) was purchased from VWR (Radnor, PA, USA). Acetonitrile (HPLC grade) was purchased from Carlo Erba (Milan, Italy).

Water (HPLC grade) was obtained from an ELGA PURELAB Ultra system (M- medical, Cornaredo, Milan, Italy).

Extraction and Quantification of the Phenolic Acids, Carotenoids, and Anthocyanins

The soluble phenolic acids (SPAs) and cell wall-bound phenolic acids (CWBPAs) were individually extracted by a two-step method previously described.[19] A common extraction step was performed using an ethanol−water solution (80:20, *v*/*v*), then the SPAs were determined after alkaline hydrolysis of the ethanol:water extract, whereas CWBPAs were determined after alkaline hydrolysis of the solid sample residue. After acidification of the hydrolysates and liquid−liquid extraction with ethyl acetate, the organic phase was evaporated to dryness under a nitrogen stream. The dry residue was reconstituted with 80:20 (*v*/*v*) methanol:water solution, filtered, and analyzed by means of high-performance liquid chromatography with diode array detection (HPLC/DAD). The detailed sample preparation procedure as well as the chromatographic conditions are described in Giordano et al.[19] Phenolic acids were identified using the retention times and the UV/vis spectra of their respective standards. Solutions of individual phenolic acid standards were prepared and diluted to different concentrations to obtain calibration curves for quantification purposes.

The carotenoids were extracted with a mixture of ethanol:acetone:hexane (1:1:2, *v*/*v*/*v*). After evaporation of the extract under vacuum at 40 °C, the dry residue was reconstituted with ethanol:acetone (3:2, *v*/*v*) containing 0.2% BHT, filtered, and analyzed by means of HPLC/DAD. The detailed method of carotenoid analysis is described in Paznocht et al.[20] Quantification was carried out by means of external calibration in the range of 0.1− 10 $\mu g \cdot ml^{-1}$.

The anthocyanins were extracted with a mixture of methanol and 1 M HCl (85:15, *v*/*v*) as reported by Ficco et al.[21] The extract was then centrifuged at 8228 rcf for 10 min (5810R, Eppendorf, Hamburg, Germany) after freezing at −18 °C and evaporated under vacuum at 45 °C (Rotavapor R-200, Büchi Labortechnik, Flawil, Switzerland). The dry residue was reconstituted with 2 mL of the extraction mixture, filtered through a PTFE microfilter (0.22 μm), and analyzed by means of HPLC/DAD (Ultimate 3000, Thermo Fisher Scientific, Waltham, MA, USA). The analytes were separated by gradient elution on a Gemini analytical column (C18, 150 × 4.6 mm, S = 3 μm) and a guard column C18 (4 × 3 mm), both from Phenomenex (Torrance, CA, USA). Mobile phase A consisted of formic acid:H2O (1:99, *v*/*v*), and mobile phase B consisted of 10% formic acid:H2O (1:99, *v*/*v*), 50% methanol, and 40% acetonitrile. The gradient was expressed in terms of mobile phase B: 0−1 min 10% B, 1−5 min 20% B, 5−20 min 50% B, and 20−23 min 95% B, and this was followed by column flushing and equilibration over the next 14 min. The operating conditions were as follows: flow rate at 0.5 $mL \cdot min^{-1}$, column temperature at 40 °C, autosampler temperature at 15 °C, injection volume at 5 μL, time of analysis at 37 min, detection wavelength at 525 nm, and spectral acquisition at 300−600 nm. Identification was performed by comparing the retention times and absorption spectra with those of analytical standards. Quantification was carried out by means of

external calibration, which was based on the peak area (0.05−10 $\mu g \cdot mL^{-1}$). The exact concentrations of the analytical standards were calculated using molar absorptivity (ε).[22]
The results of all the determined and quantified phytochemicals by means of HPLC separations (SPAs, CWBPAs, carotenoids, and anthocyanins) were expressed as $mg \cdot kg^{-1}$ of DW.

Determination of the Antioxidant Capacity by Means of ABTS (AC_{ABTS}) and FRAP Assays (AC_{FRAP})
ABTS and ferric reducing antioxidant power (FRAP) assays were employed to determine the AC of the flour following the QUENCHER procedure (direct measurement on solid samples), as described by Serpen et al.[23] Both of the methods allow the evaluation of the electron-donating potency of the antioxidant compounds of the solid matrix, by scavenging the synthetic ABTS radical cation and by reducing the Fe^{III} to Fe^{II}.
For AC_{ABTS} analysis, the whole-meal flour was mixed with the ABTS reagent, which was previously prepared by reacting an aqueous solution of ABTS with potassium persulfate and ethanol in a 1:1 *v*/*v* ratio. The mixture was allowed to react on a shaker at 20 °C for 30 min, immediately followed by centrifugation and measurement of absorbance at 734 nm. For the determination of AC_{FRAP}, the whole- meal flour was mixed with FRAP working solution (10 mM TPTZ, 20 mM $FeCl_3$, and 300 mM sodium acetate buffer of pH 3.6, 1:1:10, *v*/*v*/*v*) and methanol in a 99:1 *v*/*v* ratio. The mixture was allowed to react on a shaker at 20 °C for 120 min, immediately followed by centrifugation and measurement of absorbance at 593 nm. The detailed procedures for both assays are described in Serpen et al.[24] The results were expressed as mmol Trolox equivalents (TE)$\cdot kg^{-1}$ of DW by means of a Trolox dose−response curve.

Statistical Analyses
The obtained data were compared, by means of an analysis of variance (ANOVA), to evaluate the effect of thegenotype (G), the environment (E), the N fertilization (N), and their interactions on the yield, the grain qualitative parameters, and on the content of bioactive compounds of the whole-meal flour. Means were identified as significantly different ($p < 0.05$) on the basis of the Ryan−Einot−Gabriel−Welsch F (REGW-F) statistical test. Prior to the analysis, data were tested for normality and homoscedasticity. The analyses were carried out by means of the SPSS for Windows statistical package, version 28.0 (SPSS, Inc., Chicago, IL, USA). A principal component analysis (PCA) was performed to investigate the relationships between the bioactive compounds and the antioxidant properties of the analyzed genotypes grown under the three different environmental conditions. The data were first standardized by subtracting the means and dividing the result by the standard deviations of each variable. The multivariate analysis was carried out by means of the Past4Project for Windows data analyzer, version 4.03.

Results and discussion

Meteorological Data and Agronomic Conditions

The meteorological data recorded during the two growing seasons in the three research sites are shown in Table 3. High rainfall occurred in the second growing season (2019−20), particularly in November and December, thus leading to a probable higher N leaching at the end of winter in environment A. Overall, the rainfall distributions and the temperatures from flowering (May) to the end of the ripening stage (June) were similar in all the considered production systems, thereby resulting in comparable weather conditions across the three environments. As far as the N content of the soil is concerned (Table 2), the environment C (Carmagnola, on a silty-loam deep soil) was higher than environments B and A (Cigliano, on a sandy-loam soil). According to soil physical and chemical parameters, the expected mineralized N within the growing season increased from environment A to B and C.[25] The different plant N availability in the compared environments can be directly deduced from the crop response: the AUCGC index, which expresses both the plant vigor and the leaf chlorophyll content within the growing season and which is strictly influenced by the N availability,[17] the GY, and the GPC increased proportionally from environment A to C (Table 2).

Table 3. Monthly rainfall, rainy days, and growing degree days (GDDs)[a] from sowing (November) to the end of the ripening stage (June) in the field experiments.

Environments	Months	Rainfall (mm)	Rainy days (n°)	GDDs (Σ °C·d^{-1})
A	November	314	12	249
Cigliano (VC), 2019-20	December	132	8	193
	January	5	1	168
	February	1	0	229
	March	62	7	285
	April	81	10	414
	May	122	7	579
	June	113	7	624
	November - June	830	52	2741
	November - March	514	28	1124
	April - June	316	24	1617
B	November	124	5	292
Cigliano (VC), 2018-19	December	11	1	151
	January	6	1	141
	February	43	7	195
	March	17	4	314
	April	116	7	393
	May	178	9	478
	June	40	4	667
	November - June	535	38	2632
	November - March	200	18	1093
	April - June	335	20	1539
C	November	118	17	281
Carmagnola (TO), 2018-19	December	9	3	110
	January	7	2	62
	February	32	3	133
	March	4	1	305
	April	110	12	377
	May	97	12	484
	June	36	7	694
	November - June	413	57	2445
	November - March	170	26	890
	April - June	243	31	1555

[a]Accumulated growing degree days for each month using a 0 °C base. Source: Rete Agrometeorologica del Piemonte, Regione Piemonte, Assessorato Agricoltura, Settore Fitosanitario, Sezione di Agrometeorologia.

Grain Yield and the Physical and Qualitative Parameters

Three-way ANOVA showed significant effects of all the three investigated factors (genotype, environment, and N fertilization) on the GY and TKW of the 14 evaluated cereal genotypes (Table 4). The factor that had the most important effect on GY was the environment (around 54% of the variation), followed by the N fertilization and the genotype (29 and 6%). On the other hand, the genotype was the factor that had the most effect on TKW, accounting for almost 70% of the variation. As expected, the recently developed pigmented genotypes showed a significantly lower productivity than the common red variety (Table 5), which had been chosen as it is one of the most cultivated varieties in North Italy, and it is characterized by a high-yield potential. However, a great variation was observed for GY and TKW among and within the color lines. Compared to the control, the lowest GY (−34 and −27%) and the lowest TKW (−9 and −3%) were observed for the Ye cereals, that is, tritordeum and wheat, respectively (Figure 2A,B). Similarly, a low average GY was recorded for the Ba wheats, whereas the Pp and Pp + Ba groups showed higher GY values for the pigmented genotypes, although they were still significantly lower than the control. The TKW trait showed differences among the groups, with Pp + Ba > Ba > Pp > red > Ye wheat > Ye tritordeum, possibly due to differences in their grain shapes as a result of the strong genotypic component.

The GY and TKW were influenced by the environment to a great extent, although at different rates in relation to the genotype (Table 4). A significantly higher average yield was observed in the environment with high soil N content (C, 6.5 $t \cdot ha^{-1}$), followed by that of the B environment (4.8 $t \cdot ha^{-1}$), both significantly different (+42 and +4%) from A (4.6 $t \cdot ha^{-1}$), as shown in Table 5. In agreement with previous studies,[12,13,17,26] the N fertilization rate affected the productivity of all the tested varieties to a great extent, but it did so at a different rate in relation to the genotype (Table 4). The application of 160 and 80 kg of $N \cdot ha^{-1}$ resulted in increases in GY of about 34 and 21%, respectively, when compared to 0 kg of $N \cdot ha^{-1}$ (6.0 and 5.4 vs 4.5 $t \cdot ha^{-1}$, Table 5). As expected, TKW dropped along with the N fertilization rate and the soil N content in the environments as a trade-off between the seed number and the seed size.

The E × N was the interaction that influenced GY the most (around 9% of the variation), whereas it contributed to TKW to a lesser extent (1%), although the effect was still significant (Table 4). The increase in GY, as a consequence of N fertilization, was not significant in the C environment, presumably because of the higher N content of the soil in the site (data not shown).

Table S2 shows the qualitative parameters of the whole-meal flour of the investigated genotypes. The wheat group with Ba was characterized by the lowest mean TW (73.8 $kg \cdot hL^{-1}$, −5% with respect to the control). The pigmented genotypes showed similar or higher GPC (11.5, 12.3, 12.5, and 12.9% for the Pp, Ba, Pp + Ba, and Ye groups, respectively) than the commercial variety (11.5%), as previously observed by some other authors,[21,27] and were thus characterized by good product- based utilization features. A comparison of the technological and rheological properties of the wheat

samples is provided in Table S3. The farinograph evaluation and the baking test showed a comparable bread-making aptitude of the pigmented wheat genotypes to that of the control.

Table 4. Level of Significance of the Three-Way ANOVA Analyses Performed to Evaluate the Contribution of the Genotype, the Environment, the N Fertilization, and Their Interaction on the Agronomic Traits, Bioactive Compounds, and Antioxidant Capacity of the Whole-Meal Flour of the Investigated Wheat and Tritordeum Genotypes[a]

Factor	GY	TKW	SPAs	CWBPAs	TAC	TCC	AC_{ABTS}	AC_{FRAP}
Genotype (G)	6.4 ***	69.9 ***	44.0 ***	13.3 ***	45.5 ***	97.3 ***	1.6 ***	29.1 ***
Environment (E)	54.0 ***	21.8 ***	41.1 ***	57.9 ***	49.4 ***	1.2 ***	97.3 ***	47.2 ***
N fertilization (N)	29.1 ***	0.9 *	0.6	10.8 ***	0.6 ***	0.1 *	0.1	3.4
G × E	0.9 ***	4.8 ***	8.9 ***	10.7 ***	4.1 ***	1.0 ***	0.3 ***	12.1 ***
G × N	0.1	0.7 ***	2.3 ***	0.9	0.1	0.1 **	0.2 ***	3.1 **
E × N	9.2 ***	1.2 **	0.1	4.5 ***	0.1	0.1 **	0.3 **	0.6
G × E × N	0.1	0.4 *	2.0 ***	1.2 **	0.1	0.1	0.1 ***	2.9 **
Error	0.1	0.3	0.9	0.7	0.1	0.0	0.1	1.6

[a]The results are expressed as percentages of the total mean square. GY, grain yield; TKW, thousand kernel weight; SPAs, soluble phenolic acids; CWBPAs, cell wall-bound phenolic acids; TAC, total anthocyanin content; TCC, total carotenoid content; AC, antioxidant capacity (ABTS and FRAP assays). The marked factors are statistically significant, according to the REGW-F test [(*) p (F) < 0.05, (**) p (F) < 0.01, and (***) p (F) < 0.001].

Table 5. Effect of the Genotype, the Environment, and the N Fertilization on the Agronomic Traits, Bioactive Compounds, and Antioxidant Capacity of the Whole-Meal Flour of the Investigated Wheat and Tritordeum Genotypes[a]

Factor	Source of variation		GY ($t \cdot ha^{-1}$)	TKW (g)	SPAs ($mg \cdot kg^{-1}$)	CWBPAs ($mg \cdot kg^{-1}$)	TAC ($mg \cdot kg^{-1}$)	TCC ($mg \cdot kg^{-1}$)	AC_{ABTS} (mmol TE$\cdot kg^{-1}$)	AC_{FRAP} (mmol TE$\cdot kg^{-1}$)
Genotype	Red	Aubusson	6.6 a	43.1 f	106 gh	931 defg	< LOD	1.9 f	16.0 cd	10.0 c
	Pp	AnthoGrain™	4.8 e	51.9 b	142 cde	967 cdef	13.9 e	2.7 c	17.4 b	12.2 a
		Ceraso	5.8 bc	50.1 cd	146 bcd	992 bcde	14.9 e	1.7 g	17.0 bc	11.8 ab
		AF Jumiko	6.1 b	45.2 e	133 ef	831 hi	1.2 g	1.7 g	16.6 bc	11.3 b
		KM 106-18	5.4 cd	53.2 a	145 bcd	926 defg	1.9 g	2.1 e	16.7 bc	10.4 c
		Merlot	6.0 b	51.2 bc	137 def	891 fgh	2.5 g	1.6 g	15.6 de	10.2 c
		Rosso	5.7 bcd	49.9 d	146 bcd	995 abcd	6.4 f	2.6 d	15.0 e	11.5 ab
	Ba	Skorpion	3.6 g	51.9 b	111 g	1051 abc	18.9 d	1.9 f	15.5 de	10.3 c
		AF Oxana	5.5 cd	49.8 d	147 bcd	842 ghi	18.8 d	1.7 g	17.1 b	10.6 c
		KM 72-18	4.8 e	53.5 a	165 a	1073 a	21.9 c	1.7 g	15.6 de	10.5 c
	Pp + Ba	AF Zora	5.3 d	50.9 bcd	153 bc	1041 abc	42.9 a	1.7 g	16.7 bc	11.9 ab
		KM 98-18	5.8 bcd	53.3 a	155 ab	1066 ab	25.9 b	1.3 h	16.7 bc	11.7 ab
	Ye	Bona Vita	4.8 f	41.6 g	126 f	916 efg	< LOD	5.6 b	16.8 bc	10.2 c
	Trit	Bulel	4.3 f	39.3 h	99 h	793 i	< LOD	8.8 a	18.5 a	10.2 c
Environment		A	4.6 c	49.5 a	126 c	1022 a	22.9 a	2.5 c	12.5 c	11.3 a
		B	4.8 b	49.6 a	138 b	968 b	13.4 b	2.8 a	17.7 b	10.5 c
		C	6.5 a	47.5 b	145 a	859 c	9.8 c	2.6 b	19.5 a	10.9 b
N Fertilization (kg of N$\cdot ha^{-1}$)		0	4.5 c	48.9 ab	136 a	993 a	16.0 a	2.7 ab	16.5 a	11.0 a
		80	5.4 b	49.0 a	137 a	927 b	15.5 a	2.7 a	16.4 a	10.9 a
		160	6.0 a	48.6 b	135 a	933 b	14.6 b	2.6 b	16.6 a	10.8 a

[a]Pp, purple pericarp; Ba, blue aleurone; Pp + Ba, black grain; Ye, yellow endosperm; Trit, tritordeum; GY, grain yield; TKW, thousand kernel weight; SPAs, soluble phenolic acids; CWBPAs, cell wall- bound phenolic acids; TAC, total anthocyanin content; TCC, total carotenoid content; AC, antioxidant capacity (ABTS and FRAP assays); LOD, limit of detection. The results are expressed on a DW basis. Means followed by different letters are significantly different, according to the REGW-F test. The ANOVA level of significance is shown in Table 4.

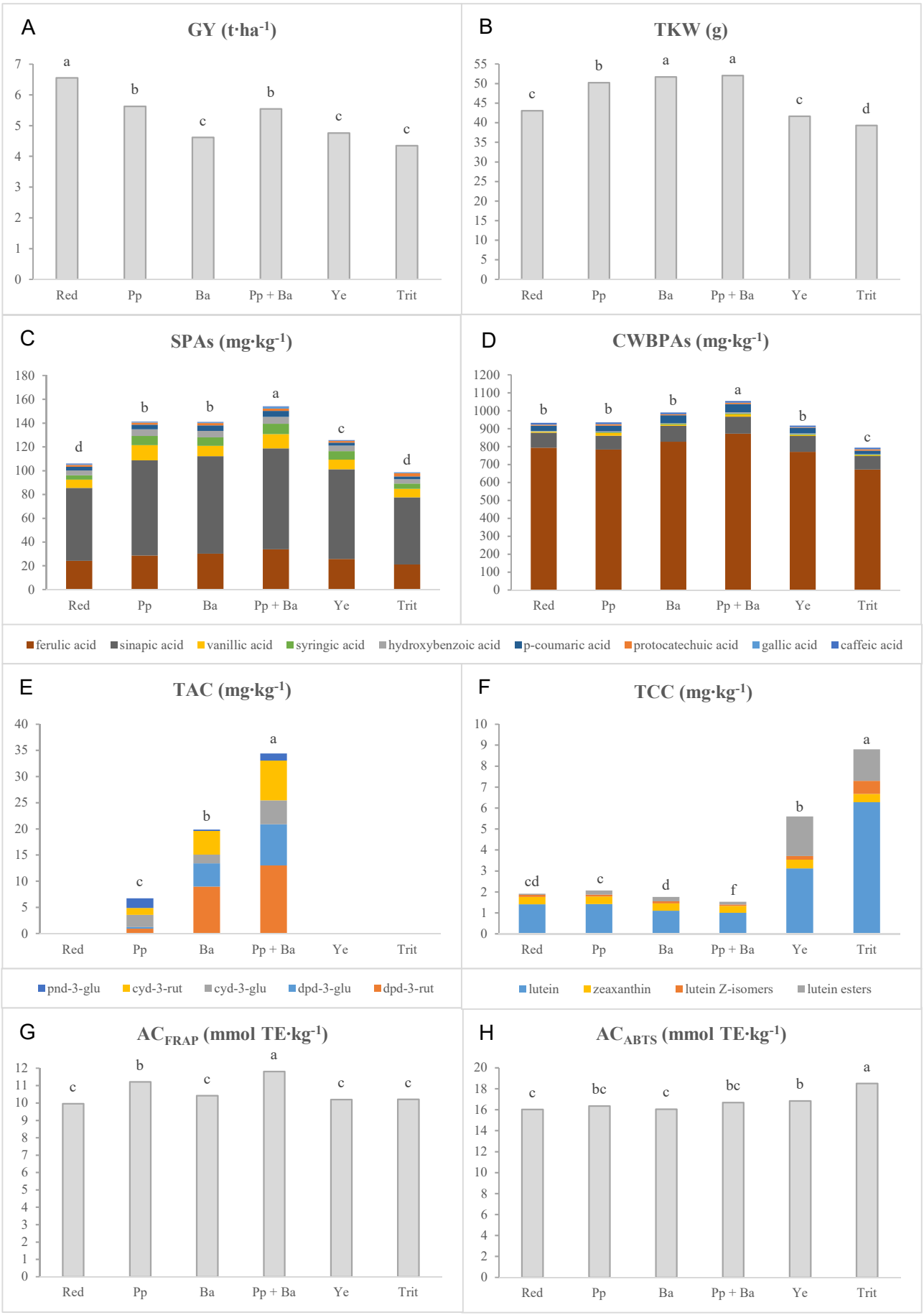

A
GY (t·ha-1)
B
TKW (g)
C
SPAs (mg·kg-1)
D
CWBPAs (mg·kg-1)
ferulic acid
sinapic acid
vanillic acid
syringic acid
hydroxybenzoic acid
p-coumaric acid
protocatechuic acid
gallic acid
caffeic acid
E
TAC (mg·kg-1)
F
TCC (mg·kg-1)
pnd-3-glu
cyd-3-rut
cyd-3-glu
dpd-3-glu
dpd-3-rut
lutein
zeaxanthin
lutein Z-isomers
lutein esters
G
ACFRAP (mmol TE·kg-1)
H
ACABTS (mmol TE·kg-1)
Red
Pp
Ba
Pp + Ba
Ye
Trit

Figure 2. Comparison of the agronomic traits (A, B), bioactive compounds (C, D, E, F) and antioxidant capacity (G, H) of the investigated wheat and tritordeum genotypes grouped according to the grain color.

Pp, purple pericarp; Ba, blue aleurone; Pp + Ba, black grain; Ye, yellow endosperm; Trit, tritordeum; GY, grain yield; TKW, thousand kernel weight; SPAs, soluble phenolic acids; CWBPAs, cell wall-bound phenolic acids; TAC, total anthocyanin content; pnd-3-glu, peonidin-3-glucoside; cyd-3-rut, cyanidin-3-rutinoside; cyd-3-glu, cyanidin-3-glucoside; dpd-3-glu, delphinidin-3-glucoside; dpd-3-rut, delphinidin-3-rutinoside; TCC, total carotenoid content; AC, antioxidant capacity (FRAP and ABTS assays). The results are expressed on a DW basis. Different letters above the columns indicate significant differences between the six groups at p (F) < 0.05, according to the REGW-F test.

Soluble and Cell Wall-Bound Phenolic Acids

Small cereals contain phenolic compounds as secondary metabolites that are naturally synthesized in response to various biotic and abiotic stress factors, and they are mainly appreciated for their antioxidant properties. Phenolic acids constitute one of the main groups of phenolic compounds that have been identified in wheat. They exist in cereals in soluble-free, soluble- conjugated, and insoluble-bound forms, with most of them being present in the aleurone layer and in the bran linked to cell wall materials, such as polysaccharides and lignans, through ester bonds.[28] The insoluble fraction has been associated with more effective health improvement than the soluble forms. In fact, phenolics bound to dietary fiber may survive upper gastrointestinal digestion and be released through the activity of the intestinal microflora, thus exerting antioxidant effects in the colon or other tissues after absorption and slow release into the bloodstream.[29] In this work, soluble-free and conjugated acids were extracted together and analyzed as free aglycones by HPLC, since the free form usually constitutes a small proportion of the total content (<1%).[28,30,31] As expected, CWBPAs represented most of the total content of the phenolic acids, on average accounting for more than 87%. The HPLC/ DAD analysis revealed that ferulic acid was the primary phenolic acid in the bound form (Figure 2D), representing more than 84% of the total CWBPAs in all the genotypes, followed by sinapic (9%) and *p*-coumaric acids (4%), whereas sinapic and ferulic acids constituted the majority of SPAs (57 and 21%), followed by a small share of vanillic acid (8%). The relative distribution of SPAs and CWBPAs did not differ to any great extent between genotypes or between color groups, although other works have described different phenolic compound profiles between wheat genotypes.[32] However, Paznocht et al.[30] observed that the share of individual phenolic acids over the total content was very similar across the analyzed pigmented wheat groups, and the shares of soluble and bound forms over the total concentration (8 and 92%) they observed were similar to those of the present study. These results are in line with the values reported by Martini et al.[31] (14 and 86%), who did not observe a great variability in the SPA and CWBPA spectrum across durum wheat genotypes (with the exception of the soluble free forms, which, however, accounted for less than 1% of the total phenolic acids). In the same way as for wheat, the CWBPA content of tritordeum was higher than those of the SPAs. Moreover, the phenolic acid profile was similar to the one observed for the pigmented wheats, a result that is consistent with previously reported findings. Giordano et al.[19] observed that the SPA and CWBPA profiles of tritordeum were closer to those of durum and bread wheat cultivars than to those of barley. Navas-Lopez et al.[33] reported a similar percentage of ferulic acid in tritordeum and in wheat, with respect to the total phenolic compounds.

All the three factors analyzed with three-way ANOVA showed significant effects on the CWBPA content, whereas only the genotype and the environment had significant effects on the SPA content (Table 4). The genotype accounted for most of the variation observed for the SPAs (44%) and, albeit to a lesser extent, for the CWBPAs (13%). Overall, all the pigmented groups had similar or higher

concentrations of SPAs and CWBPAs than the control, with the exception of tritordeum (Figure 2C,D). This result is in agreement with previous studies performed on the whole-meal flour of pigmented genotypes. Paznocht et al.[30] classified the analyzed pigmented wheat groups according to the total phenolic acids in wholegrain flour in descending order as follows: Ba > Pp > Ye > red. Ma et al.[34] reported a 20% higher phenolic acid content in Pp wheats than in control varieties. In the present study, the Pp + Ba genotypes had the highest contents of both SPAs and CWBPAs, that is, 45 and 13% more than the control, a result that is in agreement with previous works performed on whole-meal black wheat flour.[5,35] As shown in Table 5, the highest concentrations of both SPAs and CWBPAs were observed in the KM 72-18 Ba genotype (165 and 1073 mg· kg^{-1}), while tritordeum presented the lowest values (99 and 793 mg·kg^{-1}).

The marked effect of the environment factor on the SPAs and CWBPAs (41 and 58%) resulted in higher concentrations of SPAs in the C (+14%) and B (+9%) environments than in the A one (Table 5). The CWBPA content showed an opposite trend, with increases of 19 and 13% in the grains obtained from the A and B environments, compared to the C one. The N fertilization rate induced consistent CWBPA results, with the highest values being measured in the samples cultivated in the unfertilized plots (+6% with respect to plots treated with 160 kg of N·ha^{-1}), while no significant differences were detected on the SPA contents at different N rates.

The interaction that significantly influenced both the SPA and CWBPA contents the most was G × E (around 9 and 11% of the variation), followed by G × N for the SPAs (2%), with the N effect only being significant for the Ceraso and AnthoGrain™ genotypes (Pp), and E × N for the CWBPAs (4%). Specifically, the higher CWBPA content associated with lower N fertilization rates was noticeable in B and C, whereas no significant difference was observed in the A environment (data not shown).

The effect of N on the contents of the phenolic compounds of plants in greenhouse or growth chamber experiments has been studied by various authors.[35−37] As wheat phenolics seem to be more abundant in immature grains (in the milky and soft stages) and to decrease during the maturity process,[13,32] such types of stress as N deficiency, which interferes with the normal maturation process, may lead these secondary metabolites to remain in higher concentrations in mature kernels. Furthermore, according to the “C/N balance theory”, when N availability is limited, plants change their metabolism toward carbon-containing compounds, such as starch, cellulose, and non-N-containing secondary metabolites, including phenolic acids and terpenoids. Moreover, the accumulation of phenolic acids has been associated with the expression of genes involved in the phenylpropanoid path- way.[36] Under conditions of reduced N availability, the phenylalanine ammonia lyase enzyme catalyzes the deamination of phenylalanine, thereby releasing ammonia and cinnamic acid. The produced ammonia can be recycled to generate the amino acids required for the biosynthesis of proteins and to sustain plant growth,[37] while cinnamic acid can be directed toward different biosynthetic pathways to form various phenylalanine-derived phenolics, such as phenolic acids or anthocyanins.[36,38] However, the aforementioned studies monitored the effect of an N

deficiency on the leaf tissues under controlled conditions, whereas the results from experiments performed under field conditions on cereal grains offered conflicting information. N fertilization has been reported to induce little[9] or no change[8] in the phenolic content of wheat grains, while other authors have observed an increase in the soluble,[13] or even in both the soluble and insoluble,[15] phenolic acid fractions. These contrasting results may be due to the different field conditions in the above-mentioned studies, such as in the meteorological trends, in the physical and chemical characteristics of the soil and/or in the agronomic management. In the present study, the environment, by comparing experiments with different soil N contents, had a more pronounced effect on the accumulation of phenolic acids than the N fertilization rate. A first explanation is related to the role that the different meteorological trends and the complex of soil properties could have on the accumulation of these bioactive compounds.[8,9,11,13−15] Furthermore, according to the reported agronomic indexes (e.g. AUCGC, GY, and GPC), which are strictly related to the soil N availability for the plant, the environment was characterized by a more marked inter-experiment variation than that among N fertilization rates, thus suggesting a greater difference in crop N nutrition within the compared environments. However, little is known about the effect of the soil N content on the accumulation of secondary metabolites, which are assumed to have beneficial effects on human health, in pigmented wheat grains. Zrcková et al.[39] investigated the impact of the cropping system (conventional and organic, with N availability expected to be lower in the latter one) on the antioxidant content of pigmented wheats. The authors observed that the cropping system significantly affected the contents of all the determined groups of antioxidant compounds, even though to a lesser extent than the year and the genotype, and this led to higher concentrations being observed in most of individual genotypes grown under the organic system. However, they only determined the total phenolic acid component. In the present study, the SPAs and CWBPAs showed opposite trends, which were reflected by the two most abundant identified phenolics, soluble sinapic acid and insoluble ferulic acid, thus indicating that the different forms of phenolic acid may respond differently to N fertilization rates as well as to various soil N contents over the compared environments. Further studies are therefore needed to investigate the biosynthesis mechanisms of different phenolic acid types, as there might be differences in the synthesis processes of soluble and insoluble fractions due to their specific storage sites, solubilities, and their roles within the plant organism. Furthermore, the individual genotypes reacted differently to increased soil N contents and increased N applications (data not shown) in terms of SPA and CWBPA contents, in a similar way to what Ma et al.[40] observed in Pp wheat varieties supplied with different combinations of N and phosphorus fertilizer treatments, thus suggesting the impor- tance of selecting specific genotypes that are naturally rich in bioactive compounds in suitable growing environments.

Anthocyanins

Anthocyanins are a major class of red to blue flavonoid pigments that are extensively represented in plants but an unconventional and rare trait in wheat, where they differ significantly among genotypes and are highly correlated with the grain pigmentation. No pigmented forms of bread wheat existed in the past, but then breeders took advantage of and introgressed the ability to accumulate purple or blue pigments from tetraploid and diploid landraces or wild relatives of wheat.[3] Black lines were later developed through the hybridization of Pp and Ba wheats.[5] In the present study, the genotype factor had a significant impact on the total variance, on average accounting for 46% of the observed variation (Table 4). The total anthocyanin content (TAC) ranged between 1.2 mg·kg^{-1} of AF Jumiko (Pp) and 42.9 mg· kg^{-1} of AF Zora (Pp + Ba), as shown in Table 5. No anthocyanins were detected in the control variety or in the Ye genotypes, in either wheat or tritordeum. Other studies have stated that the grain color and anthocyanin content are mainly controlled by the genotype.[21,26] Considerable differences in the total contents were also observed among the individual color groups (Figure 2E). The highest TAC was observed in the Pp + Ba genotypes (34.4 mg·kg^{-1}), whose whole-meal flour was on average characterized by 5.1 and 1.7 times greater contents than those of the Pp (6.8 mg·kg^{-1}) and Ba (19.9 mg·kg^{-1}) wheats. Anthocyanins have been identified and quantified in other studies in various pigmented-grain wheats, with different contents and compositions. Ficco et al.,[21] by means of HPLC analysis, observed total mean contents of 11.8 and 68.4 mg· kg^{-1} for Pp durum wheat and Ba bread wheat genotypes, respectively, while the total content of anthocyanins observed by Hosseinian et al.[41] in Pp wheat samples was 447.6 mg·kg^{-1}. These differences between studies could be attributed to the variations in wheat genetics and physiology (which depend on the donor and recipient cultivars used for the development of the germplasm), the environmental conditions, the extraction and quantification methods, and to the number of identified compounds.[3] Overall, the results of the present study are in agreement with earlier works in which Pp + Ba genotypes were characterized by the highest TAC and were followed by Ba and Pp lines.[27,35,42] The analysis of individual anthocyanins showed that the genotypes grouped according to the grain color not only differed concerning their total content but also in the anthocyanin profile (Figure 2E). As in previous studies, the most common anthocyanin in Pp wheats was found to be cyd-3-glu, followed by pnd-3-glu.[27,42–44] Abdel-Aal et al.[43] and Escribano-Bailón et al.[44] found dpd-3-glu to be the most abundant anthocyanin in Ba wheats, while Sharma et al.[27] found cyd-3-rut made a higher contribution. In this study, dpd- 3-rut accounted for 45 and 38% of the total anthocyanins in the Ba and Pp + Ba groups, respectively, while dpd-3-glu and cyd-3-rut were the second most abundant ones. Delphinidin glycosides, which are responsible for the blue color, were mainly detected in the Ba and Pp + Ba groups, while cyanidin and peonidin glycosides were detected above all in the Pp group, similarly to what Giordano et al.[45] reported. Furthermore, the Pp + Ba wheats exhibited a larger proportion of the glycosides mainly detected in the Pp genotypes than the Ba wheats.

In addition to the genotypic component, growing conditions can also affect anthocyanin accumulation.[41] As shown in Table 4, the environment factor accounted for around 49% of the observed variation, followed by the G × E effect (4%), which was the only significant interaction observed for TAC. The smallest concentration of anthocyanins was observed in A(Table 5), and increasing values were observed in the B (1.4 times) and C (2.3 times) environments (9.8 vs 13.4 and 22.9 mg·kg^{-1}). This trend was observed in all the individual genotypes at different levels, with the exception of AF Zora (Pp + Ba), which on average showed the highest TAC for the plots in the B environment (data not shown). The effect of different N treatments was limited (0.6%), although still significant, and consistent with the effect of the environment, according to the soil N content, with higher values recorded for 0 and 80 kg of N·ha^{-1} than for the 160 supply (+9 and +6%). In agreement with the findings of the present study, Fan et al.[26] observed an increased accumulation of anthocyanins in Ba, Pp, and bread wheat grains for reduced applications of Zrcková et al.[39] reported a higher TAC in the organic cropping system and assumed that the probable N deficiency of organically cultivated plants led to a higher surface/volume ratio of the wheat kernels and, thus, to a higher percentage of the pericarp and of the aleurone layer, where the majority of antioxidant compounds, including anthocyanins, accumulate. Our data related to TKW do not support this hypothesis, as the increased soil N content in the present work led to a higher tiller capacity and, thus, to smaller kernels. The observed correlations between TKW and the analyzed bioactive compounds were dependent on the investigated genotypes (data not shown).

The greater anthocyanin accumulation observed for low N levels might be ascribable to the stimulation of the phenyl- propanoid pathway as a result of the N limitation, as previously mentioned for phenolic acids, and it might explain the much greater variation observed between environments compared to the different N fertilization rates, where differences in N limitation could probably be less marked, as reported by the plant agronomic response. Furthermore, anthocyanin-specific genes, as well as the genes involved in anthocyanin glycosylation and sequestration in the vacuole, were found to be highly expressed in response to nutrient deficiency and downregulated under a high N fertilizer application.[26,46] However, the majority of findings about anthocyanin response to nutrient signaling at a genetic and molecular level are based on *Arabidopsis* and fruit crops.[38,46] The increased interest in pigmented cereals registered globally will require the transfer of knowledge to cereal crops.

Carotenoids

Carotenoids are characterized by a wide variety of biological functions, in both plant and human tissues, and these functions are mainly determined by their molecular structure.[4] Their profile in cereals is mainly composed of xanthophylls (the oxygen-containing carotenoids), and lutein is usually the most abundant in wheat grains, followed by zeaxanthin, antheraxanthin, and small amounts of carotenes (hydrocarbons that contain no oxygen), that is, *α*- and *β*- carotenes.[3]

Carotenoids predominantly occur in plants as *E*- isomers, but *Z*-isomers of lutein and zeaxanthin can be detected, as a consequence of the photochemical isomerization of their all-*E*-isomers, which involves changes in their biological properties, bioavailability, and in their antioxidant activity.[47] Furthermore, some cereals have the ability to form xanthophyll mono- and diesters, which enables the accumulation of greater amounts of pigments in grains and, according to the findings of some authors, protects xanthophylls against degradation and improves bioaccessibility during digestion.[48] Lutein esterified with fatty acids has not been found in bread or durum wheat grains, or it has been found at low concentrations, while a much larger degree of esterification has been observed in tritordeum cultivars.[6] Three-way ANOVA (Table 4) showed significant effects of all three factors on the total carotenoid content (TCC), although genotype accounted for most of the observed variation (>97%). This is in agreement with the high heritability and the low G × E interaction that characterizes this trait, which has led to a wide variation in the content of carotenoids among cereal species and varieties.[48] As shown in Figure 2F, the anthocyanin-rich varieties were found to have a low TCC, with the lowest concentration observed in the Pp + Ba group (1.5 mg·kg^{-1}). The Ba and Pp genotypes did not differ significantly from the red-grained control, while the Ye wheat had the highest TCC among the pigmented wheats (5.6 mg·kg^{-1}). Overall, tritordeum presented the highest TCC (8.8 mg·kg^{-1}), which was 5.8, 5.0, 4.6, 4.3, and 1.6 times higher than the Pp + Ba, Ba, red, Pp, and Ye wheats. Differences were also found within the same color group (Table 5), especially among wheats with Pp, due to the wide multiplicity of evaluated genotypes, with a marked superiority of the AnthoGrain™ genotype (2.7 mg·kg^{-1}), followed by Rosso and KM 106-18 (2.6 and 2.1 mg·kg^{-1}). The carotenoid that was detected the most was lutein, which represented 66% of the total content (a 2.64 mg·kg^{-1} average of all the genotypes). Lutein esters and zeaxanthin were also found in smaller amounts, accounting for 15 and 14% of the total content, while lutein *Z*-isomers on average represented <5%. The carotenoid profiles were similar in the red, Pp, Ba, and Pp + Ba wheats, while the average zeaxanthin content of the Ye genotypes differed and was only 6% of the total carotenoids, compared to the other color groups (average of 19%). Furthermore, the Ye wheat was characterized by a higher share of lutein esters than all the other analyzed genotypes (34 vs 10%) and by the highest absolute value (1.89 mg·kg^{-1}). Lutein *Z*-isomers were mainly found in tritordeum (0.63 vs 0.09 mg·kg^{-1}).

Despite the high genetic effect on carotenoid accumulation, growing conditions have also been reported to cause changes in TCC and individual compounds.[10,48] In the present study, the environment had a significant effect on TCC, as did N fertilization, but, overall, they accounted for a small share of the total variation, that is, 1.2 and 0.1%, respectively (Table 4). The effects of the G × E, G × N, and E × N interactions were very limited (1.0, 0.1, and 0.1%, respectively), although still significant (Table 4). The findings of previous studies investigating the effect of N fertilization on the carotenoid content are contradictory, with an overall limited role for this parameter and a much more

variation being attributed to differences in weather conditions between years, mainly in the average temperature patterns and rainfall distribution.[10,15,48]

Antioxidant Capacity of the Whole-Meal Flour and Multivariate Analysis

The obtained results showed a highly significant effect of the genotype on AC, which was determined by employing ABTS and FRAP methods (2 and 29% of the observed variation; Table 4). Tritordeum showed the highest AC_{ABTS} (18.5 mmol of TE·kg^{-1}), followed by the Ye wheat (16.8 mmol of TE·kg^{-1}), while their AC_{FRAP} was significantly lower (10.2 mmol of TE·kg^{-1}, mean value of the tritordeum and Ye wheat) than the Pp + Ba and Pp groups (mean value of 11.8 and 11.2 mmol of TE·kg^{-1}), and did not differ from that of the red and Ba genotypes (Figure 2G,H). The Pp + Ba group recorded the highest AC_{FRAP}, and the two black genotypes did not differ significantly from each other (Table 5). A wide variability was observed within the Pp group, with AnthoGrain™ presenting the highest measured AC for both assays (17.4 mmol of TE·kg^{-1} of AC_{ABTS} and 12.2 mmol of TE·kg^{-1} of AC_{FRAP}). Overall, the results obtained by the ABTS assay were more heterogenous among genotypes and higher than those obtained by FRAP. These two assays are widely used in the characterization of foods, and their reactivity toward antioxidants is very different: this may explain the significant difference between their reported patterns when sorted by pigmented groups (Figure 2G,H).

The environment was the most prominent factor contribu- ting to the overall variation observed for both AC_{ABTS} and AC_{FRAP} (97 and 47%), which may be attributable to the greater effect of this factor on the individual groups of monitored analytes, except for the TCC (Table 4). Nevertheless, more marked differences were observed for AC_{ABTS} than AC_{FRAP} and the results differed, depending on the considered type of assay (Table 5), with AC_{ABTS} presenting higher levels in the C and B environments compared to A (+55 and +10%) and AC_{FRAP} being the highest in the A environment (+8% compared to B, which provided the lowest mean value). The effects of the interaction were limited (<1%) for AC_{ABTS}, although still significant, while G × E was the most important interaction for AC_{FRAP}, accounting for 12% of the observed variation (Table 4), due to different responses of the genotypes in the compared environments.

Phenolic compounds have been associated with the ability to capture reactive oxygen species in other studies on cereals, such as wheat,[21,45] and it has been pointed out that an enhanced accumulation of biologically active compounds, such as anthocyanins and carotenoids, in pigmented cereal grains can lead to additional effects.[27] In the present study, the procedure adopted for the determination of the AC with both of the assays did not involve sample treatments such as chemical or enzymatic hydrolysis prior to the measurement. As shown by Serpen et al.,[49] the direct analysis of whole-meal samples enables to measure the total AC of cereals, allowing the soluble, but also the insoluble fraction with functional groups on the surface, to simultaneously come into contact with the ABTS radical and with the ferric ions of the FRAP assay, taking advantage of the surface reaction occurring at the solid−liquid interface. The direct procedure was applied in order to retain the

synergistic effect of antioxidants that is partially lost when single compounds are extracted and analyzed for the AC[23] and to better relate the data with the antioxidant effects exerted in the human gastrointestinal tract, where a simultaneous action of all the antioxidants present in the samples is expected.[1] However, using different methods to estimate the scavenging or reducing activity of an extract containing solid matrix can lead to different results. The different response of AC_{ABTS} and AC_{FRAP} to the genotype and the environment factors can be explained by considering the chemical mechanisms and the solvent compositions of the assay solutions. Although the redox potential is comparable between the assays, the reaction conditions differ, particularly the pH at which the reaction is performed, and the steric requirements of the oxidizing molecules and the ferric *di*- TPTZ and ABTS.[50] Moreover, the reaction medium significantly affects the measured AC, as it acts not only as a reactant carrier but also as a solubilizer of the food matrix.[24] The ABTS aqueous solution was mixed with ethanol in a 1:1 (*v*/*v*) ratio in order to enhance the interaction of both hydrophilic and lipophilic antioxidants with the radicals, while the FRAP assay was performed in aqueous acetic buffer (pH 3.6) to maintain iron solubility. Thus, the ABTS radical could reach extensive range of compound polarities and react with a greater amount of compounds than the ferric ions of the FRAP assay.[24] Due to their polarity, the SPAs are highly soluble in water−alcohol solutions, such as ethanol or methanol, and are therefore highly soluble in the ABTS reaction medium. At the same time, xanthophylls exhibit low polarity and they are more likely to go into the aqueous ethanolic solution of the ABTS radical, while their solubility will be negligible in the aqueous solution of the FRAP assay.[23] In addition, the acidic environment of the FRAP system could negatively affect the stability of carotenoids.[51] On the other hand, the highly hydrophilic anthocyanins are more likely to affect the AC determined by the FRAP method. The water solvent in the FRAP system may also contribute to the opening of starch, protein, and cellulose structures that act as physical barriers to liquid diffusion and tend to shrink in ethanol environment, thereby enhancing the interactions at the solid−liquid interface between the antioxidant groups bound to the insoluble polysaccharide fraction and the ferric ions.[24] Finally, it should also be considered the different reactivity of the two assays toward other antioxidant components of the matrix not measured in the present study, such as thiol-type antioxidants, which are not effectively oxidized within the FRAP protocol time.[52]

In view of these differences, multivariate analyses were carried out to quantitatively analyze and better understand the relationships between the analyzed health-related compounds and the antioxidant properties of the genotypes studied under different growing conditions. A PCA was performed on the 14 analyzed genotypes (n = 378), and a biplot was produced, where the two first principal components explained 33 and 23% of the total variation of the samples (Figure 3). Principal component 1 (PC1) mainly appeared to differentiate the entries according to their CWBPAs and TCC and to their AC_{FRAP}, whereas the SPAs and AC_{ABTS} were the most important contributors to principal component 2 (PC2). Furthermore, variables with longer vectors (CWBPAs, SPAs, and AC_{ABTS}) were more discriminating of the entries than shorter ones. The biplot showed a characteristic clustering of

data, as TCC, SPAs, and AC_{FRAP} contributed more to separating the samples according to the genotype, while TAC, CWBPAs, and AC_{ABTS} differentiated the samples according to the environment. Indeed, samples from the C site were all placed on the lower right side of the biplot, as a result of their high concentrations of CWBPAs and anthocyanins, while the B and A plots were placed in the upper part, close to AC_{ABTS} and the SPAs. When the data are sufficiently approximated by the biplot, the cosine of the angle between the vectors of two variables approximates the correlation coefficients between them. The loading plots showed a positive correlation between the CWBPAs, TAC, and AC_{FRAP} and a negative relationship of the latter ones with TCC and AC_{ABTS}. The SPAs were the only phytochemicals to be positively correlated with the AC assessed with both methods, highlighting the different contribution of various bioactive compounds to the AC estimated by different assays, even when performed on solid samples.

