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Sottoprodotti del processo di molitura dei cereali: da residui a preziose risorse

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CEREAL MILLING BY-PRODUCTS

FROM UNEXPLOITED RESIDUES TO VALUABLE RESOURCES

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Thesis for the degree of Doctor of Food Science presented with due permission of the Department of Food and Drug, Università di Parma, Italy.

Cover and back image: Marco Spaggiari
“The powerful grain”

"As soon as science has solved one problem, new ones arise. This is the essence of science."

Otto Wallach (1847-1931), Chemist

ACKNOWLEDGEMENTS

Life in research lab is a matter of group. Such group is always bigger than one could think. During these years I realized that all people I met contributed to this path, from “yesterday” until “tomorrow”. I would like to try to thank all of them with one word, resuming what meant to me all the time passed together. From the very top, they firstly introduced me, Gianni and Chiara, inspiration and devotion. My irreplaceable lab colleagues, Laura, perseverance. Martina, strength. Luca, toughness. Tito, naivety.

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To Science. To People. To Food.

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LIST OF PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text as **Introduction**, **Chapters I** (**1.1** and **1.2**), **II** (**2.1** and **2.2**) and **III**. The publications are reproduced with kind permission from the publishers, otherwise differently specified. The contribution of each author to each work is listed in text at the end of each section or research paper.

BOOK CHAPTER

Food Safety Management of Whole Grains in Whole Grains: Processing, Product Development, and Nutritional Aspects.
Authors: Marco Spaggiari, Chiara Dall'Asta, Gianni Galaverna.
Source: CRC Press, Taylor and Francis Group. Mar 2019 4, pp. 281.
DOI: 10.1201/9781351104760

RESEARCH ARTICLES

HR-MS profiling and distribution of native and modified Fusarium mycotoxins in Tritordeum, wheat and barley whole grains and corresponding pearled fractions.
Authors: Marco Spaggiari, Laura Righetti, Gianni Galaverna, Debora Giordano, Valentina Scarpino, Massimo Blandino, Chiara Dall'Asta.
Source: *Journal of Cereal Science*. 2019. Vol. 87, pp. 178-184.
DOI: 10.1016/j.jcs.2019.03.009

The impact of processing on phenolic acids, free betaine and choline in Triticum spp. L. whole grains and milling by-products.
Authors: Marco Spaggiari, Luca Calani, Silvia Folloni, Roberto Ranieri, Gianni Galaverna, Chiara Dall'Asta.
Source: *Food Chemistry.* Volume 311,1 May 2020, 125940.
DOI: 10.1016/j.foodchem.2019.125940

Solid state lactic acid fermentation: a strategy to improve wheat bran functionality

Authors: Marco Spaggiari, Annalisa Ricci, Luca Calani, Letizia Bresciani, Erasmo Neviani. Chiara Dall'Asta, Camilla Lazzi, Gianni Galaverna.

Source: *LWT. Volume 118*, January 2020, 108668.

DOI: 10.1016/j.lwt.2019.108668

Impact of air-classification, with and without micronization, on the lipid component of rice bran (Oryza sativa L.): a focus on mono-, di- and triacylglycerols

Authors: Marco Spaggiari, Laura Righetti, Silvia Folloni, Roberto Ranieri, Gianni Galaverna, Chiara Dall'Asta.

Source: *International Journal of Food Science and Technology (accepted, under minor revision).*

Rice bran: from valorisation strategies to nutritional perspectives

Authors: Marco Spaggiari, Chiara Dall'Asta, Gianni Galaverna, María Dolores del Castillo Bilbao.

Source: *Foods* MDPI *(in submission).*

Evaluation of bioactive properties, methyl donor compounds absorption and intestinal glucose uptake inhibition of pre-fermented bran enriched bread in in vitro cell line models

Authors: Marco Spaggiari, Chiara Dall'Asta, Gianni Galaverna, María Dolores del Castillo Bilbao

Source: *Food & Function (in submission*).

ORAL COMMUNICATIONS ***presented at international and national scientific conferences***

Cereal milling by-products valorisation: from unexploited materials to valuable resources.

Authors: M. Spaggiari, C. Dall'Asta, G. Galaverna.

Conference: 24th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Firenze, Italy (September 2019).

Mono-, di- and triacylglycerols UHPLC-ESI-MS/MS analysis in turbo separated and micronized rice bran (Oryza sativa).

Authors: M. Spaggiari, L. Righetti, S. Folloni, R. Ranieri, C. Dall'Asta, G. Galaverna.

Conference: 5th ISEKI Food Conference, Stuttgart, Germany (July 2018).

Lactic acid fermentation of Triticum turgidum subsp. turanicum bran as means of nutritional improvement

Authors: M. Spaggiari, C. Lazzi, S. Folloni, R. Ranieri, C. Dall'Asta, G. Galaverna.

Conference: 1st International Conference of Wheat Landraces for Healthy Food Systems, Bologna, Italy (June 2018).

BIO^2, Triticum spp. biodiversity valorisation under organic farming: safety and quality aspects

Authors: M. Spaggiari, L. Righetti, S. Folloni, R. Ranieri, C. Dall'Asta, G. Galaverna.

Conference: 17th European Young Cereal Scientists and Technologists Workshop, Warsow, Poland (April 2018)

Determinazione del contenuto di acidi fenolici, GABA (acido γ-amminobutirrico), betaina e colina e presenza di tricoteceni in Triticum spp. e co-prodotti del processo di molitura.

Authors: M. Spaggiari, L. Righetti, S. Folloni, R. Ranieri, C. Dall'Asta, G. Galaverna.

Conference: 11° Convegno AISTEC, Rome, Italy (November 2017).

POSTERS ***presented at international and national scientific conferences***

Lactic acid fermentation of cereal milling by-products: an aroma enhancer tool.

Authors: M. Spaggiari, C. Lazzi, C. Dall'Asta, G. Galaverna.

Conference: XX EuroFoodChem Conference, Porto, Portugal (June 2019).

Deoxynivalenol vs. lactic acid bacteria: a biodegradation competition.

Authors: M. Spaggiari, M. Cirlini, B. Bottari, V. Bernini, C. Dall'Asta, G. Galaverna.

Conference: EFSA Conference 2018 - Science, Food, Society, Parma, Italy (September 2018).

Free and hidden fumonisins in yellow and white whole grain maize (Zea mays) and corresponding milling fractions.

Authors: M. Spaggiari, L. Righetti, S. Folloni, R. Ranieri, C. Dall'Asta, G. Galaverna.

Conference: 8th International Symposium on RAFA, Prague, Czech Republic (November 2017).

Fumonisins in maize (Zea mays): effect of particle size on the analytical performance.

Authors: T. Damiani, M. Spaggiari, L. Righetti, C. Dall'Asta;

Conference: 8th International Symposium on RAFA, Prague, Czech Republic (November 2017).

Phenolic acids distribution in "Carnaroli" rice (Oryza sativa L.) and corresponding milling fractions.

Authors: M. Spaggiari, S. Folloni, R. Ranieri, C. Dall'Asta, G. Galaverna.

Conference: 5th MS Food Day, Bologna, Italy

"**Best Poster Award winner**" (October 2017).

Determination of betaine, choline and γ-aminobutyric acid in Triticum durum and Triticum turanicum whole grain and milling co-products.

Authors: M. Spaggiari, L. Righetti, S. Folloni, R. Ranieri, C. Dall'Asta, G. Galaverna.

Conference: XIX EUROFOODCHEM Conference, Budapest, Hungary.

"**Best Poster Award winner**" (October 2017).

Principal phenolic acids composition of Triticum spp. whole grain and corresponding milling co-products.

Authors: M. Spaggiari, L. Righetti, S. Folloni, R. Ranieri, C. Dall'Asta, G. Galaverna.

Conference: Food Integrity 2017 Conference, Parma, Italy. (May 2017).

Table of Contents

SUMMARY/RIASSUNTO

The research presented in this Ph.D. thesis is part of the project "*Agri-food industry by-products and co-products recovery for the competitiveness improvement of local companies*" (POR/FSE 2014-2020), which is financed by Regione Emilia-Romagna through the European Social Fund.

The agri-food chains related to the most cultivated cereals in Emilia-Romagna region have produced more than 2 million tons as sum of durum wheat, bread wheat and other minor wheat species (*Triticum* spp.), maize (*Zea mays*) and rice (*Oryza sativa*), a constant trend from 2015. Following an integrated and multidisciplinary approach, the recovery and valorisation of cereal milling side-streams was the core of this project.

The main findings can be summarized according to the three research goals met in this thesis:

Impact of milling process on whole grain quality and safety traits. The outermost fractions of cereal grains, known as bran, stood out for being the most abundant in terms of bioactives, like phenolic acids and methyl donor compounds. These substances occurred mainly in bound form, linked to the cell wall polysaccharide, hence scarcely bioaccessible. Moreover, hazardous compounds like mycotoxins and their forms modified by both fungi and plants metabolism might be also highly present in these fractions. Therefore, if on one hand, the milling process was detrimental in terms of reduction of high-nutritional value compounds, on the other, it helped to mitigate the endosperm from toxic metabolite contamination.

Application of innovative technologies for the total recovery and valorisation of cereal milling by-products. The wheat bran bioprocessing using lactic acid bacteria in solid state fermentation could increase the content of soluble bioactive compounds, generating a novel matrix with different organoleptic characteristics.

Likewise, air classification and micronization of rice bran increased significantly the content of mono- and di-glycerides with emulsifying properties in finer fractions. These two techniques can be confirmed as potential valorisation technologies.

Evaluation of bioactive properties of wheat bread enriched with pre-fermented wheat bran. A wheat bread enriched with previously fermented bran was successfully formulated and compared to white and raw bran enriched bran in terms of nutritional properties. After an *in vitro* simulated gastrointestinal digestion, the soluble fractions were supplemented to two different intestinal cell culture models (IEC-6 and CaCo-2). The results showed that this bread had a potential protective effect against oxidative stress and capable to counteract the inflammation markers generation, such as nitric oxide. Moreover, it had a significant delaying effect on the glucose intestinal absorption.

"Cereal milling by-products are a very heterogeneous materials, and represent an enormous opportunity for the increasing value of the entire agri-food chain, starting from farmers ending to consumer."

La ricerca presentata nel presente percorso di dottorato fa parte del progetto "*Recupero dei sottoprodotti dell'industria agroalimentare per il miglioramento della competitività delle imprese locali*" (POR/FSE 2014-2020), che è finanziato dalla Regione Emilia-Romagna attraverso il Fondo sociale europeo. Le catene agroalimentari legate ai cereali più coltivati nella regione dell'Emilia-Romagna hanno prodotto oltre 2 milioni di tonnellate come somma di grano duro, frumento tenero e altre specie minori di grano (*Triticum* spp.), Mais (*Zea mays*) e riso (*Oryza sativa*), una tendenza costante dal 2015. Seguendo un approccio integrato e multidisciplinare, il recupero e la valorizzazione dei sottoprodotti della molitura dei cereali sono stati il fulcro di questo progetto.

I principali risultati possono essere riassunti in base ai tre obiettivi raggiunti in questa tesi:

Impatto del processo di molitura sugli aspetti di qualità e sicurezza. Le frazioni più esterne che compongono il grano intero, note come crusca, sono risultate le più abbondanti in termini di composti bioattivi come acidi fenolici e composti donatori di gruppi metilici. Questi sono principalmente stati riscontrati nella forma legata alle componenti polisaccaridiche delle cellule vegetali, per cui poco bioaccessibili. Inoltre, anche composti tossici come le micotossine e le loro forme modificate dal metabolismo di pianta e fungo, potrebbero essere altamente presenti in queste frazioni. Tuttavia, se da un lato il processo di molitura ha portato ad una diminuzione di composti bioattivi dall'altro, ha aiutato a mitigare l'endosperma dalla contaminazione dei composti tossici.

Applicazione di tecnologie innovative per il totale recupero e la valorizzazione dei sottoprodotti della molitura dei cereali. Il processo biotecnologico applicato alla crusca di grano duro che vede l'uso di batteri lattici ha potuto aumentare il contenuto di composti bioattivi solubili e bioaccessibili,

generando una nuova matrice con caratteristiche organolettiche diverse rispetto alla crusca non trattata. Allo stesso modo, la classificazione per mezzo d'aria e la micronizzazione della crusca di riso hanno aumentato significativamente il contenuto di mono e di gliceridi con proprietà emulsionanti nelle frazioni più fini, generando un potenziale ingrediente funzionale.

Valutazione delle proprietà nutrizionali di un pane arricchito con crusca di grano pre-fermentata. Un pane arricchito con crusca precedentemente fermentata è stato formulato con successo e confrontato, in termini di proprietà nutrizionali, con un pane arricchito di crusca ed un pane bianco. Dopo una digestione gastrointestinale simulata *in vitro*, le frazioni solubili sono state usate per trattare due diversi modelli di colture cellulari intestinali (IEC-6 e CaCo-2). I risultati hanno mostrato che questo pane possiede un potenziale effetto protettivo contro lo stress ossidativo e la riduzione dei marker di infiammazione, come l'ossido nitrico. Inoltre, ha avuto un significativo effetto ritardante sull'assorbimento del glucosio a livello intestinale.

"I sottoprodotti della macinazione dei cereali sono materiali molto eterogenei e rappresentano un'enorme opportunità per aumentare il valore dell'intera catena agroalimentare, a partire dal settore primario fino al consumatore."

INTRODUCTION

CEREAL GRAINS: FROM FARM TO FORK

Cereal grains are historically recognised as staple food, in fact they provide essential nutrients and energy to both human and animals diets. The term "cereal grains" includes many different species deriving from Gramineae family. Among them, three species are the most cultivated nowadays worldwide: corn (*Zea mays*), rice (*Oryza sativa*) and wheat (*Triticum* spp. L.) [1]. As fundamental part of human diet, cereals play important role in the agri-food chain, thus the system whereby the field production can reach the consumers table. In this context a crucial part is the analysis-assessment-management (AAM) of the entire chain functionality. The supply chain of cereal grains is relatively simple, however considering the wide diffusion of cereal based products and the different customer target, the AAM process must be implemented. In these following pages the aim is to give a simple overview of the cereal grain chain from field to fork.

Starting from the top, the first step before the cereal sowing is the selection of the cereal species or variety, based on determined seed characteristics, for example, a higher content of dietary fibres, micronutrients or phytochemicals, yield parameters and/or resistance to plant diseases or infections. In this way, the use of biotechnology and breeding operations have been considered as the best practices[2]. Besides, the challenge is the combination of these improvements to a proper processing quality for a high consumer acceptability, taking into consideration the sustainability and bio-diversity framework.

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[1] FAOSTAT (2018) Food and Agriculture Organization Corporate Statistical Database. www.fao.org/faostat/. Accessed 31 Aug. 2019

[2] Shewry, P. R., & Hey, S. J. (2015). The contribution of wheat to human diet and health. *Food and Energy Security*, *4*(3), 178-202.

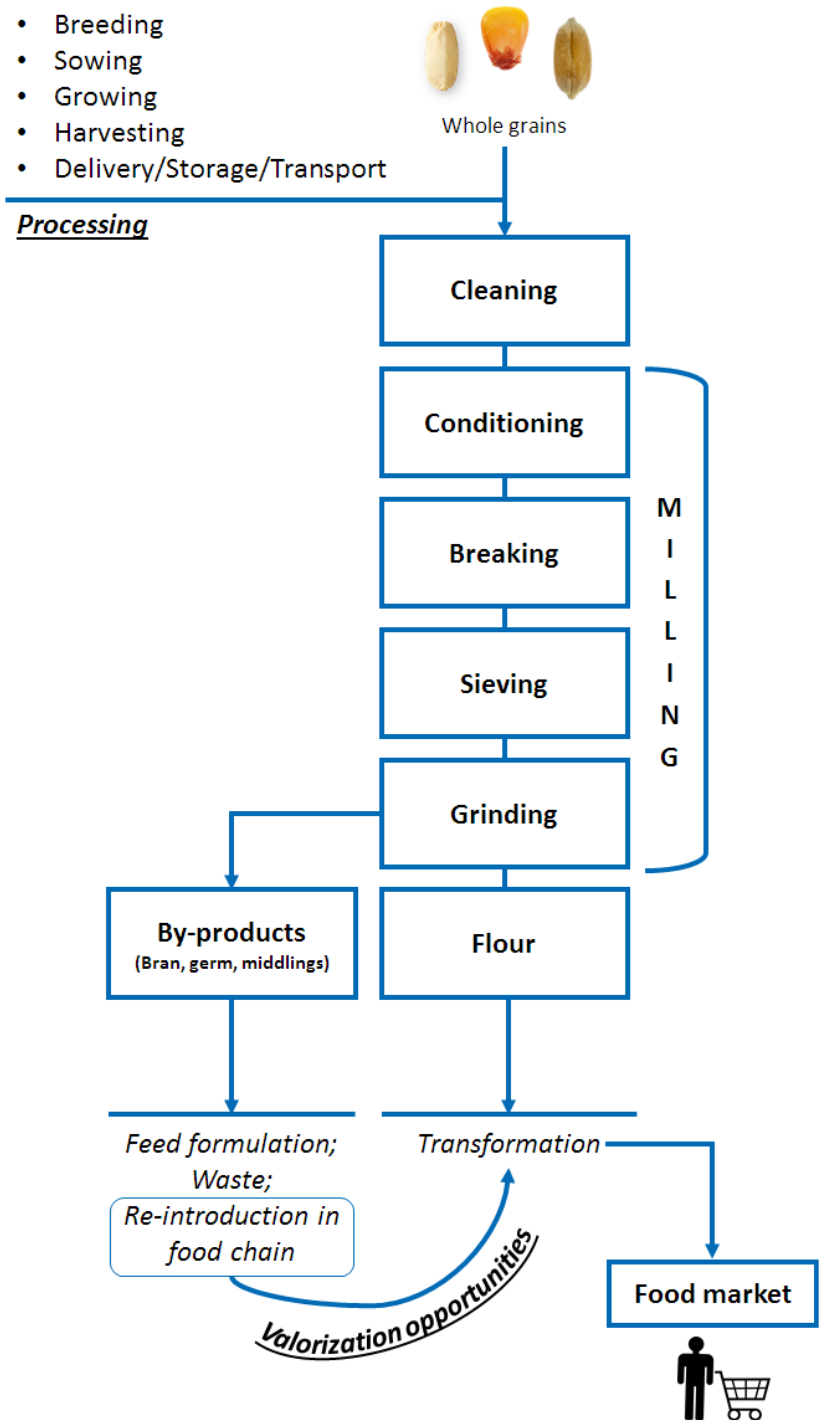

Figure 1. Schematic representation of a general cereal grain chain, highlighting the milling process and the production of by-products.

The path that cereals follow is generally similar for each grain species and includes different stages and operators [3] (**Figure 1**). In fact, after seeding and cultivation, cereals undergo to several processes different in function of the sought final product. Normally the milling process follows the harvesting and it represents the principal procedure in the cereal industry. It can be distinguished in two type, dry and wet milling. Both share the same objective: **to separate the endosperm from the outer layers of the seeds**. In dry milling, after a step of cleaning and conditioning, cereals pass through grinders and sieves to generate the milled fractions. Then, the previously conditioning step is an essential process to prevent the rupture of the outer layers structure and the overall integrity of the seeds. During this stage, the grains are moistened with tempered water during an appropriate period which can vary in function of the cereal species and type. Therefore, size, shape and hardness of kernels are important characteristics that must be taken into account in milling process [4]. Differently to dry milling, wet milling is less used, and it is aimed to the separation of grains most abundant components (i.e. starch, oil, proteins, etc.). For example, maize is predominantly treated for the starch extraction, leading to a production of syrups rich in maltodextrins and glucose, which are relevant raw materials used in food industry.

At this moment of the chain the course of cereal grain is divided into two different directions (**Figure 1**), one represented by the most used raw material, the flour, and the other formed by the cereal milling by-products. The many transactions involved in this part of the chain are focused on the production of food stuffs which have to satisfy consumers' needs; thus, these steps are strictly related to the final protagonists of food market.

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[3] Papageorgiou, M., & Skendi, A. (2018). Introduction to cereal processing and by-products. In Sustainable Recovery and Reutilization of Cereal Processing By-Products (pp. 1-25). Woodhead Publishing.

[4] Posner, E. S., & Hibbs, A. N. (2005). *Wheat flour milling* (No. Ed. 2). American Association of Cereal Chemists, Inc.

Arguably, the most important parameter in this section is the "quality" of the grains. The latter term has different meaning, however its assessment and consequently its management are crucial for the whole supply chain. As an example, a greater technological suitability of a flour rather than another is a feature deeply connected to the food manufacturing [5,6].

Finally, the trend setter is the consumer, who is the ultimate assessor of the cereal product quality. In this way, the feedback of the end users, which is variable in terms of time and magnitude, will be the last evaluation of the whole supply chain.

Regarding the cereal grain by-products which arise from the milling process represent about 27 % [7] of a general milling plant, which is translatable to huge economical losses and complicated residues management treatments. Therefore, industries put great interest in the recovery and valorisation of the residues produced during the first step of processing, since it could be an opportunity for the value increment and sustainability improvement. Indeed, policies and community funding have nowadays implemented several actions to tackle the latter issue.

[5] Miskelly, D., & Suter, D. (2017). Assessing and Managing Wheat-Flour Quality Before, During and After Milling. In *Cereal Grains* (pp. 607-634). Woodhead Publishing.

[6] Wrigley, C., Batey, I., & Miskelly, D. (2017). Grain Quality: The Future is With the Consumer, the Scientist and the Technologist. In *Cereal Grains* (pp. 691-725). Woodhead Publishing.

[7] Serna-Saldivar, S. O. (2016). *Cereal grains: properties, processing, and nutritional attributes*. CRC press.

Policy issues and legislation

The agri-food chain considered as a system, is largely regulated by international and national policies for a better organisation, continuous improvement. Nowadays a greater attention is paid to the reduction of food waste and the implementation of sustainability actions for a lower impact on the environment. In fact, there is a tendency to converge more efforts to the durable permanence of materials and resources in the economy, maintaining a high value level and generating the lowest amount of waste as possible. In this innovation-rich context, the European Union (EU) faces the problem encouraging the development of new technologies, services and processes supporting the research and the EU industries competitiveness [8].

In relation to this, there is also a need to define a clear legislative framework which can outline priorities regarding the reduction of waste, the recovery and recycling processes. In revised "Directive 2008/98/EC" [9] great relevance has been posed on the proposition of guidelines for the prevention of food waste generation coupled with the stimulation of the industries to reuse, recover and recycle by-products as valuable raw materials. The latter is true for such residues intended for human consumption, since those by-products or wastes used in feed formulation are regulated by the Regulation (EC) No. 767/2009.

[8] Horizon 2020 work program, section: "Industry 2020 in the circular economy,"

[9] European Commission, 2015. Directive of the European Parliament and of the Council (Proposal). Amendment to the Directive 2008/98/EC on Waste, COM (2015) 595 Final 2015/ 0275(COD), Brussels.

Food Safety Management of Whole Grains

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in "*Whole Grains: Processing, Product Development, and Nutritional Aspects*"

Preface

Cereals consumed as whole grains or wholemeal products are rich in health-promoting compounds. Nevertheless, the occurrence of threats is represented by the presence of contaminants and toxic compounds along the agri-food chain, which must be monitored in order to create and to maintain a "safety area" framework [1].

Moreover, the term "whole grain" includes also the external layers of cereal seed which are the mainly processing by-products. Since these fractions are the seed-environment first contact, the presence of hazardous compounds might be higher [2].

In accordance to the objectives of this project, the study of the safety related issues and the potential management measures were considered as starting point.

[1] Thielecke, F., & Nugent, A. (2018). Contaminants in Grain—A Major Risk for Whole Grain Safety? Nutrients, 10(9), 1213.

[2] Alldrick, A. J. (2017). Food safety aspects of grain and cereal product quality. In Cereal grains (pp. 393-424). Woodhead Publishing.

Abstract

Whole grain cereals are the major contributors to the daily energy intake of human and animal diet. Given their importance as food and feed, the assurance of their safety is crucial. Despite of this, food industry has the responsibility to implement good safety management system in order to contrast the potential contamination of possible hazards. These belong to different nature (chemical, physical or biological), and the most important in relation with cereal crops, are discussed in this chapter. Since the introduction of the latter contaminants could occur at all the stages of the food chain, and in accidentally or intentional way, further strategies of mitigation and control will be analysed. Finally, some recent defence tools that helps food manufacturers to prevent and respond to food crimes will be reported in this chapter.

Key words: Food safety, Cereal grains, Contaminants, Food Defence, Risk management.

Introduction

Cereals and related products have a recognized impact on both human and animal diet. The world's cereal grains production in 2016-17 has been estimated in the order of 2.4 thousand million tons (USDA, 2017). Among all the cereal species, the most important in terms of cultivated area are maize, wheat and rice. These three cereal grains directly contribute more than half of all calories consumed by human beings. Nevertheless, in some areas where the climate is not suitable for the latter crops, the population finds sustenance cultivating other so-called "minor grains" (sorghum and millet, FAO, 2016). Very often these areas coincide with the developing countries, in which the contribution by cereal grain to total daily energy intake is higher than the developed world, remarking the importance of these crops (Anon, 2003). In this context, it is obvious that food industry meets the consumer needs when products are conformed to quality and safety law requirement standards, defined by each country. "Ensuring *food: safe to eat and free from dangerous levels of harmful infectious and toxic agents (natural and accidental contamination)*" (EU, 2002), it is the definition of food safety at European level. Consequently, how food producers can ensure these conditions is based on the food safety management system. Indeed, the management process requires an identification of the hazard, followed by the analysis of the features which impact on the occurrence and degree that hazard occurs, and in addition to this, it is necessary to identify the processes useful to hazard risk reduction (Alldrick, 2010). The hazard identification step is basically the same as for other foodstuff, in which the nature of the hazard could be distinguished as chemical, physical or microbiological. Considering the aim of this chapter the risk management process is represented in a schematic form in **Figure 2.**

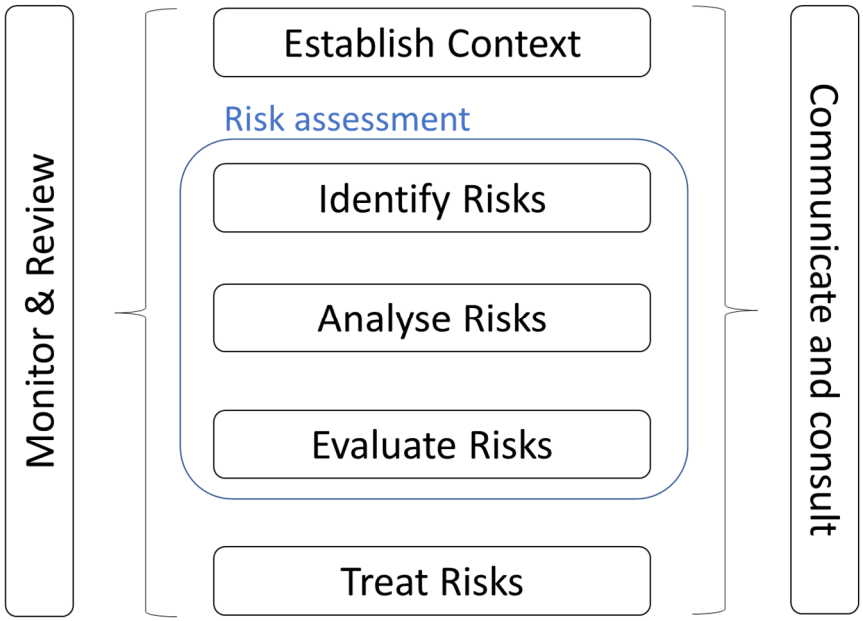

Figure 2. Risk management process (adapted to: ISO 31000:2009 (https://www.iso.org/files/live/sites/isoorg/files/archive/pdf/en/iso_31000_for_smes.pdf) https://www.iso.org/standard/43170.html

This procedure easily explains the fundamental principles for risk management, identifying opportunities and threats, and effectively use the resources for risk treatment. In addition to this it defines all actions useful for the control of the risk at a manageable level. It is also important to remember that the introduction of the hazard in the food chain is caused by either nature or human acts, intentionally (food crime) or unintentionally, and it could occur at any level of the supply chain, "*from farm to fork*" alike the safety management approach. According to this, the hazard introduction led by potential illicit activities, will be also discussed in this chapter, analysing some new tools already implemented in food safety field. Consistent with the objective of this chapter, the whole grains are considered as end products, that may then be sold directly or delivered to another processor before being sold.