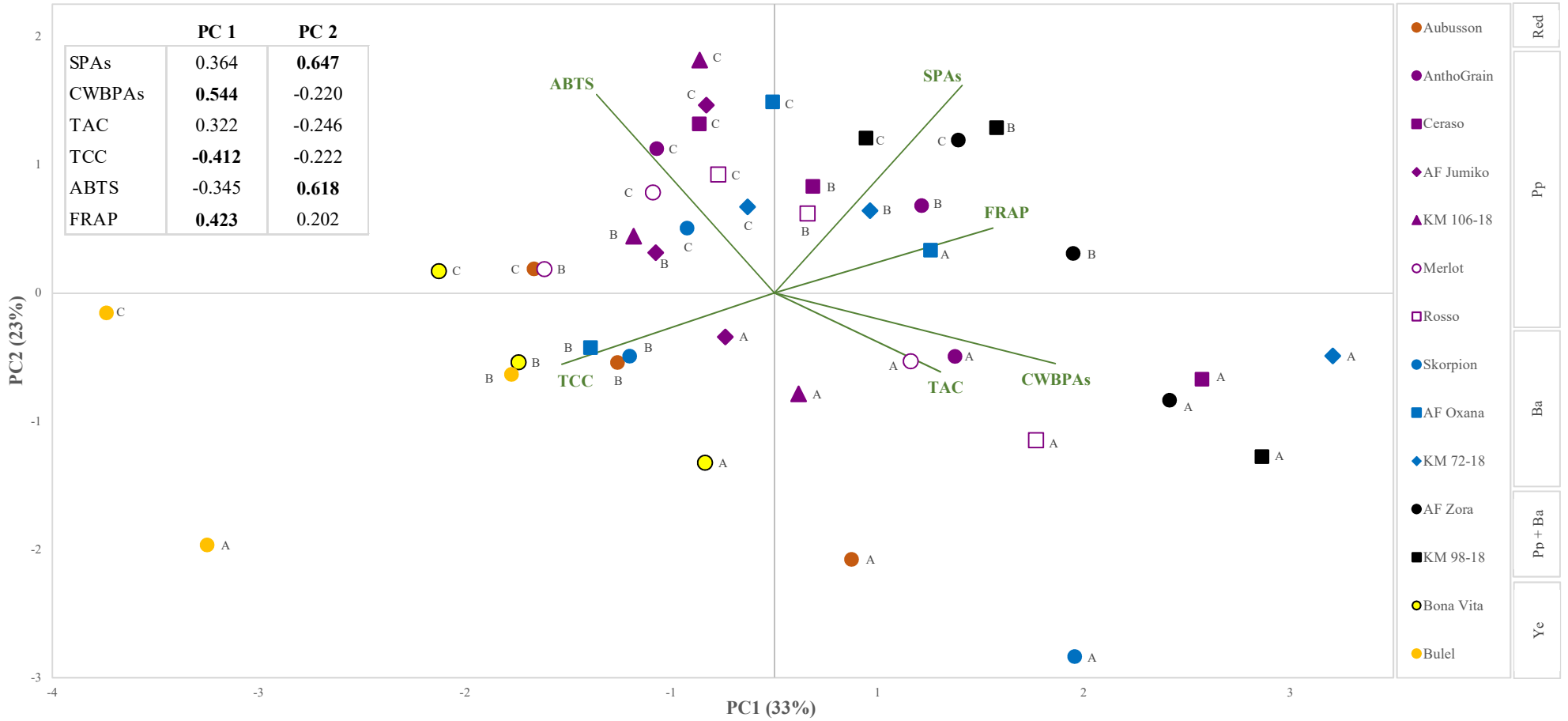


Figure 3. PCA biplot of the analyzed genotypes (*n* = 378) and phytochemical parameters associated with health-related substances.

The table on the left-hand side shows the loadings of the variables for the first two principal components. Loadings greater than 0.4 are indicated in bold. The length of each variable vector is proportional to its contribution to separating the genotypes, and the direction of the vectors indicates their relative contribution to PC1 and PC2.
The sample plots are grouped by genotype and by environment, on the basis of the average mean of 3 N levels and 3 replications.
The color names on the right-hand side indicate the color of the kernels. Pp, purple pericarp; Ba, blue aleurone; Pp + Ba, black grain; Ye, yellow endosperm.
Environments: A (Cigliano, 2019-20), B (Cigliano, 2018-19), C (Carmagnola, 2018-19).
SPAs, soluble phenolic acids; CWBPAs, cell wall-bound phenolic acids; TAC, total anthocyanin content; TCC, total carotenoid content; ABTS, antioxidant capacity determined by means of ABTS assay; FRAP, antioxidant capacity determined by means of FRAP assay.

In short, the wide array of genotypes with different genetic backgrounds analyzed in the present study provides a deeper understanding of the response of differently pigmented genotypes to environmental conditions and N fertilization rates, regarding agronomic and qualitative traits and phytochemical accumulation. Overall, the antioxidant profiles resulted to be strongly influenced by the genotype. The environment played a significant role, which could be partly ascribed to the soil N content, according to the conditions compared and the agronomic effects reported. The N fertilization treatments affected antioxidant accumulation similarly but to a lesser extent than the environment. Larger concentrations of SPAs were observed in the plots grown in soil characterized by a high N content, whereas the CWBPAs and anthocyanins were significantly reduced by a high soil N content and higher N fertilization rates. The greatest differences in the bioactive compound content were detected among the color groups, with the Pp + Ba group accumulating the highest content of anthocyanins and phenolic acids and the Ye group being the richest in carotenoids. However, a wide variability was also observed among the cultivars belonging to the same color group, and some of them appear to be promising for the development of innovative supply chains and the production of health-valued foods, as they have shown good yield and quality performances, and retained good antioxidant features in all the considered production systems. Therefore, a genetic improvement and the selection in specific environments are of key importance to develop pigmented genotypes characterized by high phytochemical accumulation and satisfactory yield potential, when compared to commercially diffused varieties. N fertilization affected the agronomic and qualitative traits, but overall had limited effects on some bioactive compounds. Thus, an optimized N management can contribute to the efficient growth of these phytonutrient-rich varieties, as it plays a fundamental role in enhancing the yield capacity and in ensuring a good technological quality and desirable product-making features, according to the intended use of the grains, without compromising their high value in terms of phytochemicals. Further studies will be necessary to improve the knowledge related to disease resistance and safety aspects of pigmented genotypes, as well as to post-harvest processing, to generate the interest of growers and producers, and to favor the large-scale adoption of such varieties.

Supplementary material

Figure S1. Appearance of the ears of the wheat and tritordeum genotypes compared in the study.

A-M – ***Triticum aestivum*** **spp.** ***aestivum*** **L.:** A, Aubusson; B, AnthoGrain™; C, Ceraso; D, AF Jumiko; E, KM 106-18 – this material has a long glume transferred from *Triticum polonicum* L.; F, Merlot; G, Rosso; H, Skorpion; I, AF Oxana; J, KM 72-18; K, AF Zora; L, KM 98-18; M, Bona Vita.
N – × ***Tritordeum martinii*** **A. Pujadas, nothosp. nov.:** Bulel.

Table S1. Timing of the main agronomic management practices applied to all the plots in the field experiments conducted in the 2018-20 period in north-west Italy.

Crop technique	**Growth stage**	**A** Cigliano (VC), 2019-20	**B** Cigliano (VC), 2018-19	**C** Carmagnola (TO), 2018-19
Sowing date		6 Nov. 2019	16 Nov. 2018	14 Nov. 2018
N fertilization	Tillering (GS 23)	5 March 2020	8 March 2019	4 March 2019
	Stem elongation (GS 32)	3 April 2020	8 April 2019	9 April 2019
Fungicide + insecticide	Anthesis (GS 65)	6 May 2020	16 May 2019	22 May 2019
Harvest date		30 June 2020	2 July 2019	10 July 2019

Table S2. Effect of the genotype, the environment, the N fertilization, and their interaction on the qualitative parameters of the whole-meal flour of the investigated wheat and tritordeum genotypes.

Factor	Source of variation		TW ($kg \cdot hL^{-1}$)	GPC (%)	Ash (%)
Genotype (G)	Red	Aubusson	78.0 de	11.5 def	1.81 efg
	Pp	AnthoGrain™	78.9 c	11.9 bcde	1.92 a
		Ceraso	78.2 cd	11.4 de	1.78 g
		AF Jumiko	81.1 a	11.3 e	1.83 cde
		KM 106-18	76.9 f	11.8 bcdef	1.90 a
		Merlot	78.7 c	11.6 cdef	1.80 fg
		Rosso	79.6 b	11.4 de	1.86 bc
	Ba	Skorpion	72.9 i	13.2 a	1.87 b
		AF Oxana	74.8 g	12.1 bcd	1.82 def
		KM 72-18	73.9 h	12.1 bc	1.84 bcde
	Pp + Ba	AF Zora	77.5 e	12.3 b	1.90 a
		KM 98-18	78.7 cd	12.3 bc	1.86 bc
	Ye	Bona Vita	79.6 b	12.9 a	1.85 bcd
	Trit	Bulel	74.6 g	12.9 a	1.90 a
		p (F)	***	***	***
Environment (E)		A	76.8 c	11.1 c	1.83 c
		B	78.1 a	11.3 b	1.84 b
		C	77.2 b	13.8 a	1.89 a
		p (F)	***	***	***
N Fertilization (N) (kg of $N \cdot ha^{-1}$)		0	77.7 a	11.5 c	1.85 b
		80	77.3 b	11.8 b	1.84 b
		160	77.2 b	12.9 a	1.87 a
		p (F)	***	***	***
G × E		*p* (F)	***	***	***
G × N		*p* (F)	*	ns	ns
E × N		*p* (F)	*	***	ns
G × E × N		*p* (F)	**	ns	ns

Pp, purple pericarp; Ba, blue aleurone; Pp + Ba, purple pericarp + blue aleurone (black); Ye, yellow endosperm; Trit, tritordeum; TW, test weight; GPC, grain protein content. The results are expressed on a DW basis. Means followed by different letters are significantly different, according to the REGW-F test [(*) *p* (F) < 0.05, (**) *p* (F) < 0.01, (***) *p* (F) < 0.001, and ns, non-significant].

Table S3. Technological parameters, farinographic assessment, and baking test of the refined flours of wheat samples from the field experiments conducted in the 2018-19 and 2019-20 periods at Cigliano, Italy, supplied with 80 kg of N·ha^{-1}.

		Technological parameters				Farinographic assessment						Baking test				Bread appearance	
Factor	**Source of variation**	**HFN (s)**	**ZT (mL)**	**GI**	**WGC (%)**	**WA (%)**	**DT (min)**	**DS (min)**	**DS$_{(10)}$ (FU)**	**DS$_{(12)}$ (FU)**	**FQN**	**LV (cm^3)**	**LH (mm)**	**LW (mm)**	**SPV (ml·100 g^{-1})**	**LS**	**CC**
Genotype	Aubusson	333	18.3	96.7	17.6	52.3	1.3	1.5	104	114	20.5	283	51	95	285	arched	normal
	AnthoGrain^{TM}	294	16.7	93.4	20.5	56.8	1.4	1.7	81	97	25.5	243	51	86	233	arched	normal
	Ceraso	217	26.5	94.5	22.9	60.3	1.5	1.0	137	162	21.0	283	55	97	268	arched	dark
	AF Jumiko	350	17.3	95.0	21.5	59.2	1.4	1.4	105	125	23.8	240	49	89	229	arched	light
	KM 106-18	226	12.0	84.5	23.8	53.8	1.2	1.3	130	151	19.5	270	45	100	266	arched	normal
	Merlot	254	26.3	97.3	19.6	62.2	1.5	1.3	104	121	25.5	273	51	92	255	arched	light
	Rosso	228	19.7	98.7	15.6	60.4	1.6	1.8	81	98	29.8	247	49	93	241	arched	normal
	Skorpion	160	29.3	85.3	28.0	59.7	1.6	1.4	127	153	25.0	263	51	102	252	arched	dark
	AF Oxana	301	30.3	96.0	25.5	60.5	1.7	1.3	119	141	25.3	263	46	98	250	arched	normal
	KM 72-18	102	27.0	68.2	28.1	59.3	1.6	1.2	143	169	23.0	300	30	134	321	flattened	normal
	AF Zora	230	21.7	65.1	28.4	58.8	1.7	1.4	115	137	25.8	257	50	101	254	arched	normal
	KM 98-18	186	24.3	89.2	24.5	61.0	1.4	1.0	135	158	21.5	297	48	99	307	arched	normal
	Bona Vita	239	35.5	95.9	26.2	58.3	1.7	1.7	87	105	26.5	270	56	94	254	arched	normal
Harvest year	2019	243	22.6	90.7	21.8	55.3	1.5	1.2	116	139	23.0	272	47	101	262		
	2020	237	24.3	87.7	24.7	62.0	1.5	1.6	110	127	25.1	265	50	96	263		

HFN, Hagberg falling number; ZT, Zeleny test; GI, gluten index; WGC, wet gluten content; WA, water absorption; DT, development time; DS, dough stability; DS$_{(10)}$, degrees of softening after 10 min; DS$_{(12)}$, degrees of softening 12 min after the maximum; FQN, farinographic quality number; LV, loaf volume; LH, loaf height; LW, loaf width; SPV, specific pastry volume; LS, loaf shape; CC, crust color; FU, farinographic unit.
HFN was determined according to ISO 3093:2009, using a Perten LM 3120 mill and 7 g of flour on a 15% moisture basis. ZT was determined according to ISO 5529:2007, using a Brabender Sedimat mill. GI and WGC were determined according to ICC Standard No. 155, using a Perten Glutomatic 2200 and Centrifuge 2015. The rheological properties were determined by means of a farinograph (ICC Standard No. 115/1), producing dough with a consistency of 500 FU. A baking test was performed using the authors' own method. The recipe is based on ICC Standard No. 131. The other components in the bread formula were instant yeast (1.8% of the total weight), salt (1.5%), sugar (1.86%), ascorbic acid (0.005%) and water. Flours with a higher falling number than 250 s were supplemented with malt flour. The dough was prepared in a farinograph with a 300 g mixer.

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Chapter V – "From paddy to polished rice: investigating antioxidant activity and phenolic compounds in Italian conventional and pigmented rice varieties and their by-products"

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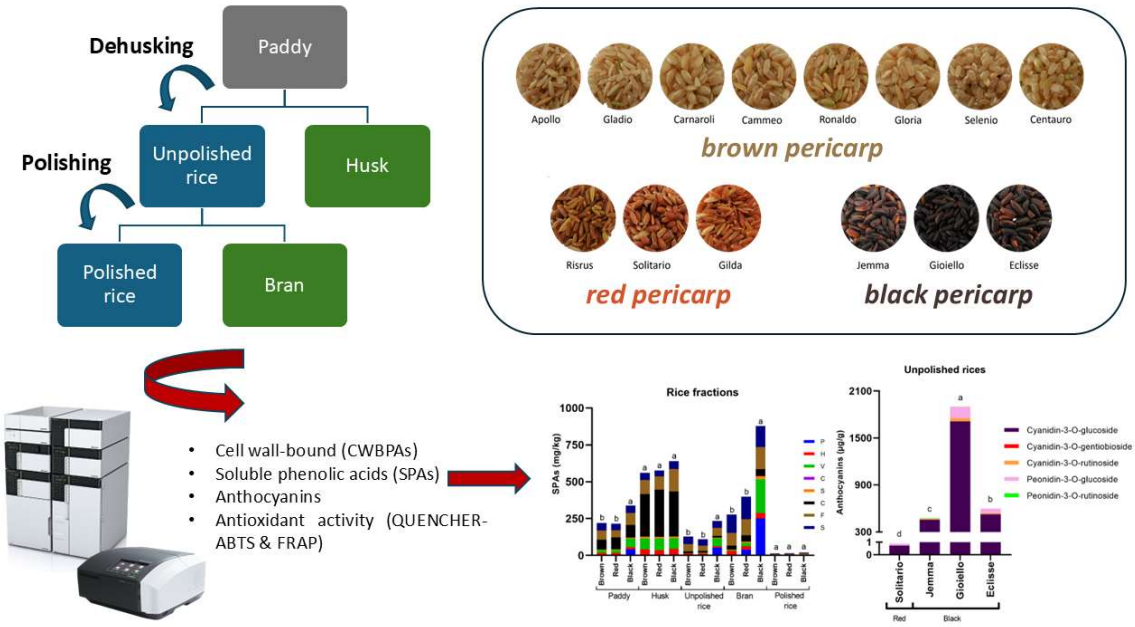
Graphical abstract



Abstract

Rice (*Oryza sativa* L.), the most widely consumed crop globally, generates substantial agricultural waste, particularly husks. Typically consumed as polished rice, the subsequent polishing process reduces the beneficial effects of bioactive compounds, especially polyphenols found in the bran. This study proposed to analyze the phenolic pattern, including soluble (SPAs) and bound phenolic acids (CWBPAs) and anthocyanins, along with the antioxidant activity of different grain parts (paddy, polished, and unpolished rice) and outer layers (husk and bran) in fourteen commercial Italian rice varieties. High-performance liquid chromatography revealed that unpolished rice samples had significantly higher phenolic compounds and antioxidant activity than polished ones. Moreover, unpolished black pericarp variety Gioiello showed high levels of SPAs (260 mg/kg), CWBPAs (655 mg/kg), and anthocyanins (1901 mg/kg) compared to red and brown pericarp varieties. Regarding external layers, rice husks, rich in SPAs (497-796 mg/kg) and CWBPAs (4060-7446 mg/kg), are a valuable by-product for bioactive compound extraction, making them a cost-effective source of natural antioxidants. Conversely, anthocyanins are specific to the bran fraction (2138-5041 mg/kg), which demonstrates high free-radical scavenging activity and significant ferric reducing ability. These findings will be relevant for future application of rice husk and bran antioxidants in functional ingredients and promote the consumption of unpolished pigmented rice varieties.

Keywords: brown rice; red rice; black rice; phenolic acids; anthocyanins; HPLC-DAD; antioxidant activity.

Abbreviations: ABTS, 2,2′-Azino-bis(3-ethylbeenzothiazoline-6-sulfonic acid) diammonium salt; CWBPAs, cell wall-bound phenolic acids; FRAP, ferric reducing antioxidant power; SPAs, soluble phenolic acids; TAA, total antioxidant activity; TE, Trolox equivalents.

Introduction

Among the cereal grains, rice (*Oryza sativa* L.) represents one of the most important human food crops consumed in the world. Based on FAO's estimates for global cereal production, 517 million tons of milled rice were produced in 2022 (FAO, 2023).

Asia represents the largest producer of rice in the world, which accounts for approximately 90% of the global rice supply chain. In Europe, rice is primarily cultivated in North Italy with a cultivation area that is mainly located in the west side of Po River valley (provinces of Novara, Vercelli, Milano and Pavia). Italy guarantees 50% of the entire EU rice production, and it is the major supplier with 1.5 million tons per year (Paoloni, 2023).

Generally, rice can be classified depending on the end-use quality, which strongly affect the commercial value of grains. The most important quality components include rice appearance, milling attitude, cooking and processing quality and nutritional composition (Koutroubas et al., 2004). Appearance is mainly determined by the grain shape and in particular grain length, grain width, the length-to-width ratio and the translucency of the endosperm, parameters that are strongly related to technological attributes (texture and stickiness and other pasting properties), and which classified European genotypes according to the following end-use groups: round, medium, long A and long B grain rice (Reg. UE 1308/2013, 2013). After harvest, rice grain is defined as paddy and may be subjected to a multitude of processing steps such as drying, dehusking, polishing, and eventually milling. About 70% of paddy is endosperm, 20% is husk and 10% is bran (Van Hoed et al., 2006). Rice is commonly consumed as polished (refined or white) rice, which is initially produced by removing the husk from the paddy to obtain unpolished (brown) rice and then removing the bran and germ fractions during the polishing or whitening process, which improved the technological characteristics. However, the subsequent removal of the outer bran layers from unpolished rice, which consist of the pericarp, aleurone, subaleurone layer, and germ, results in a loss of sensorial properties and beneficial nutrients (Saleh et al., 2019).

Rice bran, the principal edible by-product obtained from rice processing, has recently gained interest due to its high dietary fiber and protein content as well as micronutrients (minerals, vitamins) and bioactive components such as polyphenols, gamma-oryzanol, and tocopherols. The relationship between these beneficial nutrients in our diet and human health has received particular attention. As a result, the literature suggests that rice bran bound phenolics may alleviate can alleviate hyperlipidemia by regulating the uptake of cholesterol and fatty acids in the liver and their absorption in the gut (Zhao et al., 2022).

Bran contains a wide range of phenolic compounds, and this results in a highly variable amount in unpolished rice compared to polished one. The distribution of phenolics differs among the rice grain's botanical components: more than 50% of phenolic compounds existed in the bran fraction, which provides strong antioxidant ability (Ding et al., 2019; Shao et al., 2014). Given its bioactive and nutritional components, bran can be utilized in a wide range of foods as a functional ingredient or as a raw material for phenolics extraction. Polyphenols and particularly phenolic acids in rice may be present in free or esterified soluble form, and a significant amount are present in insoluble form as bound phenolic compounds (Khosravi & Razavi, 2020). These cell wall-bound polyphenols have been reported to be linked to the fibrous component. Hydrogen groups, as well as aromatic and glycosidic oxygen atoms, are found in cell wall polysaccharides and can establish hydrogen bonds and hydrophobic interactions with polyphenols (Liu et al., 2020).

In general, ferulic acid, *p*-coumaric acid and gallic acid are found to be the most abundant polyphenolic compounds in rice, quantified majorly in unpolished rice with lower levels in polished rice (Zhou et al., 2004). In addition, anthocyanins and proanthocyanidins are considered the most studied antioxidant components in pigmented rice, which give the characteristic color to the pericarp layer of red and black rice. Studies have reported that cyanidin-3-*O*-glucoside and peonidin-3-*O*-glucoside are the main anthocyanin constituents of black and red rice grains (Deng et al., 2013).

Pigmented cultivars were traditionally cultivated in Southeast Asia, but in recent years their popularity in Italy has increased due to a greater gastronomic appreciation among the Italian people as well as for their significant antioxidant capabilities as functional foods (Colasanto et al., 2021). In addition to the anthocyanin compounds, pigmented rice varieties seem to contain overall more nutrients and other bioactive compounds than conventional rice, with brown pericarp (Colasanto et al., 2023). To the authors' best knowledge, few studies have comprehensively examined the phenolic profile of rice grains throughout the entire refining process. This research aims to fill this gap by characterizing the phenolic content considering fourteen Italian rice varieties (most of which are newly introduced) and their by-products, particularly the husk and, in the case of varieties with brown pericarp, the bran, which holds significant potential for the extraction of bioactive compounds. This study represents a novel contribution to the field by providing valuable insights into the phenolic composition and utilization of rice by-products within a circular economy framework (Jaouhari et al., 2023). In accordance with the general trend, it is essential to investigate functional components often considered agro-industrial waste, like husk and bran, and to understand the active compounds that contribute toward antioxidant capacity for reducing loss and promoting functional rice varieties. Therefore, the objective of this study was to

investigate the distribution of free and bound polyphenolic compounds in different conventional brown and pigmented (red and black) pericarp rice varieties, and in their fractions obtained after processing, starting from paddy to polished rice. The antioxidant activity was also determined for each fraction.

Materials and methods

Chemicals

2,2′-Azino-bis(3-ethylbeenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, 97%), ethanol (CHROMASOLV®, 99.8%), ethyl acetate (CHROMASOLV®, 99.8%), hydrochloric acid (37%), methanol (CHROMASOLV®, 99.9%), sodium hydroxide (≥ 98%), iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$, ≥ 98%), 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ), were purchased from Sigma-Aldrich (St. Louis, MO, USA). 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). Phenolic acids (protocatechuic acid, ferulic acid, *p*-hydroxybenzoic acid, gallic acid, vanillic acid, *p*-coumaric acid, caffeic acid, syringic acid, sinapic acid, and 3,5-dichloro-4-hydroxybenzoic acid), 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 Carlo Erba (Milan, Italy). Ultrapure water (18.2 MΩ cm at 25 °C) was produced by ELGA PURELAB Ultra system (M-medical, Cornaredo, Milan, Italy).

Rice samples

The present study analyzed the entire rice processing chain, which includes paddy, unpolished, and milled rice, and the related residual by-products, such as husk and bran, of 14 Italian rice grain varieties (*Oryza sativa* L.) registered in the Italian Varietal Register. The rice varieties, cultivated in the Vercelli growing area and kindly supplied by local industrial companies, included:

- brown pericarp or conventional varieties, referring to different market group according to the actual European and Italian quality classification (Reg. UE 1308/2013, 2013 and D. Lgs. 131/2017 Annex 2, food denomination, 2017): 2 long B genotypes, Apollo and Gladio; 2 long A (high quality; referring to the denomination *superfino* from the old Italian law *Legge 18 marzo 1958, n. 325*, 1958) genotypes, Carnaroli (Carnaroli traditional group) and Cammeo (Roma-Baldo group), 2 long A genotypes, Gloria (S. Andrea Group) and Ronaldo; 2 round genotypes, Selenio and Centauro;
- red pericarp varieties: 2 long A genotypes, Risrus and Solitario; 1 medium genotype, Gilda;
- black pericarp varieties: 1 long A genotype, Jemma; 2 medium genotypes, Gioiello and Eclisse.

The main characteristics of rice varieties are reported in **Table S1 and Figure S1**.

Three replication of 1 kg each of all paddy rice cultivars were dehusked and polished through a consecutive passage using a laboratory-scale mill machine (Satake Rice Machine, Satake engineering Co.Ltd., Tokyo, Japan) to separate husk from unpolished rice and bran from milled rice.

The grain samples at different processing levels and the residual fractions were ground to a fine powder (particle size of <500 μm) with a Cyclotec TM 1093 sample mill (Foss, Padova, Italy) and then stored at −25 °C until analyses were performed. The moisture content, determined in order to express all the results on a dry weight (dw) basis, was obtained by oven-drying at 105 °C for 24 h. The thousand kernel weight (TKW) of paddy and unpolished rice was determined by weighing two sets of 100-kernels for each cultivar.

Phenolic acids characterization

Extraction of soluble and cell wall-bound phenolic acids

Extraction and quantification of the soluble and cell wall-bound phenolic acids were performed according to the procedure proposed in Giordano et al. (2019), with some modifications.

Briefly, soluble (free and conjugated) phenolic acids (SPAs) were extracted from 0.1250 g of sample two times with 1 mL of 80:20 (v/v) ethanol:water solution in a ultrasound bath (35 kHz, Sonorex Super RK 156 BH, Bandelin Electronic, Berlin, Germany) for 10 min at 4 °C. The supernatants were collected and then evaporated to dryness under a nitrogen stream. Samples were hydrolyzed with 2 M NaOH (400 μL) for 2 h under continuous stirring at 4 °C. After acidification to pH 2 with HCl 6N (160 μL), SPAs were extracted three times with 500 μL of ethyl acetate. The combined supernatants were evaporated to dryness under a nitrogen stream and then reconstituted in 100 μL of 80:20 (v/v) methanol:water solution.

For the cell wall-bound phenolic acids (CWBPAs), first the SPAs were separated following the extraction previously described. Subsequently, hydrolysis was performed by adding 400 μL of 2 M NaOH to the remaining pellet, followed by continuous stirring at 4 °C for 4 hours. After acidification to pH 2 with HCl 12 N (120 μL), the bound phenolic acids were extracted with 800 μL of ethyl acetate and then centrifuged 10,600 × *g* for 2 min at 4 °C. The extraction was repeated another time. The combined supernatants were evaporated to dryness under a nitrogen stream, and then reconstituted in 200 μL of 80:20 (v/v) methanol:water solution.

RP-HPLC-DAD quantification of soluble and cell wall-bound phenolic acids

Both SPA and CWBPA extracts were filtered through a 0.2 μm filter and then analyzed by means of a high-performance liquid chromatograph Agilent 1200 Series (Agilent Technologies, Santa Clara, CA, USA) coupled to an Agilent 1200 Series diode array

detector. Separations were carried out using a 150 × 4.6 mm, 5 µm, Gemini RP18 column (Phenomenex, Torrance, CA, USA) protected by a guard column at 35 °C. The mobile phase consisted of 0.1% acetic acid in water (solvent A) and 0.1% acetic acid in methanol (solvent B), and the separation was performed using the gradient previously described in Giordano et al. (2019). Phenolic acids were identified using the retention times and the UV/Vis spectra of their respective standards; quantifications of individual compounds were obtained through the corresponding calibration curves.

Anthocyanin characterization

Extraction of anthocyanins

Anthocyanins were extracted from pigmented rice varieties using 50:50 (v/v) water:ethanol in a sample to solvent ratio of 1:5 (w/v). The extraction was performed under magnetic stirring for 30 min. After centrifugation at 4,000 x g for 15 min, the hydroalcoholic extract was transferred in a clean tube.

RP-HPLC-DAD quantification of anthocyanins

The chromatographic method employed was the same as that proposed and validated in our previous study (Bordiga et al., 2015). Briefly, a Shimadzu LC-20A Prominence chromatographic system equipped with a diode array detector (DAD detector SPD-M20A) was used. Separation was performed on a reversed-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). Total run time was 80 min, at a constant flow rate of 400 µL/min. Anthocyanins were identified by comparison with retention times of individual authentic standard molecules and their UV–Vis spectra recorded at 520 nm, excepting for cyanidin-3-*O*-gentiobioside, which was tentatively identified based on the characteristics reported in the literature (Bordiga et al., 2014) and its UV-Vis profile. The quantification was performed based on calibration curves obtained with the corresponding standards; cyanidin-3-*O*-gentiobioside and cyanidin-3-*O*-rutinoside were expressed as equivalent of cyanidin-3-*O*-glucoside, while peonidin-3-*O*-rutinoside was expressed as equivalent of peonidin-3-*O*-glucoside.

Determination of the total antioxidant activity (TAA)

The TAA was determined adapting the FRAP (ferric reducing antioxidant power) and ABTS assays to the QUENCHER approach, as described by Serpen et al. (2012).

Briefly, FRAP reagent was prepared by mixing an aqueous solution of TPTZ (2,4,6-Tris(2-pyridyl)-*s*-triazine) and ferric chloride in sodium acetate buffer (pH 3.6) at a ratio of 1:1:10 (v:v:v). Samples (2 mg) were analyzed by adding FRAP working solution (2 mL). The reaction was carried out under stirring at 1,000 × *g* (PCMT Thermoshaker, Grant Instruments, Cambridge, UK). After 120 min, samples were centrifuged for 2 min at 20,800 × g, and the absorbance of the supernatant was measured at 593 nm (Beckman Coulter DU 370 UV-Vis Spectrophotometer). The results were expressed as mmol Trolox equivalents/kg of sample.

The ABTS assay was performed by preparing a working solution from an aqueous stock solution (7 mM ABTS, 2.45 mM potassium persulfate) with water until the absorbance at 734 nm was between 1.50±0.02. Samples (1.4 mg) were analyzed by adding 700 µL of ethanol and 700 µL of ABTS solution. The reaction was carried out under stirring at 1000 × *g*. After 30 min, samples were centrifuged for 2 min at 14,000 × *g*, and the absorbance of the supernatant was measured at 734 nm. The results were expressed as mmol Trolox equivalents/kg of sample.

Statistical analysis

All the statistical analyses and data visualizations were performed using the free statistical software R 4.2.2 version (R Core Team, 2020) and GraphPad Prism 9.5 (GraphPad Software, San Diego, CA, USA). Results were expressed as mean of 3 separated replications. 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.

Results and discussion

Although the brown pericarp varieties are the most common typology on the market, the consumption of pigmented rice and their outer fractions for the development of functional foods has piqued the interest of both researchers and the food industry in recent years. In this study, the polyphenolic patterns and the antioxidant activity of pigmented rice varieties (red and black) were analyzed and compared with the conventional brown pericarp varieties, referring to different market categories and shapes, both in the form of inedible paddy rice and after processing (as unpolished and polished rice fractions). Moreover, the composition of husk and bran fractions, mostly considered as by-products (the last one specifically for brown pericarp rice), was assessed.

The analyzed samples showed differences in their chemical content based on their color, cultivar and fraction.

Soluble phenolic acids

The chromatographic analysis permitted to identify and quantify seven phenolic acids in their soluble form within the rice fractions as shown in **Table 1**.

Total SPAs content in husk samples demonstrated a significantly greater quantity compared to other fractions, except for black varieties. Specifically, Jemma showed a similar phenolic concentration in bran and husk, while in the case of Gioiello and Eclisse, the bran exhibited a higher phenolic concentration than the husk. Our results are confirmed by literature data, based on which the husk is the rice fraction most rich in phenolics in almost varieties, followed by the bran (Wanyo et al., 2016).

Within grain fractions, the hierarchical arrangement of total SPAs across all examined cultivars is characterized by paddy rice (not edible) holding the highest position, followed by unpolished and polished rice. As shown in **Table 2**, the SPAs in paddy grains ranged from 182 mg/kg (Gloria) to 388 mg/kg (Jemma), while in unrefined and refined rice the concentration varied from 98.9 mg/kg (Gloria) to 260 mg/kg (Gioiello) and from 7.33 mg/kg (Gladio) to 36.8 mg/kg (Gioiello), respectively. According with these results, Gioiello has the greatest concentration of SPAs among the edible grains, in both unpolished and polished version. Moreover, all whole grain rice samples present values of total SPAs significantly higher than refined rice. Regarding the outer fractions, Gioiello bran (995 mg/kg) presents the higher amount of total SPAs, while Carnaroli (220 mg/kg) the lowest value. Concerning the husk fractions, the values varied from 796 mg/kg (Jemma) to 488 mg/kg (Gioiello).

Recently, unrefined rice has gained many attentions by researcher and consumers due the potential health benefits (Saleh et al., 2019), because of the higher content of fiber, proteins,

minerals and vitamins than refined rice. Unpolished rice, as more clearly illustrated in **Table 1** and **Figure S2a,** was characterized by high concentrations of ferulic and sinapic acids; in addition, pigmented varieties, especially the black ones, showed high levels of protocatechuic and vanillic acids as well. Ferulic and sinapic acids were predominant in Ronaldo unpolished rice (97.4 mg/kg and 82.4 mg/kg), while vanillic acid was abundant in the black varieties Jemma (55.5 mg/kg), Eclisse (54.8 mg/kg) and Gioiello (51.3 mg/kg). Furthermore, protocatechuic acid was quantified only in pigmented varieties, except for Risrus, and showed the highest concentration in Gioiello variety (68.5 mg/kg).

Trying to highlight a correlation between the SPAs' concentration and the color of the rice caryopsis, it can be noticed that SPAs are more concentrated in black varieties, specifically in the case of paddy and unpolished rice (**Figure 1a**). In fact, black paddy and unrefined rice samples have the highest average value (340 mg/kg and 233 mg/kg, respectively), followed by the brown (221 mg/kg and 130 mg/kg) and red pericarp varieties (215 mg/kg and 110 mg/kg), which present similar values each other's. Grouping the rice samples based on the color caryopsis, the average value of the black, red and brown polished varieties did not show any significant difference. Indeed, as illustrated in **Table 2**, the differences among varieties are not associated with color. The removal of the bran results in a reduction of polyphenols (and pigments) in polished rice and the rate of decrease of SPAs was dependent on the rice cultivar, indicating that the distribution of SPA content varies among varieties. This implies that even within the same color group, some varieties have phenolic compounds distributed more evenly throughout the kernel layers than others, as previously observed by other authors (Choi et al., 2018).

Concerning by-products, the average value of husk for the black, red and brown pericarp varieties did not show any noticeable divergence, while in the case of the bran, it was found that the black varieties showed levels of soluble phenolic acids (879 mg/kg) approximately 2 and 3 times higher compared to the red (399 mg/kg) and the brown (278 mg/kg), respectively.

Table 1. Distribution of soluble phenolic acids (mg/kg) in different fractions of rice varieties.

Pericarp color	Variety	Fraction	PRO	HYD	VAN	SYR	COU	FER	SIN	TOTAL SPAs
Brown	Apollo	Paddy	ND	17.6 b	7.75 b	2.51 b	85.1 b	57.2 c	45.8 b	216 c
		Husk	ND	33.4 a	36.0 a	9.30 a	393 a	105 a	35.9 b	613 a
		Unpolished rice	ND	16.4 b	2.41 c	1.27 c	19.3 c	50.8 c	53.4 b	143 d
		Bran	ND	37.1 a	3.20 c	2.68 b	32.6 c	88.9 b	132 a	296 b
		Polished rice	ND	3.30 c	0.373 d	0.258 d	1.04 d	6.94 d	3.95 c	15.9 e
	Gladio	Paddy	ND	15.8 b	9.95 b	2.66 b	82.3 b	48.1 bc	37.0 b	196 b
		Husk	ND	33.1 a	37.2 a	8.54 a	318 a	81.2 a	22.5 bc	500 a
		Unpolished rice	ND	11.0 bc	1.63 d	0.85 d	9.74 d	37.5 c	40.6 b	101 c
		Bran	ND	28.4 a	3.40 c	1.78 c	26.4 c	67.8 ab	112 a	240 b
		Polished rice	ND	1.50 c	0.302 e	ND	0.861 d	2.42 d	2.25 c	7.33 d
	Carnaroli	Paddy	ND	12.9 b	16.4 b	3.20 b	59.0 b	62.9 bc	51.6 b	206 b
		Husk	ND	37.6 a	74.9 a	14.9 a	268 a	97.9 a	50.5 b	544 a
		Unpolished rice	ND	7.17 c	2.29 c	1.01 b	7.24 c	53.7 c	51.1 b	122 c
		Bran	ND	16.1 b	4.87 c	1.97 b	16.0 c	75.4 b	106 a	220 b
		Polished rice	ND	1.53 d	0.543 c	0.387 b	1.30 c	10.9 d	5.83 c	20.5 d
	Cammeo	Paddy	ND	20.3 b	27.0 b	4.42 b	100 b	52.2 c	56.9 b	261 b
		Husk	ND	56.1 a	102 a	16.3 a	387 a	106 a	54.4 b	722 a
		Unpolished rice	ND	9.20 c	4.30 d	1.51 c	20.6 cd	37.8 c	55.5 b	129 c
		Bran	ND	26.8 b	6.49 c	3.46 b	37.1 c	82.0 b	141 a	297 b
		Polished rice	ND	2.06 d	0.562 e	0.318 c	1.57 d	5.65 d	4.93 c	15.1 d
	Ronaldo	Paddy	ND	23.5 bc	15.8 b	3.41 b	70.2 b	107 a	88.5 b	308 bc
		Husk	ND	36.1 ab	67.9 a	11.3 a	268 a	118 a	79.8 b	581 a
		Unpolished rice	ND	21.1 c	2.84 c	1.24 c	13.1 d	97.4 a	82.4 b	218 c
		Bran	ND	41.8 a	4.92 c	2.65 b	31.3 c	130 a	182.2 a	393 b
		Polished rice	ND	3.14 d	0.465 d	0.265 d	1.21 e	10.1 b	4.66 c	19.8 d
	Gloria	Paddy	ND	16.4 c	18.2 b	2.86 b	53.0 b	48.1 b	43.8 b	182 c
		Husk	ND	53.8 a	80.6 a	11.1 a	219 a	85.8 a	46.9 b	497 a
		Unpolished rice	ND	6.43 d	2.58 d	1.05 c	7.49 d	38.9 b	42.4 b	98.9 d
		Bran	ND	24.5 b	4.81 c	3.02 b	21.9 c	80.1 a	102 a	236 b
		Polished rice	ND	1.10 d	0.532 e	0.400 c	1.11 e	3.88 c	2.62 c	9.64 e
	Selenio	Paddy	ND	20.5 bc	21.0 b	3.78 b	47.9 b	61.3 b	54.6 c	209 c
		Husk	ND	44.7 a	94.3 a	15.2 a	212 a	94.0 a	67.3 b	527 a
		Unpolished rice	ND	14.3 c	2.32 cd	0.99 d	7.54 d	46.7 c	45.3 d	117 d
		Bran	ND	38.8 ab	4.28 c	2.52 c	23.0 c	96.0 a	107 a	272 b
		Polished rice	ND	2.96 c	0.485 d	0.226 e	1.08 d	7.39 d	4.30 e	16.4 e
	Centauro	Paddy	ND	16.9 b	20.9 b	3.62 b	60.6 b	49.0 b	40.7 b	192 c
		Husk	ND	38.7 a	89.5 a	12.8 a	247 a	77.6 a	41.2 b	507 a

		Unpolished rice	ND	11.2 b	2.51 d	1.50 c	7.21 d	43.7 b	40.6 b	107 d
		Bran	ND	38.4 a	7.74 c	3.99 b	23.8 c	79.7 a	116 a	269 b
		Polished rice	ND	2.50 c	0.604 d	0.463 c	1.07 d	6.20 c	2.69 c	13.5 e
Red	Risrus	Paddy	ND	13.0 b	23.2 b	6.30 b	74.8 b	43.9 c	41.7 b	203 c
		Husk	ND	38.8 a	82.7 a	11.7 a	297 a	80.7 b	31.9 c	543 a
		Unpolished rice	ND	6.43 d	3.63 d	2.15 c	10.1 d	33.1 d	44.4 b	99.8 d
		Bran	ND	11.6 c	6.04 c	2.55 c	23.3 c	93.8 a	110 a	247 b
		Polished rice	ND	2.17 e	1.65 e	0.751 d	4.00 d	8.50 e	10.4 d	27.5 e
	Solitario	Paddy	4.49	14.1 c	18.8 c	4.22 b	78.9 b	46.8 c	44.3 b	212 c
		Husk	1.69 b	34.0 b	78.8 a	11.3 a	353 a	92.5 b	44.7 b	616 a
		Unpolished rice	7.77 b	10.0 d	4.46 d	3.17 b	17.4 d	37.5 c	42.3 b	123 d
		Bran	85.7 a	37.0 a	47.4 b	11.8 a	60.3 c	141 a	182 a	564 b
		Polished rice	ND	2.34 e	0.555 e	0.457 c	0.974 e	4.01 d	3.78 c	12.1 e
	Gilda	Paddy	6.49 b	12.7 c	21.3 b	4.19 c	83.1 b	56.6 b	46.8 b	231 c
		Husk	ND	32.8 a	71.9 a	12.9 a	314 a	96.7 a	43.3 b	571 a
		Unpolished rice	7.10 b	5.17 d	2.73 d	2.05 d	7.82 d	38.6 c	43.9 b	107 d
		Bran	32.2 a	26.3 b	12.3 c	10.2 b	38.9 c	97.7 a	167 a	385 b
		Polished rice	ND	1.29 d	0.500 d	0.394 e	0.727 d	2.70 d	2.69 c	8.30 e
Black	Jemma	Paddy	41.8 b	16.8 c	66.0 c	6.08 c	102 b	99.3 c	53.9 b	388 b
		Husk	5.46 c	44.7 a	86.4 b	12.1 b	388 a	203 a	56.0 b	796 a
		Unpolished rice	49.3 b	7.14 d	55.5 c	4.51 c	9.71 d	54.7 d	40.9 c	222 c
		Bran	249 a	32.6 b	236 a	20.5 a	42.2 c	135 b	134 a	851 a
		Polished rice	0.282 c	1.10 e	2.80 d	0.431 d	0.644 d	1.95 e	2.21 d	9.42 d
	Gioiello	Paddy	56.6 b	14.1 b	53.2 b	7.16 b	78.6 b	70.7 c	60.0 b	340 c
		Husk	10.7 c	29.5 a	46.9 b	10.6 b	235 a	97.0 b	58.3 b	488 b
		Unpolished rice	68.5 b	7.71 c	51.3 b	6.95 b	14.9 c	57.0 d	54.0 b	260 d
		Bran	304 a	28.5 a	226 a	23.4 a	63.3 b	177 a	173 a	995 a
		Polished rice	3.18 c	2.98 d	6.71 c	1.06 c	2.35 c	8.40 e	12.2 c	36.8 e
	Eclisse	Paddy	22.4 c	17.7 c	53.1 c	5.16 c	73.1 b	77.5 c	41.1 b	290 c
		Husk	4.45 d	39.2 b	84.6 b	13.4 b	293 a	155 a	46.9 b	636 b
		Unpolished rice	41.3 b	13.2 d	54.8 c	3.58 d	8.28 d	55.8 d	41.0 b	218 d
		Bran	197 a	49.5 a	232 a	15.6 a	34.7 c	138 b	123 a	790 a
		Polished rice	ND	2.67 e	3.90 d	ND	0.870 d	3.83 e	3.18 c	14.4 e

Values are expressed as mean (n = 3). Means with different letters in the same column of each rice variety were significantly different at the level $p < 0.05$. All reported means have a relative standard deviation less than 15%. ND: not detected.
Protocatechuic acid (PRO), *p*-hydroxybenzoic acid (HYD), vanillic acid (VAN), syringic acid (SYR), *p*-coumaric acid (COU), ferulic acid (FER), sinapic acid (SIN), soluble phenolic acids (SPAs).

Table 2. Total soluble phenolic acids (mg/kg) in different fractions of rice varieties.

	Brown								Red			Black		
	Long B*	Long B	Long A (high quality)	Long A (high quality)	Long A	Long A	Round	Round	Long A	Long A	Medium	Long A	Medium	Medium
Total SPAs	Apollo	Gladio	Carnaroli	Cammeo	Ronaldo	Gloria	Selenio	Centauro	Risrus	Solitario	Gilda	Jemma	Gioiello	Eclisse
Paddy	216 ef	196 ghi	206 fgh	261 d	308 c	182 i	209 fgh	192 hi	203 fgh	212 efg	231 e	388 a	340 b	290 c
Husk	613 cde	500 gh	544 defgh	723 b	581 cdef	497 h	527 fgh	507 gh	543 efgh	616 cd	571 cdefg	796 a	488 h	636 c
Unpolished rice	143 c	101 f	122 de	129 cd	218 b	98.9 f	117 def	107 ef	99.8 f	123 de	107 ef	222 b	260 a	218 b
Bran	296 def	240 f	220 f	297 def	393 d	236 f	272 ef	269 ef	247 f	564 c	385 de	851 b	995 a	790 b
Polished rice	15.9 def	7.33 i	20.5 c	15.1 ef	19.8 cd	9.64 ghi	16.4 cde	13.5 efg	27.5 b	12.1 fgh	8.30 hi	9.42 ghi	36.8 a	14.4 ef

Values are expressed as mean (n = 3). Means with different letters in the same raw were significantly different at the level $p < 0.05$. All reported means have a relative standard deviation less than 15%. Soluble phenolic acids (SPAs).
* The reported classification complies with the current European regulation (EU Regulation 1308/2013, 2013).

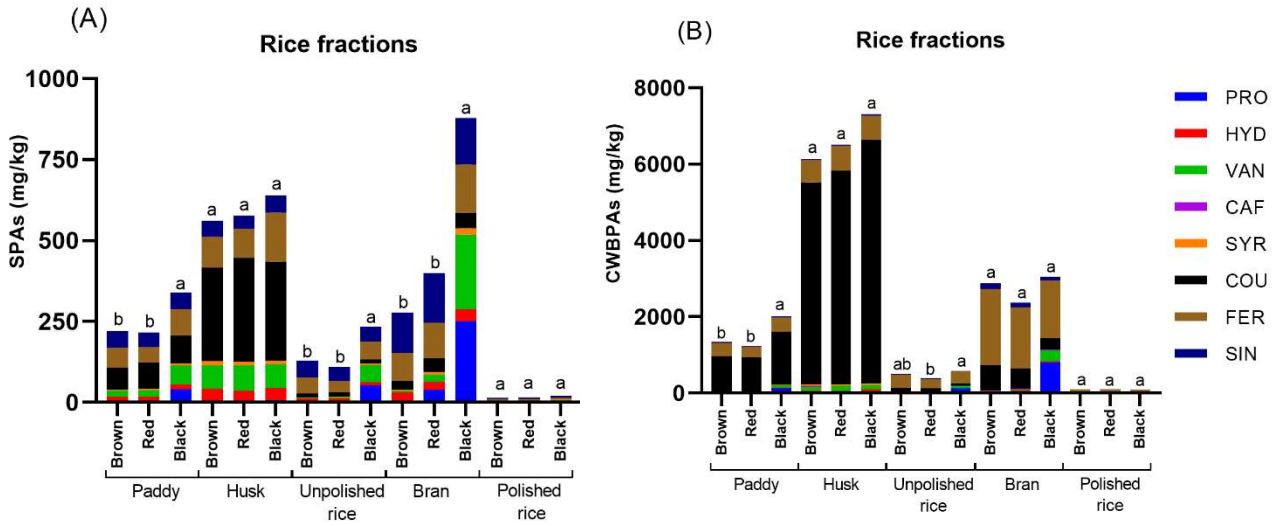


Figure 1. Average composition of soluble (SPAs) and cell wall-bound phenolic acids (CWBPAs) in rice fractions with brown and pigmented pericarp.

Different letters above the bars mean significant differences ($p < 0.05$) within the color of each fraction for SPAs (a) and CWBPAs (b).
Protocatechuic acid (PRO), *p*-hydroxybenzoic acid (HYD), vanillic acid (VAN), caffeic acid (CAF), syringic acid (SYR), *p*-coumaric acid (COU), ferulic acid (FER), sinapic acid (SIN).

Cell wall-bound phenolic acids

Phenolic acids in soluble form constitute a minority fraction in comparison to the bound counterparts, as confirmed by the existing literature in the context of rice and other cereals (Adom & Liu, 2002). Several studies have shown that bound phenolic acids, commonly linked to dietary fiber and proteins through ester bonds, reach the large intestine and undergo an intense transformation to be hydrolyzed in the soluble form, which is able to positively modulate the gut microbial ecology (Rocchetti et al., 2022). For these reasons, this study gives emphasis and importance to the bound fraction as well.

The CWBPAs quantified in the rice fractions are reported in **Table 3**. Notably, in addition of caffeic acid, all the phenolic compounds identified in the soluble fraction have been quantified in their bounded form.

Ferulic and *p*-coumaric acids were the most prevalent phenolic acids in the extracts, both representing more than 55% of the total CWBPAs quantified; instead, protocatechuic acid was only detected in pigmented samples, with the exception of Risrus (red pericarp). *p*-Coumaric acid represents in husk fractions more than 80% of total CWBPAs, while in bran samples ferulic acid ranged from 46 to 74%.

In addition, *p*-hydroxybenzoic acid, caffeic acid, syringic acid and sinapic acid were minor constituents present in all rice fractions.

As evidenced in the SPAs quantification too, sequential polishing has shown that the total bound phenolic acids decrease progressively from the inedible paddy rice to the refined rice across all examined samples. The variations observed are attributed to the notable concentration of CWBPAs within the husk, which significantly predominates across all fractions of the analyzed rice cultivars.

Considering the grain fractions, except for Carnaroli cultivar, the results obtained showed a higher total polyphenolic content in all single paddies compared to unpolished and polished rice samples.

Comparing the edible products represented by unpolished and polished rice, the unrefined samples present values 2 (Risrus) to 9 (Solitario) times higher than those of the respective polished rice. However, only Solitario, Gioiello, Apollo and Jemma in the form of unpolished rice present concentration of bound phenolic acids significantly higher than in the respective polished one (**Table 3**). This confirms that, taking in consideration edible grains, most of the bioactive compounds are located in the bran (Feng et al., 2021). For unpolished and polished rice, the concentration of total CWBPAs ranged from 655 mg/kg of Gioiello, which detained the highest level along Jemma (622 mg/kg) to 281 mg/kg (Gilda) and from 166 mg/kg (Risrus) to 59.3 mg/kg (Solitario), respectively (**Table 4**).

Gioiello paddy rice, belonging to black pericarp varieties, showed the highest bound phenolic acids content (2285 mg/kg), followed by Jemma (black, 1990 mg/kg) and Gladio (brown pericarp, long B rice 1777 mg/kg), whereas Selenio showed the lowest value (brown pericarp round rice, 1035 mg/kg).

Concerning by-products, Gioiello, Jemma and Gladio husks showed the most abundant CWBPAs (7446, 7342 and 7341 mg/kg, respectively), while the lowest value was found in Gloria husk (4060 mg/kg).

Selenio bran contains the highest amount of bound polyphenols (3364 mg/kg), while Solitario has the lowest value (1941 mg/kg).

Considering unpolished rice (**Figure S2b**), the most interesting fraction as food from the nutritional point of view, the predominant compounds are ferulic acid (representing on average about 64% of the total CWBPAs content), *p*-coumaric acid (on average 21%), protocatechuic acid (on average 6%), sinapic acid (on average 4%) and vanillic acid (on average 3%). Protocatechuic and vanillic acid are quantified in high concentration only in the black pericarp varieties. These observations are in accordance with previous studies (Shao et al., 2018).

As far as the concentration of these bound phenolic acids is concerned, high levels of *p*-coumaric and ferulic acid were found, respectively, in Apollo (239 mg/kg) and Ronaldo (425 mg/kg), while protocatechuic and vanillic acids were abundant in Gioiello (154 mg/kg) and Jemma (62.6 mg/kg).

These results confirm that the major portion of these antioxidant compounds is found in bound form. The contribution of the insoluble phenolic acids to the total (SPAs+CWBPAs) in paddy samples ranged from 90% (Gladio) to 83% (Ronaldo). While in the other fractions the CWBPAs contributed 83% (Gloria) - 69% (Eclisse) of the total phenolic acids in unpolished samples, 93% (Gladio) - 78% (Gioiello) in polished rice, 94% (Gioiello) - 79% (Gloria) in husk and 93% (Risrus) - 76% (Gioiello) in bran.

As shown in **Figure 1b**, on average considering the selected cultivars, unpolished black pericarp varieties had the highest average concentration of CWBPAs (586 mg/kg), followed by the brown (502 mg/kg) and red rice (389 mg/kg) samples; while for refined samples, the black pericarp varieties analyzed (92.7 mg/kg) did not show significant differences compared with red (95.4 mg/kg) and brown pericarp ones (97.7 mg/kg). Paddy of black pericarp varieties have shown an average concentration of CWBPAs of 2002 mg/kg, higher than those shown in brown (1334 mg/kg) and red (1236 mg/kg) one, which were almost similar. As mentioned before, waste fractions obtained from sequential rice polishing are highly rich in phenolics, followed by grain samples. There was no significant difference in the average values of husk in brown (6136 mg/kg), red (6508 mg/kg) and black pericarp

(7302 mg/kg). As well as for the husk, no differences in the average values were observed between bran of brown (2879 mg/kg), red (2367 mg/kg), and black pericarp rice (3050 mg/kg).

Table 3. Distribution of cell-wall bound phenolic acids (mg/kg) in different fractions of rice varieties.

Pericarp color	Variety	Fraction	PRO	HYD	VAN	CAF	SYR	COU	FER	SIN	TOTAL CWBPAs
Brown	Apollo	Paddy	ND	12.6 c	13.8 b	3.23 c	8.37 b	1081 b	318 c	13.9 c	1451 c
		Husk	ND	57.1 a	78.3 a	74.3 a	52.1 a	5618 a	652 b	19.6 bc	6552 a
		Unpolished rice	ND	6.31 d	3.10 c	2.76 c	2.10 c	239 d	311 c	23.5 b	587 d
		Bran	ND	44.7 b	12.1 b	19.7 b	11.4 b	727 c	1609 a	158 a	2582 b
		Polished rice	ND	0.700 e	0.462 c	0.215 c	ND	4.26 d	87.0 d	1.12 d	93.7 e
	Gladio	Paddy	ND	19.5 c	21.4 b	4.41 c	9.62 b	1334 b	373 c	14.8 bc	1777 c
		Husk	ND	65.4 a	90.8 a	14.0 b	39.4 a	6402 a	706 b	23.2 b	7341 a
		Unpolished rice	ND	5.03 d	2.54 d	2.66 d	1.22 cd	139 cd	298 c	15.0 bc	464 d
		Bran	ND	41.6 b	9.82 c	18.5 a	6.88 bc	673 c	1718 a	119 a	2587 b
		Polished rice	ND	0.634 d	0.776 d	0.206 e	0.108 d	9.00 d	87.0 d	0.818 c	98.6 d
	Carnaroli	Paddy	ND	10.7 c	18.0 b	2.71 b	5.25 b	704 b	293 c	17.5 b	1052 c
		Husk	ND	43.3 a	94.5 a	3.45 b	32.7 a	4880 a	531 b	18.4 b	5604 a
		Unpolished rice	ND	3.50 cd	1.79 c	2.44 b	0.856 c	79.8 b	346 c	21.9 b	456 c
		Bran	ND	22.8 b	11.0 bc	25.9 a	5.45 b	545 b	1858 a	140 a	2609 b
		Polished rice	ND	0.417 d	0.213 c	0.121 b	0.149 c	6.31 b	71.8 d	0.805 c	79.8 c
	Cammeo	Paddy	ND	13.0 c	23.3 b	2.04 b	8.16 b	948 b	319 bc	16.8 b	1331 c
		Husk	ND	53.0 a	116.6 a	1.78 b	35.0 a	5752 a	566 b	17.9 b	6542 a
		Unpolished rice	ND	4.58 d	3.16 cd	1.59 b	1.97 c	199 c	340 bc	22.9 b	573 d
		Bran	ND	23.1 b	10.1 c	21.1 a	7.42 b	759 b	2156 a	160 a	3137 b
		Polished rice	ND	0.431 d	0.164 d	0.073 b	0.115 c	8.99 c	83.4 c	1.02 b	94.2 d
	Ronaldo	Paddy	ND	13.1 c	19.9 b	3.13 c	7.03 b	1035 b	409 c	32.9 b	1519 c
		Husk	ND	40.7 a	93.3 a	5.56 b	25.9 a	6342 a	661 b	30.9 b	7200 a
		Unpolished rice	ND	5.12 cd	1.67 c	2.53 c	1.13 c	111 c	425 c	29.2 b	576 d
		Bran	ND	28.8 b	9.81 bc	26.4 a	7.15 b	685 b	2331 a	203 a	3291 b
		Polished rice	ND	0.518 d	0.549 c	0.182 d	0.886 c	4.43 c	96.3 d	0.803 b	104 d
	Gloria	Paddy	ND	15.9 b	26.0 b	3.86 c	7.36 b	717 b	363 b	24.8 b	1157 c
		Husk	ND	72.8 a	130 a	61.0 a	35.2 a	3332 a	402 b	26.6 b	4060 a
		Unpolished rice	ND	2.86 cd	1.89 d	2.59 c	0.93 c	79.9 c	370 b	15.2 b	473 d
		Bran	ND	12.7 bc	11.1 c	21.9 b	7.24 b	524 b	1930 a	118 a	2626 b
		Polished rice	ND	0.406 d	0.488 d	0.179 c	ND	7.64 c	81.1 c	0.774 c	90.6 d
	Selenio	Paddy	ND	12.9 b	22.5 b	2.22 c	6.33 b	668 b	312 c	11.5 c	1035 c
		Husk	ND	51.7 a	124 a	5.73 b	32.7 a	4487 a	583 b	46.2 b	5330 a
		Unpolished rice	ND	6.02 bc	2.32 c	1.70 cd	0.91 c	63.6 c	327 c	17.7 c	420 d
		Bran	ND	45.2 a	10.5 bc	26.0 a	7.81 b	719 b	2389 a	166 a	3364 b
		Polished rice	ND	0.758 c	0.557 c	0.149 d	0.094 c	5.84 c	112 d	1.37 c	121 d
	Centauro	Paddy	ND	17.4 c	36.1 b	3.57 c	9.14 b	888 b	377 c	20.7 bc	1352 c
		Husk	ND	64.5 a	173 a	10.2 b	45.8 a	5537 a	596 b	35.8 b	6463 a
		Unpolished rice	ND	5.53 d	2.76 c	1.92 cd	1.07 c	77.3 c	360 c	16.3 cd	465 d
		Bran	ND	40.0 b	10.7 c	21.3 a	6.85 b	569 b	2057 a	130 a	2835 b
		Polished rice	ND	0.663 d	0.747 c	0.140 d	0.112 c	4.34 c	93.4 d	0.816 d	100 d
Red	Risrus	Paddy	ND	13.3 c	27.2 b	2.12 c	7.17 b	740 b	257 c	15.0 b	1062 c

		Husk	ND	59.8 a	139 a	8.11 b	34.2 a	5189 a	565 b	35.3 b	6030 a
		Unpolished rice	ND	4.05 d	3.10 d	1.52 c	1.21 c	78.1 c	232 cd	21.8 b	342 d
		Bran	ND	19.3 b	8.81 c	22.8 a	5.95 b	609 b	2353 a	152 a	3172 b
		Polished rice	ND	1.76 d	1.45 d	0.803 c	0.451 c	31.2 c	124 d	6.35 b	166 d
	Solitario	Paddy	3.60 b	12.3 c	24.1 c	2.40 b	5.98 b	833 b	278 c	16.4 b	1176 c
		Husk	3.08 b	49.4 a	136 a	9.99 a	27.5 a	6172 a	730 b	25.8 b	7154 a
		Unpolished rice	4.14 b	5.92 d	5.34 d	2.13 b	1.58 c	207 d	296 c	22.2 b	544 d
		Bran	123 a	26.5 b	39.0 b	9.36 a	5.86 b	490 c	1161 a	86.2 a	1941 b
		Polished rice	0.228 b	0.596 d	0.167 d	0.133 c	0.087 c	4.333 e	52.6 d	1.16 b	59.3 e
	Gilda	Paddy	5.60 b	14.5 c	29.7 b	2.82 c	8.59 c	1108 b	281 c	19.7 bc	1470 c
		Husk	3.36 c	50.5 a	122 a	7.82 b	34.3 a	5452 a	629 b	40.9 b	6340 a
		Unpolished rice	5.26 bc	3.50 d	3.21 d	2.20 c	0.973 d	61.7 d	191 d	13.3 c	281 d
		Bran	26.8 a	22.9 b	9.95 c	17.0 a	13.7 b	490 c	1297 a	112 a	1990 b
		Polished rice	0.204 d	0.562 d	0.164 d	0.279 d	0.115 d	4.01 d	53.7 e	1.36 c	60.4 d
Black	Jemma	Paddy	115 b	14.9 c	76.2 c	4.85 b	7.55 b	1372 b	380 c	20.0 c	1990 c
		Husk	12.7 c	41.6 a	95.1 b	5.27 b	27.0 a	6474 a	655 b	30.6 b	7342 a
		Unpolished rice	121 b	2.98 d	62.6 c	4.65 bc	ND	53.7 cd	360 c	15.9 c	622 d
		Bran	701 a	21.6 b	293 a	21.9 a	7.54 b	305 c	1602 a	90.3 a	3041 b
		Polished rice	2.34 c	0.397 d	1.86 d	0.269 c	0.101 c	4.48 d	64.4 d	0.650 d	74.5 e
	Gioiello	Paddy	162 b	10.1 c	63.8 c	3.25 bc	6.66 b	1632 b	384 bc	23.8 b	2285 c
		Husk	125 b	32.3 a	102 b	4.28 b	25.1 a	6591 a	526 b	41.3 b	7446 a
		Unpolished rice	154 b	3.18 d	56.3 c	3.58 b	ND	69.3 c	342 c	26.2 b	655 d
		Bran	908 a	20.7 b	255 a	17.3 a	4.87 b	331 c	1421 a	117 a	3075 b
		Polished rice	10.6 c	0.946 d	4.32 d	0.512 c	0.304 c	11.1 c	101 d	4.14 c	132 e
	Eclisse	Paddy	88.5 b	16.7 c	68.5 c	4.35 c	10.2 b	1184 b	343 c	13.8 c	1729 c
		Husk	14.4 c	54.9 a	134 b	10.2 b	39.6 a	6122 a	715 b	27.5 b	7117 a
		Unpolished rice	106 b	4.31 d	51.8 d	2.39 cd	ND	37.0 c	267 c	11.8 c	481 d
		Bran	822 a	28.5 b	295 a	17.2 a	9.33 b	242 c	1538 a	81.0 a	3032 b
		Polished rice	1.30 c	0.626 d	1.38 e	0.359 d	0.124 c	3.04 c	63.4 d	0.911 d	71.1 d

Values are expressed as mean (n = 3). Means with different letters in the same column of each rice variety were significantly different at the level $p < 0.05$. All reported means have a relative standard deviation less than 15%.
ND: not detected.
Protocatechuic acid (PRO), *p*-hydroxybenzoic acid (HYD), vanillic acid (VAN), caffeic acid (CAF), syringic acid (SYR), *p*-coumaric acid (COU), ferulic acid (FER), sinapic acid (SIN), cell wall-bound phenolic acids (CWBPAs).

Table 4. Total cell wall-bound phenolic acids (mg/kg) in different fractions of rice varieties.

	Brown								Red			Black		
	Long B*	Long B	Long A (high quality)	Long A (high quality)	Long A	Long A	Round	Round	Long A	Long A	Medium	Long A	Medium	Medium
Total CWBPAs	Apollo	Gladio	Carnaroli	Cammeo	Ronaldo	Gloria	Selenio	Centauro	Risrus	Solitario	Gilda	Jemma	Gioiello	Eclisse
Paddy	1451 def	1777 bc	1052 hi	1331 efgh	1519 cde	1157 ghi	1035 i	1352 efg	1062 hi	1176 fghi	1470 de	1990 b	2285 a	1729 bcd
Husk	6552 abc	7341 a	5604 c	6542 abc	7200 ab	4060 d	5330 c	6463 abc	6030 bc	7154 ab	6340 abc	7342 a	7446 a	7117 ab
Unpolished rice	587 ab	464 cd	456 cde	573 abc	576 abc	473 bcd	420 de	465 cd	342 ef	544 abc	281 f	622 a	655 a	481 bcd
Bran	2582 c	2587 c	2609 c	3137 ab	3291 ab	2626 c	3364 a	2835 bc	3172 ab	1941 d	1990 d	3041 abc	3075 abc	3032 abc
Polished rice	93.7 def	98.6 de	79.8 efgh	94.2 def	104 cd	90.6 defg	121 bc	100 cde	166 a	59.3 h	60.4 h	74.5 fgh	132 b	71.1 gh

Values are expressed as mean (n = 3). Means with different letters in the same raw were significantly different at the level $p < 0.05$. All reported means have a relative standard deviation less than 15%. Cell wall-bound phenolic acids (CWBPAs).

* The reported classification complies with the current European regulation (EU Regulation 1308/2013, 2013).

Anthocyanins content

The distribution patterns of the individual anthocyanins in the red and black varieties are shown in **Table 5**. Consistent with the findings of Hosoda et al. (2018), black pericarp cultivars exhibited a notable abundance of anthocyanins, due to the limited presence of these compounds in the red varieties, particularly in the case of Solitario, and their absence in the fractions from Risrus and Gilda. The main anthocyanin detected in all fractions was cyanidin-3-*O*-glucoside, although cyanidin-3-*O*-rutinoside, cyanidin-3-*O*-gentiobioside, peonidin-3-*O*-glucoside and peonidin-3-*O*-rutinoside were also present. Across all fractions examined, cyanidin-3-*O*-glucoside represents more than 80% of the total detected anthocyanins, except for Gioiello and Eclisse husk which represent 27 and 31%, respectively. Notably, in Gioiello's husk, cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, peonidin-3-*O*-glucoside and peonidin-3-*O*-rutinoside are present approximately in comparable amounts. In contrast, the husk of Eclisse is predominantly composed of 70% peonidin-3-*O*-glucoside.

Anthocyanins were found to be located in specific layers of the pigmented rice grain. More specifically, all the individual anthocyanins identified were significantly abundant in the bran layer.

Gioiello bran showed the highest sum of individual anthocyanins (5041 mg/kg), about 11 times higher than that observed in Solitario bran, which demonstrated the lowest value (456 mg/kg).

However, in contrast to what was observed in the case of phenolic acids, it is notable that the husk is the fraction that exhibited the lowest concentration of anthocyanins. This is consistent with the fact that these red and black pericarp varieties have not pigmented husk. Nevertheless, Gioiello husk exhibited the highest value for anthocyanins, with a concentration of 4.42 mg/kg.

Considering the edible grains forms, unpolished samples showed high levels of anthocyanin content compared to the respective polished rice. This is further confirmation that the majority of pigmented antioxidant compounds reside in the bran, as confirmed by literature (Zhang et al., 2022). Only in the case of Solitario variety, the anthocyanin content of unpolished rice was similar to refined rice; this result is probably due to the low anthocyanin values in the bran, which do not permit to cause a significant increase of these compounds in the grains conserving this layer.

The high content of anthocyanins in the bran layer of the kernel makes both bran and unpolished rice, particularly considering black varieties, interesting ingredients for healthy diets and for the production of functional foods, especially given the role that these pigmented antioxidants may have on human health (Gul et al., 2015).

Among unpolished rice cultivars (**Figure S3**), Gioiello was characterized by elevated levels of total anthocyanins (1901 mg/kg), approximately 3 and 4 times higher compared to the other black pericarp varieties Eclisse and Jemma, respectively. These results emphasize that anthocyanin content is genotype dependent, in agreement with previously reported studies (Choi et al., 2018; Shao et al., 2018).

The contribution of the cyanidin-3-*O*-glucoside in the analyzed unpolished rice samples ranged from 82% (Solitario) to 96% (Jemma), while the concentration of this anthocyanin varied from 0.732 mg/kg in Solitario to 1709 mg/kg in Gioiello.

Similar to unpolished samples, cyanidin-3-*O*-glucoside emerged as the predominant compound identified in refined rice, constituting a range from 84% (Solitario) to 95% (Jemma) of the total anthocyanin content. Furthermore, the concentration of the same compound exhibited levels ranging from 0.870 mg/kg in Solitario to 212 mg/kg in Gioiello. Also in this case, Gioiello stripped from its outer layers showed the highest concentration of anthocyanins (233 mg/kg), followed by Jemma (10.1 mg/kg), Eclisse (7.35 mg/kg), and Solitario (1.03 mg/kg).

Table 5. Anthocyanin composition (mg/kg) in rice fractions of red and black varieties.

Pericarp color	Variety	Fraction	Cyanidin-3-*O*-glucoside	Cyanidin-3-*O*-gentiobioside	Cyanidin-3-*O*-rutinoside	Peonidin-3-*O*-glucoside	Peonidin-3-*O*-rutinoside	Sum of anthocyanins
Red	Risrus	Paddy	ND	ND	ND	ND	ND	ND
		Husk	ND	ND	ND	ND	ND	ND
		Unpolished rice	ND	ND	ND	ND	ND	ND
		Bran	ND	ND	ND	ND	ND	ND
		Polished rice	ND	ND	ND	ND	ND	ND
	Solitario	Paddy	ND	ND	ND	ND	ND	ND
		Husk	ND	ND	ND	ND	ND	ND
		Unpolished rice	0.732 b	ND	ND	0.165 b	ND	0.897 b
		Bran	400 a	1.12	13.2	41.1 a	0.429	456 a
		Polished rice	0.870 b	ND	ND	0.159 b	ND	1.03 b
	Gilda	Paddy	ND	ND	ND	ND	ND	ND
		Husk	ND	ND	ND	ND	ND	ND
		Unpolished rice	ND	ND	ND	ND	ND	ND
		Bran	ND	ND	ND	ND	ND	ND
		Polished rice	ND	ND	ND	ND	ND	ND
Black	Jemma	Paddy	286 c	0.576 b	8.32 c	ND	4.54 c	299 c
		Husk	0.165 d	ND	ND	ND	ND	0.165 d
		Unpolished rice	455 b	0.772 b	12.49 b	ND	7.45 b	475 b
		Bran	2088 a	11.0 a	74.75 a	1.14	45.7 a	2221 a
		Polished rice	9.66 d	ND	0.298 d	ND	0.189 d	10.1 d
	Gioiello	Paddy	1292 c	3.45 b	43.5 b	110 c	1.82 b	1451 c
		Husk	1.19 d	ND	1.21 c	0.922 d	1.10 c	4.42 d
		Unpolished rice	1709 b	4.34 b	40.1 b	145 b	2.19 b	1901 b
		Bran	4388 a	46.8 a	192 a	411 a	3.51 a	5041 a
		Polished rice	212 d	ND	4.17 c	16.9 d	0.159 d	233 d
	Eclisse	Paddy	318 b	0.32 b	9.09 c	35.8 c	0.320 b	363 b
		Husk	0.067 c	ND	ND	0.148 d	ND	0.21 c
		Unpolished rice	525 b	0.732 b	15.0 b	56.2 b	0.299 b	597 b
		Bran	1838 a	6.85 a	69.7 a	222 a	1.54 a	2138 a
		Polished rice	6.37 c	ND	0.164 d	0.821 d	ND	7.35 c

Values are expressed as mean (n = 3). Means with different letters in the same column of each rice variety were significantly different at the level $p < 0.05$. All reported means have a relative standard deviation less than 15%. ND: not detected.

Antioxidant activity

It is well noted that phenolic compounds are characterized by high antioxidant properties, thus the different rice varieties and their processed fractions were further analyzed for the antioxidant activity. The free-radical scavenging activity (ABTS assay) and the ferric reducing ability power (FRAP assay) performed in all polishing fractions of brown and pigmented pericarp rice varieties are presented in **Figure 2.**

Consistent with the results that are presented, ABTS (**Figure 2a** and **2b**) and FRAP (**Figure 2c** and **2d**) are concordant in the majority of instances, but not identical.

Considering the edible fractions, as expected, the unrefined rice fractions has shown high antioxidant activity compared to the polished rice. The highest antioxidant activity of the unpolished rice is strictly related to the bioactive compounds present in the bran fraction, which has been removed from the polished rice (Butsat & Siriamornpun, 2010). Regarding the by-products, bran samples exhibited higher antioxidant activity compared to husk. Both ABTS and FRAP assays are in accordance with the highest antioxidant ability of all bran fractions, despite husk samples showed higher levels of bound and soluble phenolic acids when compared to the bran. In literature, it has been demonstrated that bran fraction contains other bioactive molecules like tocopherols, tocotrienols and γ-oryzanol which have important contributions to the antioxidant activity (Kim, 2005; Massarolo et al., 2017). These compounds may have contributed to the increased antioxidant activity observed in the bran; in fact, antioxidant assays based on the QUENCHER approach are directly performed on the matrix without involving extraction processes, thus final results are not depending by the solubility of components. In addition, in the case of pigmented varieties, particularly the black pericarp ones, antioxidant properties of bran fractions could also be related to the presence of high anthocyanin amounts.

Analyzing the single species of unpolished rice, it becomes evident that all conventional brown pericarp cultivars exhibit lower antioxidant activity in comparison to the pigmented grains (**Figure S4**). This observation was confirmed by both FRAP and ABTS assays, which, on the other hand, gave discordant results regarding pigmented varieties. Specifically, according to the ABTS assay, Gilda (155 mmol TE/kg) had the highest antioxidant activity, followed by Gioiello (120 mmol TE/kg), Risrus (69.5 mmol TE/kg), Solitario (53.1 mmol TE/kg), Eclisse (50.9 mmol TE/kg) and Jemma (36.2 mmol TE/kg), whereas considering the FRAP assay, Gioiello (46.9 mmol TE/kg) demonstrated superior antioxidant ability, followed by Solitario (32.0 mmol TE/kg), Gilda (27.9 mmol TE/kg), Risrus (25.4 mmol TE/kg), Jemma (19.4 mmol TE/kg) and Eclisse (16.7 mmol TE/kg). However, the discordance in TAA in unpolished varieties is attributed to the different reaction mechanisms of the assays, the dilution solvents and more important the affinity of these

methods for specific antioxidants (Biskup et al., 2013; Soobrattee et al., 2005). Comparing the unpolished rice samples by their color, the statistical analysis confirmed that brown pericarp samples had the lowest antioxidant activity in both assays (**Figure 3**). Red pericarp varieties have shown an average value slightly higher than the black ones considering the ABTS assay, while considering the FRAP method the pigmented varieties are similar to each other.

It is interesting to note that in this case there is no apparent relationship between antioxidant activity and phenolic content, as the unrefined black pericarp varieties had a higher level of both bound and soluble phenolic acids, as well as anthocyanins, compared to the red varieties. The antioxidant activity in red pericarp rice is most likely due to the presence of proanthocyanidins, also known as condensed tannins, which predominantly characterize the red varieties (Gunaratne et al., 2013). Our study did not specifically focus on this particular polyphenolic fraction, which has been richly described in the literature for its biosynthetic pathway in red varieties (Furukawa et al., 2007; Shao et al., 2018). Conversely, comparing the average value of different cultivars in the form of polished rice, no significant differences have been observed among red, black and brown pericarp varieties. However, comparing the mean values of brans, it is demonstrated that black ones possess an antioxidant capacity slightly higher, as indicated by both ABTS and FRAP (430 - 202 mmol TE/kg), than red varieties (233 - 152 mmol TE/kg). Subsequently, brown pericarp cultivars demonstrate the lowest levels (63.4 - 46.7 mmol TE/kg). In contrast, there was no significant difference between the average values of husk in brown, red and black rice in both assays.

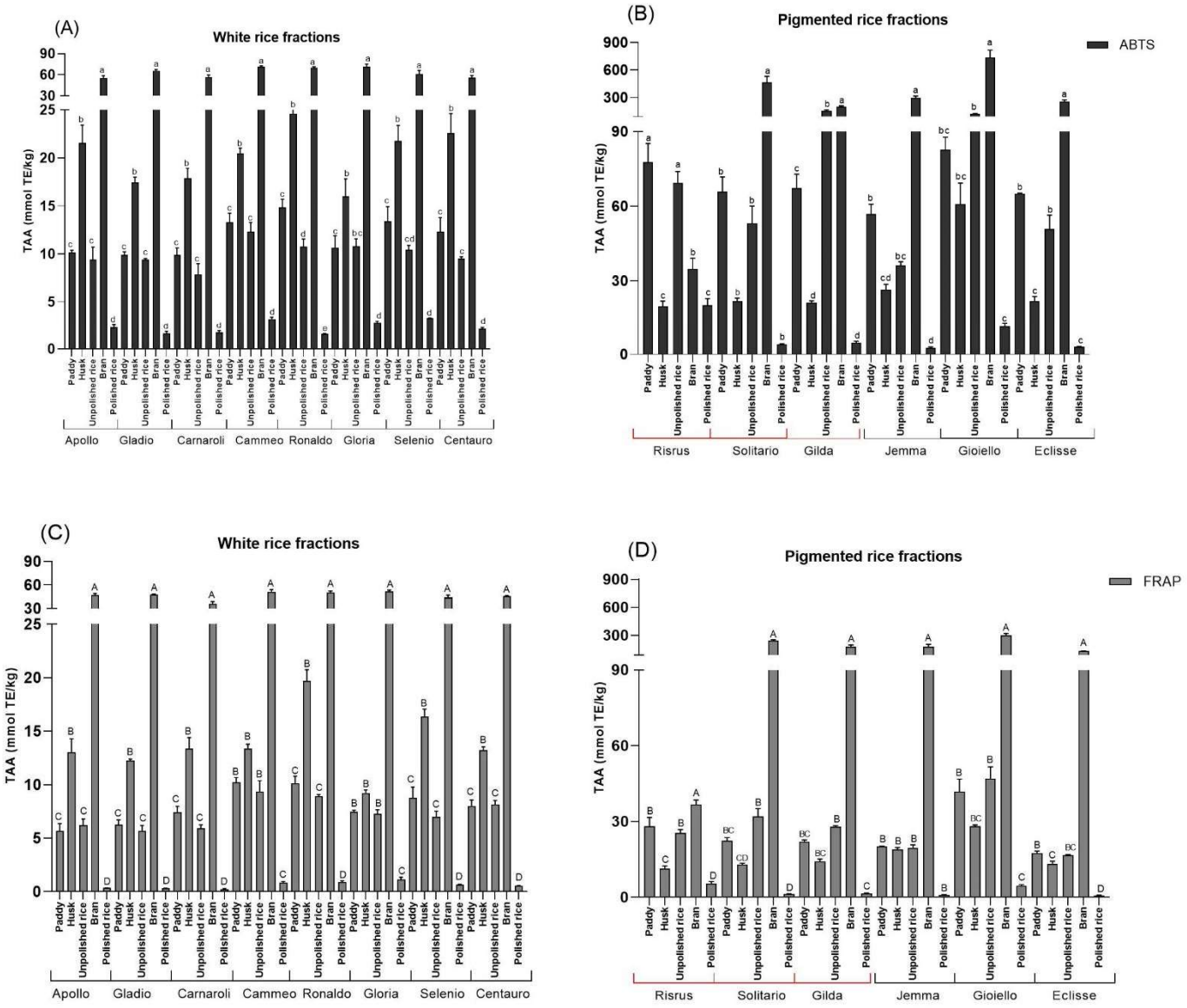


Figure 2. Total antioxidant activity (TAA) determination by ABTS and FRAP assays in conventional (brown pericarp) and pigmented rice fractions.

Pigmented rice varieties include red (Risrus, Solitario, Gilda) and black (Jemma, Gioiello, Eclisse) types. Different letters above the bars mean significant differences ($p < 0.05$) for ABTS (A, B) and FRAP (C, D) results within fractions of each variety.

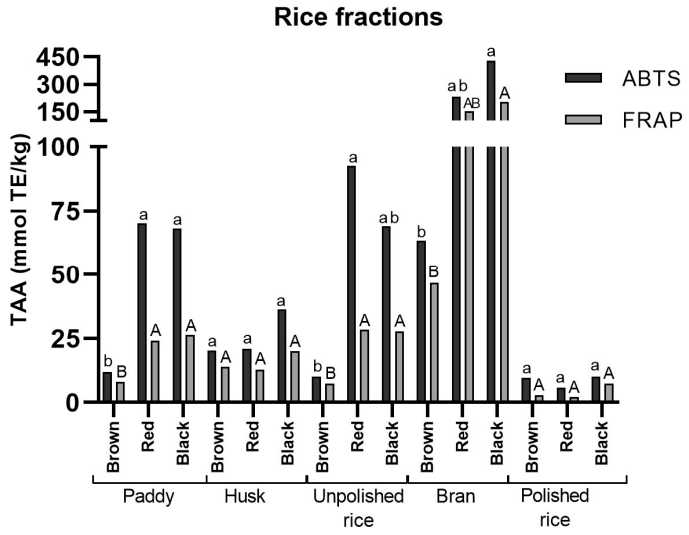


Figure 3. Average total antioxidant activity (TAA) determined by ABTS and FRAP in conventional (brown pericarp) and pigmented rice fractions.

Different smalls and capital letters above the bars mean significant differences ($p < 0.05$) for ABTS and FRAP results, respectively, within fractions.

Conclusions

This study explored the distribution of phenolic acids, in soluble and bound form, the anthocyanin profile and the antioxidant activity in different brown and pigmented pericarp rice varieties, and in their fractions, starting from paddy to refined rice. The concentration of phenolic constituents was found in greater amounts in the external layers, and the results depended on the molecular class. Soluble and bound phenolic acids were predominantly found in rice husks, which currently represent an underemployed waste and, instead, could be valorized and exploited for the extraction of bioactive compounds. As a result, rice husks could be seen as a cost-effective source of natural antioxidants. This aligns with the principles of a circular economy by adding value to agricultural waste. Contrarily, anthocyanins are characteristic of the bran fraction of black pericarp varieties. This latter is characterized by high free-radical scavenging activity and ferric reducing ability power against the ABTS and FRAP, respectively.

Moreover, this research focused on emphasizing the importance of incorporating unpolished rice into our diet over refined alternatives and exploring the value of pigmented rice in contrast to traditional brown varieties. Results evidenced that unrefined rice samples generally exhibited significantly elevated levels of phenolic compounds and antioxidant activity than their unpolished counterparts. Additionally, unrefined black pericarp varieties consistently demonstrated higher phenolic content compared to red and brown ones. Conversely, within the genotypes considered in this study, no significant correlations or associations were observed between the antioxidant polyphenol content and the market group's quality classification.

Among the 14 varieties analyzed, unrefined Gioiello from black varieties was characterized by an interesting phenolic concentration and high antioxidant activity.

These findings underscore the potential health benefits associated with pigmented unrefined rice and its external layers, often wasted, rich in phenolic compounds and anthocyanins, which could be crucial for developing functional foods and bioactive ingredients with enhanced antioxidant properties.

Supplementary material

Table S1. Main characteristics of rice varieties.

Pericarp color	Variety	Classification	Registration in the national database	Responsible for conservation in purity	Polishing yield (%)	Paddy thousand-seed weight (g)	Unpolished rice thousand-seed weight (g)
Brown	Apollo	Long B (Aromatic*)	2003	Sa.Pi.Se.	56	25.64	21.87
	Gladio	Long B	1998	Al.mo. s.p.a.	61	25.10	21.26
	Carnaroli	Long A (High quality**)	1983	Ente Nazionale Risi	59	40.24	32.70
	Cammeo	Long A (High quality**)	2015	Eugenio Gentinetta	60	39.25	33.86
	Ronaldo	Long A	2010	Lugano Leonardo s.r.l.	63	31.05	27.14
	Gloria	Long A	2010	Lugano Leonardo s.r.l.	59	40.98	34.95
	Selenio	Round	1987	Ente Nazionale Risi	68	24.85	22.00
	Centauro	Round	2003	Al.mo. s.p.a.	66	27.68	24.36
Red	Risrus	Long A	2015	Bertone Sementi	57	30.72	25.84
	Solitario	Long A (Aromatic*)	2018	Borando Daniele Sementi	56	24.90	21.39
	Gilda	Medium	2017	S.I.S. s.p.a.	55	28.14	24.35
Black	Jemma	Long A (Aromatic*)	2019	Borando Daniele Sementi	59	33.09	26.73
	Gioiello	Medium (Aromatic*)	2018	Borando Daniele Sementi	58	19.93	16.78
	Eclisse	Medium (Aromatic*)	2018	Borando Daniele Sementi	58	27.33	24.41

The reported classification complies with the current European regulation (EU Regulation 1308/2013, 2013).
* Aromatic rice classification as defined by the official Italian national database (registro varietale ENTERISI, Articolo 6 D.Lgs. 4 agosto 2017, n.131): https://www.enterisi.it/
** Quality classification according to the Italian law “Legge 18 marzo 1958, n. 325, 1958”.

Figure S1. Brown (a), red (b) and black pericarp (c) rice samples in unpolished form.

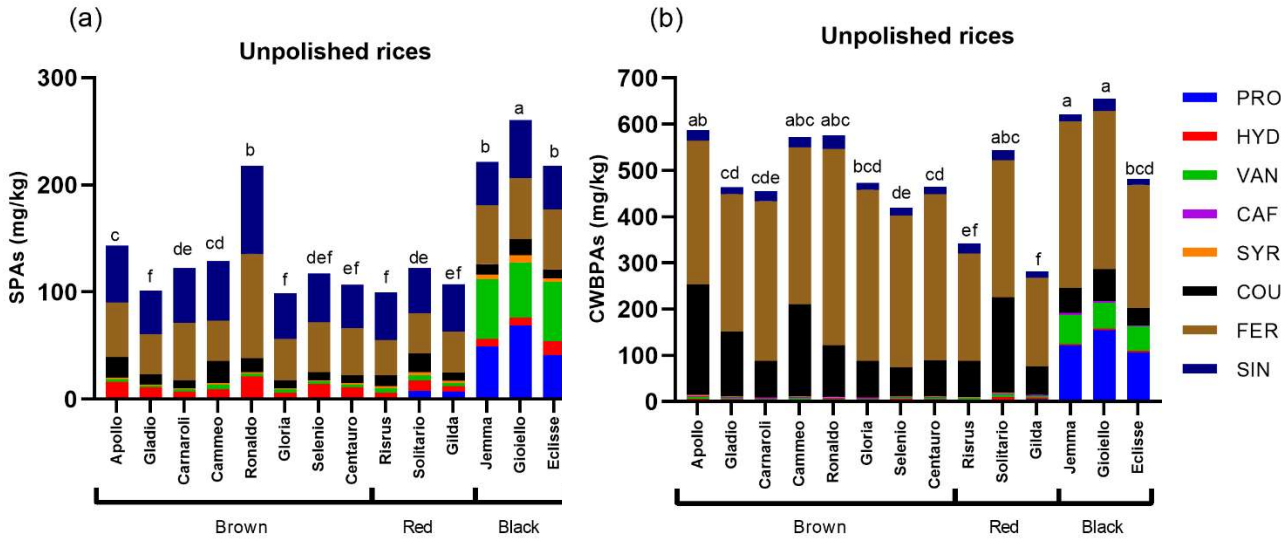


Figure S2. Soluble (SPAs; a) and cellwall-bound phenolic acids (CWBPAs; b) in unpolished rice samples (brown and pigmented pericarp).