Therefore, the main purpose is to consider the safety aspect of whole cereal grains through the characterization of the possible hazards associated with these products. The general structure of the caryopsis will be explained in order to better comprehend the distribution of the possible hazards within the seed parts. Besides this, also the processes for the risk mitigation or reduction inter-connected with management, will be elucidated (**Figure 3**).

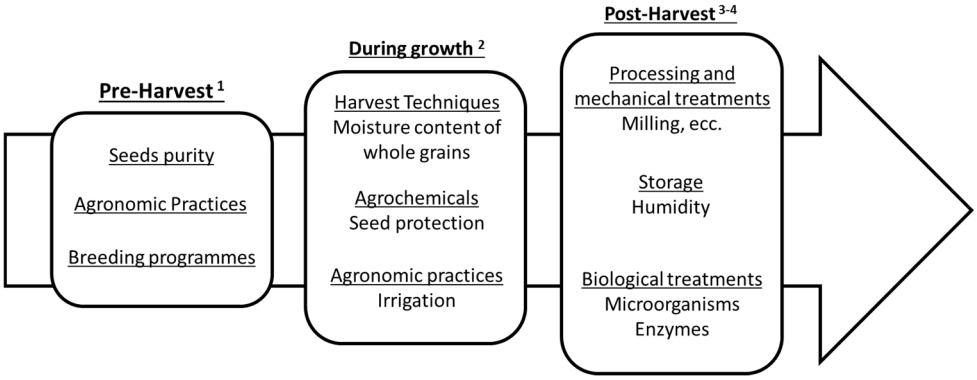

Figure 3. Risk mitigation strategies in cereal crops. 1: Cheli et al., 2017; 2: Edwards, 2004; 3-Lopez-Garcia et al., 1999; 4: Karlovsky et al., 2016.

Managing safety in food industry

A simple way to see a food enterprise is to divide all the activities into processes. Each of these processes must be described in all their parts and consequently must be controlled to ensure the proper operation. The effectiveness of management actions is determined by maximum and minimum limits. In **Figure 4** is represented a hypothetical process in which the range of work is delimited by the current food safety policies joint to the results of the internal risk assessment process (max limit). Meanwhile, the lower threshold is represented by the quality features of the product sold in the food market, that are the sum of internal decisions and consumer likes.

Since periodical controls are planned as elements of good management system, when the process goes beyond the fixed limits the management has to be revised. The alarm could be caused by several factors present in the food chain. Particularly important in the case of cereal is, for example, the continuous control of suppliers that have to respect maximum limit of raw material contamination (i.e. mycotoxins and/or contaminants).

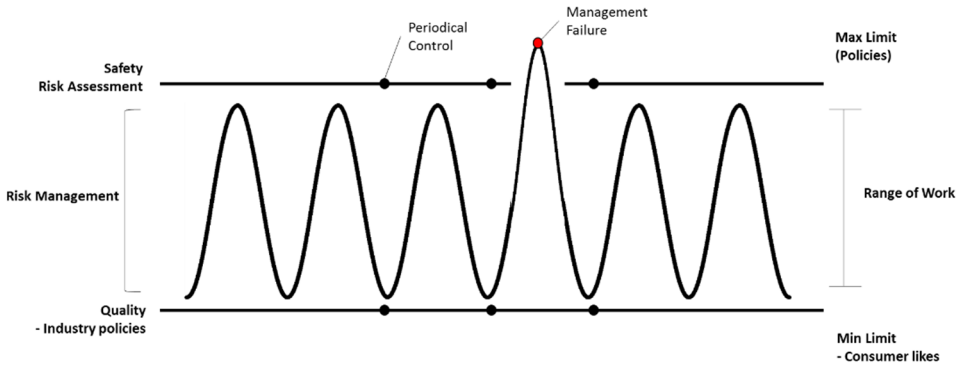

Figure 4. Simplified description of general food process and the management system.

Know the fundamentals: the overall structure of cereal grain

The anatomy of a cereal grain is basically common for all species (Evars and Millar, 2002). They are composed by three major fractions called endosperm (80-85% of the whole grain), bran layers (13-17%) and germ (or embryo, 2-3%) (**Figure 5**). These seed components differ in terms of nutrients and general composition since they have diverse functions during the kernel development. For instance, the primary role of the endosperm is to sustain the embryo during the germination process, providing essential nutrients. While the bran, composed by several thick-cell layers, have the principal physiological function of seed protection and in some cases as storage of essential nutrients.

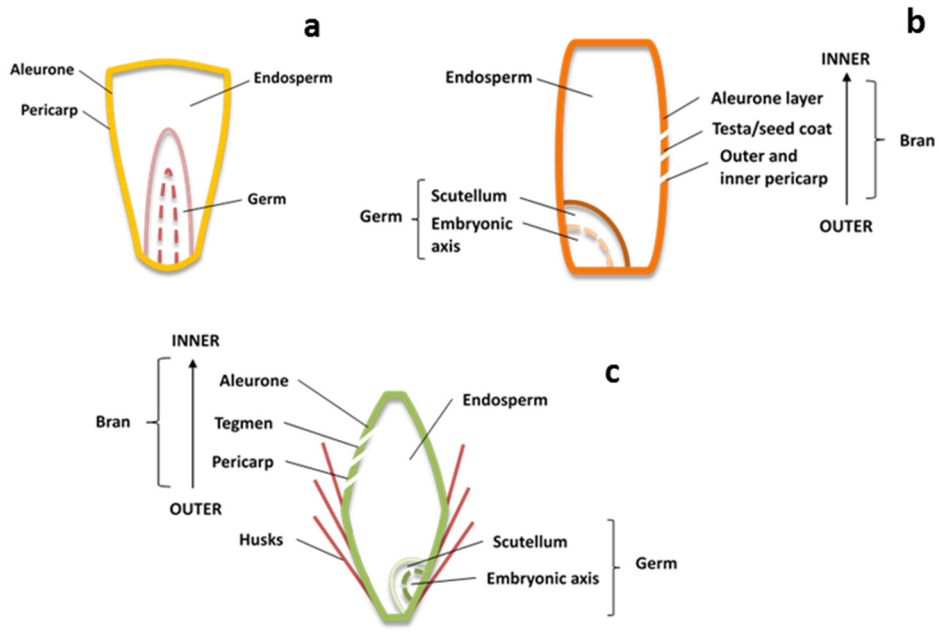

Figure 5. Simplified anatomy of Maize (*Zea mays*) (a), Wheat (*Triticum* spp. L.) (b) and Rice (*Oryza sativa*) (c) whole grains.

Seeds, intended as whole kernel, are very often consumes as they are. Different kind of processing are applied to them in order:

1) to select one of the fractions of interest (e.g. endosperm);
2) to enhance a particular technological property (e.g. gluten formation in bread-making process);
3) and to obtain the greatest organoleptic and nutritional benefit (palatability and digestibility).

The most spread early processes which whole grains are submitted are mainly physical and mechanical. These can involve the removal of outer seed fractions, milling aimed to obtain a specific particle size, and other thermal processing (i.e.: baking). In this way, the structure of the grain seeds is an essential feature to consider, that influences a possibly routes of contamination and therefore represent a probable hazard to consumers.

Whole grains "harmful" components: Phytotoxins

Not so surprisingly, cereals intended as whole grains, in addition to numerous positive bioactive compounds, are rich in so-called anti-nutritional substances. The function of such compounds in the kernel can vary from cellular structural components to microelements storage. In cereals, the most important compounds from the safety point of view are tannins and phytates. They are almost present in all cereal species, because of their roles, nevertheless the concentration could be different in function of the species and variety. In addition to this, plants are able to produce secondary metabolites which present certain level of toxicity to human health. The latter is not primarily related to cereals *per-se*, since the class of compounds in question are the alkaloids, which the cultivated cereals are not capable to synthesize. In fact, the principal issue is the co-harvesting of seed belonging to other alkaloids-producing plants.

Tannins

Tannins are secondary metabolites found in many plant species. These molecules are essentially phenolic compounds that may acts in seed as protective agents against pathogens and predators (Kaufman et al., 2013). Their high molecular weight structure is responsible to the interaction with proteins through cross-linking (Griffiths, 1991). Despite of the numerous beneficial factors of polyphenols on health, this mechanism of complexation, is reported to be the principal cause of the antinutritional effect of tannins. In this way, the condensed tannins (CT) have the ability to reduce the protein digestibility (Griffiths, 1991). Their occurrence in cereals is widely documented and, between cereal species, sorghum (*Sorghum bicolor* (L.) is the most important from this point of view. In fact, in terms of concentration, the presence of tannins in major cereals as rice, wheat and maize is negligible. Nevertheless, some strategies to reduce tannins concentration and thus their negative effects, have been studied.

Processing might be one of these. Indeed, physical treatment such as the seed external parts removal, contributes to deep reduce CT content in final products (Kruger et al., 2012). Moreover, genetic techniques are useful tools for the control of this hazard. Among them, the most promising are based on varietal selection and breeding programs, directed to the cultivation of low tannin varieties in which pigments in testa and pericarp are absent (Taylor and Duodu, 2010).

Phytates

In whole grain, phosphorus (P) is mostly stored as phytate, the myo-inositol-1,2,3,4,5,6-hexaphosphoric acid salt. This compound may exert strong chelating effects of minerals, such as iron and zinc, at physiological pH levels (Graf et al., 1986). This results in insoluble complexes that may lead to a decrease in mineral absorption and bioavailability in human. Consequently, phytates may possibly contribute to malnutrition, and to physiological-pathological conditions, like osteoporosis, and iron deficiency (Akhter et al., 2012). As described above, the content of this molecule could vary among cereal species and genotypes. In addition, the distribution of phytic acids within the kernel is also different. Their distribution in wheat is mainly concentrated in the aleurone layer, while in maize phytates are more abundant in the germ. On the contrary, in pearl millet they seem to be homogeneously distributed through the whole caryopsis (Reddy et al., 1989; Coudray et al., 2001). Phytates are also found at varying levels in different sorghum grain varieties (Wu et al., 2016). Also in this case, the knowledge of seed anatomy helps to implement strategies that are able to reduce the occurrence of the hazard. Overall, the milling process, removing the outer layers of the seed, is the most effective way to reduce phytic content in end products. Consequently, the continuous monitoring of the milling process is a crucial step to manage the issue. Furthermore, breeding for low phytate content seems to be a realistic practice to enhance nutritional quality of any crop.

The unwelcome: Tropane and Ergot Alkaloids

As mentioned at the beginning of this section, this category of hazards relies to the cross contamination of certain cereal crops with other toxic compound producing plants. The wide diffusion of cereal grain as staple foods makes them particularly important, especially because they are consumed by all the population category.

Is that buckwheat (*Fagopyrum esculentum*)?

Alkaloid-producing plants have been used by humans since ancient times. Their properties make them famous poisons, narcotics, aphrodisiacs and medicines. Nevertheless, nowadays these plants are part of human and animal diet (Koleva et al., 2012) Their occurrence is not directly correlated to cereal crops, as cited above. In fact, the co-harvest of alkaloids producing plant appears to be the mainly cause of their occurrence in foods (Mulder et al., 2016), which translates in a low-level but continuous source of exposure. In this way, cereals and cereal products, are typically characterized by the presence of atropine and scopolamine, two compounds belonging to tropane alkaloids (TA) class.

As an example, the buckwheat and buckwheat-derived foods emerging problem will be discussed because of its growing attention (Aehle & Drager, 2010). Two are the main reasons of the increasing consumption of buckwheat: a) the absence of gluten; b) a good nutritional profile.

Buckwheat is a pseudo-cereal mostly sold as flour, and it has been used as a healthy constituent in different cereal-based products including baked products and specialty food for infant nutrition (Giménez-Bastida & Zielinski, 2015). Regarding the cultivation aspects, the real issue is that both *Datura stramonium* (an alkaloid producing plant) and *Fagopyrum* grow up and produce almost at the same time their respective fruit which share similarities in shape, colour and weight (**Figure 6**).

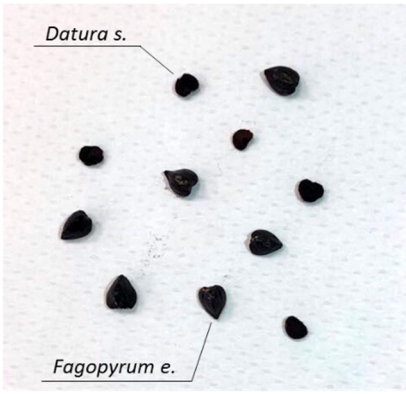

Figure 6. *Datura stromonium* and *Fagopyrum esculentum* whole seeds.