Different letters above the bars mean significant differences ($p < 0.05$) within varieties.
Protocatechuic acid (PRO), *p*-hydroxybenzoic acid (HYD), vanillic acid (VAN), caffeic acid (CAF), syringic acid (SYR), *p*-coumaric acid (COU), ferulic acid (FER), sinapic acid (SIN).

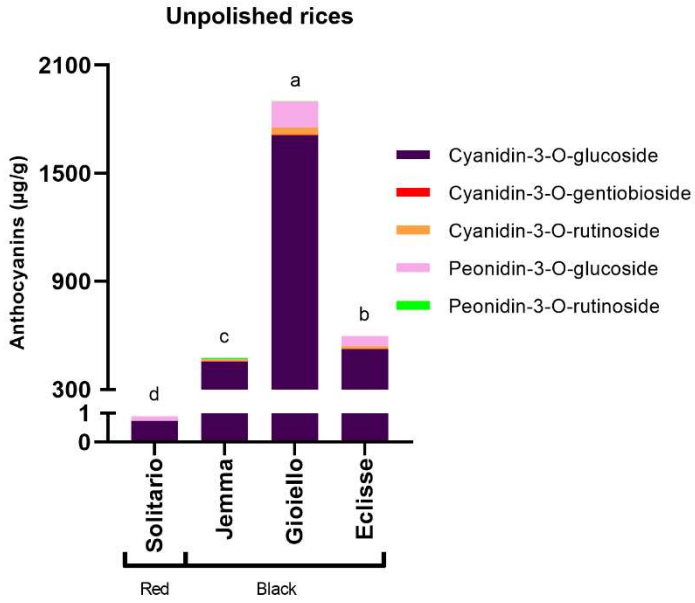


Figure S3. Anthocyanins profile in pigmented unpolished rice samples.

Different letters above the bars mean significant differences ($p < 0.05$) within varieties.

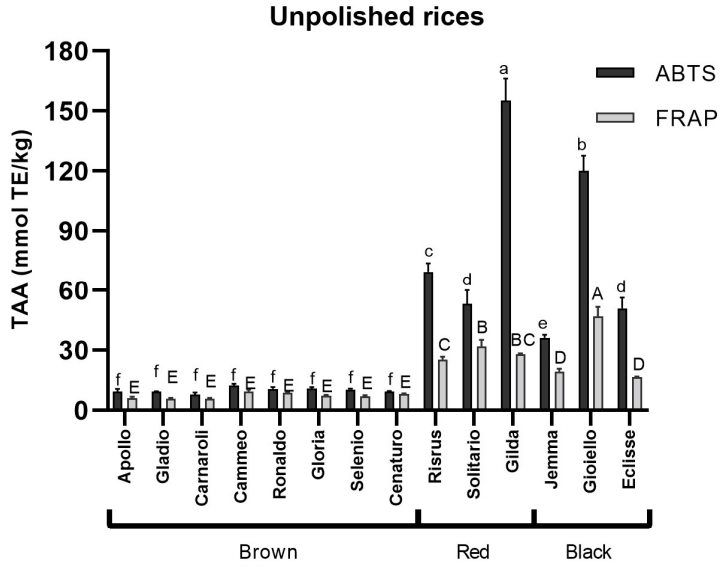


Figure S4. Total antioxidant activity (TAA) determination by ABTS and FRAP assays in unpolished rice samples (brown pericarp and pigmented).

Different smalls and capital letters above the bars mean significant differences ($p < 0.05$) for ABTS and FRAP results, respectively, within varieties.

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Chapter VI – "Balancing Nutrition and Safety: How Phytochemicals and Mycotoxins of Dent and Flint × Dent Corn are Affected by Different Dry Milling Strategies"

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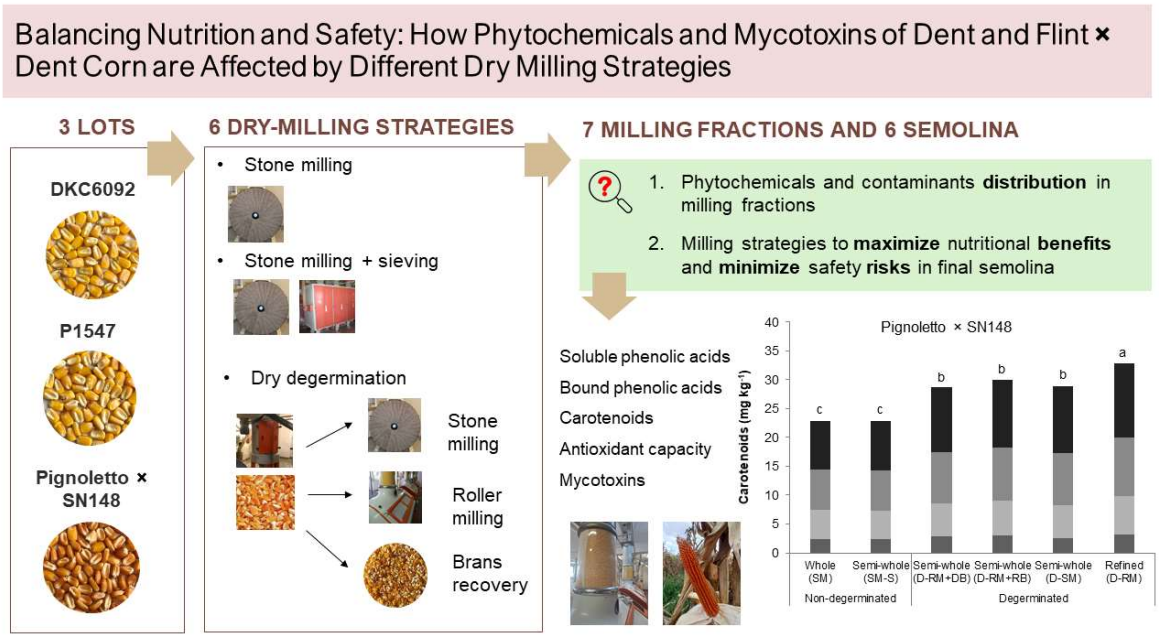
Graphical abstract



Abstract

This study assessed how different large-scale dry-milling strategies affect the redistribution of phenolic acids, carotenoids, antioxidant capacity, and mycotoxins in semolina and co-products from three corn hybrids, including a novel flint × dent genotype. The hybrids exhibit different redistribution patterns, likely influenced by different kernel hardness. Soluble and bound phenolic acids accumulated in fine and bran fractions, leading to higher retention in whole and semi-whole semolina, which also had the highest mycotoxin content. Carotenoids, mainly located in the endosperm fractions, were most abundant in the refined semolina (+45% vs. whole semolina), which also showed the greatest mycotoxin reduction. Intermediate strategies, such as stone milling after degermination or bran incorporation, offered a balance between antioxidant retention and safety, depending on the initial contamination. The flint × dent hybrid showed higher carotenoid and lower mycotoxin levels than the dent ones, supporting its potential in the dry-milling industry for by-products recovery and food enhancement purposes.

Keywords: dry degermination; stone milling; carotenoids; phenolic acids; fumonisins; deoxynivalenol; moniliformin.

Abbreviations: 3-ADON, 3-acetyldeoxynivalenol; 15-ADON, 15-acetyldeoxynivalenol; ABTS, 2,2′-Azino-bis(3-ethylbeenzothiazoline-6-sulfonic acid) diammonium salt; AC, antioxidant capacity; AF_{B1}, aflatoxin B_1; ANOVA, analysis of variance; D, degermination; DB, degermination bran; D-RM+DB, degermination followed by roller milling and degermination bran incorporation; D-RM+RB, degermination followed by roller milling and refining bran incorporation; D-RM, degermination followed by roller milling; D-SM, degermination followed by stone milling; FB_1, fumonisin B_1; FB_2, fumonisin B_2; FB_{TOT}, sum of FB_1 and FB_2; CWBPAs, cell wall-bound phenolic acids; DAD, diode array detection; DON, deoxynivalenol; DON-3-G, deoxynivalenol-3-glucoside; DON_{TOT}, total deoxynivalenol forms, sum of DON, DON-3-G, 3-ADON, and 15-ADON; DW, dry weight; FRAP, ferric reducing antioxidant power; HPLC, high-performance liquid chromatography; LOQ, limit of quantification; MON, moniliformin; MS, mass spectrometry; RB, refining bran; RM, roller milling; REGW–F test, Ryan–Einot–Gabriel–Welsch F test; SM, stone milling; SM-S, sieving followed by stone milling; SPAs, soluble phenolic acids; TCC, total carotenoid content; TE, Trolox equivalents; ZEA, zearalenone.

Introduction

Yellow corn hybrids of the dent type (*Zea mays* L.) represent a staple cereal crop worldwide, serving as a fundamental component for human nutrition, animal feed, and biofuel production, due to their versatility and high yield potential. The hardness of corn kernels, which is genetically determined and influenced by the relative proportion of vitreous to floury endosperm (Fox & Manley, 2009), allows medium-to-high-hardness dent corn to be effectively processed via dry milling. These processes yield various milling fractions and food-grade products with distinct particle size, which determines their functionality, food applications, and marketability. The corn-derived products are widely used either as is (e.g., semolina for polenta and porridge) or as ingredients (e.g., grits and flour) to be incorporated into diverse food categories, such as ready-to-eat breakfast cereals, snacks, bakery and pasta products (Gwirtz & Garcia-Casal, 2014).

The increasing consumer awareness of the benefits of a diverse and healthy diet has driven the demand for nutrient-dense foods, particularly in the gluten-free sector, where improving the nutritional profile remains a key challenge (El Khoury et al., 2018). Beyond its macronutrient composition, corn is also a rich source of bioactive phytochemicals, particularly phenolic acids and carotenoids, which are valued for their antioxidant properties and potential protective effects against chronic diseases (Siyuan et al., 2018). Corn grains contain phenolic acids at concentrations exceeding those found in many fruits and vegetables – up to ten times higher (Mattila et al., 2006; Rashmi & Negi, 2020) – and the highest among cereals (Adom & Liu, 2002; Ndolo & Beta, 2014). Additionally, corn of the yellow-orange type is the most carotenoid-rich cereal crops, with levels typically ranging from 16 to 39 mg kg^{-1} dry weight (Saenz et al., 2020), a trait strongly correlated with kernel and flour color (Kljak et al., 2014). Studies on several corn hybrids and varieties highlighted the extensive contribution of the genotype to the variation in the levels and profile of both phenolic compounds and carotenoids (De La Parra et al., 2007; Dias et al., 2021; Giordano et al., 2018; Žilić et al., 2012). As a consequence, breeding strategies have been used as a tool for biofortifying corn and enhancing its nutritional quality, as a complementary approach to food fortification and supplementation (Nestel et al., 2006).

In this context, corn landraces, characterized by genetic diversity and heterogeneity, represent a valuable resource to be exploited as genetic material for developing improved corn genotypes of enhanced kernel quality (Bernardi et al., 2018; Domínguez-Hernández et al., 2022; Puglisi et al., 2018). These genotypes, often of the flint type, usually display high kernel vitreousness (Borrás et al., 2022), which confers favorable traits such as superior milling properties (Blandino et al., 2013), lower susceptibility to damage (Dombrink-

Kurtzman & Knutson, 1997) and fungal penetration (Philippeau et al., 2000), and improved health-related benefits, such as lower *in vitro* starch digestibility (Caballero-Rothar et al., 2022) and higher carotenoid concentrations (Saenz et al., 2020). However, to enhance commercial viability and competitiveness of landraces, efforts have been made to develop flint × dent hybrids that combine the desirable attributes of landraces with the improved agronomic performance of modern high-yielding cultivars (Abdala et al., 2024). Such breeding strategies aim to preserve the kernel physical and nutritional qualities of local varieties while meeting the productivity demands and the sanitary requirements of modern agriculture and industrial food supply chains.

Nevertheless, the nutritional advantages offered by selective breeding must be preserved through post-harvest handling and processing. Milling, a necessary step for human consumption of cereal grains, often leads to significant losses of phenolic compounds, which are primarily concentrated in the bran (Butts-Wilmsmeyer et al., 2018). Conversely, more than 70% of total carotenoids are located within the corn vitreous endosperm, with the remainder distributed among floury endosperm, germ, and bran fractions (Ndolo & Beta, 2013). Thus, both the corn genotype and the type of processing employed significantly influence the final content and bioavailability of these compounds, highlighting the importance of integrated approaches to maximize their retention (Lozano-Alejo et al., 2007).

In addition to nutritional considerations, food safety is a critical concern for corn-based products, given corn high susceptibility to fungal contamination and mycotoxin accumulation (Locatelli et al., 2022). These secondary metabolites, produced by spoilage fungi of the *Fusarium*, *Aspergillus*, *Alternaria*, and *Penicillium* genera, compromise the marketability of corn-based products and pose significant risks to human and animal health (Marin et al., 2013). While regulatory frameworks primarily focus on aflatoxins, deoxynivalenol, zearalenone, and fumonisins, corn grains are frequently contaminated by several mycotoxins, including masked forms and other emerging compounds. Assessing the fate of these contaminants along the supply chain is crucial for accurate risk assessments and consumer safety. Combined strategies including genotype selection and application of specific agronomic practices are fundamental for minimizing the contamination levels prior to harvest (Blandino, Scarpino, et al., 2017). Additionally, several studies have examined the distribution of the main regulated (Bordini et al., 2017; Brera et al., 2006; Castells et al., 2008; Vanara et al., 2018) and emerging (Scarpino et al., 2020, 2021) mycotoxins during dry-milling processes of commercial lots, emphasizing the critical role of the cleaning and milling processes in redistributing the contaminants among milling co-products.

Industrial dry milling is the main process adopted for semi-vitreous dent corn destined to human consumption, particularly in regions where nixtamalization is not traditionally

applied. Among dry-milling techniques, stone milling and dry degermination offer different outcomes. Stone milling retains all anatomical parts of the kernel, including germ and bran, thereby preserving grain components such as dietary fiber, lipids, micronutrients, and phytochemicals as phenolic acids. This traditional method is gaining renewed interest among consumers seeking minimally processed, nutrient-dense products. On the other hand, degermination physically separates the kernel into endosperm, germ, and pericarp, and endosperm pieces can be further milled to obtain semolina with a reduced lipid content and longer shelf life. The choice between these milling strategies depends on the desired product characteristics, nutritional goals, market demands, and safety standards. While stone milling only allows to obtain whole or semi-whole semolina, with minimal decontamination capacity, degermination can improve food safety by redistributing contaminants in the milling by-products, and it gives the opportunity to further recombine the fractionated products to partially reconstitute the kernel anatomical features and obtain semi-whole semolina (Gwirtz & Garcia-Casal, 2014). Consequently, the integration of innovative milling strategies with selective breeding approaches is fundamental for developing corn-based products that are both nutritious and safe.

Within this framework, the present study investigates the impact of different dry-milling strategies on the retention and redistribution of both health-promoting bioactive compounds and harmful mycotoxins in corn intended for human consumption. Specifically, three mono-varietal lots, including a newly developed flint × dent hybrid developed from local germplasm, were processed using different dry-milling strategies. The antioxidant capacity of all the solid samples was assessed by measuring the free-radical scavenging and the ferric-reducing activities. In addition, the study compared the compositional characteristics, the content of phytochemicals and contaminants, and the antioxidant properties of six types of end-use cornmeal semolina generated from these processes. To the best of the authors' knowledge, this is the first study to simultaneously evaluate the effects of dry-milling strategies commonly applied by industrial mills for cornmeal semolina production on both key biologically active compounds and the main regulated/emerging contaminants. This integrated approach seeks to identify processing-genotype combinations that maximize nutritional benefits while minimizing safety risks, supporting the development of innovative corn-based food products aligned with modern dietary and regulatory demands.

Materials and methods

Samples and milling processes

The present study has compared three commercial corn lots cultivated in 2022 in the same growing area (north-west Italy, province of Turin). The lots were monovarietal and included two commercial dent × dent hybrids (DKC6092 and P1547), selected among those commonly used in the food industrial mill to produce hominy grits, and an innovative flint × dent hybrid (Pignoletto × SN148). The flint × dent hybrid was developed from local germplasm, by crossing the open-pollinated flint variety Pignoletto rosso (male parent), an old genotype still cultivated in north-west Italy for food purposes, with the dent pure line SN148 (female parent). Visual comparison of the kernels of the studied genotypes is presented in Figure S1.

Each hybrid was characterized for its kernel hardness by determining the total milling energy (TME) as reported in Blandino et al. (2013). This parameter provided an indirect evaluation of the relative amount of vitreous and soft fractions of the endosperm and allowed to discriminate the selected lots in medium, medium-high, and high hardness (1427, 1563, and 1684 W for DKC6092, P1547, Pignoletto × SN148, respectively).

All the corn lots were processed in an industrial mill by means of three milling strategies based on different dry-milling technologies (Figure 1). In all processes, the starting raw materials were cleaned grains. The cleaning was performed by a dry stoner, an intensive horizontal scourer, a vibrating aspirator, and an optical sorting equipment. The first process was based on the stone milling (Milleral, Imas Machinery, Turkey) of the cleaned grains without degermination (SM), and allowed to obtain whole semolina containing germ and bran. The second process involved stone milling, without a previous degermination step, followed by sieving (SM-S) in order to separate the animal feed flour, a mixture of impurities, bran and a portion of the mealy endosperm with a fine particle size (< 315 μm), and to obtain a semi-whole semolina containing germ and bran. Further processes were based on a dry-degermination system: an impact degerminator (Ocrim, Cremona, Italy) was used to break down the kernels into corn grits, an intermediate of the process made of pieces of the endosperm of 1000–2500 μm. After the degermination process, the main by products were the animal feed flour, the degermination bran (DB), and the germ, which were separated by means of a plansichter and a gravity separator (Ocrim, Cremona, Italy). The corn grits were then milled by applying two different processes: the first one involved stone milling and separation of the corn flour, that is, the low particle size flour derived from the softer parts of the endosperm (< 315 μm), which allowed the obtaining of semi-whole semolina without germ; the second milling process involved roller milling (RM), with a series of passages

through a sieving and classifying system composed of roller mills, plansichters, and flour purifiers (Sangati, Padova, Italy), which allowed to obtain refined semolina. These additional grinding and classifying steps resulted in a further separation of the corn flour and the refining bran (RB). The two types of bran obtained from both the degermination (DB) and refining steps (RB) were collected, milled using a Cyclotec 1093 sample mill (Foss, Padua, Italy), and blended with the refined semolina obtained from the degermination and roller-milling process in a 25:75 ratio, in order to produce other two types of semi-whole semolina. A total of 6 cornmeal semolina were obtained from the different milling strategies, and named as follows: the whole semolina, obtained through stone milling of the grains, without prior degermination (SM); the semi-whole semolina, obtained through stone milling of the grains and sieving, without prior degermination (SM-S); the semi-whole semolina, obtained through incorporation of degermination bran to degerminated refined semolina (D-RM+DB); the semi-whole semolina, following incorporation of refining bran to degerminated refined semolina (D-RM+RB); the semi-whole semolina, obtained through stone milling of degerminated grits (D-SM); the refined semolina, obtained through roller milling of degerminated grits (D-RM). All the cornmeal semolina were characterized for their particle size (Table S1) and proximal composition (Table S2).

The occurrence and distribution of biologically active compounds and mycotoxins was investigated by sampling and analyzing the grains after cleaning, 7 milling fractions (co-products and by-products including animal feed flour, bran, and corn flour), and 6 cornmeal semolina obtained from each corn lot. Each corn lot (200 t) was subjected to the previously descripted milling processes for 40 h. A total of 126 samples were collected (3 genotypes x 15 milling fractions or semolina x 3 replicates). The samples were collected from the opening slits of the milling plant according to a dynamic sampling procedure derived from the European Commission Regulation (EC) No. 401/2006. An aggregate sample was the result of a careful blending of 40 incremental samples of 100 g each, which were collected over a period of 1 h at regular intervals. A sampling lasting 1 h was performed three times for each lot and each milling process in order to obtain three replications.

The cleaned grains and the milling fractions were further ground to 0.50 mm using a laboratory mill (ZM-200, Retsch, Haan, Germany) in order to obtain a fine and homogeneous particle size, and the samples were stored at –25 °C until being analyzed.

Compositional analyses

The proximal composition of the 6 cornmeal semolina obtained is reported in Table S2. The protein and ash contents were determined according to the AOAC combustion 992.23 and direct 923.03 methods, respectively (AOAC International, 2005). The starch content was

measured according to Commission Regulation (EU) No 118/2010 of 9 February 2010, Annex I (European Commission, 2010). The fat and the total soluble and insoluble dietary fiber were measured according to Soxhlet method AOAC 2003.05 and enzymatic-gravimetric method AOAC 991.43, respectively (AOAC International, 2005). The moisture was determined by oven drying (Falc Instrument S.r.l., Treviglio, Italy) the samples at 105 °C to a constant weight, and the results of the chemical analyses were expressed on a dry-weight (DW) basis.

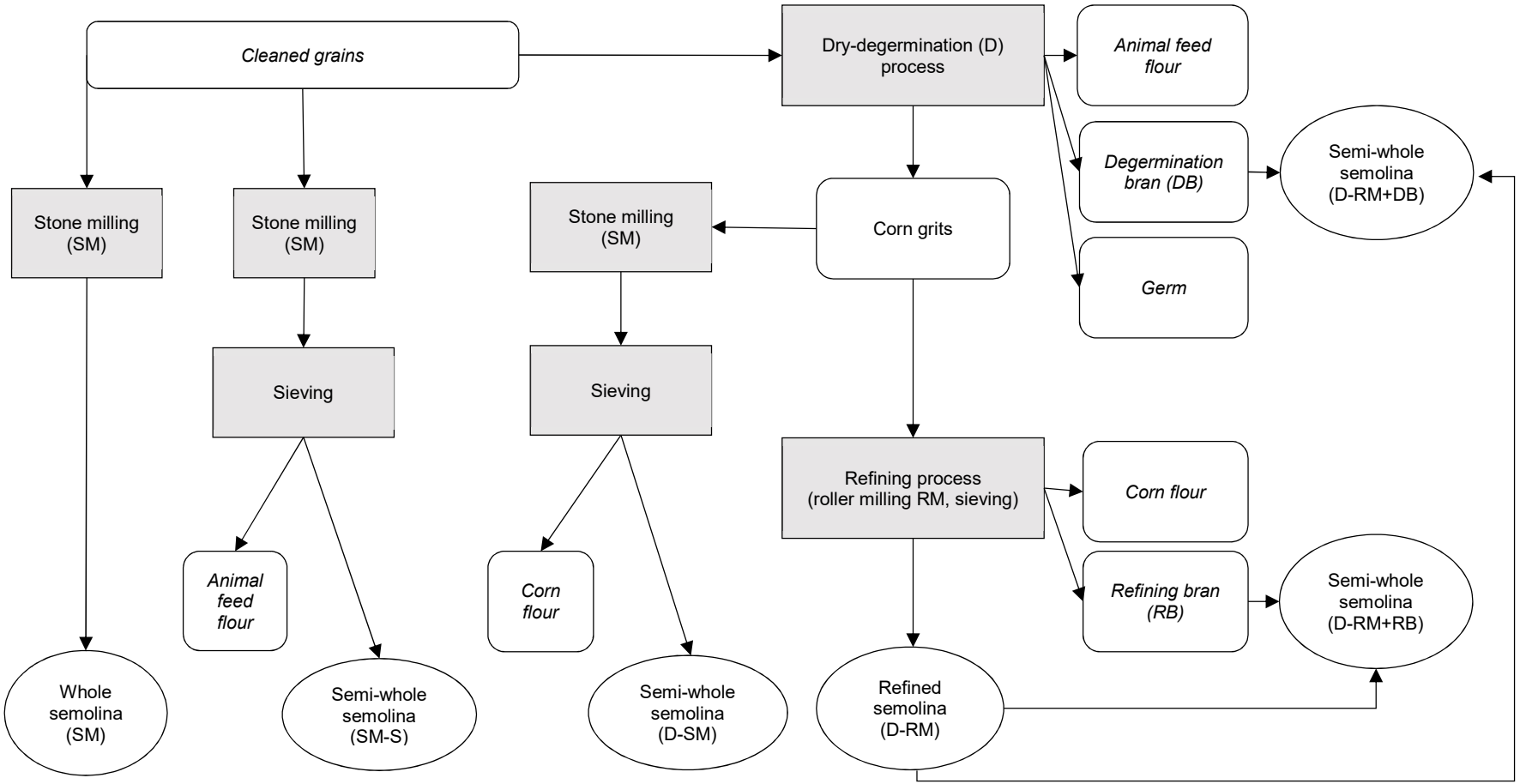


Figure 1. Flow diagram of the different milling processes.

D, degermination; SM, stone milling; SM-S, stone milling followed by sieving; D-RM+DB, degermination followed by roller milling and degermination bran incorporation; D-RM+RB, degermination followed by roller milling and refining bran incorporation; D-SM, degermination followed by stone milling; D-RM, degermination followed by roller milling. The milling strategies are reported in the rectangular shape. The raw materials and co-products collected and analyzed (italics) are reported in the semi-oval shape. The final cornmeal semolina are reported in the oval shape.

Chemical analyses

Chemicals

2,2′-Azino-bis(3-ethylbeenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), ethanol (CHROMASOLV®, 99.8%), ethyl acetate (CHROMASOLV®, 99.8%), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, 97%), hydrochloric acid (37%), iron(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$, ≥98%), methanol (CHROMASOLV®, 99.9%), potassium sulfate (≥99%), sodium hydroxide (≥98%), 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ), butylated hydroxytoluene (BHT, ≥99%), *tert*-butyl methyl ether (HPLC grade), and phenolic acid standards (caffeic acid ≥98%, *p*-coumaric acid ≥98%, ferulic acid ≥99%, gallic acid ≥99%, protocatechuic acid ≥99%, *p*-hydroxybenzoic acid ≥99%, sinapic acid ≥98%, syringic acid ≥95%, and vanillic acid ≥97%) were purchased from Merck KGaA (Darmstadt, Germany). β-Carotene and lutein were from Sigma (St. Louis, MO). β-Cryptoxanthin and zeaxanthin were from Extrasynthese (Z. I. Lyon-Nord, Genay, France). All the chemicals and analytical standards for mycotoxin LC-MS/MS analysis were purchased from Sigma Aldrich (St. Louis, MO), or from VWR (Milan, Italy). The solvents were all gradient grade or LC-MS grade. Water (HPLC grade) was prepared using an ELGA PURELAB Ultra system (M-medical, Cornaredo, Milan, Italy).

Analytical methods

The soluble (SPAs) and cell wall-bound phenolic acids (CWBPAs) were individually extracted through a two-step method and analyzed by means of HPLC–DAD. Details of the sample preparation procedure as well as of the chromatographic conditions were given in Giordano et al. (2019). The total carotenoid content (TCC) was determined as the sum of the individual compounds (β-carotene, β-cryptoxanthin, lutein, and zeaxanthin). Extraction and quantification by HPLC–DAD followed the method described by Panfili et al. (2004). Both phenolic acids and carotenoids were expressed in mg kg^{-1} DW.

The ABTS and ferric reducing antioxidant power (FRAP) assays were employed to determine the antioxidant capacity (AC), following the QUENCHER procedure (direct measurements on solid samples), as described by Serpen et al. (2008). The obtained results were expressed as mmol Trolox equivalents (TE) kg^{-1} DW.

The main regulated and emerging mycotoxins found in corn were determined by means of a multi-mycotoxin LC-MS/MS analysis. The extraction phase and the chromatographic and mass spectrometric parameters are reported in detail in Scarpino et al. (2019).

Statistical analyses

An analysis of variance (ANOVA) was run for each corn hybrid to compare the distribution of the phytochemical compounds, the antioxidant capacity levels, and the mycotoxin contamination in the analyzed milling fractions/coproducts. The final levels of phytochemical compounds, antioxidant capacity, and mycotoxins of the 6 types of cornmeal semolina obtained from the different milling strategies were compared by means of ANOVA, taking into account the effects of the hybrid (H) and the type of cornmeal semolina (S), and their interactions. Means were identified as significantly different ($p < 0.05$) on the basis of the Ryan–Einot–Gabriel–Welsch F (REGW–F) statistical test. Prior to the analysis, the data were tested for normality and homoscedasticity. The analyses were carried out by means of the SPSS for Windows statistical package, version 29.0.1 (SPSS, Inc., Chicago, IL, USA). Principal Component Analysis (PCA) was performed using the free statistical software R 4.2.2 version (R Core Team, 2020).

Results and Discussion

Distribution of phytochemicals and antioxidant capacity in the corn milling fractions

Phenolic acids

The study quantified 9 hydroxycinnamic and hydroxybenzoic acids in corn grains and milling fractions, both in their soluble (SPAs) and cell wall-bound (CWBPAs) forms (Table S3 and S4). The total contents calculated as the sum of the individual derivatives are reported in Table 1. Among the cleaned grains of the tested hybrids, DKC6092 exhibited the highest content of both SPAs and CWBPAs, followed by P1547 and Pignoletto × SN148. Overall, the total SPAs + CWBPAs (1753–2088 mg kg^{-1}) falls within the range of previously reported values of Adom & Liu (2002) for yellow corn (1749 mg kg^{-1}) and of Žilić et al. (2012) for colored corn (1556–4521 mg kg^{-1}), while it is lower than that reported for dent (4543 mg kg^{-1}) and flint corn (3455 mg kg^{-1}) by Das & Singh (2015) and for white dent corn (2198–3214 mg kg^{-1}) by Chiremba et al. (2012). The nutritional relevance of phenolic acids in both soluble and bound forms is well recognized (Călinoiu & Vodnar, 2018; Çelik & Gökmen, 2022), but their distribution across corn milling fractions is still poorly documented and limited to grain botanical fractions. This study provides new insights by analyzing their fate in various industrial dry milling processes.

The sieving procedure following stone milling led to the removal of animal feed flour, which had a higher SPA content than grains (+22%) for all hybrids. By applying dry milling following degermination, the redistribution among the fractions differed among the hybrids, even though the total SPAs were predominantly concentrated in the milling co-products generated during the degermination step. For instance, DKC6092 and P1547 had their highest SPA levels in the degermination bran (DB; 412 mg kg^{-1}; +35% vs. grains) and germ (336 mg kg^{-1}; +20% vs. grains), respectively, while Pignoletto × SN148 consistently showed in the degermination co-products the highest SPA concentrations (average of 350 mg kg^{-1}; +52% vs. grains). Subsequent milling of grits, using either stone or roller mills, further redistributed SPAs into milling co-products, with similar or lower amount than grains, resulting in a significant reduction of these compounds in the final semolina, both semi-whole and refined. The corn flour separated by stone milling accumulated more SPAs than that derived from roller milling for DKC6092 and P1547 (more than double and 43% higher, respectively), while no significant differences were detected for Pignoletto × SN148. In both DKC6092 and Pignoletto × SN148, the refining bran (RB) retained a relatively high amount of SPAs, comparable to the grains. These findings align with prior studies indicating an uneven distribution of total (Ndolo & Beta, 2014) and bound (Chiremba et al., 2012) phenolic acids in corn kernels, and differences between soluble and bound fractions (Das & Singh,

2015), with implications on the recovery of these nutritionally valuable compounds at industrial level. Das & Singh (2015) reported that the germ, which is usually discarded, contributed approximately 60% of total free phenolic acids in both dent and flint corn, followed by pericarp and endosperm. As in the present study, the same authors observed higher levels of free phenolic acids in the grains of the genotype characterized by a higher soft/hard endosperm ratio. In addition, in this work, the genotype characterized by higher kernel hardness, Pignoletto × SN148, yielded more SPAs in the degermination co-products despite lower initial grain concentrations, suggesting potential applications of its milling fractions for food fortification and value-addition purposes.

The distribution of individual SPAs differed among milling fractions (Table S3), confirming the varying concentrations observed in botanical fractions of dent and flint corn by Das & Singh (2015): ferulic, *p*-coumaric, and *p*-hydroxybenzoic acids were mainly detected in the germ (110, 58, 10 mg kg^{-1}, respectively), while others such as protocatechuic (42-48 mg kg^{-1}), sinapic (160-184 mg kg^{-1}), and syringic acids (9-11 mg kg^{-1}) were mostly found in co-products containing floury and bran parts of the kernels (animal feed flour, DB, corn flour from stone milling). Little information is available on the composition of the soluble form of phenolic acids in corn milling fractions, and, to the author's knowledge, this is the first study reporting the fate of the main hydroxycinnamic and hydroxybenzoic SPAs during different types of dry milling.

In terms of CWBPAs, the animal feed flour separated by stone milling contained significantly higher levels than the grains only in Pignoletto × SN148 (+11%), whereas the sieving of DKC6092 and P1547 yielded lower CWBPA concentrations (-24 and -15%, respectively) in the separated animal feed flour. Following degermination, DKC6092 yielded the highest concentration in the RB (2625 mg kg^{-1}; +47% vs. grains), followed by the DB (2386 mg kg^{-1}, +34% vs. grains), unlike P1547 and Pignoletto × SN148, whose CWBPAs accumulated the most in the degermination co-products. A marked increase was noticed for Pignoletto × SN148, as both the DB and the animal feed flour contained more than twice the CWBPA concentration found in the grains. For all hybrids, corn flour separated by stone milling retained higher CWBPA concentrations than that separated by roller milling. Only in the case of Pignoletto × SN148, the corn flours separated by stone and roller milling contained a significantly higher CWBPAs amount than the respective semolina. At industrial level, previous studies have observed a loss of both content and variability of bound ferulic and *p*-coumaric acids at the flaking grits stage (Butts-Wilmsmeyer et al., 2018), thus suggesting the possibility of adding back the removed phenolic acids to the processed food product by co-products extraction. Our study further delineates how different hybrids exhibit varying redistribution patterns in the milling co-products, likely influenced by different proportions of

floury and horny endosperm. Das & Singh (2015) observed a higher contribution of the pericarp of flint corn to the total bound phenolics (78%), compared to that of dent corn (55%). Comparing the ferulic acid content (Del Pozo-Insfran et al., 2006) and the bound phenolic acids (Chiremba et al., 2012) of corn genotypes varying in flour texture, higher values were reported in the relatively harder genotypes compared to the softer ones. The higher accumulation of phenolic acids of Pignoletto × SN148 in the pericarp-rich co-products observed for all the milling strategies may be linked to its flint genetic background and higher kernel hardness. Indeed, phenolic acids are thought to influence grain structural properties, as they form strong linkages within the plant cell walls to arabinoxylans and lignins, and were reported to be strongly correlated with corn kernel hardness and other physical properties (Chiremba et al., 2012). Although the present study analyzed a limited number of hybrids with varying kernel vitreousness, the results suggest that the milling of genotypes characterized by higher kernel hardness could result in enhanced accumulation of phenolic acids in specific milling co-products, which could be recovered as a cost-effective source of antioxidants and used to serve either as functional ingredients for food addition purposes or for bioactive compound extraction, when sanitary standards are not met. The distribution within milling fractions of individual bound phenolic acids was less heterogenous than that of the soluble form (Table S4), with ferulic acid being mostly present in the DB (average of 1963 mg kg^{-1}) and consistently holding the highest share (77-88%), followed by *p*-coumaric and sinapic acid, which were also mainly accumulated in the DB (321 and 100 mg kg^{-1}). The share of *p*-coumaric increased in the germ fraction (17%) and decreased to 6% in the fine fractions (corn flour and refined semolina); the average contribution of sinapic acid was around 4% in all fractions with the exception of corn flour and refined semolina (8%).

Table 1. The distribution of soluble (SPAs) and cell wall-bound (CWBPAs) phenolic acids, carotenoids (TCC), and antioxidant capacity (AC) in the products and coproducts of different milling processes and different maize hybrids.

Hybrid	Degermination process (D)	Dry milling process	Milling fraction	SPAs (mg kg^{-1})	CWBPAs (mg kg^{-1})	TCC (mg kg^{-1})	AC_{ABTS} (mmol TE kg^{-1})	AC_{FRAP} (mmol TE kg^{-1})
DKC6092			Corn grains	305 bc	1782 c	19.3 bcd	10.5 c	13.2 b
	None	SM-S	Animal feed flour	350 b	1350 e	13.9 e	11.3 bc	12.0 bc
			Semi-whole semolina (SM-S)	268 c	1747 cd	20.2 bc	9.7 c	11.2 c
	D		Animal feed flour	330 b	1552 d	19.2 bcd	10.4 c	11.0 c
			Degermination bran (DB)	412 a	2386 b	23.7 b	10.5 c	13.3 b
			Germ	345 b	1280 e	14.6 de	13.8 a	13.7 b
		SM	Corn flour	350 b	772 f	18.2 cde	10.7 c	9.3 d
			Semi-whole semolina (D-SM)	122 e	651 f	23.6 b	7.0 d	5.4 ef
		RM	Corn flour	166 d	266 g	21.8 bc	6.6 d	3.8 f
			Refining bran (RB)	330 b	2625 a	22.4 bc	12.3 b	15.7 a
			Refined semolina (D-RM)	186 d	396 g	31.7 a	5.8 d	5.5 e
			p (F)	***	***	***	***	***
P1547			Corn grains	279 bc	1552 a	21.8 bc	10.3 de	11.8 c
	None	SM-S	Animal feed flour	356 a	1325 b	20.0 bcd	11.9 c	14.1 b
			Semi-whole semolina (SM-S)	258 cd	1628 a	22.7 b	9.6 ef	10.9 c
	D		Animal feed flour	235 de	1237 b	17.1 d	11.8 cd	11.8 c
			Degermination bran (DB)	300 b	1685 a	21.5 bc	13.9 b	17.8 a
			Germ	336 a	1207 b	5.5 e	17.8 a	18.6 a
		SM	Corn flour	227 e	531 d	21.2 bc	8.5 f	8.4 d
			Semi-whole semolina (D-SM)	130 g	440 de	24.4 ab	6.7 g	6.7 ef
		RM	Corn flour	159 f	329 e	17.7 cd	5.1 h	6.5 f
			Refining bran (RB)	172 f	1063 c	23.7 b	11.0 cde	7.8 de
			Refined semolina (D-RM)	116 g	333 e	27.7 a	6.6 g	5.0 g
			p (F)	***	***	***	***	***
Pignoletto × SN148			Corn grains	231 c	1522 c	22.8 d	13.5 d	13.1 c
	None	SM-S	Animal feed flour	291 b	1690 b	16.3 f	16.4 c	17.3 b
			Semi-whole semolina (SM-S)	232 c	1471 c	22.9 d	12.2 d	12.8 c
	D		Animal feed flour	363 a	3097 a	18.2 ef	16.9 bc	20.6 a
			Degermination bran (DB)	349 a	3165 a	19.9 e	18.6 ab	20.6 a
			Germ	339 a	1185 d	13.2 g	19.2 a	19.0 ab
		SM	Corn flour	192 d	758 e	23.7 cd	10.1 e	8.7 d
			Semi-whole semolina (D-SM)	67 f	433 f	28.9 b	6.6 fg	4.6 ef
		RM	Corn flour	181 d	360 f	24.4 cd	7.8 f	5.9 e
			Refining bran (RB)	206 cd	1140 d	26.2 bc	13.1 d	12.8 c
			Refined semolina (D-RM)	103 e	181 g	32.8 a	5.2 g	3.7 f
			p (F)	***	***	***	***	***

D, degermination; SM, stone milling; SM-S, stone milling followed by sieving; RM, roller milling; DB, degermination bran; RB, refining bran; SPAs, soluble phenolic acids; CWBPAs, cell wall-bound phenolic acids; TCC, total carotenoids content; AC, antioxidant capacity by means of QUENCHER-ABTS and -FRAP assays. The results are expressed on a DW basis. Means followed by different letters are significantly different, according to the REGW-F test [(*) *p* (F) < 0.05, (**) *p* (F) < 0.01, and (***) *p* (F) < 0.001, and ns, non-significant].

Carotenoids

Comparing the cleaned grains, the highest TCC, calculated as the sum of β-carotene, β-cryptoxanthin, zeaxanthin, and lutein (Table 1), was found in Pignoletto × SN148, followed by P1547 and DKC6092, in agreement with previous studies indicating higher carotenoid concentration in hard endosperm yellow corn (Saenz et al., 2021). Unlike phenolic acids, carotenoids were most concentrated in the refined semolina obtained via roller milling, rather than in milling co-products. The animal feed flour separated after stone milling was characterized by similar (P1547) or lower (-28%, DKC6092 and Pignoletto × SN148) TCC than the grains. The lowest TCC was found in the germ (-24%, -75%, -42% vs. grains for DKC6092, P1547, and Pignoletto × SN148, respectively). For DKC6092 and P1547, characterized by lower kernel hardness than Pignoletto × SN148, degermination co-products like bran and feed flour retained carotenoid levels comparable to the cleaned grains, probably due to adulteration of endosperm fraction during separation, so that only the refining step via roller milling resulted in a semolina with significantly higher TCCs than cleaned grains. In contrast, Pignoletto × SN148 showed low TCC in all degermination by-products, with semolina (both semi-whole and refined) having higher levels than the grains. Only in Pignoletto × SN148 did the two bran types differ significantly, with RB containing 32% more TCC than DB (26.2 vs 19.9 mg kg^{-1}). These observations at industrial level reflect the distribution pattern observed for hand-dissected corn fractions by other authors, who reported higher carotenoid concentrations in the endosperm and aleurone layer rather than the germ, unlike in other cereals like wheat, oats, and barley (Ndolo & Beta, 2013). Negligible or low (1.8-6.5 mg kg^{-1}) amounts of TCC were observed in the outer pericarp by Ndolo & Beta (2013) and Kean et al. (2008), respectively, in mechanically separated bran fraction. Ortiz et al. (2018) found a lower content of total carotenoids of about 15% in the flour with particle size < 212 μm compared to meal and grits fractions of biofortified pro-vitamin A cultivars, in good accordance with the findings of the present study. However, the same authors also observed a loss of about 14% for each carotenoid after mechanical decortication and removal of pericarp and germ, although the differences were only significant for the orange-flint genotype and not for the yellow-dent genotype. Other works focusing on carotenoid retention during dry milling of biofortified pro-vitamin A corn found no significant carotenoid losses during the production of corn debranned (Mugode et al., 2014) or degerminated flours (Kean et al., 2011). Although direct comparisons are not possible due to differences in the dry milling method and co-products particle sizes, the distribution pattern observed in the present study is similar to what Kean et al. (2011) found for decorticated bran fractions, with significantly lower carotenoid content (18.0 mg kg^{-1}) than decorticated flour fractions (30.8 mg kg^{-1}) and corn grains(22.5 mg kg^{-1}). Similarly,

Blandino et al. (2017) reported the highest xanthophyll content in the fractions derived from the endosperm obtained after dry degermination for the production of cornmeal semolina with different particle sizes, and the lowest in the germ and animal feed flour. However, the same authors observed for the semolina and corn flour a xanthophyll content similar to that of the grains, whereas in this work the analysis of the individual compounds highlights the effectiveness of roller milling in maximizing the carotenoid concentration in the final refined semolina. High-performance liquid chromatography revealed differential distribution of individual compounds within milling fractions (Table S4). A high ratio of xanthophyll/carotenes was observed in both types of bran and degerminated semolina, while corn flour, animal feed flour, and germ were characterized by lower values. The animal feed flour and degermination co-products (with the exception of the germ) showed a higher content of zeaxanthin than lutein, the latter one mainly accumulated in the milling fractions derived from the endosperm following degermination. The floury parts of the endosperm (animal feed and corn flour) were characterized by a higher β-cryptoxanthin/β-carotene ratio, while the relative abundance of β-carotene was greater in germ and bran.

Table 2. The distribution of total fumonisins (FUM_{TOT}), total deoxynivalenol (DON_{TOT}), moniliformin (MON), aflatoxin B_1 (AF_{B1}), and zearalenone (ZEA) in the products and coproducts of different milling processes and different corn hybrids.

Hybrid	Degermination process (D)	Dry milling process	Milling fraction	FB_{TOT} (µg kg^{-1})	DON_{TOT} (µg kg^{-1})	AF_{B1} (µg kg^{-1})	ZEA (µg kg^{-1})	MON (µg kg^{-1})
DKC6092			Corn grains	2998 d	3998 d	<LOQ	85 d	725 c
	None	SM-S	Animal feed flour	4071 b	4344 c	<LOQ	93 c	983 b
			Semi-whole semolina (SM-S)	2332 e	3569 e	<LOQ	77 e	603 d
	D		Animal feed flour	6748 a	5204 a	<LOQ	119 a	1663 a
			Degermination bran (DB)	3394 c	5048 b	<LOQ	110 b	948 b
			Germ	875 g	3214 f	<LOQ	61 f	330 f
		SM	Corn flour	1453 f	2824 g	<LOQ	39 g	493 e
			Semi-whole semolina (D-SM)	503 h	2565 h	<LOQ	41 g	361 f
		RM	Corn flour	1108 fg	2654 h	<LOQ	39 gh	590 d
			Refining bran (RB)	486 h	1995 i	<LOQ	33 i	217 g
			Refined semolina (D-RM)	383 h	1887 i	<LOQ	36 h	182 g
			p (F)	***	***	ns	***	***
P1547			Corn grains	5269 d	3078 d	3.2 e	51 d	505 c
	None	SM-S	Animal feed flour	7068 c	3403 c	4.6 b	55 c	612 b
			Semi-whole semolina (SM-S)	4034 e	2851 e	2.7 f	45 e	444 d
	D		Animal feed flour	10783 a	4290 a	3.8 d	71 a	1025 a
			Degermination bran (DB)	7543 b	4050 b	4.3 c	67 b	628 b
			Germ	1597 h	2096 f	5.3 a	37 f	249 g
		SM	Corn flour	2051 g	1933 gh	<LOQ	21 g	364 e
			Semi-whole semolina (D-SM)	1307 h	2000 g	<LOQ	22 g	299 f
		RM	Corn flour	3411 f	1877 h	1.7 g	22 g	479 cd
			Refining bran (RB)	617 i	1399 l	<LOQ	16 h	163 h
			Refined semolina (D-RM)	565 i	1503 i	<LOQ	18 h	154 h
			p (F)	***	***	***	***	***
Pignoletto × SN148			Corn grains	1593 c	2244 d	<LOQ	42 d	379 c
	None	SM-S	Animal feed flour	2168 b	2391 c	<LOQ	45 c	648 b
			Semi-whole semolina (SM-S)	1383 d	1981 e	<LOQ	33 e	308 d
	D		Animal feed flour	5131 a	2999 a	<LOQ	57 a	1156 a
			Degermination bran (DB)	2210 b	2586 b	<LOQ	53 b	647 b
			Germ	800 f	1967 e	<LOQ	28 f	168 e
		SM	Corn flour	803 f	1405 g	<LOQ	17 g	189 e
			Semi-whole semolina (D-SM)	403 g	1174 h	<LOQ	18 g	283 d
		RM	Corn flour	1113 e	1562 f	<LOQ	16 g	203 e
			Refining bran (RB)	301 g	865 i	<LOQ	10 i	115 f
			Refined semolina (D-RM)	262 g	920 i	<LOQ	13 h	90 f
			p (F)	***	***	ns	***	***

D, degermination; SM, stone milling; SM-S, stone milling followed by sieving; RM, roller milling; DB, degermination bran; RB, refining bran; FB_{TOT}, total fumonisins, sum of fumonisins B_1 and B_2); DON_{TOT}, total deoxynivalenol, sum of DON, deoxynivalenol-3-glucoside (DON-3-G), 3-acetyldeoxynivalenol (3-ADON), and 15-acetyldeoxynivalenol (15-ADON); AF_{B1}, aflatoxin B_1 (LOQ = 1.6 µg kg^{-1}); ZEA, zearalenone; MON, moniliformin; LOQ, limit of quantification. 3-ADON, nivalenol (NIV), and enniatin B (ENN B) were <LOQ (LOQ = 10, 10, and 1.4 µg kg^{-1}, respectively). Means followed by different letters are significantly different, according to the REGW-F test [(*) *p* (F) < 0.05, (**) *p* (F) < 0.01, and (***) *p* (F) < 0.001, and ns, non-significant].

Antioxidant capacity

The milling products/co-products were also evaluated for their free-radical scavenging potential (ABTS assay) and ferric-reducing activity (FRAP assay) (Table 1). Using ABTS, the germ of all hybrids exhibited the highest antioxidant capacity (AC). By means of both assays, in P1547 and Pignoletto × SN148 the animal feed flour and the DB along with germ consistently exhibited higher AC than the grains. Overall, a progressive reduction of AC was observed in all hybrids after degermination, as corn flour and RB yielded similar or lower values than the grains, except for the RB of DKC6092, whose AC was found to be significantly higher than that of the grains by means of both assays, most likely due to the high amount of CWBPAs (Cömert & Gökmen, 2017). On the other hand, the RBs of P1547 and Pignoletto × SN148 were consistently characterized by a lower AC than that of DB. The effectiveness in the recovery of antioxidant-rich fractions differed at the refining step, as the corn flour separated by stone milling had a superior AC than the corn flour separated by roller milling, the latter one showing the lowest AC among all the milling co-products.

Distribution of mycotoxins in corn fractions

The fate of the main regulated and emerging mycotoxins detected in the different milling fractions/co-products of the three hybrids is reported in Table 2. In 2023, the rainy and cooler weather conditions from May to September favored the growth of *Fusarium* spp., leading to significant contamination by *Fusarium*-derived metabolites in all corn hybrids. Meanwhile, *Aspergillus*-derived metabolites were only found in the form of aflatoxin B_1 (AF_{B1}) in P1547 grains (below the maximum level of 5.0 μg kg^{-1} established by the European Commission Regulation (EU) 2023/915), and some milling fractions. Among hybrids, Pignoletto × SN148 consistently had the lowest contamination levels in both grains and milling fractions. P1547 exhibited the highest FB_{TOT} contents, reaching 5269 μg kg^{-1} in grains, thus exceeding the European Commission Regulation (EU) 2023/915 limit for unprocessed corn grains (4000 μg kg^{-1}). The grains of all the hybrids turned out to be contaminated with DON at levels (data not shown) exceeding established limit in unprocessed corn grains (1500 μg kg^{-1}), with DKC6092 exhibiting the highest levels (3062 μg kg^{-1}), followed by P1547 and Pignoletto × SN148 (2364 and 1726 μg kg^{-1}, respectively). In addition, some of DON plant metabolites were also detected, i.e. DON-3-G and 15-ADON, while 3-ADON was always under the limit of quantification (<LOQ). The relative abundance, compared to DON_{TOT}, was 77% for DON, 15% for DON-3-G, and 8% for 15-ADON. DKC6092 also exhibited the highest levels of ZEA (85 μg kg^{-1}), another regulated mycotoxin produced by *Fusarium* spp., although the limit (100 μg kg^{-1}) was not exceeded. Being MON an emerging mycotoxin, no regulatory limits have been established. However,

it is noteworthy to mention the different susceptibility to MON accumulation between hybrids, and the overall higher contamination of cleaned grains of the present study (379-725 µg kg^{-1}) when compared to previously reported data of the same growing area (215 µg kg^{-1}; Scarpino et al., 2020).

The animal feed flour obtained after degermination represented the fraction with the highest contents of FB_{TOT}, DON_{TOT}, ZEA, and MON for all hybrids. The inverse relationship between the particle size of corn milling fractions and the FB_{TOT} content is well known in the literature (Bordini et al., 2017; Vanara et al., 2018) and was recently described for more than 30 mycotoxins and other fungal metabolites, including DON and ZEA, with their masked or modified forms, and MON (Scarpino et al., 2021). Compared to the grains, FB_{TOT} and MON levels increased by 2.3-fold in DKC6092 and P1547, and by 3.1-fold in Pignoletto × SN148. DON_{TOT} and ZEA concentrations also increased across all hybrids, although to a lesser extent (average of 1.3- and 1.4-fold, respectively). Conversely, the increase of *Fusarium* toxins was less pronounced in the animal feed flour obtained following stone milling and sieving, by 1.4-fold for FB_{TOT} and MON, and by 1.1-fold for DON_{TOT} and ZEA. This strategy resulted in a significant reduction of mycotoxin contamination in the semi-whole semolina compared to the grains. However, the decontamination was far less pronounced than that achieved by applying degermination. Indeed, degermination was effective in redistributing the contamination in the animal feed flour as well as in the DB, with FB_{TOT}, DON_{TOT}, and ZEA increasing by 1.3-fold, and MON by 1.4-fold. The germ instead presented significantly lower FB_{TOT}, DON_{TOT}, ZEA, and MON (-63, -21, -30, and -54%, respectively) contents compared to the grains. The only exception was AF_{B1} found in P1547 milling fractions, among which the germ showed the highest increase (1.7-fold). Mycotoxins are produced at the site of fungal growth, which occurs mainly at the pedicel and on the surface layers (Zheng et al., 2024). Although located on the outside of the kernel, the germ was observed to be not a suitable substrate for the production of FB_{TOT} by *F. verticilloides* (Shim et al., 2003), and this is consistent with the germ concentration levels of the metabolites produced by *Fusarium* spp. of the *Liseola* section, i.e. FB_{TOT} and MON, observed in the present study. On the contrary, mycotoxins produced by *Fusarium* spp. of the *Discolor* section, such as DON and ZEA, were mainly found in high-fat fractions, such as germ, with increased contents of about 4 (Schollenberger et al., 2008) and 2.8 (Scarpino et al., 2021) times higher for DON, and 3.1 (Brera et al., 2006) and 5.2 (Scarpino et al., 2021) times higher for ZEA, compared to the cleaned grains. However, in the present study ZEA was mostly found in the finer size fractions and bran. The high contamination rates in animal feed flour and DB are attributable to the fact that these fractions are obtained, respectively, from the fine particles generated mainly from the floury endosperm surrounding the germ, where a higher

development of fungal species is possible (Bluhm & Woloshuk, 2005; Philippeau et al., 2000), and from the milling of the outer layers, i.e. pericarp and tip cap, which act as a physical barrier to mycelial penetration (Castells et al., 2008). The germ's higher contamination with aflatoxins than with the other investigated mycotoxins aligns with previous redistribution data showing a more superficial accumulation, mostly affecting the germ (Brera et al., 2006; Castells et al., 2008; Scarpino et al., 2021). The data collected in the present study suggest that mycotoxins concentrations in both milling co-products should be carefully evaluated, as they pose a risk to both animals and consumers when incorporated in feed and food formulations, either to recover a milling by-product within a circular economy framework or to increase the nutritional value of the final product. Following degermination, stone milling led to higher FB_{TOT}, DON_{TOT}, and MON accumulation in the corn flour than in the semi-whole semolina, while no significant differences were observed for ZEA. Roller milling following degermination produced corn flour that was generally more contaminated than that obtained through stone milling. The additional bran fraction separated during roller milling (RB) exhibited low contamination levels, with significant reductions of 8.9, 2.8, 3.3, and 4.6-fold for FB_{TOT}, DON_{TOT}, ZEA, and MON, respectively, compared to the DB. The RB fraction also displayed the lowest mycotoxin concentrations among all milling products and did not significantly differ from those observed in refined semolina obtained through the same process, making it a suitable food-grade milling fraction to be used as a functional ingredient for food fortification purposes.

Nutritional and health-related traits of the cornmeal semolina

The application of different milling strategies and the addition of bran to the refined flour resulted in 6 types of final semolina with different proximal composition (Table S2), which reflected the degree of refinement. The whole (SM) and semi-whole (SM-S) semolina obtained by stone milling were characterized by higher contents of dietary fiber, lipids, and ash compared to the semi-whole (D-SM) and refined (D-RM) semolina obtained after degermination, which instead had a higher starch content. The bran incorporation to the refined D-RM semolina allowed to obtain semolina (D-RM+DB and D-RM+RB) with higher protein and fiber contents than the starting D-RM semolina, while the lipid content remained comparable.

The content of biologically active compounds as well as of mycotoxins of both the hybrids (H) and the cornmeal semolina (S) were significantly differentiated by ANOVA. However, differing response levels were observed due to consistently significant S × H interactions (Table 3 and 4).

Phytochemicals and antioxidant capacity

As shown in Table 3, the content of SPAs in DKC6092 was significantly higher than in P1547 (+26%) and Pignoletto × SN148 (+56%), as well as the content of CWBPAs (+19% than both other hybrids). For all hybrids, a similar amount of SPAs and CWBPAs to that of whole SM semolina was retained in the semi-whole SM-S semolina, since sieving removed only a small fraction (about 10%) of bran, impurities, and floury endosperm. On the other hand, all the semolina obtained by degermination had lower concentrations of phenolic acids compared to those obtained by the stone milling of the grains, in agreement with the redistribution observed in the milling co-products. However, considering the most refined semolina (D-RM), the decrease was more pronounced for CWBPAs (-81%) than for SPAs (-50%). By applying degermination, the bran incorporation resulted to be the most effective strategy in maximizing the concentration of these compounds, although the results varied based on the used bran (DB vs. RB). The high SPA concentration in the DB of DKC6092 resulted in a semi-whole semolina (D-RM+DB) with a SPA level similar to that of the SM semolina (Figure 2A). On the other hand, for the other two hybrids a significant decrease was recorded (-35%), although DB incorporation remained the best strategy to enhance the SPA content when degermination was applied. The inclusion of RB did not offer advantages in terms of SPAs, as the semi-whole semolina (D-RM+RB) obtained did not differ significantly from the D-RM one. On the contrary, it did differ in terms of CWBPAs (Figure S2), with different results depending on the hybrid. The increase was more pronounced for DKC6092 and Pignoletto × SN148 (+203%) than for P1547 (+50%), compared to the D-RM semolina. For P1547 and Pignoletto × SN148, the semi-whole D-RM+RB semolina did not differ from the semi-whole D-SM one, but the inclusion of DB was always more advantageous in terms of CWBPAs than the inclusion of RB, showing the lowest decrease (-29%) compared to the SM semolina. On the other hand, for DKC6092 the lowest decrease (-35%) was observed when RB was used for incorporation, due to the high initial bran CWBPA content.

The three hybrids did not differ in the relative abundance of individual compounds. Within the soluble fraction, the hydroxybenzoic sinapic acid was the most abundant, its content ranging 52-56%, followed by ferulic acid (12-17%), protocatechuic (12-16%), and *p*-coumaric (7-9%). These findings contrast with those of Butts-Wilmsmeyer et al. (2018), who only detected in large flaking grits the cinnamic acid, a precursor to phenolic acids, and low contributions of ferulic and *p*-coumaric. The bound fraction was dominated by ferulic acid (86-88%), followed by *p*-coumaric (8-9%) and sinapic (3-4%), while the other compounds accounted for < 0.5% to the total amount. The relative abundance of some compounds varied as a function of the milling strategy, particularly in the soluble form (Figure 2A),

reflecting the observed composition of the milling co-products (Table S3). The shares of soluble sinapic, ferulic, and *p*-coumaric acids were similar in the SM and SM-S semolina, whereas semolina from degerminated grains showed a decrease in ferulic and *p*-coumaric acids, and an increase in sinapic acid. To the best of the author's knowledge, this is the first study to report the distribution of phenolic acids in cornmeal semolina obtained by different dry-milling strategies. While other studies have explored the impact of nixtamalization and extrusion on phenolic acids in cooked kernels and related products (De La Parra et al., 2007; Del Pozo-Insfran et al., 2006), research on dry milling has largely focused on laboratory-scale studies of either colored corn grits for value-added coproducts recovery (Ali et al., 2025; Li et al., 2017), or grits used to produce cornflakes (Butts-Wilmsmeyer et al., 2018) and snack foods (Ortiz et al., 2018; Thakur et al., 2017), or mechanically decorticated fractions to obtain porridge and traditional products (Kean et al., 2011; Mugode et al., 2014; Pillay et al., 2014). However, dry milling processes are widely employed to also obtain cornmeal semolina, which is characterized by smaller particle size than grits, and is commonly used for polenta, leavened baked or fried products (Blandino, Alfieri, et al., 2017), and gluten-free alternatives (Bresciani et al., 2021).

The highest TCC (Table 3) was observed in the semolina of Pignoletto × SN148 (+10%), which differed significantly from the other two hybrids, although the results varied depending on the milling strategy (Figure 2B). The lowest TCCs were observed in the SM and SM-S semolina, with no significant differences between the two, while the application of degermination and roller milling was the most effective strategy to maximize the TCC in the D-RM semolina (+45%; Table 3). Significant differences among semolina types were only significant for DKC6092 and Pignoletto × SN148 (Figure 2B), with D-RM semolina showing the greatest TCC increase (+64 and +44% vs. SM semolina, respectively). For DKC6092, bran incorporation increased the TCC of about 55%, regardless of the type of bran, reaching a similar amount to that of the D-RM semolina. The application of stone milling following degermination led to an average TCC increase in the D-SM semolina (+20%), when compared to stone milling without prior degermination. This increase was more pronounced and significant only for Pignoletto × SN148 (+27%). Given the carotenoids localization primarily in the vitreous endosperm (Ndolo & Beta, 2013), the roller milling was the most effective strategy in maximizing the TCC, and especially in the Pignoletto × SN148 (32.8 mg kg^{-1} in its D-RM semolina; Figure 2B). The incorporation of bran led to a slight but significant drop (-10%) compared to the D-RM, regardless of the type of bran.

Pignoletto × SN148 semolina differed in carotenoid profile, with higher β-carotene (10% vs. 6%), β-cryptoxanthin (20% vs. 15%), similar lutein (31% vs. 29%), and lower zeaxanthin (39% vs. 50%) compared to DKC6092 and P1547. Consequently, both the

xanthophylls/carotenes and zeaxanthin/lutein ratios were lower for Pignoletto × SN148 (2.3 vs. 3.8, 1.3 vs. 1.7, respectively), while the β-cryptoxanthin/β-carotene ratio remained similar (2.1 vs. 2.3). Higher kernel hardness, as in Pignoletto × SN148, has been associated with an improved ability of storing more carotenoids (Kljak et al., 2024; Saenz et al., 2020). Moreover, the composition of carotenoids was observed to account for the vitreous endosperm formation, influencing the stability of amyloplast membranes during kernel desiccation and affecting the interactions between starch granules and proteins bodies (Wang et al., 2020). Saenz et al. (2021) reported higher levels of pro-vitamin A carotenoids in genotypes with increasing kernel hardness, similarly to what has been observed in the present study (Pignoletto × SN148 > P1547 > DKC6092). However, greater kernel hardness was associated with higher concentration and proportion of all β-branch carotenoids, zeaxanthin included (Kljak et al., 2024; Saenz et al., 2021). The higher levels of zeaxanthin usually reported in the hard endosperm flint genotypes when compared to the dent ones (Borrás et al., 2022; Nuss & Tanumihardjo, 2010) were not observed for the flint × dent hybrid analyzed in the present study, highlighting differences in the expression of the main enzymes regulating the biosynthetic pathway favoring either the α- or the β-branch. Notably, the pro-vitamin A carotenoid content in Pignoletto × SN148 D-RM semolina (9.8 mg kg^{-1}) exceeded that of conventional dent hybrids (6.1 mg kg^{-1}), approaching the 12–15 mg kg^{-1} target that is considered nutritionally relevant for biofortification purposes (Menkir et al., 2017).

Carotenoid distribution across milling fractions influenced semolina profiles (Figure 2B): higher xanthophylls/carotenes and lower zeaxanthin/lutein ratios were observed in all the degerminated semolina. The shares of β-criptoxantina and β-carotene were found to be higher in non-degerminated semolina, while the relative abundance of zeaxanthin was greater in the D-RM+DB semolina. Previous studies reported lower levels of zeaxanthin, β-cryptoxanthin, and β-carotene (Ortiz et al., 2018; Pillay et al., 2014), or only β-cryptoxanthin (Mugode et al., 2014), in degerminated and dehulled endosperm fractions, likely due to thermal degradation induced by the high temperatures reached by dehulling abrasion at lab- or semi-industrial scale, which is less relevant at industrial-level processing (Prabhasankar & Haridas Rao, 2001).

The AC_{ABTS} was observed to be significantly higher in Pignoletto × SN148 semolina compared to the other hybrids analyzed (+15%), while no significant differences were found with AC_{FRAP} (Table 3). However, compared to SM semolina, Pignoletto × SN148 was characterized by more pronounced percentage decreases by increasing degree of refinement (Figure S3). The SM semolina exhibited the highest AC, and a drop was observed after sieving (-8%), although it was significant only for Pignoletto × SN148

(AC_{ABTS}) and for DKC6092 (AC_{FRAP}). Among degerminated semolina, the D-RM semolina retained the lowest absolute values (-48 and -63%, with AC_{ABTS} and AC_{FRAP}, respectively, vs. SM semolina). The D-RM+DB semolina was always found to have a higher AC than that obtained with RB, which together with the D-SM maintained similar or slightly higher values than the D-RM semolina, depending on the hybrid and the assay.

Table 3. The contents of soluble (SPAs) and cell wall-bound (CWBPAs) phenolic acids, carotenoids (TCC), and the levels of antioxidant capacity (AC) of the cornmeal semolina of each corn hybrid obtained by different milling strategies.

Factor	Degermination process (D)	Source of variation	SPAs (mg kg^{-1})	CWBPAs (mg kg^{-1})	TCC (mg kg^{-1})	AC_{ABTS} (mmol TE kg^{-1})	AC_{FRAP} (mmol TE kg^{-1})
Hybrid (H)		DKC6092	231 a	1072 a	25.7 b	8.3 b	8.6 a
		P1547	184 b	910 b	24.8 b	8.1 b	8.4 a
		Pignoletto × SN148	148 c	891 b	27.7 a	9.4 a	8.3 a
		p (F)	***	***	**	***	ns
Cornmeal semolina (S)	None	Whole (SM)	272 a	1619 a	21.3 d	11.4 a	12.7 a
		Semi-whole (SM-S)	253 b	1599 a	21.9 d	10.5 b	11.6 b
	D	Semi-whole (D-RM+DB)	205 c	1034 b	28.0 b	9.2 c	9.4 c
		Semi-whole (D-RM+RB)	156 d	741 c	28.9 ab	7.8 d	6.6 d
		Semi-whole (D-SM)	107 f	508 d	25.6 c	6.8 e	5.6 e
		Refined (D-RM)	135 e	303 e	30.8 a	5.9 f	4.7 f
		p (F)	***	***	***	***	***
H × S		*p* (F)	***	***	*	***	***

D, degermination; SM, stone milling; SM-S, stone milling followed by sieving; D-RM+DB, degermination followed by roller milling and degermination bran incorporation; D-RM+RB, degermination followed by roller milling and refining bran incorporation; D-SM, degermination followed by stone milling; D-RM, degermination followed by roller milling; SPAs, soluble phenolic acids; CWBPAs, cell wall-bound phenolic acids; TCC, total carotenoids content; AC, antioxidant capacity by means of QUENCHER-ABTS and -FRAP assays. The milling strategies applied to obtain the cornmeal semolina are detailed in Figure 1. The results are expressed on a DW basis. Means followed by different letters are significantly different, according to the REGW-F test [(*) *p* (F) < 0.05, (**) *p* (F) < 0.01, and (***) *p* (F) < 0.001, and ns, non-significant].

Mycotoxins

Across all semolina types, Pignoletto × SN148 consistently exhibited the lowest levels of mycotoxin contamination (Table 4). The FB_{TOT} contamination followed a similar reduction trend among hybrids: compared to SM semolina, sieving already led to a significant reduction (-21%); among degerminated semolina, the reduction percentage was less marked for the D-RM+DB semolina (-46%) than for the D-SM semolina (-78%). The D-RM+DB and D-RM semolina presented the lowest FB_{TOT} levels (-87%), and significant differences in the absolute values were observed only in P1547 (794 vs. 565 μg kg^{-1}), since higher contamination levels of the grains led to more marked differences among final semolina (Figure 3A). FB_1 remained the dominant fumonisin but showed a reduced share (75% vs. 80%) in D-RM and D-RM+RB semolina. Most notably, for all hybrids, only degermination followed by roller milling, or RB incorporation resulted in semolina compliant with EU regulations for cornmeal semolina destined to consumers (1000 μg kg^{-1}). Only in the case of low contamination levels of the initial grains (DKC6092 and Pignoletto × SN148), degermination followed by stone milling also met the legal limits.

AF_{B1} was only found in the SM and SM-S semolina of P1547. Only the D-RM+RB and D-RM semolina of Pignoletto × SN148 followed the EU limit of 750 μg kg^{-1} for DON (Figure S4A), while all the semolina complied with the 100 μg kg^{-1} limit for ZEA (Figure S4B) set for corn destined for the final consumer. The DON_{TOT} decontamination trend was similar to that of FB_{TOT}, but the decrease percentages were less pronounced (Table 4). A weaker decontamination of DON, and particularly of DON-3-G, than FB_{TOT} has been already noticed by previous publications, in which the variable effects of the process were ascribed to the degree of fungal penetration in the endosperm (Brera et al., 2006; Scarpino et al., 2021). The sieving after stone milling led to a modest but significant reduction in the SM-S semolina of about 10 and 12% of DON_{TOT} and ZEA, respectively, for all hybrids, while stone milling after degermination achieved a larger reduction in the D-SM semolina (-38 and -54%). The highest decontamination was reached in the D-RM semolina (-54 and -63%, respectively). Incorporating RB into refined flour resulted in significantly higher levels of DON_{TOT} only for the D-RM+RB semolina of P1547 (1334 vs. 1146 μg kg^{-1}; Figure S4A), similar to FB_{TOT}, when compared to D-RM semolina. Additionally, it resulted in higher ZEA contents for both P1547 and Pignoletto × SN148. The DB incorporation posed a risk of increasing the contamination level of the refined flour even higher compared to the use of RB. This was especially evident when highly contaminated bran was used, as in the case of DON_{TOT} for DKC6092, where the final content was slightly lower (-4%) than that of the SM semolina. Although the DON-3-G/DON was similar across hybrids in the SM flour (20%), it varied

according to the degree of refinement and the hybrid. Specifically, higher percentages were observed for P1547 and Pignoletto × SN148 in the D-RM semolina, reaching up to 22%. The D-RM+DB semolina was the second most contaminated with MON after SM semolina (average of 9% reduction; Table 4). Specifically, the levels were statistically similar to SM semolina for P1547 (Figure 3B). That was followed by SM-S (-16%), D-SM (-41%), D-RM+RB (-68%), and D-RM semolina (-74%). Lower abatement levels were observed by Scarpino et al. (2020) for pearl and break meals (-57%) when compared to cleaned grains. A relatively high MON contamination was found in the RB, so that the derived D-RM+RB semolina always showed significantly higher levels than those of the D-RM one. It is worth mentioning that the occurrence of MON has mainly been monitored in grains and there is little information about the occurrence in corn milling products and foods. The herein obtained data for the food-grade corn milling products destined for human consumption ranged from 142 to 536 μg kg^{-1} and they are within the range reported by Gutema et al. (2000) 26–774 μg kg^{-1} for food-grade commercial corn samples, but they are higher than more recent findings in flour and grits (126, 190, 82–214 μg kg^{-1}) (Herrera et al., 2017; Scarpino et al., 2020; Von Bargen et al., 2012). The limited reduction of MON relative to FB_{TOT} raises concerns about exposure, especially since co-occurrence of the two toxins has been already reported (Scarpino et al., 2020). This is particularly true if the process does not effectively remove fine particles size or if highly contaminated bran is recovered.

Table 4. The contents of total fumonisins (FUM_{TOT}), total deoxynivalenol (DON_{TOT}), moniliformin (MON), aflatoxin B_1 (AF_{B1}), and zearalenone (ZEA) of the cornmeal semolina of each corn hybrid obtained by different milling strategies.

Factor	Degermination process (D)	Source of variation	FB_{TOT} (µg kg^{-1})	DON_{TOT} (µg kg^{-1})	AF_{B1} (µg kg^{-1})	ZEA (µg kg^{-1})	MON (µg kg^{-1})
Hybrid (H)		DKC6092	1347 b	2955 a	<LOQ	54 a	452 a
		P1547	2440 a	2236 b	2.9 a	30 b	347 b
		Pignoletto × SN148	830 c	1477 c	<LOQ	24 c	255 c
		p (F)	***	***	ns	***	***
Cornmeal semolina (S)	None	Whole (SM)	3287 a	3107 a	3.2 a	59 a	536 a
		Semi-whole (SM-S)	2583 b	2801 b	2.7 b	52 b	452 c
	D	Semi-whole (D-RM+DB)	1769 c	2600 c	<LOQ	30 c	490 b
		Semi-whole (D-RM+RB)	455 e	1479 e	<LOQ	24 e	172 e
		Semi-whole (D-SM)	738 d	1913 d	<LOQ	27 d	314 d
		Refined (D-RM)	403 e	1437 e	<LOQ	22 f	142 f
		p (F)	***	***	*	***	***
H × S		*p* (F)	***	***	ns	***	***

D, degermination; SM, stone milling; SM-S, stone milling followed by sieving; D-RM+DB, degermination followed by roller milling and degermination bran incorporation; D-RM+RB, degermination followed by roller milling and refining bran incorporation; D-SM, degermination followed by stone milling; D-RM, degermination followed by roller milling; SPAs, soluble phenolic acids; CWBPAs, cell wall-bound phenolic acids; TCC, total carotenoids content; AC, antioxidant capacity by means of QUENCHER-ABTS and -FRAP assays. The milling strategies applied to obtain the cornmeal semolina are detailed in Figure 1. The results are expressed on a DW basis. Means followed by different letters are significantly different, according to the REGW-F test [(*) *p* (F) < 0.05, (**) *p* (F) < 0.01, and (***) *p* (F) < 0.001, and ns, non-significant].

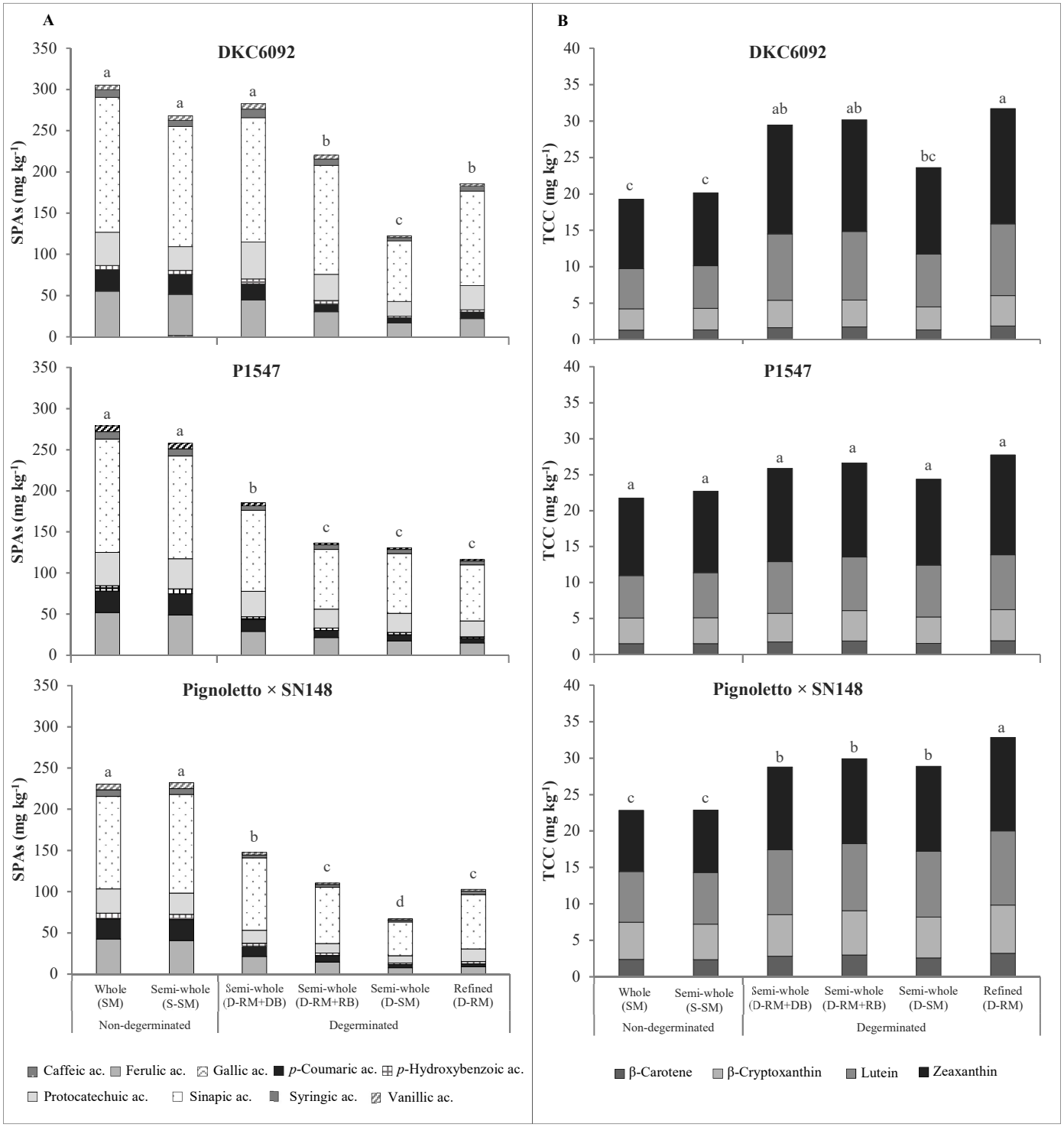


Figure 2. Comparison of the content of soluble phenolic acids (SPAs; A) and carotenoids (TCC; B) in the cornmeal semolina of different milling processes and different corn hybrids.

SM, stone milling; SM-S, stone milling followed by sieving; D-RM+DB, degermination followed by roller milling and degermination bran incorporation; D-RM+RB, degermination followed by roller milling and refining bran incorporation; D-SM, degermination followed by stone milling; D-RM, degermination followed by roller milling. The results are expressed on a DW basis. Different letters above the columns indicate significant differences between the six groups at p (F) < 0.05, according to the REGW-F test.

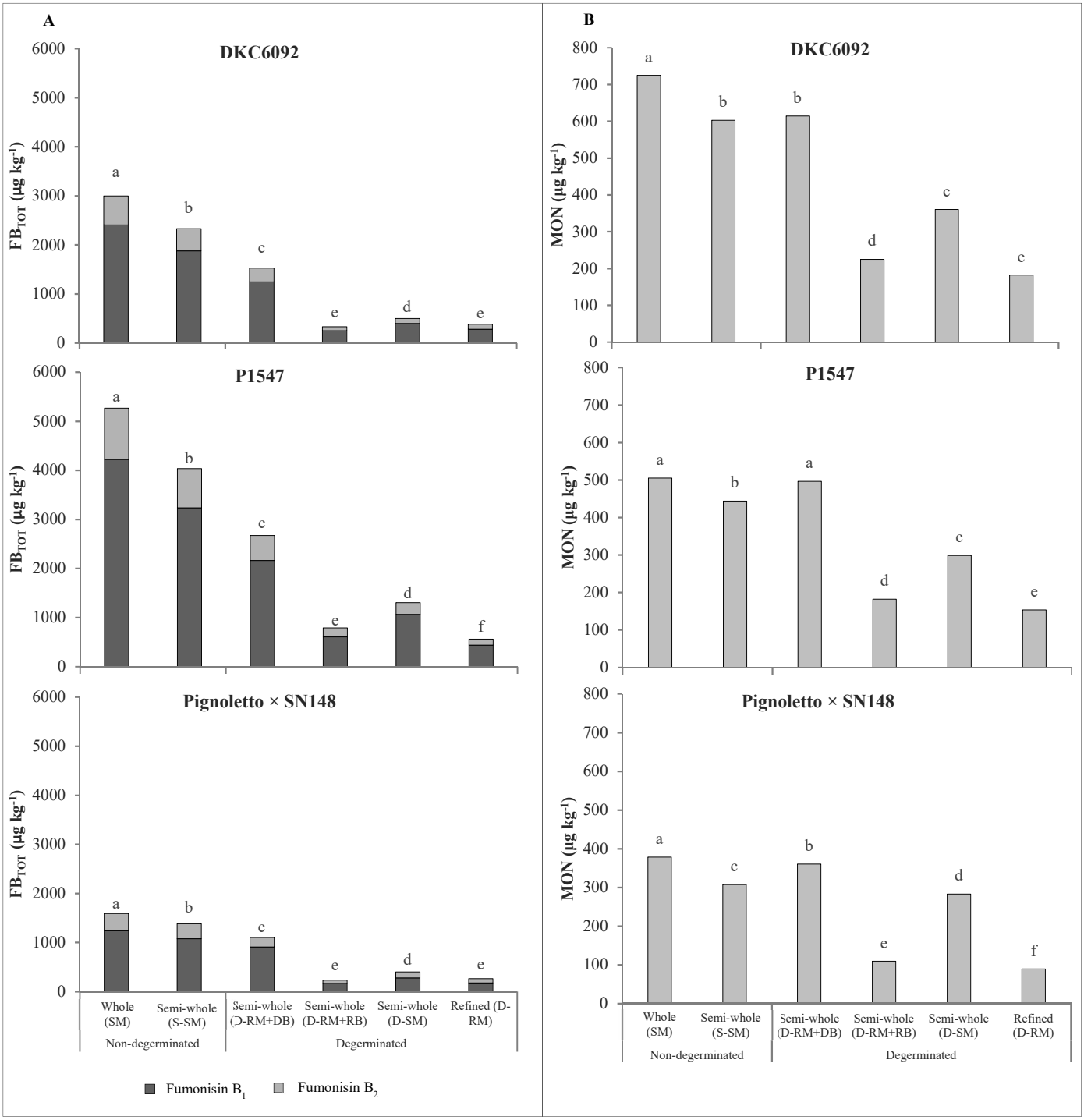


Figure 3. Comparison of the content of fumonisins (FB_{TOT}; A) as the sum of B_1 and B_2, and moniliformin (B) in the cornmeal semolina of different milling processes and different corn hybrids.

SM, stone milling; SM-S, stone milling followed by sieving; D-RM+DB, degermination followed by roller milling and degermination bran incorporation; D-RM+RB, degermination followed by roller milling and refining bran incorporation; D-SM, degermination followed by stone milling; D-RM, degermination followed by roller milling. The results are expressed on a DW basis. Different letters above the columns indicate significant differences between the six groups at p (F) < 0.05, according to the REGW-F test.

Multivariate analysis

Further information on differences in the contents of phytochemical compounds and mycotoxins between the three species and the types of semolina was given by multivariate analysis. PCA partially separated the three species on PC2, with semolina of Pignoletto × SN148 all placed on the upper side of the score plot, as opposed to those of DKC6092 and P1547 that are all placed on the lower side, whereas the types of semolina were mainly differentiated on PC1 (Figure 4A). The loading plot (Figure 4B) and the loadings of the variables (Figure S5A and S5B) showed that Pignoletto × SN148 was particularly rich in β-carotene, β-cryptoxanthin, AC_{ABTS}, but poor in zeaxanthin and less contaminated by mycotoxins, as opposed to the conventional hybrids. Indeed, SM and SM-S semolina of all hybrids and D-RM+DB semolina of DKC6092 and P1547 were richer in phenolic acids and characterized by higher antioxidant properties, but also greater sanitary risks. The other strategies applied to obtain semi-whole and refined semolina, all following degermination, were more effective in maximizing both carotenes and xanthophylls concentration and reducing mycotoxin contamination.

Conclusions

This study highlights that corn grains can be an excellent source of phytochemicals, and that the breeding of novel hybrids from flint corn germplasm with high kernel hardness holds promise for the dry-milling industry to produce ingredients with enhanced nutritional value (e.g., higher protein content) and health-promoting compounds, particularly carotenoids. Nevertheless, the careful selection of appropriate dry-milling strategies is crucial to simultaneously meet specific nutritional targets and ensure food safety. Tailoring milling approaches to the specific characteristics of each corn lot, especially in growing environments and harvest seasons with higher fungal pressure, is essential to comply with safety standards. In low contamination scenarios, strategies such as stone milling (as is or with sieving) or the incorporation of degermination bran into refined flour, each with progressively greater mycotoxin abatement capacity, can be adopted to maximize the antioxidant potential of derived semolina and deliver the highest contents of phenolic acids, minerals, and dietary fiber. When contamination levels are higher, especially with mycotoxins characterized by weaker decontamination such as DON_{TOT} and MON, strategies such as stone milling of degerminated grains or selective recovery of refining bran can be adopted, as they are progressively more effective in reducing mycotoxin levels, and they are able to preserve a portion of phenolic acids and further enhance carotenoid contents. For highly contaminated lots, the roller refining of degerminated grains remains the best strategy to ensure food safety, minimizing mycotoxin risk, while best preserving the carotenoid composition.

The improved mycotoxin resistance observed in the new flint × dent hybrid supports its potential for the dry-milling industry, as it allows the recovery of less contaminated by-products, in line with circular economy principles. Future studies should assess the agronomic performance of this genotype and investigate the bioavailability and stability of its bioactive compounds during food processing, with the ultimate goal of supporting the development of healthier and sustainable corn-based foods.

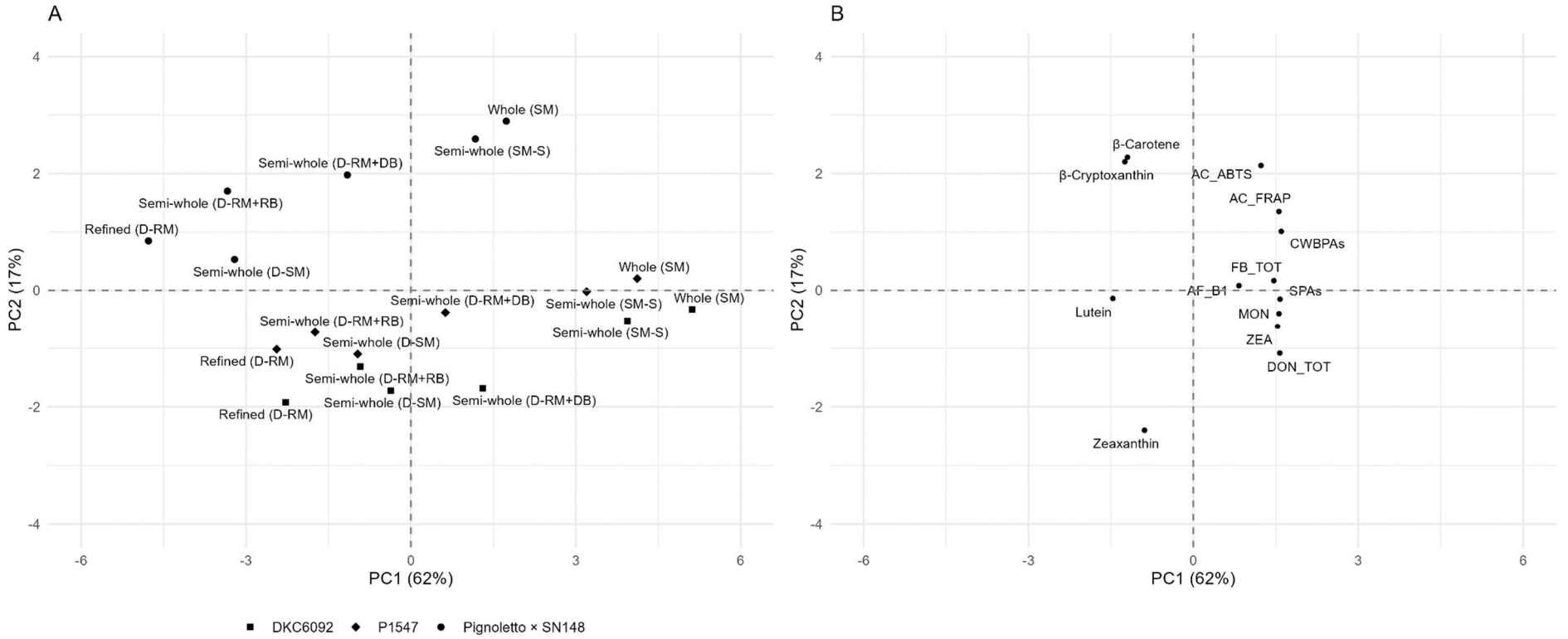


Figure 4. Multivariate analysis of the contents of phytochemical compounds and fungal metabolites in the cornmeal semolina of the three corn hybrids evaluated in this study. A, score plot of the principal component analysis (PCA); B, loading plot for the separation in A. The sample plots are grouped on the basis of the average mean of 3 replications. The loadings of the variables of PC1 and PC2 are shown in Figure S5.

SM, stone milling; SM-S, stone milling followed by sieving; D-RM+DB, degermination followed by roller milling and degermination bran incorporation; D-RM+RB, degermination followed by roller milling and refining bran incorporation; D-SM, degermination followed by stone milling; D-RM, degermination followed by roller milling; FB_TOT, total fumonisins; DON_TOT, total deoxynivalenol; AF_B1, aflatoxin B_1; ZEA, zearalenone; MON, moniliformin; SPAs, soluble phenolic acids; CWBPAs, cell wall-bound phenolic acids; AC, antioxidant capacity by means of QUENCHER-ABTS and -FRAP assays.

Supplementary material

Table S1. Distribution of the particle sizes of the cornmeal semolina of each corn hybrid.