The occurrence of *Datura stramonium* is particularly significative in organic agriculture, where the use of agrochemicals against growth of wild plant is restricted (Bond and Grundy, 2001). Actually, the presence of TA in foods has been recently reconsidered, so that the European Commission has emanated a specific recommendation (European Commission, 2016). From the food safety management point of view, it is fundamental to break up the food chain in little section, or much better, in steps. Therefore, a strict control at the very beginning of the food chain is the first point to consider. Additionally, detection of seed contamination may be as simple as a visual inspection. Moreover, the application of technological treatment like sorting and cleaning steps upstream the production process may support the selection process together with an adequate management in post-harvest handling and control. Serious episodes of TA intoxication following the consumption of buckwheat products have been reported recently in Slovenia (2005) and France (2012). These outbreaks ended up with the setting up of survey programs at EU level, whose results highlighted a wide range of contamination including feed and food flours (Perharic et al., 2013; Perharic, 2005).

Once upon a time: Ergot alkaloids and cereals

Ergot alkaloids (EA) and its producer (the fungus *Claviceps purpurea*) have a long history, being known in China since 100 BCE and in Europe since Middle Ages (Schiff, 2006) Although ergot alkaloids are secondary metabolites of fungi and therefore they may be classified as mycotoxins, they can be structurally described as alkaloids sharing a lysergic acid nucleus. The adverse health effects vary from hallucinations, burning sensation to convulsions. *Claviceps purpurea* infection and growth leads to the formation of sclerotia, a dark mycelial mass rich in alkaloids, that replace the healthy seeds (**Figure 7**)

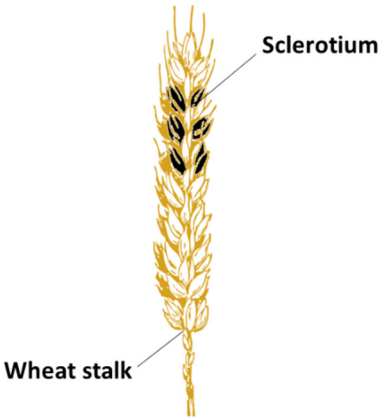

Figure 7. Wheat sclerotium.

Arguably, the risk associated to EA contamination is not only limited to humans, and many cases of livestock intoxication due to the consumption of contaminated feed have been reported (Bryden, 2012). The presence of sclerotia and, therefore, EA occurrence is more frequent in wheat and rye compared to other cereals (Stoll et al., 1970; EFSA 2012). In particular, rye, a cross-pollinator with characteristic open florets, is particularly susceptible toward *C. purpurea* infection (Lorenz and Hosney, 197). This leads to a generally higher content of EA in rye and rye-derived products compared to other cereal grains (EFSA 2012).

Nowadays the management processes are directed to limiting the occurrence of EA and, consequently, the presence of sclerotias in cereal grains, at the upstream of the food production chain. In a recent study, authors studied several interactions and influencing attributes on plant infection (Orlando et al. 2017). They found that the host plant, the previous cropping system, the presence of grassweed as well as the tillage system, all had a significant influence on the overall alkaloid content in the final product. Despite of this, the main factor driving *C. purpurea* infection is still related to extreme weather conditions (Orlando et al 2017). Since the presence of sclerotia in cereals is almost visible, visual inspection followed by a selection process is often applied in the cereal transformation industry. This can be achieved with simply sorting steps based on colour and seed density (BfR 2004; Franzmann et al. 2011).

Heavy Metals: do not be taken "lightly"

Heavy metals are worldwide recognized as chemical contaminants in food and feed. Among metals, Arsenic and Cadmium have a documented importance as contaminant in cereal crops and their dietary intake from cereal-based products, represents the higher contribution in human exposure, as recently reported by the European Food Safety Authority (EFSA, 2014). The inorganic elements profile of plants is strictly dependent from the soil composition. Consequently, the environment surrounding the crops and the pedoclimatic conditions are the main factors influencing the plant uptake of metals. Despite this, the development of cereal varieties with reduced capacity to accumulate toxic elements in their edible tissues requires a deep molecular understanding of the mechanisms in charge of this accumulation.

Careful with those: Arsenic (As) and Cadmium (Cd)

Arsenic and Cadmium are microelements that occur naturally in the environment; thus, they can be considered as ubiquitous. Human activities or/and natural phenomenon can however affect their presence in environment. Arsenic can be found in various form, organic and inorganic, depending on the presence of carbon or not, respectively. The inorganic forms have gained more attention because of their toxic health effects. In fact, these substances have been classified as Group I carcinogens (EFSA, 2009). Intoxication due to As and Cd has been related with increased incidence of lung, breast and bladder cancers, and other pathologies (Huang et al., 2006; Li et al., 2011; Liao et al., 2005). Their wide distribution in the environment, especially in soil, makes them easily absorbable by plant crops. In these terms, cereals with high cultivated areas play an important role as contributors for the human exposure to these metals. For this reason, it is essential understanding the possible factors that might affect the absorption of As and Cd in plants, and their distribution within plant organs. This provides more reliable information about the element accumulation in those parts of the seed that are consumed, taking into account that co-occurrence of multiple toxic elements increases the burden of risk exposure. Among cereal species, Rice (*Oryza sativa*) is well known for its capacity to accumulate As in tissues (Rahman and Hasegawa, 2011, Davis et al., 2012). Nevertheless, also wheat is reported to be a constant accumulator of As from highly contaminated soil and water (Liu et al., 2005). Similarly, corn plants can transfer As in the areal parts, and this relies more on livestock health, since the feed preparations are mainly composed by cereal by-products (Requejo and Tena, 2014; Rosas-Castor et al., 2014). Taking rice crops into more deep consideration, As repartition among different plant tissues appears to be higher in roots, with a gradient of concentration from husks to kernels (Liu et al., 2006). This indicates that mobility pathways of these elements start from the soil. Indeed, contaminated water used for irrigation leads during a long period leads to an elevated As concentration in cereal crops (Dahal et al., 2008).

It is important to remark the importance of the agricultural practices in the food risk management. Irrigation and fertilization practices contribute in greater degree on the occurrence of As and Cd in food products, in particular the massive use of highly concentrated phosphorus fertilizers is reported to be the central factor (Bandara et al., 2010).

The old acquaintances

Mycotoxins in whole grains: a real threat

Mycotoxins are the widely known toxic compounds in cereal crops. They are defined as the secondary metabolites of various filamentous fungi belongings to species such as *Fusarium* spp, *Aspergillus* spp and *Penicillium* spp. Alike their reputation, mycotoxins play a significant role in both food and feed safety. Approximately 25% of the total world's cereal production is reported to be contaminated with at least one or more members of this particular group of contaminants (Charmley et al., 1995). In addition to health-related issues, these compounds are responsible of enormous economic losses, caused by the diminished livestock productivity as well as the direct losses in crop yield and stored agricultural products. Chemically speaking, these compounds are structurally different and – as a consequence - the range of both acute and chronic toxic effects is also widely diverse. Several studies reported the impact of mycotoxicosis on human health, recognising the importance of these class of substances (Reddy, 2010; da Rocha, 2014). As mentioned above, these compounds are ubiquitous in cereal crops and they could be distinguished as "field" or "storage" contaminants (Miller, 1995). This distinction implies that, in some cases, fungal infection and growth takes place prevalently during the vegetal developing stage (mainly *Fusarium* spp.). In other cases, fungal infection and mycotoxin accumulation may occur also at post-harvest and storage (e.g., *Aspergillus* spp. and *Penicillium* spp.).

Due to the wide occurrence in cereals and the increasing trend of fungal infection related to climate change, regulatory bodies, worldwide, have set dedicated regulations and guidelines to ensure food safety and allow a possible mitigation. For instance, at European level, the European Commission deeply regulate both food and feed products (European Commission, 2006). The first strategy for mycotoxins safety management is represented by the full compliance of the good manufacturing practices (GMP's) and HACCP (Hazard Analysis of Critical Control Point) principles as mean of prevention (Codex Alimentarius, 2012), and this approach must be integrated along the entire food chain. Therefore, a monitoring plan that guarantees the continuous ideal operation of these practices, is essential to confirm quality and safety standards for food products. The verification by audit or inspection (internal or external) is widely applied within cereal industry. However, these activities have the main objective to check if the entire management plan, established in advance, is working within the settled limits. Hence, direct and reliable analysis of the raw material is a fundamental measure of control, that must be undertaken according to proper guidelines defining analytical steps from sampling to final measurements. In addition, strategies of mitigation of mycotoxin contamination are well implemented by food industry. These methods are a vital part of an overall management strategy and they have to be considered as such. In their systematic review, Karlovsky et al. considered the potential impact of food processing and detoxification treatments on mycotoxin contamination, ranking the physical, chemical and biological mechanisms that are used today in cereal industrial processing (Karlovsky et al. 2016).

Who protects plants? Agrochemicals

Agrochemicals are, as the word says, chemical preparations widely used in agricultural practices. They represent a strong defence mean in order to prevent pests, diseases or wild plants infestation in cultivated soils, providing an improved yield and quality of the produce (Oerke et al., 2004).

At the same time, intensive use of crop protection chemicals has attracted greater attention, since this can lead to food and environment contamination. There is no unique chemical compound used in agriculture. As a matter of fact, depending on the problem to contrast, a specific pesticide is available. Regarding this, regulations are implemented at European level, in which agrochemicals, their use and the maximum concentration of permitted residues, are specified (European Union Regulation 396/2005, European Parliament and Council, 2005). Given the relevance of cereals as staple food, residues of agrochemicals and their metabolites pose a health risk on the consumers. As the high variability of chemicals used, also the possible toxic health effects are different. Although an increasing trend of organic farming has been observed over the last decade, the most effective way to limit the use of agrochemicals while tackling plant diseases, seems to be the implementation of an Integrated Crop Management (ICM). In particular, ICM may support farmers in understanding why and how chemicals have to be used, and in producing safer food while safeguarding the environment (Burger et al., 2008). Furthermore, guidelines for the application of good agricultural practices (GAP), for environmentally friendly activities and safety and traceability measures are embedded in the ICM system (Chandler et al., 2008). Nevertheless, farmers do not always have a complete knowledge and sufficient degree of awareness in respect to the toxic effects of pesticide misuse (Atreya, 2008).

“Trust is good, control is even better”

Consumer trust is a fundamental parameter for food business. People confidence on food products is the sum of multiple factors. One of this, is the guarantee of safety of the product sold. From this point of view, also the producers are exposed to certain threats. In this picture, a prevention system that profoundly analyse the whole food chain is the key. Protection of manufacturers, and indirectly of consumers, to criminal activities is today a hot topic, that continuously gain interest.

The words “criminal activities” enclose a number of deliberate contaminations of food chain that can results in enormous economic losses and, in worst case, in safety related issues (reviewed by Davidson et al., 2017). These can vary from fraud and sabotage to food terrorism (Gyles. 2010). The motivations of these acts are many, based also on the societal and psychological significance of food, though the most widespread is the economic gain. Given the impact on the consumers, instruments which contrast negative attacks and provide defence and protection, are today largely implemented. These are relatively easily to use as they could be completely integrated in the modern safety management system currently applied by enterprises.

Food Defence for prevention

Defence systems have to be proactive as definition. They have the main objective of mitigation and prevention to potential attacks made by fraudsters and criminals (GFSI, 2014). Incidents, introduced perpetually along the food chain, are imminent and occur without warning. As an example, in 2007, melamine, already known in China as adulterant of milk and powdered milk, was added to increase nitrogen content in wheat bran directed to pet food (US FDA, 2014). Defending something embed in its description the assessment of the vulnerability of a process combined with the potential threat which is subjected. This is the fundamental approach applied by the CARVER+ Shock analysis (Manning and Soon., 2016). CARVER is the acronym (Criticality, Accessibility, Recognizability, Vulnerability, Effect, and Recoverability) of a system currently applied in USA military services, but today also adapted to food (Yadav and Sharma 2011). Additional instruments that rely on vulnerability and threat assessment, are more familiar and get inspired by HACCP, such as VACCP and TACCP (vulnerability and threat analysis of critical control point, respectively). The latter focuses on three important aspect: Criticality, Accessibility, and Vulnerability (FDA, 2015), and are simple tools to identify sources of vulnerability/ies and the means to mitigate them.

Obviously, as in all management system, the assessment of the risk associated with threats is also expected (BSI, 2017). In few words, these processes act as solid countermeasures to respond and prevent an attack, through a decomposition (ideologically) of the food chain into little “joints”, to which finally, a score of “critic degree” is given in order to identify potential source of weakness.

Conclusion

The importance of whole grains in human and animal diets has been repeatedly emphasized in this chapter. Therefore, although these products are less susceptible to hazards than other foodstuffs, they represent an important source of risk for the consumer. The implementation of robust food safety management plans is essential in order to verify the compliance established by the food legislation. Such risk management system relies on a continuous study of the potential hazards concurring in cereal chain and, consequently, on the strategies to limit their occurrence or prevent their presence in final product.

Author contribution

CD, GG and MS designed the work. MS wrote the manuscripts. All the authors contributed to the critical review of the manuscript.

References

Aehle, E. and Drager, B. 2010. Tropane alkaloids analysis by chromatographic and electrophoretic techniques: an update. *Journal of Chromatography B*, 878, 1391–1406.

Akhter, S., Saeed, A., Irfan, M., Malik, K. A. 2012. In vitro dephytinization and bioavailability of essential minerals in several wheat varieties. *Journal of Cereal Science*, 56, 741–746.

Alimentarius, C., 2008. Standard for foods for special dietary use for persons intolerant to gluten 11 8e1979. FAO/WHO.

Alimentarius, C., 2012. Prevention and Reduction of Food and Feed contamination. World Health Organization Food and Agriculture Organization of the United Nations, Rome.

Alldrick, A. J. 2010. Food safety aspects of grain and cereal product quality. In *Cereal grains: assessing and managing quality*, ed Colin Wrigley, 342-366. Elsevier.

Anon, A. 2003. Diet, nutrition and the prevention of chronic diseases. In *WHO Technical Report Series*, Geneva, Vol. 916, pp 1−150.

Araújo, J., Delgado, F. I. and Paumgartten. F. J. R. 2016. Glyphosate and adverse pregnancy outcomes, a systematic review of observational studies. *BMC Public Health* 16:472.

Atchison, J., Head, L., Gates, A. 2010. Wheat as food, wheat as industrial substance; comparative geographies of transformation and mobility. *Geoforum*, 41, 236–246.

Atreya, K. 2008. Health costs from short-term exposure to pesticides. *Nepal. Soc. Sci. Med.*, 67, 511-519.

Bond, W. and Grundy, A.C., 2001. Non-chemical weed management in organic farming systems. *Weed research*, 41(5), pp.383-405.

Boukid, F., Mejri, M., Pellegrini, N., Sforza, S., Prandi, B. 2017. How Looking for Celiac-Safe Wheat Can Influence Its Technological Properties. *Comprehensive Reviews in Food Science and Food Safety*, 16(5), 797-807.

British Standard Intitute. 2017. PAS 96:2014. Guide to Protecting and Defending Food and Drink from Deliberate Attack. BSI Standards Ltd. London. https://www.food.gov.uk/sites/default/files/pas962017.pdf (accessed September 16, 2017).

Bryden, W.L., 2012. Mycotoxin contamination of the feed supply chain: Implications for animal productivity and feed security. *Animal Feed Science and Technology*, 173(1), pp.134-158.

Bürger, J., de Mol, F. and Gerowitt, B., 2008. The "necessary extent" of pesticide use—thoughts about a key term in German pesticide policy. *Crop Protection*, 27(3), pp.343-351.

Chandler, D., Davidson, G., Grant, W.P., Greaves, J. and Tatchell, G.M., 2008. Microbial biopesticides for integrated crop management: an assessment of environmental and regulatory sustainability. *Trends in Food Science & Technology*, 19(5), pp.275-283.

Charmley, L.L., Trenholm, H.L., Prelusky, D.B., Rosenberg, A., 1995. Economic losses and decontamination. *Nat. Toxins* 3, 199-203.

Cheli, F., Battaglia, D., Gallo, R. and Dell'Orto, V., 2014. EU legislation on cereal safety: An update with a focus on mycotoxins. *Food Control*, 37, pp.315-325.

Cheli, F., Pinoti, L., Campagnoli, A., Fusi, E., Rebuci, R. and Baldi, A., 2008. Mycotoxin Analysis, Mycotoxin producing Fungi Assays and Mycotoxin Toxicity Bioassays in Food Mycotoxin Monitoring and Surveillance. *Italian Journal of Food Science*, 20(4).

Cheli, F., Pinotti, L., Novacco, M., Ottoboni, M., Tretola, M. and Dell'Orto, V., 2017. Mycotoxins in Wheat and Mitigation Measures. In *Wheat Improvement, Management and Utilization*. InTech.

Commision, E.C., 2006. Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuff. 2006R1881-EN-01.09. 2014-014.001-1.

Commision, E.C., 2006. Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuff. 2006R1881-EN-01.09. 2014-014.001-1.

Commission, E. C. 2015. Commission recommendation No 2015/976 of 19 June 2015 on the monitoring of the presence of tropane alkaloids in food. Official Journal of the European Union, 157, 97–98.

Coudray, C., Levrat-Verny, M.A., Tressol, J.C., Feillet-Coudray, C., Horcajada-Molteni, N.M., Demigné, C., Rayssiguier, Y. and Rémésy, C., 2001. Mineral supplementation of white wheat flour is necessary to maintain adequate mineral status and bone characteristics in rats. *Journal of trace elements in medicine and biology*, 15(2-3), pp.131-137.

Crevel, R.W., Baumert, J.L., Luccioli, S., Baka, A., Hattersley, S., Hourihane, J.O.B., Ronsmans, S., Timmermans, F., Ward, R. and Chung, Y.J., 2014. Translating reference doses into allergen management practice: Challenges for stakeholders. *Food and chemical toxicology*, 67, pp.277-287.

Dahal, B.M., Fuerhacker, M., Mentler, A., Karki, K.B., Shrestha, R.R. and Blum, W.E.H., 2008. Arsenic contamination of soils and agricultural plants through irrigation water in Nepal. *Environmental pollution*, 155(1), pp.157-163.

Davidson, R.K., Davidson, R.K., Antunes, W., Antunes, W., Madslien, E.H., Madslien, E.H., Belenguer, J., Belenguer, J., Gerevini, M., Gerevini, M. and Torroba Perez, T., 2017. From food defence to food supply chain integrity. *British Food Journal*, 119(1), pp.52-66.

Davis, M.A., Mackenzie, T.A., Cottingham, K.L., Gilbert-Diamond, D., Punshon, T. and Karagas, M.R., 2012. Rice consumption and urinary arsenic concentrations in US children. *Environmental health perspectives*, 120(10), p.1418.

De Saeger, S., Audenaert, K. and Croubels, S., 2016. Report from the 5th International symposium on mycotoxins and toxigenic Moulds: challenges and perspectives (*MYTOX*) held in Ghent, Belgium, May 2016.

Edwards, S.G., 2004. Influence of agricultural practices on Fusarium infection of cereals and subsequent contamination of grain by trichothecene mycotoxins. *Toxicology letters*, *153*(1), pp.29-35.

European Food Safety Authority (EFSA). 2015. The EFSA comprehensive European food consumption database. http://www.efsa.europa.eu/en/food-consumption/comprehensive-database

European Food Safety Authority, 2009. Scientific opinion on arsenic in food. EFSA J. 7 (10), 1351, 1e191.

European Food Safety Authority, 2012. Scientific opinion on ergot alkaloids in food and feed. EFSA J. 10, 2798–2956.

European Food Safety Authority, 2014. Dietary exposure to inorganic arsenic in the European population. EFSA J. 12 (3), 3597.

European Parliament, 2003 Directive 2003/89/EC of the European Parliament and of the Council of 10 November 2003 amending Directive 2000/13/EC as regards indication of the ingredients present in foodstuffs. Official Journal of the European Union. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2003:308:0015:0018:EN:PDF

European Parliament, 2005. Regulation No, E.R., 2005. 396/2005 of the European Parliament and of the Council of 23 February 2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin and amending Council Directive 91/414/EEC.

Evers, T. and Millar, S., 2002. Cereal grain structure and development: some implications for quality. *Journal of Cereal Science*, 36(3), pp.261-284.

Fasano, A., 2009. Surprises from celiac disease. *Scientific American*, 301(2), pp.54-61.

Food and Agriculture Organization (FAO), FAOSTAT 2017. In http://faostat.fao.org/site/291/default.aspx (accessed October 21, 2017).

Food and Drug Administration (FDA). 2015. Vulnerability assessment software.https://www.fda.gov/Food/FoodDefense/ToolsEducationalMaterials/ucm295900.htm (accessed September 29, 2017)

Food Drink Europe. (2013). Guidance on food allergen management for food manufactures. http://www.fooddrinkeurope.eu/uploads/press-releases_documents/temp_file_FINAL_Allergen_A4_web1.pdf (accessed November 3, 2017)

Food Standard Agency (FSA), 2006. Guidance on allergen management and consumer information. https://www.food.gov.uk/sites/default/files/multimedia/pdfs/maycontainguide.pdf (accessed October 11, 2017).

Franzmann, C., Schröder, J., Münzing, K., Wolf, K., Lindhauer, M.G. and Humpf, H.U., 2011. Distribution of ergot alkaloids and ricinoleic acid in different milling fractions. *Mycotoxin research*, 27(1), pp.13-21.

für Risicobewertung, B., 2004. Mutterkornalkaloide in Roggenmehl, Stellungnahme des BfR vom 22. January 2004. Via internet: http://www. bfr. bund. de/cm/208/mutterkornalkaloide_in_roggenmehl. pdf.

Gibson, R.S., 2006. Zinc: the missing link in combating micronutrient malnutrition in developing countries. *Proceedings of the Nutrition Society*, 65(1), pp.51-60.

Giménez-Bastida, J.A. and Zieliński, H., 2015. Buckwheat as a functional food and its effects on health. *Journal of agricultural and food chemistry*, 63(36), pp.7896-7913.

Global Food Safety Initiative (GFSI) (2014), GFSI Position on Mitigating the Public Health Risk of Food Fraud. Global Food Safety Initiative Position Paper, Issy les Moulineaux, 4pp,

https://www.mygfsi.com/files/Technical_Documents/Food_Fraud_Position_Paper.pdf (accessed December 7, 2017).

Graf, E., 1986. Chemistry and applications of phytic acid: an overview. In Phytic Acid: Chemistry & Applications (pp. 1-21).

Griffiths, D.W., 1991. Condensed tannins. In: D'Mello, J.P.F., Duffus, C.M., Duffus, J.H. (Eds.), Toxic Substances in Crop Plants. Royal Society of Chemistry, Cambridge, pp. 180–201.

Gyles, C. 2010. "Agroterrorism", *Canadian Veterinary Journal*, Vol. 51 No. 4, pp. 347-348.

Huang, R.Q., Gao, S.F., Wang, W.L., Staunton, S. and Wang, G., 2006. Soil arsenic availability and the transfer of soil arsenic to crops in suburban areas in Fujian Province, southeast China. *Science of the Total Environment,* 368(2), pp.531-541.

Jouanin, A., Gilissen, L.J., Boyd, L.A., Cockram, J., Leigh, F.J., Wallington, E.J., van den Broeck, H.C., van der Meer, I.M., Schaart, J.G., Visser, R.G. and Smulders, M.J., 2017. Food processing and breeding strategies for coeliac-safe and healthy wheat products. *Food Research International*.

Karlovsky, P., Suman, M., Berthiller, F., De Meester, J., Eisenbrand, G., Perrin, I., Oswald, I.P., Speijers, G., Chiodini, A., Recker, T. and Dussort, P., 2016. Impact of food processing and detoxification treatments on mycotoxin contamination. *Mycotoxin research*, 32(4), pp.179-205.

Karlovsky, P., Suman, M., Berthiller, F., De Meester, J., Eisenbrand, G., Perrin, I., Oswald, I.P., Speijers, G., Chiodini, A., Recker, T. and Dussort, P., 2016. Impact of food processing and detoxification treatments on mycotoxin contamination. *Mycotoxin research*, *32*(4), pp.179-205.

Kaufman, R.C., Herald, T.J., Bean, S.R., Wilson, J.D. and Tuinstra, M.R., 2013. Variability in tannin content, chemistry and activity in a diverse group of tannin containing sorghum cultivars. *Journal of the Science of Food and Agriculture*, 93(5), pp.1233-1241.

Koleva, I.I., van Beek, T.A., Soffers, A.E., Dusemund, B. and Rietjens, I.M., 2012. Alkaloids in the human food chain–natural occurrence and possible adverse effects. *Molecular nutrition & food research*, 56(1), pp.30-52.

Koning, F., 2012, July. Celiac disease: quantity matters. In *Seminars in immunopathology* Vol. 34, No. 4, pp. 541-549. Springer-Verlag.

Kruger, J., Taylor, J.R. and Oelofse, A., 2012. Effects of reducing phytate content in sorghum through genetic modification and fermentation on in vitro iron availability in whole grain porridges. *Food chemistry*, 131(1), pp.220-224.

Li, G., Sun, G.X., Williams, P.N., Nunes, L. and Zhu, Y.G., 2011. Inorganic arsenic in Chinese food and its cancer risk. *Environment international*, 37(7), pp.1219-1225.

Liao, X.Y., Chen, T.B., Xie, H. and Liu, Y.R., 2005. Soil As contamination and its risk assessment in areas near the industrial districts of Chenzhou City, Southern China. *Environment International*, 31(6), pp.791-798.

Liu, W.J., Zhu, Y.G., Hu, Y., Williams, P.N., Gault, A.G., Meharg, A.A., Charnock, J.M. and Smith, F.A., 2006. Arsenic sequestration in iron plaque, its accumulation and speciation in mature rice plants (*Oryza sativa* L.). *Environmental Science & Technology*, 40(18), pp.5730-5736.

Liu, X., Zhang, S., Shan, X. and Zhu, Y.G., 2005. Toxicity of arsenate and arsenite on germination, seedling growth and amylolytic activity of wheat. *Chemosphere*, 61(2), pp.293-301.

Lopez-Garcia, R., Park, D.L. and Phillips, T.D., 1999. Integrated mycotoxin management systems. *Food Nutrition and Agriculture*, pp.38-48.

Lorenz, K.; Hoseney, R. C. 1979. Ergot on cereal grains. *CRC Crit. Rev. Food Sci. Nutr*., 11, 311−354.

Madsen, C.B., Hattersley, S., Allen, K.J., Beyer, K., Chan, C.H., Godefroy, S.B., Hodgson, R., Mills, E.N.C., Muñoz-Furlong, A., Schnadt, S. and Ward, R., 2012. Can we define a tolerable level of risk in food allergy? Report from a EuroPrevall/UK Food Standards Agency workshop. *Clinical & Experimental Allergy*, 42(1), pp.30-37.

Manning, L. and Soon, J.M., 2016. Food safety, food fraud, and food defense: A fast evolving literature. *Journal of food science*, 81(4).

Moechnig, M. and Deneke, D., 2011. Harvest aid weed control in small grain. Ed South Dakota Cooperative Extension Service.

Mulder, P.P., Nijs, M., Castellari, M., Hortos, M., MacDonald, S., Crews, C., Hajslova, J. and Stranska, M., 2016. Occurrence of tropane alkaloids in food. *EFSA Supporting Publications*, 13(12).

Oerke, E.C. and Dehne, H.W., 2004. Safeguarding production—losses in major crops and the role of crop protection. *Crop protection*, 23(4), pp.275-285.