Hybrid	Degermination process (D)	Cornmeal semolina	Particle size distribution (%)						
			>2000 µm	1500-2000 µm	1000-1500 µm	710-1000 µm	500-710 µm	315-500 µm	<315 µm
DKC6092	None	Whole (SM)	0.0	1.3	16.2	12.7	13.9	38.3	17.7
		Semi-whole (SM-S)	0.0	2.0	19.7	16.7	18.3	33.9	9.4
	D	Semi-whole (D-RM+DB)	0.0	0.3	11.3	17.5	23.7	37.9	9.3
		Semi-whole (D-RM+RB)	0.0	0.0	10.3	16.5	20.8	39.8	12.6
		Semi-whole (D-SM)	0.0	5.1	30.6	25.4	20.4	16.3	2.2
		Refined (D-RM)	0.0	0.0	15.5	21.6	24.2	37.0	1.6
P1547	None	Whole (SM)	0.0	2.3	20.3	12.7	12.6	32.2	19.9
		Semi-whole (SM-S)	0.0	2.7	23.1	15.2	16.0	31.6	11.4
	D	Semi-whole (D-RM+DB)	0.0	5.6	25.8	19.0	17.1	20.5	12.0
		Semi-whole (D-RM+RB)	0.0	4.8	24.0	18.1	20.3	20.6	12.2
		Semi-whole (D-SM)	0.0	2.7	19.3	15.6	19.8	28.2	14.4
		Refined (D-RM)	0.0	7.3	37.0	20.4	18.2	15.8	1.2
Pignoletto × SN148	None	Whole (SM)	0.0	1.0	12.4	16.9	15.5	37.4	16.7
		Semi-whole (SM-S)	0.0	1.2	14.1	19.4	17.4	35.1	12.8
	D	Semi-whole (D-RM+DB)	0.0	0.0	2.9	12.2	28.9	42.2	13.8
		Semi-whole (D-RM+RB)	0.0	0.0	2.7	12.6	29.1	42.1	13.5
		Semi-whole (D-SM)	0.0	4.1	20.6	13.6	27.1	32.7	1.9
		Refined (D-RM)	0.0	0.0	3.7	16.8	36.0	41.8	1.6

D, degermination; SM, stone milling; SM-S, stone milling followed by sieving; D-RM+DB, degermination followed by roller milling and degermination bran incorporation; D-RM+RB, degermination followed by roller milling and refining bran incorporation; D-SM, degermination followed by stone milling; D-RM, degermination followed by roller milling.

Table S2. Proximal composition of the cornmeal semolina of each corn hybrid.

Hybrid	Degermination process (D)	Cornmeal semolina	Proximal composition (g 100 g^{-1})				
			Carbohydrates	Proteins	Dietary fiber	Lipids	Ash
DKC6092	None	Whole (SM)	62.7	7.9	10.4	4.1	1.27
		Semi-whole (SM-S)	63.8	7.7	10.0	3.5	1.10
	D	Semi-whole (D-RM+DB)	69.3	7.2	6.8	1.4	0.45
		Semi-whole (D-RM+RB)	67.2	7.6	7.7	2.3	0.99
		Semi-whole (D-SM)	73.1	6.6	4.8	0.9	0.32
		Refined (D-RM)	72.4	6.8	4.8	1.1	0.39
P1547	None	Whole (SM)	63.4	7.9	10.5	3.8	1.20
		Semi-whole (SM-S)	63.7	7.7	10.0	3.9	1.16
	D	Semi-whole (D-RM+DB)	66.1	7.6	10.0	1.6	0.48
		Semi-whole (D-RM+RB)	70.3	8.0	5.2	2.2	0.76
		Semi-whole (D-SM)	72.5	7.0	4.7	1.8	0.38
		Refined (D-RM)	72.1	7.4	5.2	1.2	0.28
Pignoletto × SN148	None	Whole (SM)	62.6	9.4	9.3	5.2	1.22
		Semi-whole (SM-S)	62.8	9.3	10.1	4.1	1.00
	D	Semi-whole (D-RM+DB)	66.4	8.9	8.3	2.5	0.48
		Semi-whole (D-RM+RB)	69.7	8.9	5.2	2.5	0.86
		Semi-whole (D-SM)	72.2	8.0	4.4	1.4	0.34
		Refined (D-RM)	71.9	8.3	4.3	1.4	0.28

D, degermination; SM, stone milling; SM-S, stone milling followed by sieving; D-RM+DB, degermination followed by roller milling and degermination bran incorporation; D-RM+RB, degermination followed by roller milling and refining bran incorporation; D-SM, degermination followed by stone milling; D-RM, degermination followed by roller milling.

Table S3. The profile of analyzed soluble phenolic acids (SPAs; mg kg^{-1}) in the products and coproducts of different milling processes and different corn hybrids.

				Hydroxycinnamic SPAs				Hydroxybenzoic SPAs				
Hybrid	**Degermination process (D)**	**Dry milling process**	**Milling fraction**	**Caffeic acid**	**Ferulic acid**	**Gallic acid**	***p*-Coumaric acid**	***p*-Hydroxybenzoic acid**	**Protocatechuic acid**	**Sinapic acid**	**Syringic acid**	**Vanillic acid**
DKC6092			Corn grains	<LOD	55.3 c	<LOD	26.0 cd	5.3 de	40.3 bc	163 cd	9.3 b	5.6 bc
	None	SM-S	Animal feed flour	1.7 a	57.6 c	<LOD	24.6 d	6.3 c	43.1 b	201 ab	10.5 ab	5.2 bc
			Semi-whole semolina (SM-S)	1.4 a	50.1 c	<LOD	24.2 d	4.8 e	28.8 d	146 d	7.5 c	5.5 bc
	D		Animal feed flour	<LOD	54.6 c	<LOD	29.9 c	4.5 e	51.1 ab	178 bc	7.4 c	5.2 bc
			Degermination bran (DB)	<LOD	72.8 b	0.3 a	38.5 b	6.0 cd	58.9 a	217 a	9.4 b	10.0 a
			Germ	1.7 a	103.2 a	<LOD	48.7 a	10.0 a	14.6 f	152 cd	6.8 c	7.4 ab
		SM	Corn flour	<LOD	36.8 d	<LOD	12.5 f	5.1 de	57.2 a	221 a	12.1 a	5.2 bc
			Semi-whole semolina (D-SM)	<LOD	16.6 e	<LOD	6.5 g	1.9 f	17.8 ef	74 f	3.7 d	2.1 d
		RM	Corn flour	<LOD	22.6 e	<LOD	8.1 g	2.3 f	28.6 de	96 ef	6.2 c	2.4 d
			Refining bran (RB)	<LOD	61.3 c	<LOD	19.1 e	7.8 b	40.4 bc	182 bc	10.6 ab	9.6 a
			Refined semolina (D-RM)	<LOD	22.1 e	<LOD	7.8 g	2.7 f	29.6 cd	115 e	6.4 c	2.6 cd
			p (F)	ns	***	ns	***	***	***	***	***	***
P1547			Corn grains	<LOD	51.8 bcd	<LOD	26.3 c	6.4 c	40.7 cd	138 b	9.0 b	7.0 b
	None	SM-S	Animal feed flour	<LOD	58.3 bc	<LOD	27.9 c	8.3 b	55.6 a	186 a	11.8 a	7.9 b
			Semi-whole semolina (SM-S)	<LOD	48.9 cd	<LOD	25.8 c	6.1 cd	36.4 d	125 b	8.4 b	7.0 b
	D		Animal feed flour	<LOD	43.9 de	<LOD	24.1 c	3.8 fg	44.8 bc	108 c	6.3 d	4.2 c
			Degermination bran (DB)	<LOD	62.3 b	<LOD	33.2 b	5.1 de	48.0 b	137 b	7.4 c	7.4 b
			Germ	<LOD	122.8 a	0.1 a	62.1 a	11.1 a	12.0 g	108 c	8.3 b	11.8 a
		SM	Corn flour	<LOD	22.6 f	<LOD	11.6 de	4.4 ef	43.4 bc	133 b	9.3 b	3.0 d
			Semi-whole semolina (D-SM)	<LOD	17.3 f	<LOD	7.9 f	2.6 h	23.2 f	73 ef	5.0 e	1.9 e
		RM	Corn flour	<LOD	17.1 f	<LOD	8.2 ef	3.0 gh	29.2 e	92 d	6.5 cd	2.1 de
			Refining bran (RB)	<LOD	36.0 e	<LOD	15.2 d	4.5 ef	20.8 f	85 de	6.3 d	4.3 c
			Refined semolina (D-RM)	<LOD	14.9 f	<LOD	5.3 f	2.3 h	19.4 f	68 f	4.7 e	1.7 e
			p (F)	ns	***	ns	***	***	***	***	***	***
Pignoletto × SN148			Corn grains	<LOD	42.6 c	0.1 a	24.5 d	6.6 c	29.6 de	112 c	8.2 bc	6.8 cd
	None	SM-S	Animal feed flour	<LOD	39.3 c	<LOD	23.8 d	8.3 b	37.6 b	166 b	10.4 a	5.9 d
			Semi-whole semolina (SM-S)	<LOD	40.8 c	<LOD	25.5 d	6.0 c	26.0 ef	119 c	7.3 de	7.0 cd

D		Animal feed flour	<LOD	55.8	b	<LOD	37.9	c	9.2	ab	45.9	a	197	a	9.4	ab	8.4	bc
		Degermination bran (DB)	<LOD	60.6	b	<LOD	42.5	b	9.5	a	35.8	bc	181	ab	8.0	bc	11.8	a
		Germ	<LOD	103.9	a	1.2	a	64.5	a	10.3	a	14.0	gh	128	c	7.7	bcd	9.2	b
	SM	Corn flour	<LOD	18.9	d	<LOD	7.7	e	4.6	d	26.2	ef	125	c	6.0	e	3.3	e
		Semi-whole semolina (D-SM)	<LOD	7.9	e	<LOD	3.6	ef	1.9	e	9.0	h	41	e	2.4	g	1.3	e
	RM	Corn flour	<LOD	13.9	de	<LOD	5.5	ef	4.6	d	32.4	bcd	115	c	7.1	de	2.4	e
		Refining bran (RB)	<LOD	38.5	c	<LOD	23.0	d	6.1	c	20.4	fg	106	c	6.4	de	5.4	d
		Refined semolina (D-RM)	<LOD	9.0	e	<LOD	3.5	f	2.8	e	15.5	gh	66	d	4.2	f	1.9	e
		p (F)	ns	***		ns	***		***		***		***		***		***	

D, degermination; SM, stone milling; SM-S, stone milling followed by sieving; RM, roller milling; DB, degermination bran; RB, refining bran; LOD, limit of detection. The results are expressed on a DW basis. Means followed by different letters are significantly different, according to the REGW-F test [(*) p (F) < 0.05, (**) p (F) < 0.01, and (***) p (F) < 0.001, and ns, non-significant].

Table S4. The profile of analyzed cell wall-bound phenolic acids (CWBPAs; mg kg^{-1}) in the products and coproducts of different milling processes and different corn hybrids.

				Hydroxycinnamic CWBPAs				Hydroxybenzoic CWBPAs				
Hybrid	**Degermination process (D)**	**Dry milling process**	**Milling fraction**	**Caffeic acid**	**Ferulic acid**	**Gallic acid**	***p*-Coumaric acid**	***p*-Hydroxybenzoic acid**	**Protocatechuic acid**	**Sinapic acid**	**Syringic acid**	**Vanillic acid**
DKC6092			Corn grains	3.7 a	1581 c	<LOD	141 b	1.0 cde	4.3 b	47 c	1.5 cdef	2.1 bc
	None	SM-S	Animal feed flour	3.5 a	1164 e	<LOD	108 c	1.3 cd	5.0 ab	64 bc	1.7 bcde	1.7 bcd
			Semi-whole semolina (SM-S)	<LOD	1521 cd	<LOD	156 b	1.2 cd	4.6 ab	62 c	1.5 cdef	1.9 bcd
	D		Animal feed flour	2.6 a	1355 d	<LOD	130 bc	1.5 c	7.1 ab	51 c	2.2 abc	2.5 b
			Degermination bran (DB)	3.2 a	2044 b	<LOD	222 a	2.2 b	9.6 a	98 a	3.0 a	3.8 a
			Germ	3.1 a	1054 e	0.5 a	154 b	2.8 a	2.7 b	57 c	2.1 bcd	2.6 b
		SM	Corn flour	1.1 a	691 f	0.3 a	48 d	0.9 def	3.5 b	24 d	1.1 def	1.2 def
			Semi-whole semolina (D-SM)	0.7 a	582 f	1.0 a	47 d	0.5 f	1.5 b	16 d	0.8 f	0.9 ef
		RM	Corn flour	0.7 a	229 g	<LOD	14 e	0.6 ef	2.6 b	17 c	1.0 ef	0.6 f
			Refining bran (RB)	2.6 a	2313 a	<LOD	212 a	2.4 ab	3.4 b	85 ab	2.5 ab	3.9 a
			Refined semolina (D-RM)	<LOD	346 g	<LOD	24 de	0.5 f	2.1 b	22 c	0.8 f	0.7 f
			p (F)	ns	***	ns	***	***	**	***	***	***
P1547			Corn grains	5.2 a	1355 a	<LOD	139 d	1.0 de	3.8 abc	45 b	1.4 bc	1.8 d
	None	SM-S	Animal feed flour	6.7 a	1151 b	<LOD	106 e	1.3 bc	6.4 a	50 b	1.3 c	1.8 d
			Semi-whole semolina (SM-S)	5.3 a	1415 a	<LOD	155 d	1.1 bcd	4.3 abc	44 b	1.2 c	1.8 d
	D		Animal feed flour	7.2 a	988 c	<LOD	178 c	1.6 b	5.8 ab	52 b	1.9 abc	2.7 c
			Degermination bran (DB)	10.4 a	1230 b	<LOD	348 a	2.2 a	6.5 a	81 a	2.9 ab	4.4 a
			Germ	2.4 a	903 c	0.4 a	239 b	2.3 a	4.4 abc	50 b	2.7 abc	3.5 b
		SM	Corn flour	2.0 a	460 d	0.3 a	37 f	0.7 de	4.2 abc	24 c	1.3 c	0.9 e
			Semi-whole semolina (D-SM)	1.8 a	384 de	0.9 a	33 f	0.5 e	1.5 c	16 c	0.9 c	0.7 e
		RM	Corn flour	<LOD	279 e	0.6 a	19 f	0.6 e	2.1 abc	26 c	1.2 c	0.5 e
			Refining bran (RB)	3.6 a	916 c	0.4 a	89 e	1.2 bc	4.9 abc	44 b	3.1 a	1.7 e
			Refined semolina (D-RM)	2.5 a	283 e	0.2 a	19 f	0.6 e	2.1 bc	24 c	1.1 c	0.4 e
			p (F)	ns	***	ns	***	***	**	***	**	***
			Corn grains	11.6 abc	1308 bc	<LOD	140 e	1.5 de	5.2 bcd	54 cde	2.0 cde	2.1 def

Pignoletto × SN148	None	SM-S	Animal feed flour	8.1	bcd	1421	b	<LOD		180	d	3.2	b	7.2	b	64	c	3.2	c	3.1	cd
			Semi-whole semolina (SM-S)	5.2	bcd	1273	c	<LOD		136	e	1.3	def	5.8	bc	46	cde	1.6	def	2.4	de
	D		Animal feed flour	16.0	a	2574	a	0.7	a	331	b	5.5	a	11.8	a	147	a	5.7	a	6.3	b
			Degermination bran (DB)	13.8	ab	2615	a	0.6	a	392	a	3.5	b	6.7	bc	122	b	4.4	b	7.2	a
			Germ	5.3	cd	880	d	0.3	a	234	c	2.4	c	4.9	bcd	55	c	2.5	cd	3.2	c
		SM	Corn flour	3.0	d	652	e	1.0	a	54	f	1.7	d	5.0	bcd	37	e	1.9	def	1.6	efg
			Semi-whole semolina (D-SM)	1.6	d	375	f	1.3	a	31	g	0.9	ef	2.1	cd	20	f	1.0	f	1.1	g
		RM	Corn flour	<LOD		287	f	0.9	a	22	g	1.9	cd	5.9	bc	38	e	2.6	cd	1.3	fg
			Refining bran (RB)	4.4	cd	967	d	0.3	a	120	e	1.5	de	2.9	bcd	40	de	2.2	cd	2.4	cde
			Refined semolina (D-RM)	<LOD		147	g	0.7	a	10	g	0.9	f	1.8	d	19	f	1.0	ef	0.7	g
			p (F)	***		***		ns		***		***		***		***		***		***	

D, degermination; SM, stone milling; SM-S, stone milling followed by sieving; RM, roller milling; DB, degermination bran; RB, refining bran; LOD, limit of detection. The results are expressed on a DW basis. Means followed by different letters are significantly different, according to the REGW-F test [(*) p (F) < 0.05, (**) p (F) < 0.01, and (***) p (F) < 0.001, and ns, non-significant].

Table S5. The profile of analyzed carotenoids (mg kg^{-1}) in the products and coproducts of different milling processes and different corn hybrids.

Hybrid	Degermination process (D)	Dry milling process	Milling fraction	β-Carotene	β-Cryptoxanthin	Lutein	Zeaxanthin
DKC6092			Corn grains	1.3 bc	2.9 bcd	5.5 def	9.5 bcd
	None	SM-S	Animal feed flour	1.1 c	2.6 cd	3.7 g	6.5 f
			Semi-whole semolina (SM-S)	1.3 bc	3.0 bcd	5.8 cde	10.0 bcd
	D		Animal feed flour	1.2 bc	3.2 bcd	5.8 def	9.0 de
			Degermination bran (DB)	1.4 b	3.0 bcd	7.6 b	11.7 bc
			Germ	1.2 bc	2.5 cd	4.2 fg	6.8 ef
		SM	Corn flour	1.1 bc	2.9 bcd	5.1 efg	9.1 cde
			Semi-whole semolina (D-SM)	1.3 bc	3.2 bc	7.2 bc	11.9 b
		RM	Corn flour	1.3 bc	3.7 ab	6.5 bcde	10.4 bcd
			Refining bran (RB)	1.4 bc	2.4 d	7.1 bcd	11.4 bcd
			Refined semolina (D-RM)	1.9 a	4.2 a	9.8 a	15.8 a
			p (F)	***	***	***	***
P1547			Corn grains	1.5 b	3.5 b	5.9 cde	10.8 bc
	None	SM-S	Animal feed flour	1.5 b	3.7 b	5.3 def	9.5 de
			Semi-whole semolina (SM-S)	1.5 b	3.6 b	6.3 cd	11.3 bc
	D		Animal feed flour	1.1 c	3.1 bc	4.6 ef	8.4 ef
			Degermination bran (DB)	1.5 b	2.9 c	6.1 cd	11.0 bc
			Germ	0.7 d	1.3 d	1.3 g	2.2 f
		SM	Corn flour	1.4 bc	3.6 b	6.3 bcd	10.0 bcd
			Semi-whole semolina (D-SM)	1.6 b	3.7 b	7.2 abc	11.9 b
		RM	Corn flour	1.2 bc	3.5 b	4.6 f	8.4 ef
			Refining bran (RB)	1.5 b	2.6 c	7.5 ab	12.1 b
			Refined semolina (D-RM)	1.9 a	4.3 a	7.6 a	13.9 a
			p (F)	***	***	***	***
Pignoletto × SN148			Corn grains	2.4 b	5.1 cd	7.0 d	8.4 d
	None	SM-S	Animal feed flour	1.8 c	4.2 e	4.6 f	5.8 f
			Semi-whole semolina (SM-S)	2.3 b	4.9 d	7.1 d	8.6 cd
	D		Animal feed flour	1.8 c	4.2 e	5.2 f	7.0 e
			Degermination bran (DB)	1.9 c	3.6 f	6.1 e	8.4 d
			Germ	1.7 c	3.4 f	3.6 g	4.5 g
		SM	Corn flour	2.3 b	5.0 cd	7.1 d	9.3 cd
			Semi-whole semolina (D-SM)	2.6 b	5.6 bc	9.0 b	11.6 b

RM	Corn flour	2.5 b	6.0 ab	7.1 d	8.9 cd
	Refining bran (RB)	2.9 a	5.4 bcd	8.2 c	9.8 c
	Refined semolina (D-RM)	3.2 a	6.6 a	10.2 a	12.8 a
	p (F)	***	***	***	***

D, degermination; SM, stone milling; SM-S, stone milling followed by sieving; RM, roller milling; DB, degermination bran; RB, refining bran; LOD, limit of detection. The results are expressed on a DW basis. Means followed by different letters are significantly different, according to the REGW-F test [(*) *p* (F) < 0.05, (**) *p* (F) < 0.01, and (***) *p* (F) < 0.001, and ns, non-significant].

Figure S1. Visual comparison of the kernels of the corn hybrids evaluated in this study: DKC6092 (A), P1547 (B), Pignoletto × SN148 (C).

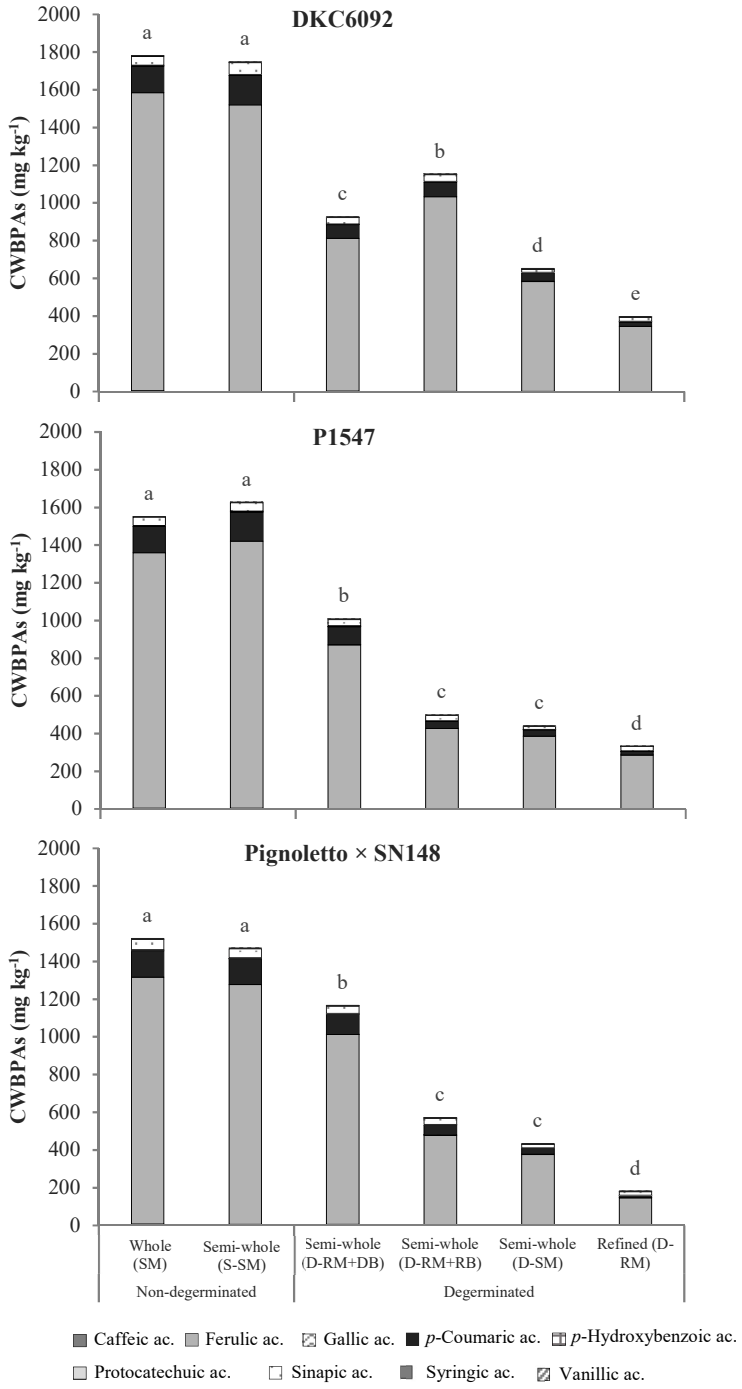


Figure S2. Comparison of the content of cell wall-bound phenolic acids (CWBPAs) in the cornmeal semolina of different milling processes and different corn hybrids.

SM, stone milling; SM-S, stone milling followed by sieving; D-RM+DB, degermination followed by roller milling and degermination bran incorporation; D-RM+RB, degermination followed by roller milling and refining bran incorporation; D-SM, degermination followed by stone milling; D-RM, degermination followed by roller milling. The results are expressed on a DW basis. Different letters above the columns indicate significant differences between the six groups at p (F) < 0.05, according to the REGW-F test.

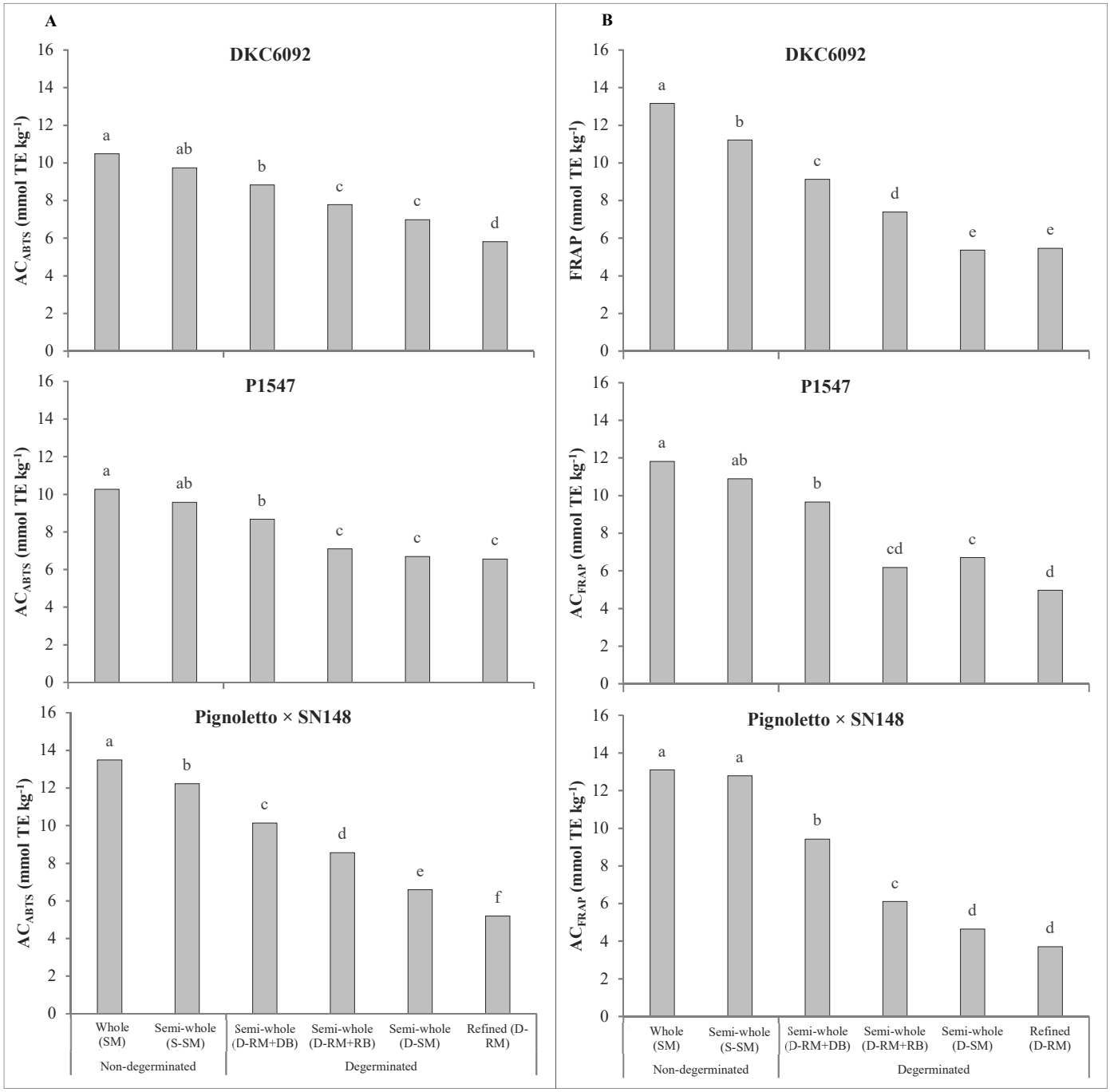


Figure S3. Comparison of the antioxidant capacity (AC) by means of ABTS and FRAP assays in the cornmeal semolina of different milling processes and different corn hybrids.

SM, stone milling; SM-S, stone milling followed by sieving; D-RM+DB, degermination followed by roller milling and degermination bran incorporation; D-RM+RB, degermination followed by roller milling and refining bran incorporation; D-SM, degermination followed by stone milling; D-RM, degermination followed by roller milling. The results are expressed on a DW basis. Different letters above the columns indicate significant differences between the six groups at p (F) < 0.05, according to the REGW-F test.

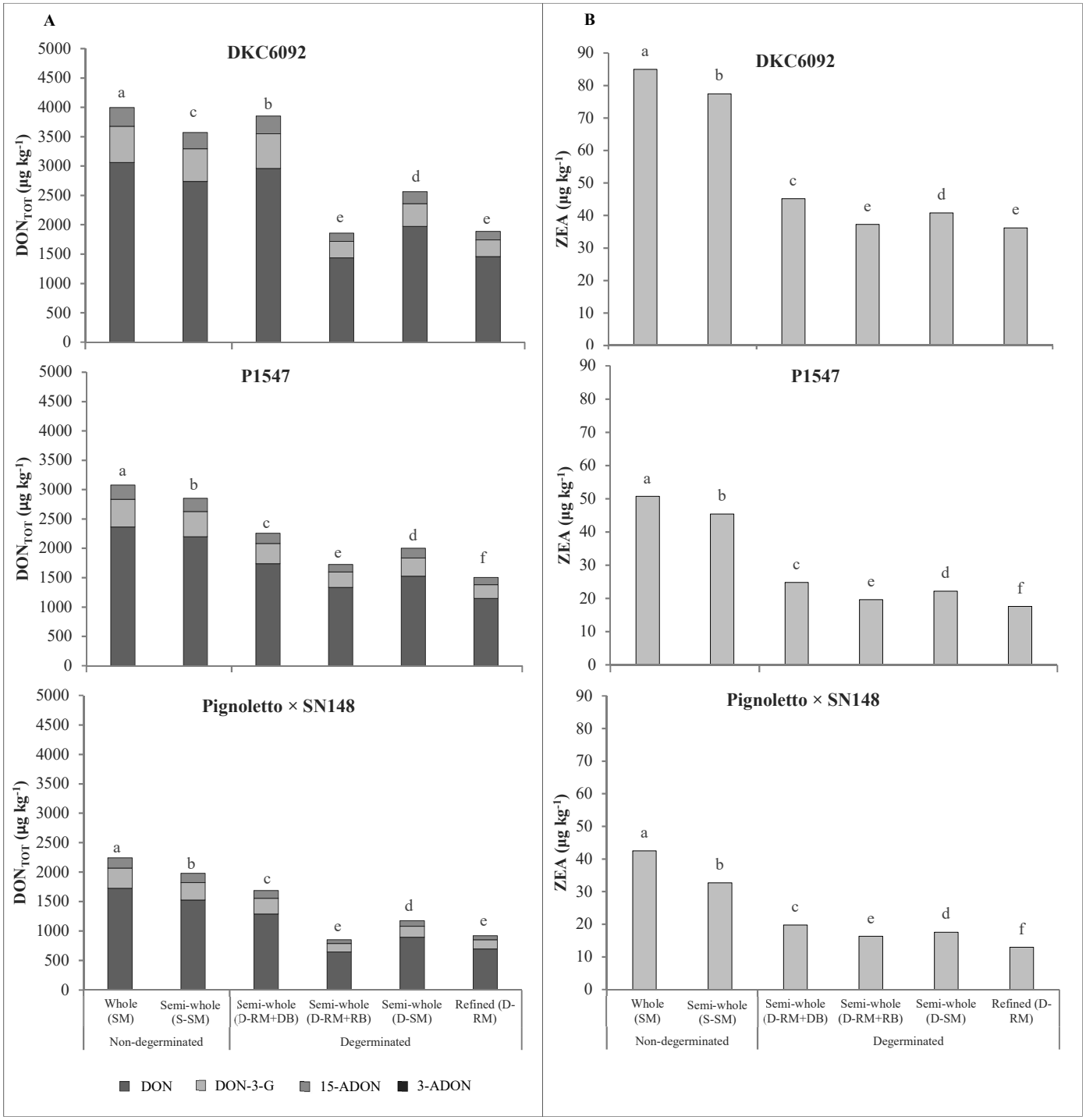


Figure S4. Comparison of the content of deoxynivalenol (DON_{TOT}; A) as the sum of DON, DON-3-G, 15-ADON, and 3-ADON (<LOQ; LOQ = 10 µg kg^{-1}), and zearalenone (ZEA; B) in the cornmeal semolina of different milling processes and different corn hybrids.

SM, stone milling; SM-S, stone milling followed by sieving; D-RM+DB, degermination followed by roller milling and degermination bran incorporation; D-RM+RB, degermination followed by roller milling and refining bran incorporation; D-SM, degermination followed by stone milling; D-RM, degermination followed by roller milling. The results are expressed on a DW basis. Different letters above the columns indicate significant differences between the six groups at p (F) < 0.05, according to the REGW-F test.

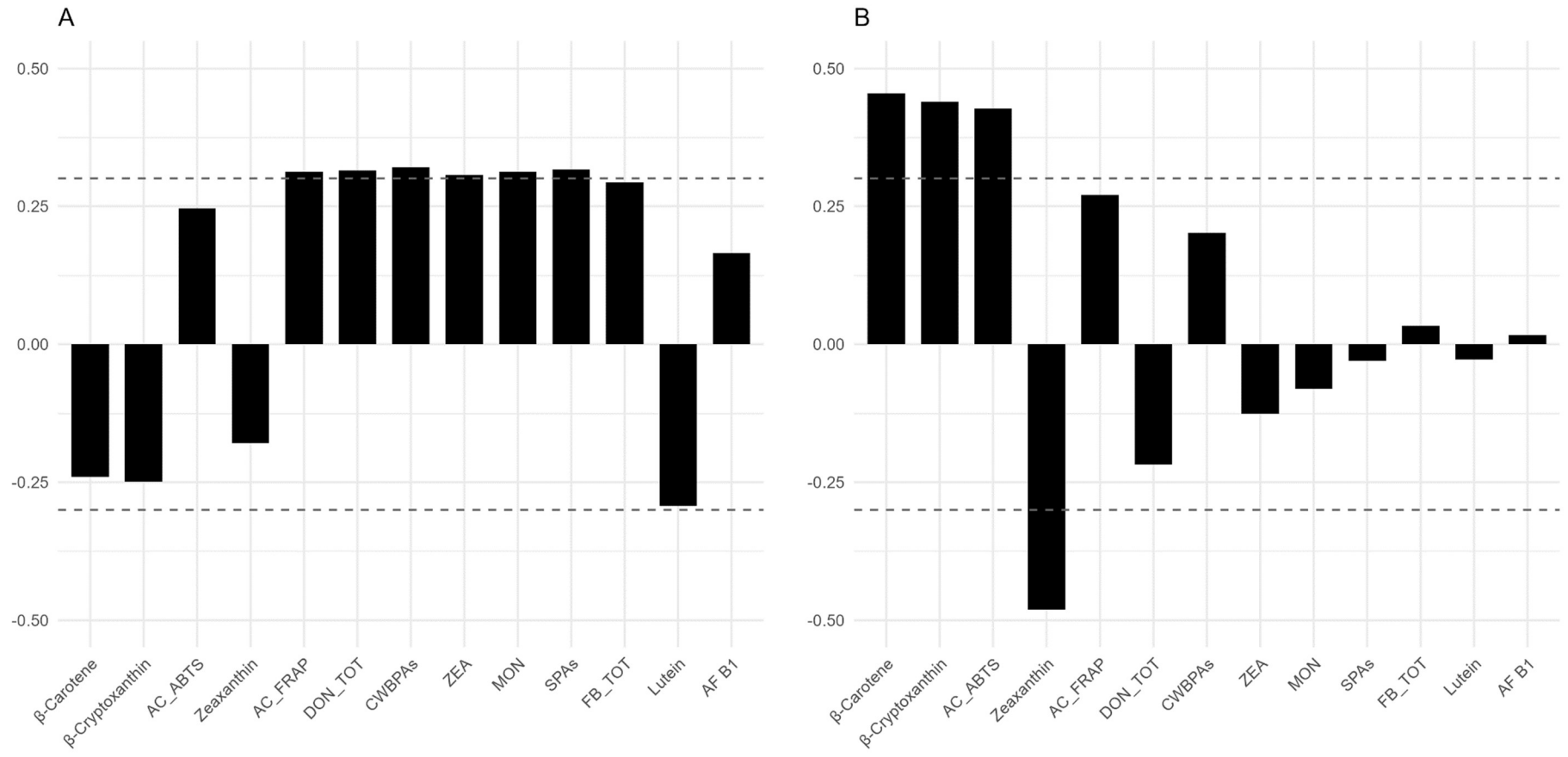


Figure S5. Loadings on Component 1 (A) and on Component 2 (B) of the principal component analysis reported in Figure 4.

The dotted lines delineate loadings greater than 0.3. FB_TOT, total fumonisins; DON_TOT, total deoxynivalenol; AF B1, aflatoxin B_1; ZEA, zearalenone; MON, moniliformin; SPAs, soluble phenolic acids; CWBPAs, cell wall-bound phenolic acids; AC, antioxidant capacity by means of QUENCHER-ABTS and -FRAP assays.

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Chapter VII – "The potential of cereals as key sources of bioactive compounds: the role of milling and processing in wheat and tritordeum"

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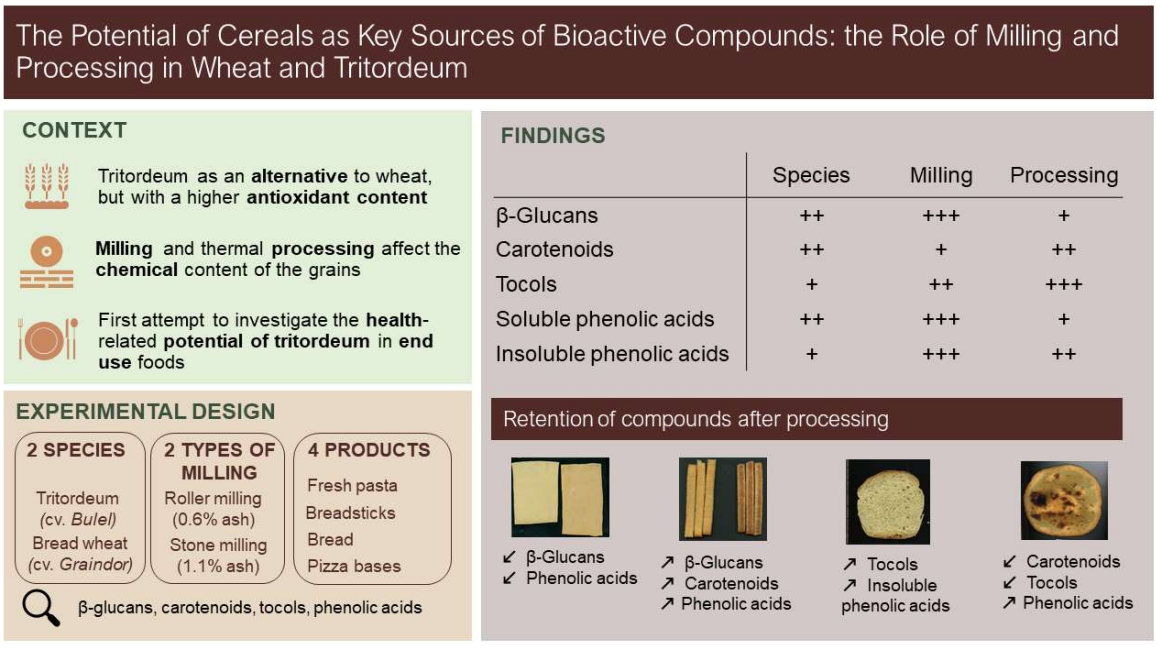
Graphical abstract



Abstract

The β-glucan, carotenoid, tocol, and phenolic acid contents of tritordeum and of bread wheat, the latter of which was used as a control, was determined in roller or stone milled flour and in four end products made using different processing techniques. Carotenoid retention varied more according to the species and processing method, with the highest levels being found in roller milled tritordeum breadsticks (32% of flour; 2.48 $mg \cdot kg^{-1}$). Milling accounted for most of the β-glucan, soluble and bound phenolic acid, and antioxidant capacity variations, while processing explained most of the tocol variations. The tritordeum flours and products showed a 34% higher total antioxidant capacity than bread wheat, thereby offering opportunities of providing higher concentrations and diverse profiles of carotenoids, tocols, and phenolic acids in food commodities.

Keywords: × *Tritordeum martinii*, phenolic acids, carotenoids, tocols, antioxidant capacity, stone milling.

Abbreviations: ABTS, 2,2′-Azino-bis(3-ethylbeenzothiazoline-6-sulfonic acid) diammonium salt; AC, antioxidant capacity; ANOVA, analysis of variance; CWBPAs, cell wall-bound phenolic acids; DAD, diode array detection; DW, dry weight; FLD, fluorescence detector; FRAP, ferric reducing antioxidant power; FW, fresh weight; HPLC, high-performance liquid chromatography; LOD, limit of detection; REGW–F test, Ryan–Einot–Gabriel–Welsch F test; SPAs, soluble phenolic acids; TCC, total carotenoid content; TE, Trolox equivalents; TTC, total tocol content.

Introduction

Tritordeum (× *Tritordeum martinii* A. Pujadas, nothosp. nov.) is a novel, man-made, cereal species that was first generated in the 1970s by following a similar hybridization pathway to that of triticale (Martín et al., 1999). The hexaploid genotypes obtained from crossing perennial wild barley (*Hordeum chilense* Roem. et Schultz.), as the seed parent, and durum wheat (*Triticum turgidum*, var. *durum*), as the pollen donor, are currently mainly cultivated for human consumption (Ávila et al., 2021). In recent years, tritordeum has piqued the interest of researchers and food chain operators because of some unique characteristics. The most distinctive feature of tritordeum is the intense yellow color of its grains and derived products, which confer a clear differentiation from standard bread wheat. This trait is caused by an exceptionally high concentration of carotenoids, mainly lutein, in the endosperm, which has been reported to be up to 5 times higher in tritordeum lines than in durum wheat (Atienza et al., 2007). Despite the yellow pigmentation of its flour, tritordeum is considered an alternative cereal to bread wheat, as its rheological and technological properties have been shown to be suitable for bread-making (Martín et al., 1999). Its end-use products are also characterized by good organoleptic properties, which in turn lead to a high level of consumer acceptability (Sánchez-León et al., 2020; Vaquero et al., 2018). Other applications have also been suggested for this species, such as the production of quality beer as an alternative to barley malt (Yding et al., 2023). Furthermore, tritordeum has been promoted for its potential health benefits. Having a lower number of gluten immunogenic peptides than bread wheat, it has been suggested to be more suitable for individuals who suffer from adverse reactions to wheat (Sánchez-León et al., 2020; Vaquero et al., 2018). In terms of grain composition, significantly higher contents of proteins, some minerals, total phenolics, and methyl donors were observed by Shewry et al. (2023). However, a high within-species variability was also pointed out, with overlapping contents between tritordeum, bread, and durum wheat. The enhanced concentration of phytochemicals makes tritordeum a valuable commodity for producing novel functional foods, which could contribute to the daily intake of beneficial compounds, thereby potentially improving consumers' health (Elvira-Torales et al., 2019; Gupta et al., 2021). However, these beneficial effects depend on the type of grain-based foods that are consumed, as the grains necessarily undergo some form of processing to achieve the desired sensory properties, and such a processing can affect its components. Grain milling is one of the main factors that determines the bioactive compound content and the antioxidant capacity of a final product, because of the uneven distribution of these compounds among the botanical components of the kernel (Giordano et al., 2019). In addition, any subsequent thermal

processing may affect the chemical composition and overall nutritional value of the product, with different results, depending on the technological steps and the considered substance. Indeed, bioactive compounds show different degrees of stability to various factors, such as oxidation and temperature, which in turn affect their final bioavailability and antioxidant activity at the moment of consumption (Burešová et al., 2023). To date, few studies have explored the content and composition of health-related compounds in tritordeum, such as minerals (Phuong et al., 2017; Shewry et al., 2023), phenolic acids (Giordano et al., 2019), tocols (Lachman et al., 2018), carotenoids (Paznocht et al., 2018), and β-glucans and arabinoxylans (Rakha et al., 2012; Shewry et al., 2023). Although tritordeum exhibits promising characteristics, the effects of milling and processing on its bioactive compounds have not yet been investigated. Indeed, the aforementioned studies primarily focused on grain composition, and overlooked the health-related potential of tritordeum in the final products and the corresponding benefits for consumers. Therefore, the objectives of the present study have been (1) to investigate the effect of two milling processes on the β-glucan, carotenoid, tocol, and phenolic acid contents and on the total antioxidant capacity of tritordeum, and (2) to determine the retention levels of these compounds and of the antioxidant capacity in four standard food prototypes (fresh pasta, breadsticks, bread, and pizza bases) prepared with similar ingredients but using different technological parameters.