Orlando, B., Maumené, C. and Piraux, F., 2017. Ergot and ergot alkaloids in French cereals: occurrence, pattern and agronomic practices for managing the risk. *World Mycotoxin Journal*, 10(4), pp.327-338.

Perharic, L., 2005. Mass tropane alkaloid poisoning due to buckwheat flour contamination. *Journal of Toxicology: Clinical Toxicology*, 43(5), p.413.

Perharič, L., Koželj, G., Družina, B. and Stanovnik, L., 2013. Risk assessment of buckwheat flour contaminated by thorn-apple (Datura stramonium L.) alkaloids: a case study from Slovenia. *Food Additives & Contaminants:* Part A, 30(2), pp.321-330.

Rahman, M.A. and Hasegawa, H., 2011. High levels of inorganic arsenic in rice in areas where arsenic-contaminated water is used for irrigation and cooking. *Science of the Total Environment*, 409(22), pp.4645-4655.

Reddy, K.R., DeLaune, R. and Craft, C.B., 2010. Nutrients in Wetlands: implications to water quality under changing climatic conditions*. Report to US Environmental Protection Agency*, EPA Contract No. EP-C-09, 1, pp.1-48.

Reddy, N.R., Pierson, M.D., Sathe, S.K. and Salunkhe, D.K., 1989. Phytates in cereals and legumes. Boca Raton CRC Press.

Requejo, R. and Tena, M., 2014. Intra-specific variability in the response of maize to arsenic exposure. *Environmental Science and Pollution Research*, 21(18), pp.10574-10582.

Rosas-Castor, J.M., Guzmán-Mar, J.L., Alfaro-Barbosa, J.M., Hernández-Ramírez, A., Pérez-Maldonado, I.N., Caballero-Quintero, A. and Hinojosa-Reyes, L., 2014. Evaluation of the transfer of soil arsenic to maize crops in suburban areas of San Luis Potosi, Mexico. *Science of the Total Environment*, 497, pp.153-162.

Sanz-Penella, J.M., Tamayo-Ramos, J.A. and Haros, M., 2012. Application of bifidobacteria as starter culture in whole wheat sourdough breadmaking. *Food and Bioprocess Technology*, 5(6), pp.2370-2380.

Schiff Jr, P.L., 2006. Ergot and its alkaloids. *American journal of pharmaceutical education*, 70(5), p.98.

Shewry, P.R. and Tatham, A.S., 2016. Improving wheat to remove coeliac epitopes but retain functionality. *Journal of cereal science*, 67, pp.12-21.

Smulders, M.J.M., Jouanin, A.A., Schaart, J.G., Visser, R.G.F., Cockram, J., Leigh, F., Wallington, E., Boyd, L.A., van den Broeck, H.C., van der Meer, I.M. and Gilissen, L.J.W.J., 2014. Development of wheat varieties with reduced contents of celiac-immunogenic epitopes through conventional and GM strategies. In *Proceedings of the 28th meeting of the Working Group on Prolamin Analysis and Toxicity* (pp. 47-56).

Stoll, A. and Hofmann, A. 1970. The chemistry of the ergot alkaloids. Ed. Van Nostrand Reinhold: New York, 1970; pp 267−301.

Taylor, J.R.N. and Duodu, K.G., 2010. Sorghum and millets: characteristics and quality requirements. Cereal Grains, pp.237-263. Ed. Cereal Grains: Assessing and Managing Quality. Woodhead Publishing Limited, Oxford, pp. 237–263.

Taylor, S.L., Baumert, J.L., Kruizinga, A.G., Remington, B.C., Crevel, R.W., Brooke-Taylor, S., Allen, K.J., of Australia, T.A.B. and Houben, G., 2014. Establishment of reference doses for residues of allergenic foods: report of the VITAL expert panel. *Food and chemical toxicology*, 63, pp.9-17.

US Department of Agriculture, Oct. 12, 2017. Grain World Markets and Trade. https://apps.fas.usda.gov/psdonline/circulars/grain.pdf (accessed December 1, 2017).

US Food & Drug Administration, 2009. CARVER+Shock Primer. https://www.fda.gov/Food/FoodDefense/FoodDefensePrograms/ucm376791.htm (accessed November 4, 2017)

USDoANAS: 2016. Agricultural Chemical Usage - Field Crops and Potatoes.http://usda.mannlib.cornell.edu/usda/current/AgriChemUsFC/AgriChemUsFC-05-15-2013.txt. (accessed November 21, 2017.)

Van Egmond, H.P., Schothorst, R. And Jonker, M., Review Regulations relating to mycotoxins in food Perspectives in a global and European context. *Analytical and Bioanalytical Chemistry*, pp.147-157.

Wu, G., Johnson, S. K., Bornman, J. F., Bennett, S. J., Singh, V., Simic, A., & Fang, Z. (2016). Effects of genotype and growth temperature on the contents of tannin, phytate and in vitro iron availability of sorghum grains. *PloS One*, 11, e0148712.).

Yadav, V. and Sharma, A., 2011. A free software for food industries to ensure food safety: CARVER+ Shock. *Comprehensive Reviews in Food Science and Food Safety*, 10(2), pp.109-117.

Cereal milling by-products:

Toward a zero-waste valorisation approach

Preface

"A successful food product: nutritious, good to consume and sustainable."

In this "challenge-opportunity" context the main goals are to strengthen the worldwide food processing system improving the nutrition aspect, without forgoing the impact on the environment creating modern and satisfactory food products [1]. The chance to recover agri-food by-products for the waste reduction with a concomitant generation of indirect profits is nowadays an "open door" [2]. On the hand, residues generated by food industries are characterised by poor stability, mainly due to their organic nature and sensitivity to degradation processes, which cause a great ecosystem load [3].

Multiple paths can be followed to attempt the reduction of by-products, most of them end in their re-introduction in food system for a specific improvement: nutritional, technological or sensory-related. The ideal situation would be the replacement of "chemical" ingredient/s, mostly additives, from the food label by a part or the entire raw or treated by-product. The results could lead to an enormous strategic market advantage: **the clean label** [4].

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[1] Fan, S., & Brzeska, J. (2016). Sustainable food security and nutrition: Demystifying conventional beliefs. Global food security, 11, 11-16.

[2] Sharma, S. K., Bansal, S., Mangal, M., Dixit, A. K., Gupta, R. K., & Mangal, A. K. (2016). Utilization of food processing by-products as dietary, functional, and novel fiber: A review. Critical Reviews in Food Science and Nutrition, 56(10), 1647-1661.

[3] Helkar, P. B., Sahoo, A. K., & Patil, N. J. (2016). Review: Food industry by-products used as a functional food ingredients. International Journal of Waste Resources, 6(3), 1-6.

[4] Ayala-Zavala, J., Vega-Vega, V., Rosas-Domínguez, C., Palafox-Carlos, H., Villa-Rodriguez, J. A., Siddiqui, M. W., ... & González-Aguilar, G. A. (2011). Agro-industrial potential of exotic fruit byproducts as a source of food additives. Food Research International, 44(7), 1866-1874.

The added value related to nutritional profile improvement, besides potential matrix-correlated beneficial effects, is mainly derived from the content of bioactive compounds present in a specific raw material, in relation to their biological activity after all the physiological functions of the organism (i.e. digestion and metabolism). Cereal grains are mostly composed by polysaccharides, proteins and lipids, however there are several compounds which can exert a bioactivity. Therefore, cereals and cereal-related products are considered as valuable sources of nutrients because of their high distribution, utilization and easy affordability. In fact, cereals intended as principal ingredient in food, provide a relative higher amount of nutrients in respect to other sources [5].

Plants develop metabolisms in response to external stimulus which end in a great and diverse abundance of substances depending on their functions (i.e. defense or communication). These functions can have a relevant bioactivity also in human organisms, where they can modulate a wide range of biological processes. Regarding this, such compounds present antioxidant and anti-inflammatory functions. Arguably, their occurrence in cereal is drastically variable due the genetic diversity, environment, cultivation and finally the method of analysis. Nowadays, the bioactive properties related to cereal and derived products can be ascribed to phytate, phenolic compounds, methyl donors, vitamins and micronutrients.

The inflammation status, at molecular level, is considered as a defense mechanism to a toxic stimulus. This process is usually well-regulated, but when errors happen, a cascade reaction occurs, and the effects are detrimental for the cell homeostasis. In this context, bioactive compounds can play a relevant role during the inflammation prevention and counteraction, for example the protection against reactive oxygen species (ROS) or the down regulation of several enzymes [6].

[5] Curtis BC. Wheat in the world. In: Curtis BC, Rajaram S, Macpherson HG, editors. Bread wheat improvement and production. Rome, Italy: Plant production and protection series. FAO 2002. p. 1-19.

[6] Medzhitov R. Origin and physiological roles of inflammation. Nature. 2008; 454:428-35

Therefore, putting together the potential embedded in cereal grain by-products and the challenge to improve sustainability, more efforts are currently spent for the development of innovative and valuable food products. During the last decades, a part of research in food science which deals with the relationship between consumer and foods has been prioritised [7]. At the same time the offer of food with high functional level increased rapidly. This joint situation led to a higher consciousness of consumer towards these products, resulting in a different attitude and greater expectation in terms of taste and health [8]. However, this segment of the market is highly competitive, and it is strictly regulated by several policies. In fact, with the perspective of by-products utilization, opportunely treated, as functional ingredients in food formulation, they must respect the European regulation No. 258/97, concerning the novel foods and novel ingredient: materials for human consumption not used in Europe before 1997 [9]. Therefore, certain food by-products used for a specific function and, therefore included in a recipe have to be previously authorised.

[7] Iriondo-DeHond, M., Miguel, E., & Del Castillo, M. D. (2018). Food byproducts as sustainable ingredients for innovative and healthy dairy foods. Nutrients, 10(10), 1358

[8] Verbeke, W. (2006). Functional foods: Consumer willingness to compromise on taste for health? Food quality and preference, 17(1-2), 126-131.

[9] Regulation, H. A. T. (1997). Regulation (EC) No 258/97 of the European Parliament and of the Council of 27 January 1997 concerning novel foods and novel food ingredients. Off. J. Eur. Communities, 40, 1-7

The bioeconomy concepts

In last years the concepts of bio and circular economy have risen up [10]. This term refers to a set of actions related to the development, production and processing improvement which impact on the overall society economy system. The transition to an integrated bioeconomy model uses of renewable biological resources and the corresponding conversion to a high-value goods/services as mainly goal. All these is strongly correlated to a business vision and a sustainable environment fixed in a long period [11]. The latter process relies firstly, on the interactions between local resources and both public and private shareholders, under the European policy umbrella.

Food industry is not the only sector, but is largely the most important, at European level, characterised by a growth increase in traditional and innovative industries. The initial point to start the bioeconomy integration is the identification of the challenges regarding the by-products reutilisation:

- The formulation of novel foods or innovative ingredients with high-nutritional value;
- The study of new food processes for the production of valuable resources;
- Strengthen the economy by reducing costs or creating new market segments;
- To maintain as low as possible the impact on the environment during the previous steps.

The following section is dedicated to the literature review regarding the innovative technologies applied to the cereal milling by-product “rice bran”, the analysis of their effects in terms of chemical and physical changes and the nutritional properties when included in food system and supplemented in a diet.

[10] http://www.clusterspring.it/wp-content/uploads/notizie/BIT_v4_ENG_LUGLIO_2017.pdf
[11] Closing the loop - An EU action plan for the Circular Economy, COM/2015/0614 final

Rice bran, in these terms, has been studied because of their high content of potentially health beneficial compounds such as, phenolics, prebiotics oligosaccharides and arabinoxylans. Moreover, rice bran can be used as raw material in gluten-free production, leading to a conceivable improvement in terms of nutritional quality of such food products.

However, the enormous potential of the technologies here described is that they are completely transferable to other agri-food side streams, such as wheat bran or maize germ, making appropriate modifications. Although the degree and nature of matrix alterations must be determined with further studies. Meanwhile other research should be focussed in the sensory aspect improvement, capable to connect and study new textural and flavour properties for the achievement of healthier and taster products correctly placed in the modern market.

Rice bran: from valorisation strategies to nutritional perspectives

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Abstract

Rice bran (*Oryza sativa*) is the main by-products of rice grain processing. Produced in large quantities, it contains a high amount of valuable nutrients and bioactive compounds with important health-related effects. Despite that, its applications in food industry is still poor because of its oxidation sensitivity, instability and bad technological suitability. However, many innovative techniques have been proposed during the last decades in order to convert this by-product into a valuable food ingredient with the final aim to produce high-added value food products which are summarized in the present review. The health-related effects pre-treated rice bran are also presented considering the up to date literature focused on both *in vivo* and *in vitro* studies. Finally, in relation to this aspect a brief description of rice bran arabinoxylans is described.

Keywords: Rice bran; cereal by-products, food bioprocessing; bioactive compounds, nutritional value, rice bran arabinoxylans.

Introduction

Food processing is a set of operation that permits to transform raw materials into valuable food products. Cereal crops, for example, are rarely consumed as whole grain and during their transformation process a huge amount of residues are produced. Rice bran (RB) is a by-product that derives from the milling of rice grain, the third most consumed cereal overworld [1]. It represents around 12% of the total kernel weight and it is composed by the external layer of the seed (i.e. pericarp, tegmen and aleurone), translatable in almost 68 million tons of unmanageable material per year, worldwide [2]. Similarly to other cereal species, the kernel surrounding layers are richer in bioactive compounds, minerals, vitamins, dietary fibers, proteins and lipids than the core endosperm, which is characterized by simply carbohydrates and starch granules [3,4]. In particular, rice bran has a not negligible amount of lipids (15-20 g/100 g of RB) where some of the most important bioactive compounds, such as γ-oryzanol, ferulic acid, tocopherols and polyunsaturated fatty acids could be found, thus for this reason RB has been used for oil extraction [5]. Despite that, RB is highly sensitive to lipid oxidation due to the rapid activity of lipolytic endogenous enzymes which makes a thermal stabilization step usually required [6]. However, the first fate of rice bran is the feed formulation industry, losing the opportunity for the recovery of its potential. Therefore, the study of innovative recovery strategies focused on the valorization of agro-industrial by-products is nowadays an interesting growing sector directed to an improved sustainability and dietary habits of the whole food system. In this work, the strategies for the valorization of rice bran and its recent nutritional evidences will be briefly reviewed (**Figure 8**).

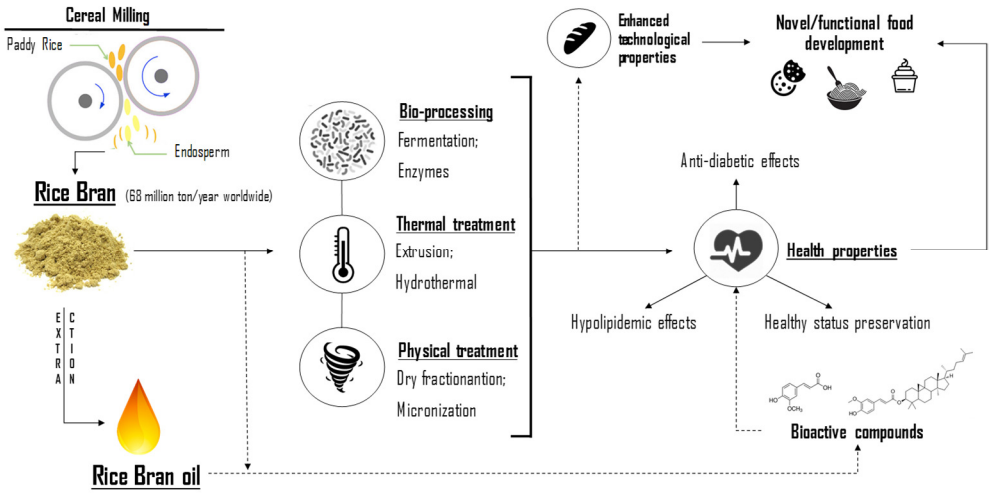

Figure 8. Rice bran production, the treatments applied for its improvement and the nutritional evidences for the formulation of novel or functional food ingredients.

Strategies for the recovery of rice bran potential

The smart recovery of food processing residues is an important aspect for the food industry which always is in search of value generation. In this context, a great benefit may derive from different branches of science, such as biotechnology, chemistry or physics applied to the food scenario. The most studied innovative techniques applied to produce a high-value ingredient useful for the food manufacturers are summarized in **Table 1.**

Table 1. Innovative technologies applied to rice bran by-product and corresponding outcomes.

Treatment		Study outcomes	Reference
Bioprocessing	*Microorganism/complex enzymes*		
SSF of autoclaved RB for 12 days	*Rhizopus oligosporus* and *Monascus purpureus*	Increased phenolic acid content and AOA.	[7]
SSF of RB during 120 h	*Rhizopus oryzae (CCT 1217)*	Two-fold increased phenolic compound content and increased inhibition for DPPH and for the peroxidase enzyme.	[8]
SSF of autoclaved RB during 120 h	*Rhizopus oryzae (CCT 7560)*	Changes in lipid, fatty acids and phospholipids. Decreased saturated fatty acids content and increased unsaturated ones.	[9]
SSF of heat stabilized RB during 24 h	*Saccharomyces boulardii*	Generation of novel metabolite with bioactivity	[10]
Fermentation of defatted RB water extract for 9 days	*Grifola frondosa*	Polysaccharides molecular weight changes from 10^3-10^4 Da to 10^2-10^3 Da, after fermentation. Enhanced antioxidant activities and effects on the production of NO significantly	[11]
RB enzyme treatment coupled to SSF for 35 h.	*Lactobacillus acidophilus* (GIM 1.731) and *Lactobacillus plantarum* (GIM 1.648); α-amylase, 0.5% glucoamylase, 1.5% acid protease and 1.5% acid cellulase	Improved total phenolic content and antioxidant activity.	[17]

(Table 1 continued)

Bioprocessing	***Microorganism/complex enzymes***	**Study outcomes**	**Reference**
RB enzyme treatment	Treated with cellulase and xylanase during 90 min	Higher soluble and total polyphenols, flavonoids, γ-oryzanol content, free and total antioxidant activity.	[15]
RB enzyme treatment	Treated with alcalase for 30 min and endoglucanase for 1 h	Higher γ-oryzanol, α-tocopherol and polyphenols recovery improved total antioxidant activity. Increased SCFAs (acetic acid and propionic acid) content. Total recovery of B group vitamins after enzyme treatment.	[16]
Thermal treatment	***Conditions***	**Study outcomes**	**Reference**
Hot-air and FIR of RB	120° C for 30 min using hot-air oven; FIR intensity of 2 kW/m^2 and dried at 40 °C for 2 h.	FIR radiated RB had higher content of bioactive compounds such as tocopherols phenolic acids and antioxidant activities, compared with hot air dried	[21]
Ex and Ex in combination with enzymes of native, defatted and stabilized RB	Xylanase 55 °C for 5 h; screw speeds 50 and 100 rpm barrel temperature of the first three zones constant at 60 °C die temperature at 100 °C.	Improved solubility of RB dietary fiber and other soluble components, especially the sequential extrusion-enzyme treatment.	[18]

(Table 1 continued)

Thermal treatment	***Conditions***	**Study outcomes**	**Reference**
Extrusion of defatted RB	Screw-speeds 80 and 160 revolution per minute. The barrel temperature was set at 80–140° C	Extraction of arabinoxylans increased significantly with an increase in screw speed	[22]
Extrusion	Single-screw extruder. Optimum processing conditions determined through response surface methodology.	Improvement of the emulsifying and foaming properties of extruded rice bran protein.	[23]
Physical treatment	***Conditions***	**Study outcomes**	**Reference**
Dry fractionation of defatted RB	Pin mill at ambient temperature, 22,000 rpm, air flow of 75m^3/h. Electrostatic separation flow rate of the carrier nitrogen gas 20 L/min, the applied voltage to the positive electrode 20 kV and the distance between the electrodes 10 cm. Air jet sieving.	Dietary fiber enrichment of the RB fractions.	[24]
HHP and HHP in combination with enzymes	100 MPa pressure at 50 °C for 24 h. Commercial pectinolytic and cellulolytic enzymes.	Increasing of the extractability of ferulic acid; inhibition of linoleic acid oxidation	[19]
Defatted RB enzymatic treatment and enzymatic treatment combined to micronization	Cellulase and xylanase; wet-milling with a planetary ball mill at 3000 r/min for 2 h.	Modified structural and functional properties of rice bran dietary fibers.	[20]

Abbreviations: SSF, solid-state fermentation; RB, rice bran; AOA, antioxidant activity; NO, nitric oxide; SCFAs, short chain fatty acids; FIR, far-infrared radiation; Ex, extrusion; HHP, high hydrostatic pressure.

Bioprocessing of rice bran: solid-state fermentation and enzymatic treatment

Food bioprocessing is that term used to define an operation which combines living cells or their components to a normal procedure in order to obtain a different food product. Nowadays, solid-state fermentation (SSF) and the use of specific enzymes are widely studied and applied in food industry, having the principal aim to improve the characteristics of the raw materials. The most important, from nutritional point of view, is the fact that the microorganism metabolism or selective enzymes can disrupt the vegetable cell matrix releasing those compounds that present a bioactivity, enhancing their bioavailability in human organism. Regarding the fermentation, both fungi and bacteria can take part to this process. Obviously, the metabolic characteristics of each are different, as well as the resulting raw material modifications. For rice bran fermentation, fungus and yeasts are the mostly used microorganisms. For example, in the study of Abd Razak *et. al.* [7], they used *Rhizopus oligosporus* and *Monascus purpureus* both alone and in combination, for the fermentation of sterilized rice bran. The study analysed the overall antioxidant capacity (AOA) and bioactive compounds enhancement caused by the treatment. Interestingly the two microorganisms used in co-culture resulted in a significantly higher AOA and phenolic acids in respect to the non-fermented bran. Similar outcomes were obtained by Schmidt *et. al.* [8], where rice bran was fermented with *Rhizopus oryzae (CCT 1217)* during 120 hours. They measured AOA, phenolic acids content and the inhibition activity against peroxidase enzyme, which is the mainly responsible for the lipid oxidation. The AOA increased in respect to the non-treated rice bran and phenolic compounds, such as ferulic and gallic acids, doubled their initial concentration. This fact can only be attributed to the cell-wall disrupting metabolism of microorganisms, and not to their direct biosynthesis, since fungi cannot produce such secondary metabolites. Also Oliveira *et. al.* [9] used the same fungus (different strain), but they focused the study on the lipid compounds.

Interestingly, the total lipid content of fermented rice bran diminished after the fermentation, but the phospholipids slightly increased, indicating that the microorganism could metabolize such molecules. Moreover, the saturated and unsaturated fatty acids content was altered, probably as a combined action of oxidation and fungus metabolism. Ryan *et al.* [10], followed a different but innovative approach to characterize fermented rice bran from three different rice varieties using *Saccharomyces boulardii*, a yeast. Employing a metabolomic gas chromatography coupled to mass spectrometry (GC-MS) based technique they could identify and quantify new bioactive metabolites and assess their action on normal human peripheral blood lymphocytes (PBL) and malignant human B-cell lymphoma cell lines, resulting in a growth reduction of the latter ones. Besides phenolic compounds, rice bran polysaccharides (RBPSs) have gained more interest due to their multiple biological benefits. In the study carried out by Liu *et al.* [11], they fermented a defatted rice bran water extract using the fungus *Grifola frondosa*, aiming to evaluate the composition of the oligosaccharides fraction, AOA and production of nitric oxide (NO) in macrophages cells. They reported a substantial shift of the polysaccharides molecular weight, from higher to lower, beside the increased AOA and the adjustment of the NO production compared to the non-fermented sample.

Enzymes that have specific activities are widely spread in food industry [12]. Their capacity to improve functional and sensory features of food products is accompanied by the fact that their impact on the native composition is very low [13]. Regarding cereal related products, enzymes are used to modify the principal component of that foods, such protein, lipids and carbohydrates [14]. In fact, in the study conducted by Prabhu *et al.* [15], they treated rice bran with cellulase and xylanase with the aim to solubilize polyphenols with the corresponding improving of the AOA. Similarly, Vallabha *et al.* [16], treated rice bran with alcalase and endoglucanase, resulting in an increased content of bioactive molecules with no effect on the retention of B group vitamins after the enzymatic treatment.

They also reported an increment of the short chain fatty acids (SCFAs) content in respect to the non-treated rice bran. Moreover, enzymatic processes are usually utilized in combination with other techniques such as fermentation [17], extrusion [18], high hydrostatic pressure (HHP) [19] or micronization [20], leading to an increased extractability of the bioactive component, a solubilization of dietary fibers and inhibition of lipid oxidation.

Thermal treatment of rice bran

The conscious manipulation of heat has been always used for the transformation of food material in, generally, quality-improved products. In current years, several new technologies have arisen thanks to their potential. Extrusion, hot air and far infrared irradiation are some of these. For example, Wanyo *et al.* [21] used hot air and far-infrared radiation (FIR), alone and in combination to assess the effects on the antioxidant properties and bioactives content of rice bran and husk. They reported that the use of FIR technology conferred to rice bran a higher content of bioactive compounds, mainly phenolics, and AOA in respect to the classic hot air treatment. Unfortunately, no mention on the sensorial characteristics were present in latter study. Furthermore, in the experiment carried out by Dang and Vasanthan [18], they performed a sequential enzyme treatment and extrusion, concluding in an increased total soluble pentosan content compared to the to individual or simultaneous treatments. Moreover, Fadel *et al.* [22] and *Liu et al.* [23] applied extrusion techniques at different conditions reporting an increased extraction of arabinoxylans and improved foaming and emulsifying properties of the extruded bran, respectively.

Physical treatment of rice bran

Food science is continuously modernizing studying innovative techniques with minimum impact on both products and environment. Physical treatments are those operations based on physics principals applied for many objectives.

In the case of cereal products, such rice grain, the properties of porous and fine material (i.e. flour) is the main aspect to be taken into account. Separation, concentration and physical properties modification of specific food component are the main paths followed by researchers, achieved using dry fractionation, air classification and particle size reduction technologies. For example, Wang *et al.* [24], applied a pin mill fractionation coupled to an electrostatic separation to defatted rice bran. The mainly outcome they reported was a dietary fibre enrichment of the coarse fraction of rice bran, remarking the redistribution effect proportioned by this type of technology. Nevertheless, more studies must be carried out in order to optimize this type of treatments.

Rice bran application in food industry

The most important step for the recovery of food by-product is the incorporation of such raw material in food product formulation. The primary objective is to add value to the final product, both enhancing nutritional properties or sensorial characteristics, such as colour, taste, smell and texture. The latter functions are also important form the labelling point of view, since the lack of additives which are replaced by the recovered material lead to a so called “green-label”. Rice bran have also another advantage, represented by its suitability for gluten-free products manufacturing. These products usually are poor in term of sensorial quality but have a high value in market. Nevertheless, is highly recommendable that rice bran must be stabilized or defatted before its use as an ingredient, considering its sensibility to peroxidation and off-flavour formation. This can be easily achieved through the application of thermal and non-thermal techniques, as reported in Section 2. Sairam *et al.* [25] has analysed the addition of different concentration of defatted rice bran (DRB) in bread, aiming to a bread nutritional profile improvement. Total dietary fibers, AOA and shelf-life of bread increased with no repercussions on the sensorial properties.