Materials and methods

Samples and technological processes

One variety of tritordeum (Bulel) and one of bread wheat (Graindor), used as a control, were cultivated in the 2020–21 growing season in north-west Italy. Tritordeum and wheat grains were ground by either roller or stone milling in an industrial plant (Molini Bongiovanni S.p.a., Cambiano, Italy) to obtain refined and semi-whole flours, respectively, which were then used to make 4 different types of products (fresh pasta, breadsticks, bread, and pizza). The products were selected because they have similar formulations but different processing procedures, i.e. specific technological parameters in the kneading, leavening, and cooking/baking stages, as summarized in Table S1.

For the preparation of fresh pasta, an aliquot of 2 kg of flour was mixed with water (40 °C) at different ratios, depending on the type of flour and on the basis of their farinograph water absorption (roller milled tritordeum 46.5% and wheat 40.0%, stone milled tritordeum 50.0% and wheat 44.1%), and the obtained dough was then processed in a planetary kneading machine (Kenwood, De' Longhi Appliances S.r.l., Treviso, Italy). The dough was left to rest under vacuum and then rolled into 1 mm thick pasta sheets using a continuous press (Imperia & Monferrina S.p.a., Moncalieri, Italy). The sheets were shaped into 10×10 cm squares and stored at 4 °C. The obtained pasta was cooked in boiling distilled water (pasta:water ratio = 1:10) for the optimum time, which had been determined by ten individuals through a sensory evaluation of the products, cooked for different time periods.

Breadsticks were produced in a local factory by means of an industrial process in which 15 kg of flour was mixed with corn oil (4%), fresh yeast (3%), salt (1%), and water in different ratios, depending on the type of flour (roller milled tritordeum 27.9% and wheat 26.7%, stone milled tritordeum 29.6% and wheat 27.5%). The dough was left to rest and then sheeted and folded for about 10 min. After leavening, the dough was shaped using stainless steel molds (Torneria Automatica Tealdi S.n.c., Clavesana, Italy) at 27 °C and 45% relative humidity. The breadsticks were baked in a continuous tunnel oven (Tibiletti Forni S.r.l., Novara, Italy).

For the preparation of bread, an aliquot of 3 kg of flour was mixed with fresh yeast (3%), salt (2.5%), and water in different ratios, depending on the type of flour (roller milled tritordeum 60.3% and wheat 53.6%, stone milled tritordeum 66.6% and wheat 58.4%) and processed in a spiral kneading machine (Esmach Ali Group S.r.l., Grisignano di Zocco, Italy). The dough was left to rest at 70% relative humidity in a proofing chamber (Esmach Ali Group S.r.l) and then manually divided into 750 g loaves, which, after leavening, were baked in a bakery oven (Moretti Forni S.p.A., Mondolfo, Italy).

For the preparation of pizza, an aliquot of 3 kg of flour was mixed with fresh yeast (1%), salt (2.5%), and water in different ratios, depending on the type of flour (roller milled tritordeum 60.3% and wheat 53.6%, stone milled tritordeum 66.6% and wheat 58.4%) and processed in a spiral kneading machine. The dough was left to rest and then manually divided into 300 g loaves. After leavening for 17 h, the loaves were kneaded by hand and left to rest for another 2 h. The loaves were then shaped and baked in a bakery oven without the addition of any ingredients as toppings.

Samples of the flours and cooked/baked products were collected and immediately stored at –25 °C for analytical determination. The final products were freeze-dried using a Lyovapor L-200 Freeze Dryer (Buchi, Flawil, Switzerland), ground to a fine powder (particle size <500 µm) with a Cyclotec 1093 sample mill (Foss, Padua, Italy), and stored at –25 °C until being analyzed.

Composition analyses

The proximal composition of the 4 flours used in this study is reported in Table S2. The protein content was determined according to AACC method 39-10.01 (AACC International, 2008) by means of an NIR System Model 6500 (FOSS Italia S.r.l., Padua, Italy), as was the ash content. The starch content was measured according to Commission Regulation (EU) No 118/2010 of 9 February 2010, Annex I (European Commission, 2010). The fat and the total soluble and insoluble dietary fiber were measured according to Soxhlet method AOAC 2003.05 and enzymatic-gravimetric method AOAC 991.43, respectively (AOAC International, 2005). The moisture content of the flours and lyophilized products was determined by oven-drying (Falc Instrument S.r.l., Treviglio, Italy) the samples at 105 °C to a constant weight, and the results of the chemical analyses were expressed on a dry weight (DW) basis.

Chemical analyses

Chemicals

2,2′-Azino-bis(3-ethylbeenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), ethanol (CHROMASOLV®, 99.8%), ethyl acetate (CHROMASOLV®, 99.8%), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, 97%), hydrochloric acid (37%), iron(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$, ≥98%), methanol (CHROMASOLV®, 99.9%), potassium sulfate (≥99%), sodium hydroxide (≥98%), 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ), butylated hydroxytoluene (BHT, ≥99%), *tert*-butyl methyl ether (HPLC grade), and phenolic acid standards (caffeic acid ≥98%, *p*-coumaric acid ≥98%, ferulic acid ≥99%, gallic acid ≥99%, protocatechuic acid ≥99%, *p*-hydroxybenzoic acid ≥99%, sinapic acid ≥98%, syringic acid

≥95%, and vanillic acid ≥97%) were purchased from Merck KGaA (Darmstadt, Germany). Lutein (≥95%) and zeaxanthin (≥98%) standards were obtained from Extrasynthese (Genay, France). A tocotrienol and tocopherol mixed standard solution was obtained from ChromaDex (Irvine, CA, USA). Ethanol, acetone, hexane, ethyl acetate, sodium chloride, potassium hydroxide, and pyrogallol (all GR grade), and methanol (HPLC grade) were obtained from Lachner (Neratovice, The Czech Republic). Water (HPLC grade) was prepared using an ELGA PURELAB Ultra system (M-medical, Cornaredo, Milan, Italy).

Analytical methods

All analyses were performed in triplicate. The β-glucan content (AACC International, 2008) was determined using a Megazyme mixed-linkage β-glucan assay kit, according to the producer's instructions (Megazyme International Ireland Ltd, Wicklow, Ireland). The total β-glucan was expressed in g·100 g^{-1} DW.

The carotenoids were extracted and analyzed by means of HPLC–DAD, according to the method described by Paznocht et al. (2019). The total carotenoid content (TCC) was expressed as the sum of all the determined lutein isomers, zeaxanthin, and xanthophyll esters (mg·kg^{-1} DW). The extraction, chromatographic separation, identification, and quantification of the tocols, by means of HPLC–FLD, were described in detail in a previous study by Burešová et al. (2021). The total tocol content (TTC) was expressed as the sum of all the determined tocopherols (T) and tocotrienols (T3) (mg·kg^{-1} DW). The soluble (SPAs) and cell wall-bound phenolic acids (CWBPAs) were individually extracted through a two-step method, analyzed by means of HPLC–DAD, and expressed in mg·kg^{-1} DW. Details of the sample preparation procedure as well as of the chromatographic conditions were given in Giordano et al. (2019). ABTS and ferric reducing antioxidant power (FRAP) assays were employed to determine the antioxidant capacity (AC) of the flours and derived products, following the QUENCHER procedure (direct measurements on solid samples), as described by Serpen et al. (2008), and the obtained results were expressed as mmol Trolox equivalents (TE)·kg^{-1} DW.

Statistical analyses

The obtained data were compared by means of an analysis of variance (ANOVA) to evaluate the effect of the species (S), the milling degree (M), the type of processing (P), and their interactions on the bioactive compound content, and on the AC of the flours and of the final products (n = 60). Means were identified as significantly different ($p < 0.05$) on the basis of the Ryan–Einot–Gabriel–Welsch F (REGW–F) statistical test. Prior to the

analysis, the data were tested for normality and homoscedasticity. The analyses were carried out by means of the SPSS for Windows statistical package, version 28.0 (SPSS, Inc., Chicago, IL, USA).

Table 1. Level of significance of the three-way ANOVA analysis performed to evaluate the contribution of the species, degree of milling, type of processing, and their interaction to the biologically relevant compounds and the antioxidant capacity of the analyzed samples. The results are expressed as percentages of the total mean square.

Factors	β-glucans	TCC	TTC	SPAs	CWBPAs	AC_{ABTS}	AC_{FRAP}
Species (S)	14.7 ***	43.8 ***	0.3 ***	9.2 ***	0.1 *	27.7 ***	17.6 ***
Milling degree (M)	71.4 ***	0.4 ***	8.7 ***	72.7 ***	98.2 ***	43.5 ***	61.5 ***
Processing type (P)	10.6 ***	44.2 ***	85.2 ***	6.5 ***	1.2 ***	23.4 ***	6.9 ***
S × M	0.2	0.5 ***	0.4 ***	7.7 ***	0.2 *	0.4	5.2 ***
S × P	2.3 ***	10.7 ***	0.01	1.5 ***	0.02	0.6 *	6.4 ***
M × P	0.7 ***	0.2 ***	5.1 ***	1.8 ***	0.2 ***	2.9 ***	1.6 ***
S × M × P	0.0	0.2 ***	0.3 ***	0.5 **	0.04	1.4 ***	0.7 **
Error	0.1	0.01	0.01	0.1	0.02	0.2	0.1

TCC, total carotenoid content; TTC, total tocol content; SPAs, soluble phenolic acids; CWBPAs, cell-wall bound phenolic acids; AC, antioxidant capacity (ABTS and FRAP assay). The indicated factors are statistically significant, according to the REGW-F test [(*) p (F) < 0.05, (**) p (F) < 0.01, and (***) p (F) < 0.001].

Results and discussion

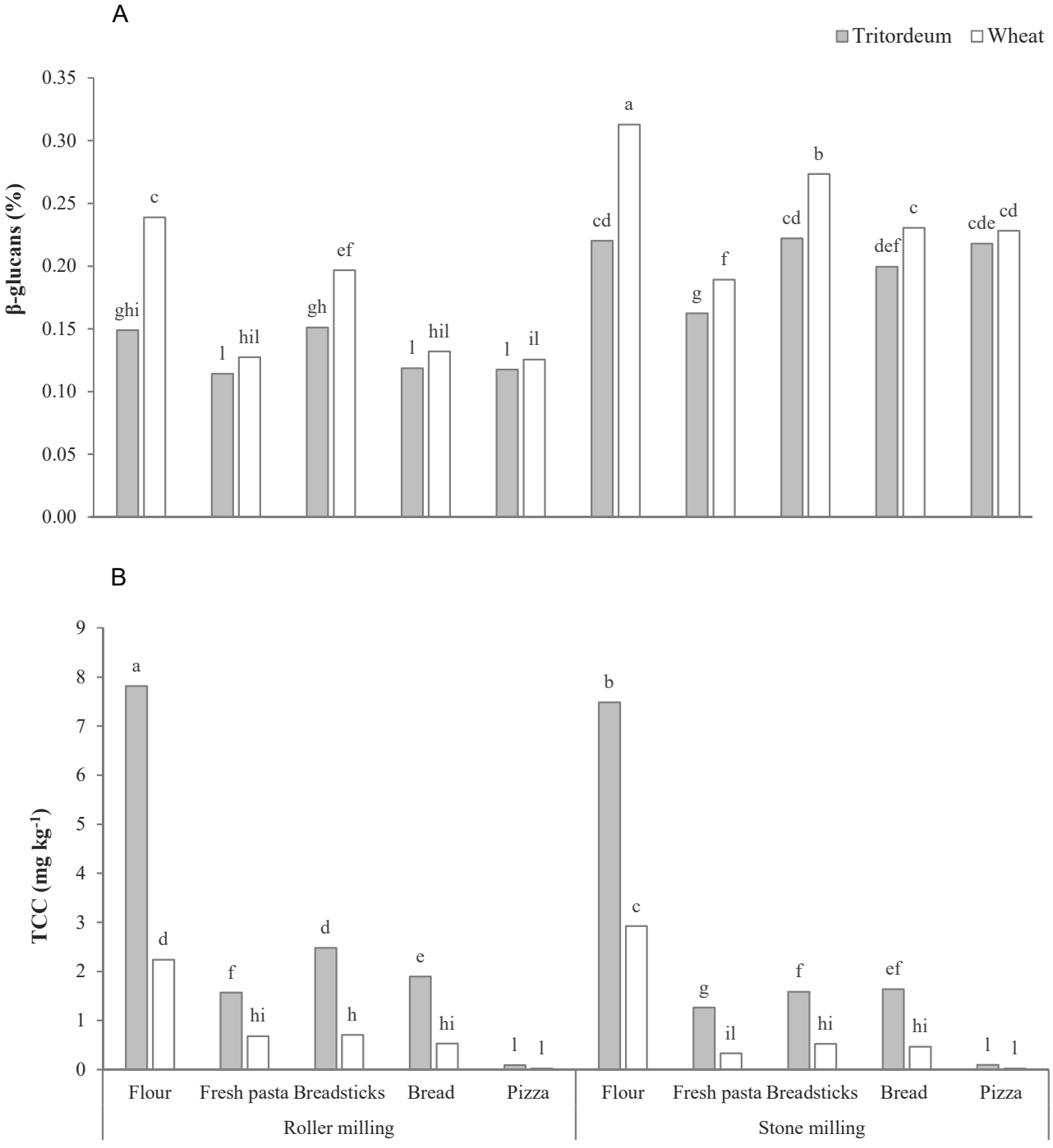
β-glucans

Three-way ANOVA showed significant effects of all of the three investigated factors (species, milling degree, and processing type) on the β-glucan contents of the analyzed samples (Table 1). The milling degree accounted for most of the observed variation (71.4%), and this was followed by the species (14.7%) and the processing type (10.6%). The S × P and M × P interactions were significant, although they only accounted for a small share of the total variation.

The wheat samples showed a higher β-glucan content (+18%) than the samples made of tritordeum (average value of 0.20 vs 0.17 g·100 g^{-1}; data not shown). The highest β-glucan value (Figure 1A) was observed in the stone milled wheat flour (0.31 g·100 g^{-1}), which was 41% higher than its tritordeum counterpart (0.22 g·100 g^{-1}), while the values were 0.24 and 0.15 g·100 g^{-1} when the wheat and tritordeum grains were roller milled. Consistently with other literature data (Giordano et al., 2019; Rakha et al., 2012), the β-glucan content of the novel cereal tritordeum was observed to be lower than that of bread wheat, closer to that of durum wheat, and not as high as that of barley varieties. However, the aforementioned studies focused solely on the grain content, and they reported a β-glucan concentration for tritordeum grains that was 3 times higher than that observed in the stone milled tritordeum flour analyzed in the present study. It is worth mentioning that the stone milling method used in this work involved sieving, which meant that some of the intermediate layers, where β-glucans in tritordeum have been reported to accumulate the most (Giordano et al., 2019), were discarded. The stone milled samples were characterized by a higher average β-glucan content (+64%), and this was reflected in both the flour and in the derived products, which showed lower percentage losses than the roller milled samples (Figure 1A). Furthermore, the initial β-glucan contents observed in the flours decreased significantly, at different rates, according to the species and the type of process. The higher levels observed in wheat than in tritordeum flour were maintained throughout production, although wheat showed higher percentage losses than tritordeum, especially when roller milled, and this led to less marked differences between the wheat and tritordeum derived products (Figure 1A). The production of breadsticks proved to be the most β-glucans-sparing technology for both cereal species, as it reduced the β-glucan content of the wheat samples to 83% of its initial value (i.v.) in the flour, while statistically similar values to the i.v. were maintained for the tritordeum samples. The lowest retained amount was observed in the pasta samples (65% i.v.). However, statistically relevant differences in the β-glucan content of pasta, compared to the

bread and pizza samples, were only observed for the stone milled tritordeum and wheat flours.

Cereal β-glucans are linear long-chain homopolymers of high molecular weight, formed by D-glucose residues linked by β-(1,4) bonds and interspersed with β-(1,3) linkages (Lante et al., 2023). It has been observed that certain factors induced during processing and cooking primarily affect the polymer structure, that is, they change the molecular weight and extractability, and therefore viscosity, which is associated with the physiological functions of cereal β-glucans (Henrion et al., 2019). De Paula et al. (2017) found the highest viscosity in cooked pasta with the lowest β-glucan concentration among samples with different barley flour substitutions, highlighting that the solubility and molecular weight of β-glucans play a more significant role in determining viscosity than the total content (Regand et al., 2009). Indeed, it will be particularly important to consider the physiochemical properties of β-glucan in tritordeum flours and products to better characterize this novel cereal.


A
Tritordeum
Wheat
β-glucans (%)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
ghi
c
l
hil
gh
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l
hil
l
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a
g
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hi
ef
hi
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Flour
Fresh pasta
Breadsticks
Bread
Pizza
Roller milling
Flour
Fresh pasta
Breadsticks
Bread
Pizza
Stone milling

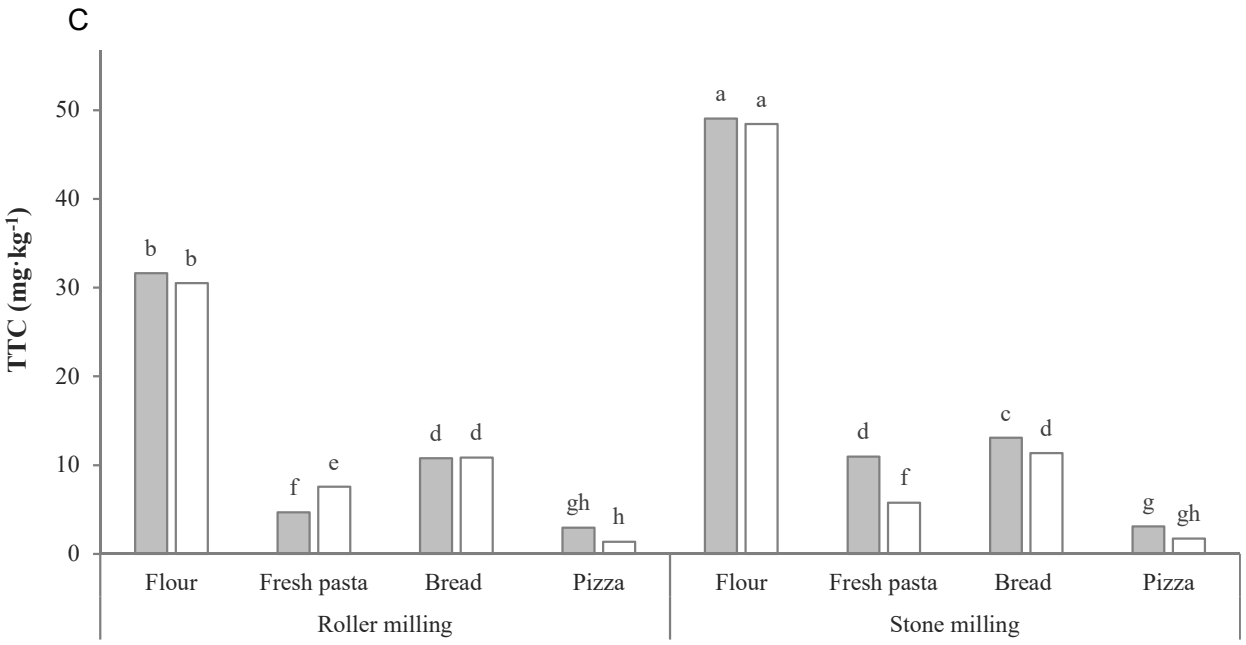

Figure 1. The β-glucan (A), carotenoid (TCC; B), and tocol (TTC; C) contents of the tritordeum and wheat samples of flour, fresh pasta, breadsticks, bread, and pizza, obtained from roller and stone milling.

The results are expressed on a DW basis. Different letters above the columns indicate significant differences for p (F) < 0.05, according to the REGW-F test.

Carotenoids

The three investigated factors all had significant effects on the TCC, as did their interactions (Table 1). Most of the observed variation was explained by the species (43.8%) and the manufacturing process (44.2%). The TCC was found to be 2-4 times higher in the tritordeum samples than in the wheat counterparts (Figure 1B). Moreover, the higher TCC in the roller milled samples (+10%) was statistically relevant, as was the average decrease caused by the technological processing of the flour, although different rates were observed, depending on the applied process and on the species, as S × P accounted for a large share of variation (10.7%).

The high initial TCC of tritordeum flour (7.65 $mg \cdot kg^{-1}$ DW as the average of roller and stone milling, Table 2) is in agreement with the observations of previous authors (Giordano et al., 2019; Rodríguez-Suárez et al., 2024) and falls within the ranges previously observed for einkorn (*T. monococcum* L. subsp. *monococcum*), which has the highest carotenoid content of all cultivated wheats (Hidalgo et al., 2010). Significantly higher values of TCC were found in the whole-grain flour obtained from a tritordeum breeding line by Paznocht et al. (2018) (12.16 $mg \cdot kg^{-1}$ DW) or, and even more so, in that obtained by Suchowilska et al. (2021), who reported an average of 17.09 $mg \cdot kg^{-1}$ DW for eleven spring tritordeum lines. The variability in the TCC of cereal flours observed in different studies may be due to the different growing conditions and genetic backgrounds, as well as to the environmental conditions during cultivation, differences in the milling degrees, the analytical methods, and the individual determined compounds. Overall, the TCC of tritordeum flour was about 3 times higher than that of the control bread wheat variety, being in line with previous data (Giordano et al., 2019; Paznocht et al., 2018).

The carotenoid profiles revealed that all-*E*-lutein was the most abundant carotenoid in free form in both tritordeum and wheat flour, accounting for 68 and 61% of the TCC, respectively (Figure S1), with average contents of 5.17 and 1.58 $mg \cdot kg^{-1}$ (Table 2), accompanied by its *Z*-isomers (10 vs 7%; 0.73 vs 0.18 $mg \cdot kg^{-1}$). The results of the present study confirm the relatively larger amounts of xanthophylls esterified with fatty acids in tritordeum than in wheat (1.50 vs 0.70 $mg \cdot kg^{-1}$), which were dominated by lutein monoesters, as reported in previous works (Atienza et al., 2007; Mellado-Ortega & Hornero-Méndez, 2018; Paznocht et al., 2018). The proportion of xanthophyll esters was lower in tritordeum than in wheat (20 vs 27%), but in agreement with the findings of other authors (Mattera et al., 2017; Mellado-Ortega & Hornero-Méndez, 2012; Paznocht et al., 2018), who reported that lutein esters represented 14-41% of the TCC in tritordeum.

The statistically significant higher amount of TCC found in the refined tritordeum flour than in the semi-whole one (7.81 vs 7.48 $mg \cdot kg^{-1}$; Figure 1B) is consistent with the distribution

pattern of the dominant lutein, whose concentration tends to increase from the outermost to the innermost kernel fractions, with a significant drop only in highly refined flour (Giordano et al., 2019). This larger amount of TCC was preserved after the roller milling procedure applied in the present study. Similarly, 75% of lutein esters have been found in the endosperm tissue of tritordeum (Mellado-Ortega & Hornero-Méndez, 2018). On the other hand, the lutein content of the wheat flour was very low (Table 2), and the contribution of zeaxanthin, which had been found in high concentration in fractions of wheat grains retained in stone milling and discarded in roller milling (intermediate layers and germs) (Mellado-Ortega & Hornero-Méndez, 2018), therefore led to a larger amount of carotenoids in the stone milled wheat flour (Figure 1B).

Overall, the TCC retention rates were higher for the roller milled products than for the stone milled ones (20 vs 14% i.v.). Different shares of preserved carotenoids were found for the final products from the i.v. of the flour: breadsticks > bread > fresh pasta > pizza (26 > 22 > 19 > 1%). The reduction percentage was similar for both tritordeum and wheat, but tritordeum supplied considerably more carotenoids than wheat to the end products, due to the significantly higher initial content. This was evident for the breadstick samples made from roller milled flour, whose TCC dropped to 32% of the i.v. for both cereals. However, the tritordeum breadsticks were characterized by the greatest preserved amount of TCC, with values statistically similar to the i.v. of the wheat flour. Significant carotenoid losses were reported during the manufacturing of different end products in previous publications. A lower TCC decrease was found by Hidalgo et al. (2010) in the breadcrumbs (up to 74%) and in the bread crust (up to 45%) of wheat flour, while a comparable retention was observed for raw dry pasta (up to 22%). The different outcomes observed for products characterized by different time-temperature processing conditions confirm the important role of heat and of the cooking stage on the stability of these bioactives. The authors of two bread-making experiments observed a similar TCC retention for unleavened flatbreads (26%; Burešová et al., 2021) and leavened buns (27%; Paznocht et al., 2019). In the present study, the breadsticks benefited from milder baking conditions than the bread and pizza (Table S1). The most significant drop occurred in the pizza-making process, where no differences were observed between tritordeum and wheat, probably due to the high baking temperatures, which the thin product reached much faster than the other products. The dough kneading time has also been reported to play a role in carotenoid degradation (Burešová et al., 2021; Fratianni et al., 2012; Hidalgo et al., 2010), since the incorporation of water and oxygen during kneading promotes the lipoxygenase-catalyzed oxidation of polyunsaturated fatty acids, which in turn leads to the formation of peroxides and other free radicals that can react with carotenoid compounds (Paznocht et al., 2019). A smaller

decrease in TCC was observed for the baked products when the dough was kneaded and primarily leavened for a shorter time (breadsticks < bread < pizza, as shown in Table S1), a result that is consistent with previous studies (Leenhardt et al., 2006; Paznocht et al., 2019). As far as pasta is concerned, previous publications reported a greater role of kneading than extrusion and drying, with the total carotenoids showing a significant loss, that is, from 22 to 35%, only during this step (Chiremba et al., 2015; Fratianni et al., 2012). In the present study, the pasta procedure was characterized by a lower kneading time, but also by a higher carotenoid degradation than the breadsticks, and the losses should therefore mainly be ascribed to the cooking phase, thereby highlighting the importance of considering the impact of the cooking phase on the retention and bioaccessibility of phytochemicals at the moment of consumption (Cilla et al., 2018). The effect of boiling was studied in previous experiments, although different outcomes were obtained, depending on the starting raw materials, the processing parameters, and the detected analytes. Burešová et al. (2023) observed losses of 13-*Z*-lutein and 13'-*Z*-lutein after boiling colored-wheat grains, but increased contents of 9-*Z*-lutein and 9-*Z*'-lutein, thus indicating a pronounced stability and/or isomerization of the two *Z*-forms. Carotenoid (*E*- to *Z*-) isomerization, especially of lutein, was previously reported during the boiling of corn (De Oliveira & Rodriguez-Amaya, 2007). However, Oduro-Obeng et al. (2021) observed a decrease in 9-*Z* and 13'-*Z*-lutein after boiling pasta. They attributed this reduction to the degradation of carotenoids into apocarotenoids, and they only noted an increase in (*E*- to *Z*-) isomerization after prolonged boiling. No carotenoid isomerization was detected in any of the products in the current study, a result that is consistent with the findings of other studies (Kean et al., 2011; Kotíková et al., 2016) on sorghum porridge and cooked potatoes.

Esterified forms of xanthophylls, whose concentration were significantly higher in the tritordeum flour and derived products than in the wheat counterparts (Table 2), were detected along with free forms. However, the extent of degradation was different, depending on the species and the process (from 21 to 26% i.v. for tritordeum products and from 0 to 32% for wheat products), but was similar to that of their free form and to what Paznocht et al. (2019) and Burešová et al. (2021) observed in other baking experiments (up to 21 and 28% i.v., respectively). A less extensive degradation was observed in the wheat grains after boiling (up to 58% i.v.; Burešová et al., 2023). In the present study, as in the cited works, xanthophyll esters were not found to be more resistant to technological processing, therefore the hypothesis of higher stability of xanthophyll esters presented by other authors (Mattera et al., 2017; Mellado-Ortega & Hornero-Méndez, 2012) was not confirmed.

Table 2. The carotenoids (mg·kg^{-1}) detected in the tritordeum and wheat samples of flour, fresh pasta, breadsticks, bread and pizza, expressed as the average of roller and stone milling.

Species	Processing type	Lutein	Lutein *Z*-isomers	Xanthophyll esters	Zeaxanthin	TCC[a]
Tritordeum	Flour	5.17 a	0.73 a	1.50 a	0.25 a	7.65 a
	Fresh pasta	0.86 d	0.13 d	0.39 c	0.03 cde	1.42 c
	Breadsticks	1.35 bc	0.30 b	0.31 cd	0.07 c	2.03 b
	Bread	1.10 cd	0.22 c	0.39 c	0.05 c	1.77 bc
	Pizza	0.09 f	< LOD e	< LOD e	< LOD e	0.09 e
Wheat	Flour	1.58 b	0.18 c	0.70 b	0.12 b	2.58 b
	Fresh pasta	0.29 ef	0.01 e	0.21 d	< LOD e	0.51 d
	Breadsticks	0.48 e	0.09 d	< LOD e	0.04 cd	0.62 d
	Bread	0.25 ef	0.03 e	0.22 d	< LOD e	0.50 d
	Pizza	0.02 f	< LOD e	< LOD e	< LOD de	0.02 e
	p (F)	***	***	***	***	***

LOD, limit of detection; TCC, total carotenoid content. The results are expressed on a DW basis. Means followed by different letters are significantly different, according to the REGW-F test [(*) p (F) < 0.05, (**) p (F) < 0.01, and (***) p (F) < 0.001, and ns, non-significant].
[a]The sum of all the determined carotenoids.

Tocols

The processing type significantly contributed to the variation in the levels of TTC, and this was followed by the milling degree (85.2 and 8.7%; Table 1). The species and the S × M, M × P, and S × M × P interactions all showed significant effects, but only explained a small share of the variation.

As tocols are more abundant in the germ and less in the endosperm, the TTC of the stone milled flour was on average higher than that of the roller milled flour (48.75 vs 31.07 mg·kg^{-1}), with a slightly larger amount (but not significant) being observed in the tritordeum flours (+1 and +4%; Figure 1C) than in the wheat flours. To the best of the authors' knowledge, very little information is available on the concentration and composition of tocols in tritordeum. The concentration in the tritordeum stone milled flour of the present study (49.05 mg·kg^{-1}) was higher than that observed by Lachman et al. (2018) in spring tritordeum (30.26 mg·kg^{-1}). The variations in the TTC may have been caused by both the environmental conditions of the growing season and genetic factors (Lachman et al., 2018; Lampi et al., 2010). Similarly to the work of Lachman et al. (2018), four of the eight forms of tocol were detected in tritordeum (Table 3 and Figure S1): β-T3 (25.35 mg·kg^{-1}; 63% of the TTC) > α-T (6.39 mg·kg^{-1}; 16%) > α-T3 (5.57 mg·kg^{-1}; 14%) > β-T (2.94 mg·kg^{-1}; 7% of the TTC). δ-T3 was also identified, but at much lower levels (0.09 mg·kg^{-1}). Greater differences between wheat and tritordeum flour were observed for the β-T and α-T3 proportions (12 vs 7% and 6 vs 14%, respectively), and this resulted in a higher overall proportion of tocotrienols (77%) in the TTC of the tritordeum than in the wheat flour (71%), both of which were dominated by β-T3.

Most of the variation was explained by considering the process, which led to significant losses and different levels of retained TTC, depending on the product. Because of the specific formulation of breadsticks, which, in this case, involved the addition of 4% corn oil to the dough, these products were not included in the statistical comparison, as the addition of oil led to the detection of some compounds (γ-T3, δ-T, and γ-T) that are typically detected in corn oil (Moreau & Hicks, 2005; Wen et al., 2020), and which were not present in the initial flour (data not shown). The adopted processing caused a significant degradation of TTC in all the pasta, bread, and pizza samples, with bread retaining the largest amount (11.52 mg·kg^{-1}; Table 3), and fresh pasta and pizza preserving the least (7.25 and 2.29 mg·kg^{-1}, respectively). The milling degree influenced the share of preserved TTC (Figure 1C), with the largest proportion being retained in the products prepared with roller milled flour (21% i.v.) and the smallest in the products prepared with stone milled flour (16% i.v.). The stone milled tritordeum flour showed higher retention levels in the cooked and baked products (18% i.v.) than the stone milled bread wheat counterparts (13% i.v.). The

degradation of tocols and carotenoids seems to have been caused by the same factors, i.e. direct oxygenation and enzyme-controlled oxidation during kneading and leavening as well as heat destruction during baking/cooking (Burešová et al., 2021). The lower level of TTC degradation in the roller-milled products could be ascribed to a lower lipoxygenase activity and a lower polyunsaturated fatty acid content (Leenhardt et al., 2006) than the stone-milled flour containing the germ, as well as to a higher concentration of other lipophilic antioxidants, such as carotenoids, as observed in tritordeum, thus suggesting that regeneration phenomena may have occurred (Çelik & Gökmen, 2022). The limited retention in the pizza could be ascribed to the fermentation step, during which the oxidative enzymes may be active for longer, and by the more intensive heat treatment. The retention of tocols in the cooked fresh pasta reached an intermediate level for the bread and pizza. Cooking the pasta in large amount of water did not increase the tocol level, which can occur as a result of the leaching of soluble dry matter and the release of bound tocols, as has been reported after the conventional boiling of rice and vegetables (Finocchiaro et al., 2007; Fratianni et al., 2021). However, it did result in a loss of TTC, which could be ascribed to oxidative destruction because of heating, as reported in previous works that focused on the cooking of brown rice (Pascual et al., 2013). In all the processes, and especially after pizza baking, the tocotrienol and tocopherol ratio increased, and tocotrienols were found to be the main forms of retained vitamin E and to be more resistant to the thermal conditions. Cereal grains are the second most important source of tocols in diets, after vegetable oils, and are considered a unique source due to the predominance of tocotrienols, which have a variety of beneficial functions, over tocopherols (Tiwari & Cummins, 2009). The degradation of tocols in all the considered processes was found to be lower than that of carotenoids, thereby supporting the higher thermostability suggested by some other authors (Burešová et al., 2021; Fratianni et al., 2012, 2021; Hidalgo & Brandolini, 2010; Leenhardt et al., 2006).

Table 3. The tocols (mg·kg^{-1}) detected in the tritordeum and wheat samples of the flour, fresh pasta, bread, and pizza, expressed as the average of roller and stone milling.

Species	Processing type	Tocotrienols				Tocopherols				TTC[a]
		δ-T3	γ-T3	β-T3	α-T3	δ-T	γ-T	β-T	α-T	
Tritordeum	Flour	0.09 a	< LOD a	25.35 a	5.57 a	< LOD a	< LOD a	2.94 b	6.39 a	40.34 a
	Fresh pasta	< LOD d	< LOD a	6.25 bc	0.40 c	< LOD a	< LOD a	0.72 c	0.46 b	7.83 bcd
	Bread	0.02 c	< LOD a	9.16 b	0.91 c	< LOD a	< LOD a	0.98 c	0.85 b	11.93 b
	Pizza	< LOD d	< LOD a	2.58 d	0.26 c	< LOD a	< LOD a	0.18 c	0.00 b	3.02 cd
Bread wheat	Flour	0.06 b	< LOD a	25.65 a	2.41 b	< LOD a	< LOD a	4.59 a	6.77 a	39.48 a
	Fresh pasta	< LOD d	< LOD a	5.74 c	< LOD c	< LOD a	< LOD a	0.93 c	0.00 b	6.67 bcd
	Bread	< LOD d	< LOD a	8.89 bc	0.17 c	< LOD a	< LOD a	1.39 c	0.66 b	11.11 bc
	Pizza	< LOD d	< LOD a	1.44 d	< LOD c	< LOD a	< LOD a	0.11 c	< LOD b	1.55 d
	p (F)	***	ns	***	***	ns	ns	***	***	***

δ-T3, δ-tocotrienol; γ-T3, γ-tocotrienol; β-T3, β-tocotrienol; α-T3, α-tocotrienol; δ-T, δ-tocopherol; γ-T, γ-tocopherol; β-T, β-tocopherol; α-T, α-tocopherol; LOD, limit of detection; TTC, total tocol content. The results are expressed on a DW basis. Means followed by different letters are significantly different, according to the REGW-F test [(*) p (F) < 0.05, (**) p (F) < 0.01, and (***) p (F) < 0.001, and ns, non-significant].
[a]The sum of all the determined tocols.

Phenolic acids

The three investigated factors and their interactions significantly affected the SPAs (Table 1), which were extracted and analyzed as a combination of the soluble-free and soluble-conjugated fractions. Regarding the bound fraction (CWBPA), the three factors and the S × M and M × P interactions showed significant effects, although the milling degree explained most of the observed variation.

The highest concentration of SPAs was found in the tritordeum samples (+33% compared to the wheat samples), and in particular in the stone milled flour (+85%, 91.90 vs 49.79 $mg \cdot kg^{-1}$ in wheat; Figure 2A). Differences in the CWBPA content were significant, although far less pronounced (+3% in the tritordeum samples; Figure 2B), and only accounted for a small share of the variation (0.1%; Table 1). The SPAs contributed less to the total phenolic acid content than the CWBPAs, but the SPAs in the flour represented a higher proportion of the total phenolic acids than the bread wheat (29 vs 19%). Differences were observed in the phenolic acid profiles of the two species, but both were characterized by a prevalence of sinapic acid in the soluble fraction, which averaged 23.99 and 18.30 $mg \cdot kg^{-1}$ in the tritordeum and wheat flours, respectively (Table 4), corresponding to 39 and 53% of the total SPAs (Figure S1), and by the predominance of ferulic acid in the bound fraction (127.03 and 119.00 $mg \cdot kg^{-1}$; 84 and 82% share of the total CWBPAs). Interestingly, gallic acid was the second most abundant SPA in the tritordeum flour (16.78 $mg \cdot kg^{-1}$; 28% of the total SPAs), but its content was much lower in the wheat flour (0.52 $mg \cdot kg^{-1}$; 2%), with ferulic acid being the second most predominant SPA (8.66 $mg \cdot kg^{-1}$; 25%). Vanillic acid represented 6 and 7% of the total SPAs in the tritordeum and wheat flours, with no significant differences between species (3.19 $mg \cdot kg^{-1}$ on average). The soluble protocatechuic, hydroxybenzoic, caffeic, syringic, and *p*-coumaric acid contents were each < 3.00 $mg \cdot kg^{-1}$, and they accounted for less than 4% in all the flours. The CWBPA composition was less heterogeneous (Figure S1). After ferulic acid, sinapic acid accounted for 10-12% of the total CWBPA content, with no significant differences between tritordeum and wheat flour (15.90 $mg \cdot kg^{-1}$ on average; Table 4), followed by *p*-coumaric acid (4.87 $mg \cdot kg^{-1}$; 3%). Only a few works have investigated the phenolic acid profile of tritordeum whole grains and compared it with that of durum wheat (Giordano et al., 2019; Montesano et al., 2021; Suchowilska et al., 2021) or with that of bread wheat or barley (Giordano et al., 2019). Giordano et al. (2019) observed SPA and CWBPA concentration in tritordeum of 58 and 872 $mg \cdot kg^{-1}$, which were 33% higher and 12% lower than in bread wheat. The phenolic acid concentration ratio in tritordeum determined by these authors was similar to that obtained in our study, although they did not identify gallic acid. In contrast to our findings, Montesano et al. (2021) reported a markedly different profile of bound phenolic acids, with cinnamic and gentisic acids being

the most abundant in tritordeum lines. Suchowilska et al. (2021) also found a large abundance of cinnamic acid (the second most abundant acid after ferulic acid). It is worth noting that the phenolic acid content observed in the present study (Table 4) was overall lower than in the cited experiments, in which whole grains were analyzed.

The milling degree was responsible for most of the observed variation (72.7% for SPAs and 98.2% for CWBPAs; Table 1) for both fractions. Giordano et al. (2019) reported a decreasing concentration of tritordeum SPAs and CWBPAs from the outermost to the innermost kernel layers, with a similar pattern to that of durum and bread wheat, thereby supporting the significantly higher content of both forms observed in the stone milled samples of the present study (+130 and +160%, respectively). The cited authors also observed a 36% higher concentration of CWBPAs in the residual pearled grain of tritordeum than bread wheat, thus suggesting the use of both whole and refined tritordeum flour for enhanced health-promoting properties.

Furthermore, processing had significant effects on the total SPA and CWBPA contents, even though it only accounted for a small share of the variation (6.5 and 1.2%; Table 1), with different outcomes related to the species, the type of milling, and the type of product, and different impacts on the individual compounds. To the best of the authors' knowledge, this is the first study to have investigated the fate of tritordeum phenolic acids during different types of processing. Processing did not cause any significant losses of the total contents of either fraction in the roller milled samples (Figure 2), except for tritordeum pasta-making, during which the highest proportion of SPAs was degraded (to 35%). Only in the case of breadsticks did a marked increase in CWBPAs occur (up to 119 and 128% for tritordeum and wheat, respectively) during production, although the level was only significantly greater than the i.v. of the flour for the wheat breadsticks. More pronounced differences were observed in the stone milled samples, due to the higher initial amount. The SPA content of the tritordeum flour significantly decreased during processing, with the largest amount being retained in the breadsticks and pizza samples (69% i.v. on average) and the lowest in the bread and pasta samples (49% i.v. on average). The only significant drop in SPAs in wheat occurred after the pasta making process (47% i.v.). In terms of CWBPA content, the bread and pasta samples achieved similar retention rates, with a limited but significant drop to 89% i.v., and statistically comparable final levels (194 $mg \cdot kg^{-1}$ on average; Figure 2B). No significant variation in the total content was detected in the pizza samples (224 $mg \cdot kg^{-1}$ on average), compared to the flours, or between tritordeum and wheat. Interestingly, significant increases of up to 117% (tritordeum) and 112% (wheat) were observed in the breadsticks, as in the case of roller milling resulting in final concentrations of 259 and 240 $mg \cdot kg^{-1}$, respectively. Changes in phenolic acid contents

during processing have already been reported in the literature, albeit with conflicting results, as the effect of baking or cooking on free or bound phenolic acids seems to be dependent on the species or variety as well as on the process conditions (Abdel-Aal & Rabalski, 2013; Tian et al., 2021). Among the products obtained from tritordeum, free sinapic acid was found to be preserved the most in the case of breadsticks, while a decrease, although not significant, occurred in all the other cases (Table 4). The gallic acid content decreased significantly in the tritordeum products, except for in the pizza, by as much as 8 times (bread). On the other hand, the gallic acid in the wheat products increased to various extents, albeit not significantly, with the highest increase in the pizza (19 times higher than the flour). Regarding the CWBPAs, the predominant ferulic and sinapic acids were generally not influenced by processing, although the concentration of ferulic acid was observed to be higher in the breadsticks than in the flour for both species (Table 4). The gallic acid level increased significantly in the breadstick, pizza and pasta samples, while it reached slightly higher but comparable levels after bread making than the flour i.v. Different steps of the technological processes may contribute to the changes in the individual phenolic acid content. As suggested by Tian et al. (2021), the metabolic activity of yeast may partially degrade the phenolic-carbohydrate complex during the fermentation process, thereby increasing the amount of certain soluble phenolic acids, but it may also consume phenolic acids or convert them into other types of phenolic acids or compounds. This process was found to be highly dependent on the wheat variety (Tian et al., 2021). The same authors found a positive correlation between the decrease of soluble ferulic acid and its polymerization, and its incorporation in Maillard reaction products (MRPs) during thermal processing, which they suggested led to reduced extractability but increased antioxidant activity. High temperatures during the baking process can lead to decomposition of the free fractions, as observed in dry-extruded corn snacks (Bresciani et al., 2021b), as well as to the release of some phenolic acids from the phenolic-carbohydrate complex (Zeng et al., 2016). Abdel-Aal and Rabalski (2013) reported an increase in free ferulic acid and a concomitant decrease of its bound form during the baking of an unleavened one-layer flat bread. On the other hand, no changes in the insoluble phenolic acid levels (Mattila et al., 2005) or even a slight increase in the total ferulic acid and total phenolic acids (Lu et al., 2014) were observed during a bread-making process. In the present study, the variations in the level of the individual compounds were different throughout processing, and appeared to depend on both the species and the milling degree of the flour. Overall, the phenolic acids were better preserved after the baking processes, which, as in the case of breadsticks, proved to be beneficial for the bound fraction. As for the only boiled product, i.e. fresh pasta, the process generally reduced the total phenolic acid content, with overall losses of all the

soluble compounds and of the bound ferulic acid. As no extrusion or drying process was involved, the losses may be ascribed to degradation, due to the heat treatment, and to leaching into the cooking water. Moreover, hydrolysis of the methoxy group in bound ferulic acid may have occurred in the hot, aqueous environment, and this could have led to the formation of caffeic acid. The only significant increase observed in the bound fractions of the pasta after cooking, as well as in the baked products, was in gallic acid, which could have been due to the enhanced availability and extractability of hydrolysable tannins (gallotannins), as a result of the disruption of cell membranes caused by thermal stress. Rocchetti et al. (2017) reported significant losses of both soluble and bound phenolic acids after the cooking of different types of gluten-free pasta, although not for black rice pasta, which showed a significant increase in free phenolics. A diminished amount of phenolic compounds was also reported for both buckwheat pasta (Verardo et al., 2011) and brown and polished rice (Ti et al., 2015) after boiling. Conversely, Bresciani et al. (2021a) observed an increase in free phenolic acids and either a reduction or no changes in the bound fractions after the cooking of high-amylose gluten-free pasta. Understanding simultaneous incorporation and release of phenolic acids during processing is crucial for assessing the overall antioxidant capacity of final products, as the structure of these compounds could eventually affect the bioavailability for absorption and the subsequent physiological effects (Çelik & Gökmen, 2022).

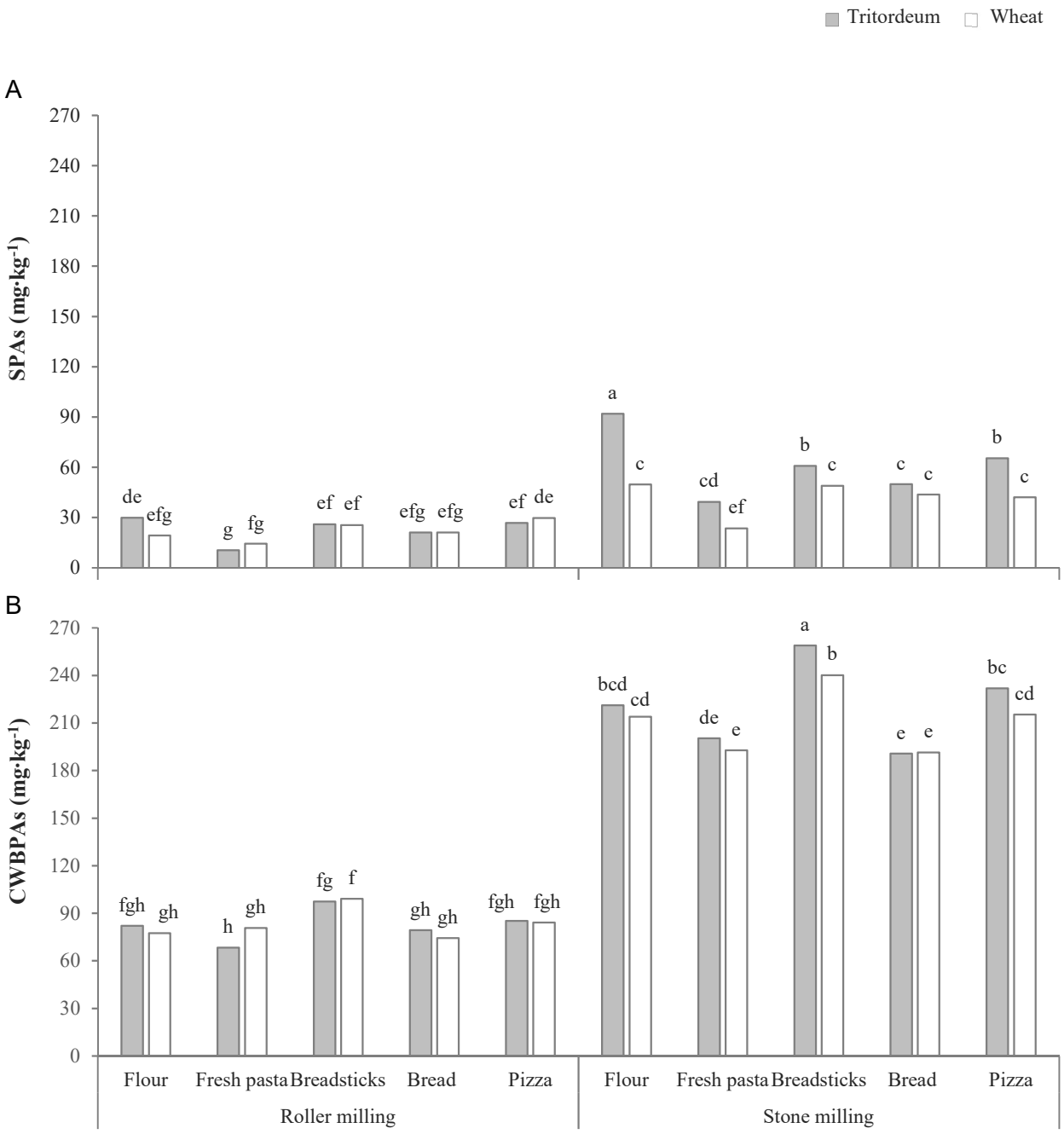

Figure 2. The soluble (SPA; A) and cell wall-bound (CWBPA; B) phenolic acid contents of the tritordeum and wheat samples of flour, fresh pasta, breadsticks, bread, and pizza, obtained from roller and stone milling.

The results are expressed on a DW basis. Different letters above the columns indicate significant differences for p (F) < 0.05, according to the REGW-F test.

Table 4. The soluble (SPAs) and cell wall-bound (CWBPAs) phenolic acids (mg·kg^{-1}) detected in the tritordeum and wheat samples of flour, fresh pasta, breadsticks, bread, and pizza, expressed as the average of roller and stone milling.

Phenolic acids	Species	Processing type	Gallic acid	Protocatechuic acid	Hydroxybenzoic acid	Vanillic acid	Caffeic acid	Syringic acid	*p*-Coumaric acid	Ferulic acid	Sinapic acid	Total content[a]
SPAs	Tritordeum	Flour	16.78 a	2.22 a	1.74 a	3.79 a	0.58 ab	1.35 a	1.10 a	9.36 a	23.99 a	60.91 a
		Fresh pasta	2.21 b	0.28 a	1.48 a	3.24 a	0.16 c	0.99 a	0.62 a	8.46 a	7.45 ab	24.89 ab
		Breadsticks	4.08 b	0.96 a	1.99 a	3.61 a	0.49 ab	1.43 a	1.09 a	9.16 a	20.64 a	43.45 ab
		Bread	2.01 b	< LOD a	1.72 a	3.72 a	0.74 a	1.09 a	1.00 a	8.50 a	16.68 ab	35.47 ab
		Pizza	7.78 ab	1.91 a	1.96 a	3.82 a	0.39 abc	1.23 a	0.97 a	10.38 a	17.66 ab	46.11 ab
	Wheat	Flour	0.52 b	0.61 a	1.28 a	2.58 a	0.47 abc	1.13 a	0.94 a	8.66 a	18.30 ab	34.50 ab
		Fresh pasta	1.05 b	0.47 a	1.16 a	2.44 a	0.17 bc	1.02 a	0.53 a	8.19 a	3.93 b	18.97 b
		Breadsticks	8.10 ab	0.81 a	1.60 a	2.19 a	0.52 ab	1.28 a	0.92 a	7.32 a	14.46 ab	37.21 ab
		Bread	4.54 ab	0.43 a	1.32 a	2.54 a	0.72 a	0.99 a	0.87 a	7.83 a	13.11 ab	32.34 ab
		Pizza	10.06 ab	0.50 a	1.26 a	2.40 a	0.41 abc	0.98 a	0.75 a	8.57 a	10.95 ab	35.88 ab
		p (F)	**	ns	ns	ns	***	ns	ns	ns	**	*
CWBPAs	Tritordeum	Flour	1.84 d	< LOD b	0.68 a	1.11 a	0.69 abc	0.84 a	4.74 a	127.03 a	14.63 a	151.57 a
		Fresh pasta	6.93 c	< LOD b	0.69 a	1.06 a	0.64 abc	0.75 a	4.41 a	106.01 a	13.82 a	134.32 a
		Breadsticks	11.19 ab	< LOD b	0.86 a	1.44 a	0.00 d	0.98 a	5.35 a	143.02 a	15.28 a	178.12 a
		Bread	2.16 d	0.06 b	0.57 a	0.91 a	0.37 abcd	0.46 a	3.72 a	112.17 a	14.58 a	134.99 a
		Pizza	11.09 b	< LOD b	0.81 a	1.45 a	0.25 cd	0.97 a	4.88 a	123.46 a	15.54 a	158.45 a
	Wheat	Flour	1.09 d	< LOD b	0.66 a	1.30 a	0.78 a	0.67 a	5.00 a	119.00 a	17.17 a	145.67 a
		Fresh pasta	14.38 a	0.70 ab	0.82 a	1.06 a	0.70 ab	0.84 a	4.77 a	97.85 a	15.64 a	136.76 a
		Breadsticks	13.43 ab	1.32 a	0.84 a	1.43 a	0.43 abcd	1.04 a	5.74 a	127.97 a	17.47 a	169.68 a
		Bread	2.89 d	< LOD b	0.63 a	0.99 a	0.33 bcd	0.58 a	4.28 a	106.66 a	16.53 a	132.88 a
		Pizza	14.26 a	< LOD b	0.74 a	1.33 a	0.34 bcd	0.86 a	4.87 a	108.05 a	19.35 a	149.80 a
		p (F)	***	**	ns	ns	***	ns	ns	ns	ns	ns

LOD, limit of detection; SPAs, soluble phenolic acids; CWBPAs, cell wall-bound phenolic acids. The results are expressed on a DW basis. Means followed by different letters are significantly different, according to the REGW-F test [(*) p (F) < 0.05, (**) p (F) < 0.01, and (***) p (F) < 0.001, and ns, non-significant].

[a]The sum of all the determined SPAs and CWBPAs.

Changes in the antioxidant capacity

Two antioxidant methods, ABTS and FRAP, were employed to measure the different antioxidant phenomena and provide a comprehensive assessment of the net antioxidant potential of the considered types of food. An extraction-independent procedure was adopted to account for any interactions between antioxidants. Most of the observed variation (Table 1) was explained, in both types of assay, by the degree of milling (43.5 and 61.5%, for ABTS and FRAP, respectively), followed by the species (27.7 and 17.6%), and the type of processing (23.4 and 6.9%).

Overall, tritordeum exhibited a superior antioxidant capacity than bread wheat, with similar average increases being observed in both the ABTS and FRAP assays (+33 and +35%). This supports the greater antioxidant potential of its flour and final products, which is imparted by a higher concentration of health-promoting phytochemicals than the control wheat. Significantly higher AC_{ABTS} values (Figure 3A) were observed in both the roller and stone milled tritordeum flour than in the wheat flour. An advantage of tritordeum over wheat after processing was observed for roller milled breadsticks and pizza, as well as for stone milled bread and pizza. The FRAP assay revealed significantly higher values for tritordeum than for wheat for the roller milled pizza, and for the stone milled fresh pasta, breadsticks, and pizza (Figure 3B). The stone milled samples generally showed a higher AC than the roller milled ones, with an increase of 40% for ABTS and 95% for FRAP. This can be attributed to both the hydrophilic and lipophilic phytochemicals of the bran and germ parts of the grain (Lu et al., 2014), which were preserved in the semi-whole flour obtained from stone milling, but removed in the refined flour produced through conventional roller milling.

As shown in Figure 3A, AC_{ABTS} was significantly reduced after processing in all the considered cases, except for the breadsticks, which retained similar values to those of the flours. The fresh pasta, bread, and pizza showed no significant differences for either the species or the milling methods, except for lower values in the stone milled tritordeum fresh pasta than in the bread and pizza. The i.v. of the flours was lower in the FRAP method than in the ABTS assay (Figure 3), thus indicating a different reactivity of the two assays toward the various antioxidant compounds of the matrix (Serpen et al., 2012. Unlike the ABTS results, the FRAP assay consistently showed that the AC after processing was similar to or even higher than that of the flours, thus suggesting a reactivity toward heat-induced compounds that exhibit antioxidant properties, such as MRPs. MRPs are in fact formed during high temperature processing and consist of different chemical species that have shown *in vitro* antioxidant activities, including the ability to bind with transition metal ions such as iron and copper, as reported in the literature on sugar-amino acid model systems (Cao et al., 2022; Mondaca-Navarro et al., 2017) and bakery products (Shen et al., 2019).

The roller and stone milled tritordeum pizza samples showed AC_{FRAP} values that were 2.2 and 1.8 times higher than their respective flours, thus suggesting a possible role of the tritordeum amino acid profile in the formation of antioxidant-rich compounds in the pizza-baking condition.

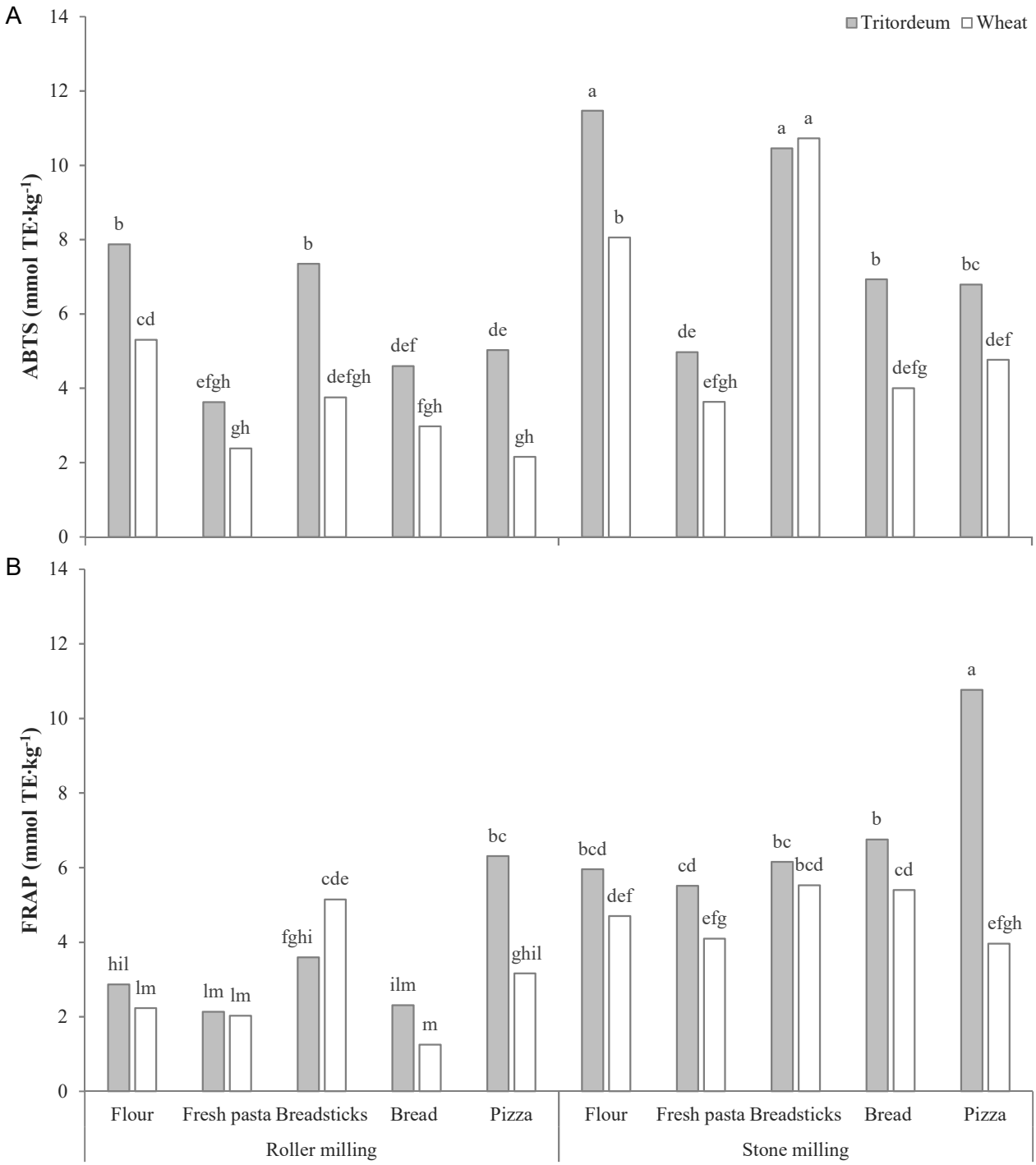

Figure 3. The antioxidant capacity (AC) measured considering the QUENCHER-ABTS (A) and -FRAP (B) of the tritordeum and wheat samples of flour, fresh pasta, breadsticks, bread, and pizza, obtained from roller and stone milling.

The results are expressed on a DW basis. Different letters above the columns indicate significant differences for p (F) < 0.05, according to the REGW-F test.

Conclusions

This study has demonstrated that tritordeum contains significantly higher concentrations of key phytochemicals, especially carotenoids and the soluble fraction of phenolic acids, than conventional bread wheat flour, which is commonly used in bakery and fresh pasta production. Stone milling helped preserve the tocols, phenolic acids, β-glucans, and the total antioxidant capacity of both the tritordeum and bread wheat. However, roller milling was more effective in maximizing carotenoid retention in tritordeum flour. Using tritordeum flour instead of bread wheat in fresh pasta and bakery products proved to be the best way of maintaining the highest levels of phytochemicals and antioxidant capacity of the end products. Nevertheless, processing resulted in a substantial reduction of the monitored phytochemicals in both species, the extent of which varied according to the processing techniques and culinary treatments that were adopted. Lipophilic antioxidants, such as carotenoids and tocols, benefited from a shorter fermentation and a gentler heat transfer (as adopted for the breadsticks and bread production). The phenolic acids seemed to be more susceptible to degradation during prolonged baking (e.g., for the bread) and to leaching during cooking (e.g., for the fresh pasta). However, it should be noted that, when flour was stone milled, the bound fraction of phenolic acids did not change significantly after the baking of pizza, and it even increased during breadstick production. Overall, tritordeum flour, whether roller or stone milled, could represent an opportunity to enhance food commodities as it includes higher concentrations and diverse profiles of carotenoids, tocols, and phenolic acids. Nevertheless, further research is needed to explore alternative processing conditions that would be capable of preserving higher levels than those observed in the present study in order to fully deliver these benefits at the moment of consumption. It is also crucial to ensure that any potential health benefits are not counterbalanced by the formation of harmful compounds, such as acrylamide, during processing. Additionally, it is essential to investigate the bioavailability and antioxidant activity of these preserved compounds during the subsequent digestion process to fully comprehend their ultimate health impact.

Supplementary material

Table S1. Technological steps of the preparation of fresh pasta, breadsticks, bread and pizza.

Processing type	Kneading time (min)	Resting time (min)	Leavening time (min/h)	Cooking/baking time (min)	Cooking/baking temperature (°C)
Fresh pasta	6 (25 °C)	30 (4 °C)	-	1.5 (roller milled) 1 (stone milled)	100
Breadsticks	10 (29 °C)	20 (27 °C - 45% RH)	35 min (36 °C - 75% RH)	8.5	280
Bread	11 (25 °C)	30 (30 °C - 70% RH)	60 min (30 °C - 70% RH)	40	220
Pizza	13 (25 °C)	30 (24 °C - 60% RH)	17 h (4 °C) + 2 h (24 °C - 60% RH)	5	320

RH, relative humidity.

Table S2. Proximal composition (g·100 g-[1]) of the flours used in the study.

Species	Milling degree	Proteins	Ash	Lipids	Starch	Dietary fiber	
						Insoluble	Soluble
Tritordeum	Roller milled	15.1	0.8	1.7	63.7	2.4	1.4
	Stone milled	15.1	1.1	2.3	60.0	4.5	1.6
Wheat	Roller milled	10.8	0.6	1.3	73.0	1.8	1.1
	Stone milled	11.7	1.0	1.7	65.3	3.8	1.3

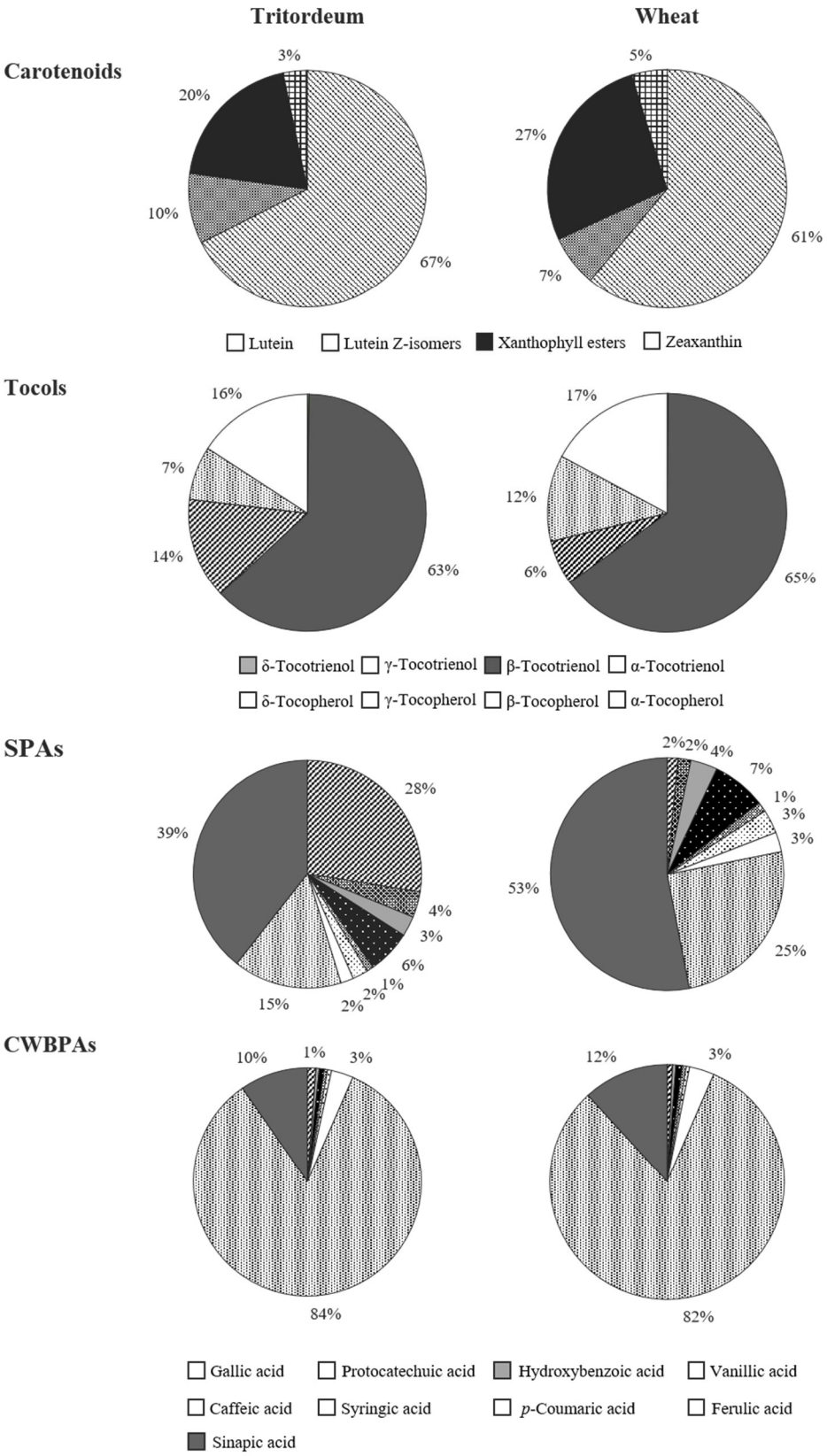

Figure S1. Composition of the carotenoid, tocol, soluble (SPA) and cell wall-bound (CWBPA) phenolic acid contents of the tritordeum and wheat flours, expressed as the average of the roller and stone milling processes.

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Chapter VIII – Conclusion and perspectives

The studies conducted throughout the doctoral research period provided a comprehensive understanding of the specialty cereal supply chain, especially those characterized by health-related added value, encompassing different topics, strategies, and methodological analyses. The research carried out in this PhD thesis was aimed at improving the knowledge about opportunities and implications for developing alternative food chains in the cereal sector by evaluating and proposing a wide array of cereal crops – ranging from rye and ancient wheat varieties to bread wheat, rice, maize, and tritordeum – as product innovations. The traits influencing the feasibility of restoring or introducing old and new genotypes, encompassing agronomic and technological performance, nutritional and phytochemical profiles, and safety aspects, were assessed under various agronomic, environmental, milling, and processing conditions, in order to account for the role of different factors on the overall sustainability and competitiveness of the product chain. The insights contribute significantly to the understanding of how minor species or newly developed biofortified genotypes can be cultivated and utilized to improve health-related aspects of modern food systems, especially within the Italian production landscapes.

Ancient and minor crops and the role of breeding

The study on Alpine rye landraces in Chapter II highlighted the potential of reintroducing historic supply chains of rye in marginal environments and producing landrace-based food products with higher nutritional and health-related values compared to modern wheat or rye cultivars. No differences in yield potential were found between rye and an ordinary bread-making wheat variety in both hilly and mountain environments. When comparing rye landraces and modern commercial rye cultivars, the higher potential yield of the hybrid cultivar was the most evident trait, even in marginal environments, although it should be noted that it was also the most susceptible to sanitary risks, particularly for the occurrence of ergot sclerotia, and thus potentially alkaloids. Overall, agronomically and qualitatively speaking, the rye landraces diverged significantly from the conventional rye cultivars and among each other, thus reflecting their genetic distinctiveness. Although certain landraces (e.g., the one from Maira Valley) exhibited morphologic and agronomic limitations such as lodging susceptibility and high plant size, or reduced rheological performances due to high content of fibers components (e.g., the one from Susa Valley), the overall agronomic traits and grain composition (8.4-8.9% DW of arabinoxylans vs 6.1-6.4% in wheat, and 1.9-2.2% DW of β-glucans vs. 0.8-0.9% in wheat; 110-202 mg/kg DW of soluble phenolic acids vs.

81-107 mg/kg DW in wheat), as well as the health-related potential of the derived bread (up to +116% than wheat in terms of *in vitro* antioxidant capacity) suggests their potential for niche market value chains.

In contrast, as detailed in Chapter III, different outcomes were observed when ancient wheat species (einkorn, emmer, and spelt) and old bread wheat varieties were cultivated in non-marginal cropping systems and subjected to standard agronomic practices. The experiment was designed in order to explore the genetic variability of minor species and old varieties and to evaluate the expansion of their cultivation to non-marginal production situations and their end-use applications, as a response to the growing interest of the market and the industrial supply chain. However, the 9 investigated genotypes of hulled wheats and old varieties of bread wheat performed poorly under non-marginal cropping systems when compared to modern varieties of bread wheat. They also displayed limited responsiveness to diverse environmental conditions and improved inputs, e.g. increasing rates of N fertilization, in terms of both agronomic and rheological properties. Moreover, the bioactive compound content varied extensively intra-group and was closely related to the growing conditions. A higher level of biodiversity is generally pointed out as a strength of landraces and old genotypes; on the basis of the cultivars that we analyzed in different production situations, a clearly greater variability of many of the studied traits was observed for the modern varieties of bread wheat than for the old ones. On the other hand, the analyzed hulled wheat cultivars (einkorn, emmer, and spelt) were selected from among improved varieties released after 2000 varieties. Despite suffering from agronomic deficiencies as the old varieties, they exhibited a wide range for all the agronomic and qualitative traits. This finding shifts the narrative around genetic erosion and supports the idea that modern breeding can enhance both agronomic and nutritional traits, provided it is guided by appropriate goals. Targeted breeding can allow the progressive identification of new modern genotypes, capable of ensuring high yields and technological performances, even with a low N supply. These genotypes can be further improved, in terms of sanitary risk tolerance and phytochemical quality, in accordance with the requirements of the food supply chain.

Grain nutritional composition and phytochemical profiles

One of the main focus of the research activity has been the comprehensive analysis of the key fiber components and biologically active compounds more relevant in cereals for human nutrition. It included a screening of the most promising crops and genotypes in terms of *in vitro* antioxidant properties, total content of arabinoxylans and β-glucans, concentration and

profile of phenolic compounds (phenolic acids, alkylresorcinols), anthocyanins, carotenoids, and vitamins such as tocopherol and tocotrienols. Although the investigation has been carried out in different growing and agronomic conditions, the extensive analytical characterization highlights that grains of rye landraces, hulled wheats, and old bread wheat varieties can offer advantages in terms of dietary fibers components and phenolic acids, particularly in soluble form. However, in some cases, modern varieties outperformed the old ones in the content of certain phytochemicals, such as the lipophilic alkylresorcinols. The bound phenolic acids resulted in being more stable among genotypes and were more influenced by environmental factors, i.e. different meteorological conditions across the years, and by genotype × year interactions. The analysis of the antioxidant capacity by means of *in vitro* assays did not always allow to discriminate among genotypes groups, as in the case of old and modern varieties of bread wheat. On the other hand, marked differences emerged when tritordeum and pigmented genotypes of bread wheat, rice, maize were analyzed.

Opportunities for pigmented genotypes

The study on pigmented wheat genotypes and tritordeum (Chapter IV) emphasized the significant impact of genotype and environmental conditions on grain antioxidant potential. Among pigmented varieties, certain groups (e.g., black-grained bread wheat) accumulated particularly high levels of anthocyanins and phenolic acids (34.4 and 1207 mg/kg DW, respectively), others (yellow endosperm group, including tritordeum and yellow-grained bread wheat) were characterized by significantly higher carotenoid content (5.6 and 8.8 mg/kg DW, respectively), offering great promise for functional food development. Intra-group variability allows the selection of the most promising varieties for cultivation and end-use applications, as some of them showed good yield and quality performances (grain protein content and rheological quality), and retained good antioxidant features in all the considered production systems. The N fertilization affected both yield and milling and technological-related traits, and overall had limited effects on bioactive compounds. Therefore, N inputs can be efficiently modulated to enhance yield capacity and lower the gap observed between conventional non-pigmented and novel pigmented genotypes, without compromising the high value in terms of phytochemicals.

The study on pigmented rice (Chapter V) underlined how black pericarp varieties are rich in phenolic compounds and anthocyanins, although the nutritional advantages tends to be concentrated in the edible bran (especially anthocyanins) and in the not-edible husk (especially phenolic acids). For instance, the black rice cultivar Gioiello stood out for its high

antioxidant activity, reinforcing the value of such varieties for the development of functional foods. Specifically, the highest levels of phytochemicals were found in the unpolished part (260 mg/kg DW of soluble phenolic acids; 655 mg/kg DW of bound phenolic acids; 1901 mg/kg DW of anthocyanins). The bran fraction exhibited the highest concentration of anthocyanins (5041 mg/kg DW). These findings support a shift toward less refined, whole-grain consumption. Pigmented varieties of both wheat and rice can be exploited as whole grains or as bran fractions to enhance the attractiveness of whole-grain products and stimulate consumer's interest and acceptance towards whole-grain consumption. The biofortification of staple foods such as bread by means of phytonutrient or phytochemical-rich varieties, such as rye or cereal bearing a pigmentation in the bran, seems to be even more promising and advantageous than other fortification strategies, such as enriching white bread with fiber alone, as it allows to further enhance the phytochemical profiles and the antioxidant properties.

The high levels of phenolic acids and antioxidant capacity observed in the rice husks indicate that this inedible external layer could be considered as cost-effective source of natural antioxidants for bioactive compound extraction. The valorization of by-products was also taken into account when evaluating maize milling and redistribution of phytochemicals and mycotoxins in milling fractions (Chapter VI). Specifically, a high concentration of phenolic acids was observed in maize milling by-products, and especially in those of a novel maize hybrid developed from local germplasm (365 mg/kg DW of soluble phenolic acids and 3131 mg/kg DW of bound phenolic acids in the animal feed flour and degermination bran; 339 mg/kg DW of soluble phenolic acids and 1185 mg/kg DW of bound phenolic acids in the germ; 206 mg/kg DW of soluble phenolic acids and 1140 mg/kg DW of bound phenolic acids in the refining bran). Their recovery as functional ingredients for incorporation into refined flour, or for extraction when safety standards are not met, can contribute to a more sustainable food system by reducing waste and enhancing milling yields from the same raw material.

Effects of processing and health-related implications

The works on maize (Chapter VI) and on wheat and tritordeum (Chapter VII) offered practical insights into how milling strategies affect the nutritional and phytochemical profiles of the derived semolina and flour, respectively, to understand whether they can be used as functional ingredients for food production.

As observed in maize milling (Chapter VI), integrated approaches between milling strategies and the raw material can provide a compromise between maximizing phytochemical

retention and minimizing contamination risks. Indeed, breeding can provide improved genotypes with enhanced kernel quality, but the benefits offered by selective breeding must be preserved through post-harvest handling and processing and should not be compromised by food safety risks, of which mycotoxins in cereals are of primary concern. Therefore, in low contamination scenarios, strategies such as stone milling (as is or with sieving) or the incorporation of degermination bran into refined flour, each with progressively greater mycotoxin abatement capacity, can be adopted to deliver the highest contents of ash (1.23 g/100g), dietary fiber (10.1 g/100g), proteins (8.4 g/100g), and both soluble (272 mg/kg DW) and bound phenolic acids (1619 mg/kg DW). When mycotoxins levels are higher, strategies such as stone milling of degerminated grains or selective recovery of refining bran can be adopted. For highly contaminated lots, the roller refining of degerminated grains remains the best strategy to ensure food safety, minimizing mycotoxin risk. This strategy led to the greatest lossess in terms of phenolic acids, which were more marked for the bound (-81%) than for the soluble fraction (-51%), and antioxidant capacity, while best preserving the carotenoid content (30.8 mg/kg DW) and composition. In this case, the genotype innovation, i.e., a new flint × dent hybrid developed from local germplasm, turned out to be promising for dry-milling purposes, as it was characterized by enhanced levels of carotenoids, reduced susceptibility to mycotoxins contamination, and higher kernel hardness. This reinforce the fundamental role of breeding for improving both food safety and nutritional quality of cereal crops, adding up the key role of post-harvest handling, and especially milling, to ensure food safety while preserving the health-promoting compounds.

Finally, in Chapter VII, the higher content of carotenoids and other phytochemicals in tritordeum compared to bread wheat (as already seen in the field trial reported in Chapter IV) was further highlighted, empathizing its potential in pasta and bakery applications. Yet, the study underscored the challenges associated with grain milling and processing into derived products, as phytochemicals losses remain a major issue. As already observed for maize milling, stone milling helped preserve the overall antioxidant capacity, as tocols, phenolic acids, β-glucans of both the tritordeum and bread wheat were mostly preserved in the derived semi-whole flour. This is due to the mechanical fractionation of the kernel layers, which accumulates the phytochemical composition mainly found in the germ and bran in the whole or semi-whole flour obtained by this type of milling. However, as already observed for maize, roller milling was more effective in maximizing carotenoid retention in the refined flour, especially for tritordeum (7.65 mg/kg DW). Using tritordeum flour instead of bread wheat in fresh pasta and bakery products proved to be the best way of maintaining the highest levels of phytochemicals and antioxidant capacity of the end products.

Nevertheless, heat and prolonged cooking were observed to be detrimental to the phytochemical profile, and especially to carotenoids, with average retention from 26% to 1% depending on the type of product and technological process. Further research is needed to explore alternative processing conditions that would be capable of preserving higher levels than those observed in the present study in order to fully deliver these benefits at the moment of consumption.

Limitations and future perspectives

While the studies offer robust comparative data, several limitations should be acknowledged.

The number of investigated genotypes per category was representative but limited, and this may reduce the generalizability of the findings. Especially in field experiments, multi-site trials with multiple genotypes allow to account for the significant effects of the genotype, environment, and genotype × environment on the investigated traits (Shewry & Hey, 2015), and a sufficient and balanced number of genotypes across group categories, including the reference, will better allow for robust comparisons.

Moreover, as observed here and in previous works (Tian et al., 2022; Yang et al., 2018), the accumulation of secondary metabolites is strongly influenced by environmental factors, including climate, water availability, temperature, and growing season. This highlights the need to evaluate the phytochemical accumulation of different varieties by considering multiple harvests at the same site, while limiting other possible influencing factors.

Since there is no standard protocol for complete extraction and analysis of phytochemicals, data comparison between studies may be problematic. The analysis of specific compounds is suggested and encouraged within the scientific community rather *in vitro* antioxidant assays (Tian et al., 2022), as many of them are all extraction dependent and often uses radicals that are not biologically relevant, thus they could be not relevant to address *in vivo* redox balance and other mechanisms other than antioxidation under most conditions. For this reason, more emphasis in the context of the present research was given to the quantification of specific compounds by chromatographic analysis. Moreover, the measurement of the antioxidant activity was always performed by a combination of two methods characterized by different biochemical mechanisms and by following a QUENCHER approach (Serpen et al., 2012). However, it should be noted that cereals are a source of a wide variety of phytochemicals with potential health effects, and it is quite difficult to establish the health advantages by making a comprehensive assessment of all of them in the context of a single study. Moreover, the extraction conditions and the

individual compounds identified often differ among laboratories, challenging comparisons between studies and highlighting the need for harmonization of standardized procedures.

Given the prominent role of the genotype on both agronomic and qualitative traits, extending the genotype screenings will broaden the germplasm pools in order to overcome deficiencies, primarily agronomic but also sanitary, and further enhance the phytochemical content. Indeed, old and newly-developed species such as hulled wheats and tritordeum did not benefit from the extensive genetic improvement to which major crop such as bread wheat, rice, and maize have been subjected over time. Our results on hulled wheat corroborate previous findings on the lower grain yields of spelt, emmer, and einkorn than those of bread wheat under conventional management. Pigmented genotypes maintained a limited but significant yield gap compared to the reference, which was more pronounced for the tritordeum variety, in good agreement with the literature (Landolfi et al., 2023).

Further works aimed to improve tritordeum yield by means of both breeding programs and optimization of agricultural practices in multiple locations will be beneficial to its adoption at a wider scale. Although inferior to modern wheat cultivars, tritordeum has shown a 34% higher yield than the wheat landraces that were cultivated in the last century (Landolfi et al., 2023). Therefore, it would be interesting to carry out an extensive comparison between tritordeum and other minor crops which could potentially compete for market allocation, to identify differences and similarities in terms of agronomic, sanitary, rheological, and nutritional quality. A comparative study has been recently published (Shewry et al., 2023), but it only analyzed grain composition and fiber components in tritordeum, durum wheat, and bread wheat and did not take into account the phytochemical profile.

As compliance with food safety requirements is a prerequisite for the marketability of new species and varieties, sanitary risks have been included in three chapters. Specifically, the occurrence of fungal spoilage in small cereals leads to yield losses, lower test weights and, consequently, lower milling extraction yield, and the accumulation of mycotoxins, i.e., toxic compounds for which legislative thresholds have been established in several countries (EC 2023/915), while the accumulation of heavy metals is the main concern for rice. Disease susceptibility of either pigmented wheat or tritordeum was not evaluated in the works of the present thesis. A high susceptibility to Fusarium head blight has been reported for tritordeum cultivars grown in temperate areas prone to this disease, as well as a significantly higher content of trichothecenes than bread wheat (Spaggiari et al., 2019). Therefore, research should focus on genetic screening and agronomic management optimization to significantly reduce this major issue. Furthermore, differently-pigmented wheat genotypes have been observed to possess significantly different susceptibility to deoxynivalenol accumulation in a recent field experiment (Gozzi et al., 2023). Varieties accumulating

anthocyanins in the pericarp (purple coloration) had significantly lower deoxynivalenol content compared to those in which aleurone was involved (blue coloration). A future research line could be the investigation of the role of the pigmentation on fungal spoilage and mycotoxin accumulation. Specifically, the comparison of near-isogenic lines, only differing for genes responsible for individual grain colors, will be of particular interest to fully ascertain the effect of anthocyanins in wheat grain on mycotoxin accumulation.

Milling operations were found to be a crucial step for phytochemical retention and mycotoxins abatement (Chapter VI and VII), in good accordance with the discussed literature, with conflicting results between group of compounds (e.g., phenolic acids, anthocyanins, tocols vs. carotenoids). The preservation after cooking and baking was found to be more challenging for lipophilic compounds such as carotenoids and tocols, underscoring the need for alternative and gentler methods. Moreover, it should be taken into account that processing can modify the chemical structure of phytochemical molecules, and other components generated during processing, such as bioactive peptides and polysaccharides, might also contribute to changes in antioxidant capacity. Indeed, in Chapter VII, discordant results were observed between *in vitro* antioxidant capacity assays after cooking/baking, with a decrease in ABTS antioxidant capacity and a concomitant increase in FRAP values. Therefore, since the ultimate goal is to increase the content of bioavailable phytochemicals, caution should be used when interpreting results regarding the effects of processing procedures. The research activity of this thesis did not evaluate the bioavailability and *in vivo* activity of the phytochemicals, leaving open the question of the actual health impact at the moment of consumption. Exploring the amount of phytochemicals preserved after *in vitro* simulated digestion process (Burešová et al., 2023) as well as the global antioxidant response of foods (Pastoriza et al., 2011; Pérez-Burillo et al., 2018), by replacing the chemical extraction with simulated digestion methods, would be of particular significance to corroborate potential health effects of phytochemical-rich matrix and help clarifying data interpretation in future studies.

Finally, one of the major issues related to the expansion of phytochemical-rich cereal genotypes, and the large-scale adoption from the industry, regards the difficulties in marketing functional products and effectively communicating with consumers without misleading advertisement. As already discussed in Chapter II, health-related claims are strictly regulated, and the content of antioxidant compounds, such as polyphenols and carotenoids, cannot be marketed on the labels of food products. For instance, the claim proposed for anthocyanin-rich foods (“contains naturally occurring antioxidants, which may help to protect against the damage caused by free radicals, as part of a healthy lifestyle”) has been rejected by the European Commission (EC, 2025) based on the EFSA opinion

that the effect was not substantiated by sufficient scientific evidence (EFSA, 2010). Despite experimental studies suggest a protective role of dietary phytochemicals of cereal grains (Singh et al., 2024), observational epidemiological studies for estimating the intake, thus contributing to dietary recommendations, are limited and inconsistent (Dias et al., 2021; Ficco et al., 2024). Some animal and human investigations on pigmented cereal intake have already been conducted (Gamel et al., 2019, 2020; Liu et al., 2018), but more clinical evidence is needed to make a step further and support potential health-related claims.

Final considerations

The creation of specialties based on undifferentiated and low-value crops is a response to the demand expressed by the markets and can contribute to the distribution of added value along the stages of the chain. Product innovation can be driven either by the final actors in the supply chain, i.e. consumer trends, or at intermediate levels, i.e. as a form of product differentiation for manufacturers or millers, or at the first levels (cultivation and storage) to support farmers' competitiveness and profitability. The possible strategy of enhancing cereal-based products with phytochemical-rich varieties should be supported by adequate scientific data and placed in a more complex framework, considering the productive and qualitative implications. The restoration or introduction of new genotypes as a product innovation may require technical, organizational, and economic implications for key actors in the supply chain. Therefore, it should not be detrimental to the overall sustainability of the supply chain in terms of agronomic productivity, food safety, and input use efficiency, in order to maintain a limited price gap with a commodity; furthermore, there is also a need to bridge the gap between agronomic research and consumer-oriented product development – e.g., collaboration with the food industry to find suitable technological uses and market allocation, and varietal selection based on end-use applications – to ultimately favour consumer acceptance. Indeed, biofortified cereal genotypes, especially those rich in pigment compounds, are promising alternatives to stimulate whole grain consumption and promote a diverse and healthy diet, but the generation of market and consumer awareness will be crucial for their widespread adoption.

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Chapter IX – Scientific production

Posters and oral communications

“Frumenti e mais pigmentati: selezione varietale, gestione colturale e molitoria per l’ottenimento di farine ricche in composti antiossidanti - Claudia Sardella, Francesca Vanara, Barbora Bureŝová, Petr Martinek, Massimo Blandino”, first author, oral presentation, AISTEC Conference, June 15-17, 2022, Napoli, Italy.

“Confronto produttivo, reologico, nutrizionale e sanitario di cereali minori a confronto con il frumento tenero - Massimo Blandino, Laura Righetti, Raffaele Meloni, Marco Gozzi, Matteo Donna, Chiara Dall’Asta, Claudia Sardella”, first author, poster, AISTEC Conference, June 15-17, 2022, Napoli, Italy.

“Influence of agronomic practices on antioxidant compounds of pigmented wheat and tritordeum cultivars - Claudia Sardella, Francesca Vanara, Barbora Bureŝová, Petr Martinek, Massimo Blandino”, first author, oral presentation, ICC Conference, July, 05-07, 2022, Vienna, Austria.

“Role of environmental conditions and genotypes on qualitative traits of rye in marginal Alpine environments - Claudia Sardella, Marco Mucciarelli, Luca Capo, Martino Adamo, Matteo Donna, Francesca Vanara, Massimo Blandino”, first author, poster, ICC Conference, July, 05-07, 2022, Vienna, Austria.

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"Competitività di un nuovo cereale, il tritordeum: confronto agronomico, nutrizionale e sanitario rispetto a genotipi di frumento e di cereali minori", Christian Marazzi. Relatore: Prof. Massimo Blandino, Correlatore: Dott.ssa Claudia Sardella. Anno Accademico 2021-2022.

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