In relation to this, Al-Okbi *et al*. [26] formulated tortilla chips and corn flakes adding different amount of rice bran to the original recipe noticing an organoleptic and rheological amelioration of the final product when the protein content decreased. Premakumari *et al*. [27] also tried to develop high fiber content ready mixes replacing the classic cereal flours with a different amount of previously stabilised RB evaluating their overall acceptability through 20 semi trained panel members. The mainly outcome showed that at least 25 % replacement did not affect the quality of the standard recipe. Moreover, Younas *et al.* [28] developed cookies using both heat and acid stabilized RB, optimizing the recipe with a 10% RB substitution. Since RB oil is considered a high added value product, the RB solid exhaust can still be used as low-fat content ingredient. For example Charunuch *et al.* [29] utilized DRB at different rate for the preparation of extruded breakfast cereal. However, the use of pre-treated rice bran by means of bioprocessing, thermal and physical treatments in food preparation is still scarce, possibly due to the lack of industrial-scale or at least pilot-scale studies.

Rice bran: nutritional evidences

The relationship between diet and health is becoming stronger. In relation to this, also the dietary patterns of developed countries are changing, showing a growing incidence of many diet-related disorders and diseases (cardiovascular disease (CVD), high cholesterol, diabetes, bowel inflammation, etc.). Rice bran is a milling processing by-product with high content of relevant bioactive compounds which can play an important role to maintain a healthy status and thus promote a beneficial living style. However, this raw material is currently discarded notwithstanding its potential as functional ingredient [30] or its potential chemo-preventive and immunomodulatory properties [31–34]. On the other hand, despite scientific proved evidences, foods and its constituents should not be considered as medical replacements but as a complementary part of the overall treatment.

Rice bran against hyperlipidemia, hyperglicemia and other health disorders

Recently, an increasing number of studies analyzed the multiple health-related properties of rice bran supplemented diet [34]. These evidences are summarized in **Table 2**. Besides its high nutrient and bioactive compounds content, rice bran can be administered in different ways, such as stabilized RB, fermented RB, enzymatically treated RB or as oil. Each of these products differ from the raw RB in terms of nutrients and non-nutrients compounds. For this reason, a previous profiling of the product under study is highly recommended in order to define the chemical compound or mixture of compounds that deliver the effects in a pre-determined diet.

Health conditions that determine an increased concentration of lipid molecules and cholesterol in blood, are a branch of a disease known as hyperlipidemia. It can raise from both genetic conditions or bad dietary habits, which could finally lead to an increased risk of CVD. For this reason, nutrition is the most important variable that must be taken under control for the treatment of such ailment. From this perspective, rice bran and its oil are rich in components which can contrast the accumulation of lipids in blood stream, such as phytosterols, unsaturated fatty acids (FAs) and tocotrienols [32,35]. In fact, the oil recovered from rice bran include glycolipids, phospholipids, free fatty acid and triglycerides with healthy profile, since oleic, linoleic (mono- and polyunsaturated) and palmitic acids (saturated) are the most abundant FAs. In a recent study, Perez-Ternero *et al.* [36] evaluated the lipid-lowering properties of rice bran enzymatic extract (RBEE) diet supplementation in apolipoprotein E-knockout (ApoE−/−) mice. They reported a higher high-density lipoprotein (HDL) serum value and an increased cholesterol excretion. The same authors reported also other protective properties of RBEE such as a restored endothelial function, prevention of high lipid in blood, oxidative stress, inflammation and cell apoptosis reduction in ApoE−/− mice aorta [37,38].

Revilla *et al.* [39] also used RBEE as diet supplementation in male Wistar rats, which resulted in an increased HDL and lower blood cholesterol concentrations in plasma. Besides, Wilson *et al.* [40] settled up a comparative study in which they evaluated the potential of trans-ferulic acid, γ-oryzanol and rice bran oil supplementation to lower the cholesterol concentration in primates. Among the groups studied, the one fed with γ-oryzanol showed the lower levels of low and very low-density lipoprotein (LDL and VLDL) and plasma cholesterol. Similar outcomes were reported by Ausman *et al.* [41] and Ha *et al.* [42], where they fed hypercholesterolemic hamsters and Male Sprague-Dawley rats, respectively, with different amount of rice bran oil. Moreover, Accinni *et al.* [43] studied the effects of various dietary supplementation, including γ-oryzanol, on the lipid profile of dyslipidemic subjects. Interestingly, results reported by the latter study indicate that the subjects which followed a supplementation diet with γ-oryzanol and niacin recovered a normal lipid blood pattern, better than the other food supplementation studied.

The presence of high level of glucose in blood system is a disturb that nowadays is extremely spread in developed country populations which is mainly associated with bad diet habits and thus could lead to a harmful healthy status, such as diabetes and hyperglycemia. In this way, vegetable origin food products, like rice bran, that contains a low free sugar and high dietary fiber content can contribute to maintain a lower glycemic index and help to the illness prevention. For example, Son *et al.* [44] studied the regulation effects of γ-oryzanol on the insulin secretion and glucose concentration in plasma, supplemented to male C57BL/6N mice. Same results were reported in the study of Somsuvra and Ghatak [45], in which adult Wistar rats with a γ-oryzanol supplemented diet had the lower serum glucose content. Moreover, Qureshi *et al.* [46] expanded the study to humans volunteers with Type I and II diabetes mellitus. Rice bran was administered as stabilized and as water extract, leading to an increased insulin serum level, decreased glucose and glycosylated hemoglobin concentration.

Since in rice bran are present a wide range of bioactive molecules, also the effects on the oxidative stress are currently object of study. Justo *et al*. [47], have reported a reduction of microvascular inflammation status in obese Zucker rats which follow a diet supplemented with RBEE. Moreover, Perez-Ternero *et al.* [48] also studied the supplementation of RBEE in Wistar rats diet, showing an inactivated superoxide production caused by an increased content of phenolic compounds in blood.

Other researches have been made focusing on the multiple effects of rice bran supplemented under various forms. For example, fermented brown rice bran using *Aspergillus oryzae* which induced the apoptosis in human acute lymphoblastic leukemia cells [49], or the anti-stress and anti-fatigue effects delivered by *Saccharomyces cerevisiae* fermented rice bran [50].

Rice bran arabinoxylans: immunomodulatory properties

Recently, the saccharide component of foods is receiving more attention. The mainly reason of this is the fact that always carbohydrates have not been studied in depth, since their major analytical issues [51]. Despite that, many biological activities have been attributed to molecules like arabinoxylans of low and high molecular weight. As widely known, carbohydrates are major components of cereals, in fact they have the primary function of seed energy storage. In detail, arabinoxylans are composed by xylan backbone (β-(1,4)-D-xylopyranose) linked to α-L-arabinofuranosyl substitutions, which differs among cereal species, conferring them different arrangement and thus different functions [52]. In fact, huge effort in terms of research are made to study the rice bran arabynoxylans-immunomodulatory relationship, capable to enhance the activity of the innate and adaptative responses. These mechanisms are responsible to activate the B-lymphocytes production, our first defence line from foreign molecules [53,54].

In this way, rice bran is usually enzymatically treated in order to break high molecular weight arabinoxylans into more little pieces ranging 30-50 Da, producing a product commercially called BioBran or MGN-3 [55]. These fraction appears to act as an enhancer of natural killer cell, macrophage phagocytosis, B and T cells functions [30]. Ghoneum *et al*. studies in depth these effects [56–62]. Furthermore, *Choi et al*. [63] reported similar outcomes after supplementation of the soluble arabinoxylans fraction of wheat bran in mice diet. A proposed theory of the mechanism of action has been proposed [64], stating that the complexity of the structure of the heteropolysaccharides like galactan, arabinan and β-1,3:1,4- glucan is the basis of the immunomodulatory actions of rice bran arabinoxylans.

Other positive effects (Table 2) of rice bran arabinoxylans were reported by Salama *et al.* [65], where the MGN-3 supplementation in patient with chronic hepatitis C virus suppressed the level of viremia. Or Zheng *et al.* [66,67] studies which reported protective effects against acute liver injury. Besides, Wang *et al.* [68] showed an improved anti-complementary activity of DRB under *in vitro* conditions.

Table 2. Studies reporting the nutritional evidences of rice bran as functional food, the mainly compounds responsible for such functions and the type of experiment followed.

Health claim	*Effect or evidence*	*Supplementation*	*Type of experiment*	*Reference*
Hypolipidemic effects	Regulation of the metabolism-related gene expression; Lower blood lipid concentration	RBEE	ApoE−/− mice	[36]
	Lower LDL, VLDL and total plasma cholesterol levels.	Trans-ferulic acid, γ-oryzanol, and rice bran oil.	Cynomologus monkeys (*Macaca fascicularis*)	[40]
	Reduced LDL cholesterol and serum total cholesterol levels.	Rice bran oil	Hypercholesterolemic hamsters	[41]
	Abnormal to normal shifting of blood lipids profile.	γ-Oryzanol, tocols, niacin, and omega-3 polyunsaturated fatty acids	Human volunteers with abnormal blood lipid levels	[43]
	Increased HDL and decreased cholesterol levels.	RBEE	Male Wistar rats	[39]
	Increased serum HDL. Recovered serum aspartate aminotransferase activity.	Rice bran oil	Male Sprague-Dawley rats	[42]
Hypoglycaemic potential	Regulated secretion of insulin and glucose. Normalized liver enzyme activities	γ-Oryzanol	Male C57BL/6N mice	[44]
	Serum glucose decrement.	γ-Oryzanol	Adult Wistar rats	[45]
	Glucose levels and glycosylated haemoglobin; Increased serum insulin levels.	Stabilised rice bran and RB water extract	Humans with diabetes mellitus Types I and II	[46]

(Table 2 continued)

Health claim	***Effect or evidence***	***Supplementation***	***Type of experiment***	***Reference***
Reduction of oxidative stress	Substantial reduction of microvascular inflammation and superoxide anion formation.	RBEE	Obese Zucker rats	[47]
	Inactivated superoxide production through phenolic compound-enriched plasma.	RBEE	Wistar rats	[48]
Cardiovascular status improvement	Amelioration of the oxidative stress related to atherosclerosis	RBEE	ApoE−/− mice	[37]
Virus-contrasting therapy	Suppression of viremia level.	Rice bran arabinoxylan (Biobran)	37 chronic Hepatitis C Virus patients were randomized into two groups	[65]
Suppressive action on GalN-induced hepatitis	Protective effect on acute liver injury. Inhibition of NF-κB and JNK/MAPK expression.	Low molecular weight arabinoxylans	Wistar rats	[67]

Abbreviations: RBEE, rice bran enzymatic extract; LDL, low density lipoprotein; HDL, high density lipoprotein; VLDL, very low-density lipoprotein, NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; JNK, c-Jun N-terminal kinases; MAPK, mitogen-activated protein kinase.

Conclusions and future perspective

Rice bran represents a massive industrial by-product with a relevant "hide" potential value. Its composition in terms of nutritional features comprehend a wide range of bioactive compounds which have been studied along current years. Furthermore, with the aid of most advanced food related technologies the quality parameters of RB can be highly improved, making it a valuable and sustainable resource for a healthy diet promotion. In fact, huge efforts have been made in order to include this ingredient in widely consumed food products, mainly baked goods. Then, several health claims suggested the importance of including RB, in its various forms, in diet, but the mechanism of action underlying such positive effects should be studied more in depth. In this way, is important to stress the fact that foods and its components must not be intended as the main instruments to struggle with an illness status. Finally, also different type of novel products can be studied, such as innovative beverages or dairy products with high nutritive quality.

Authors contribution

MdC, CD, GG and MS designed the manuscript. MS wrote the review. All the authors contributed to the critical review of the paper.

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References

1. Food and Agriculture Organization of the United Nations: Statistical Databases (FAOSTAT) Production/Crops. (Accessed on 12/12/2018)

2. Kahlon, T. Rice bran: Production, composition, functionality and food applications, physiological benefits. In *Fiber ingredients: Food applications and health benefits. S*; 2009 ISBN 9780124017160.

3. Fritsch, C.; Staebler, A.; Happel, A.; Márquez, M.A.C.; Aguiló-Aguayo, I.; Abadias, M.; Gallur, M.; Cigognini, I.M.; Montanari, A.; López, M.J.; et al. Processing, valorization and application of bio-waste derived compounds from potato, tomato, olive and cereals: A review. *Sustain.* 2017.

4. Schieber, A. Side Streams of Plant Food Processing As a Source of Valuable Compounds: Selected Examples. *Annu. Rev. Food Sci. Technol.* **2017**.

5. Gul, K.; Yousuf, B.; Singh, A.K.; Singh, P.; Wani, A.A. Rice bran: Nutritional values and its emerging potential for development of functional food - A review. *Bioact. Carbohydrates Diet. Fibre* **2015**.

6. Gopinger, E.; Ziegler, V.; Catalan, A.A. da S.; Krabbe, E.L.; Elias, M.C.; Xavier, E.G. Whole rice bran stabilization using a short chain organic acid mixture. *J. Stored Prod. Res.* **2015**.

7. Abd Razak, D.L.; Abd Rashid, N.Y.; Jamaluddin, A.; Sharifudin, S.A.; Long, K. Enhancement of phenolic acid content and antioxidant activity of rice bran fermented with Rhizopus oligosporus and Monascus purpureus. *Biocatal. Agric. Biotechnol.* **2015**.

8. Schmidt, C.G.; Gonçalves, L.M.; Prietto, L.; Hackbart, H.S.; Furlong, E.B. Antioxidant activity and enzyme inhibition of phenolic acids from fermented rice bran with fungus Rizhopus oryzae. *Food Chem.* **2014**.

9. Oliveira, M. dos S.; Feddern, V.; Kupski, L.; Cipolatti, E.P.; Badiale-Furlong, E.; De Souza-Soares, L.A. Changes in lipid, fatty acids and phospholipids composition of whole rice bran after solid-state fungal fermentation. *Bioresour. Technol.* **2011**.

10. Ryan, E.P.; Heuberger, A.L.; Weir, T.L.; Barnett, B.; Broeckling, C.D.; Prenni, J.E. Rice bran fermented with Saccharomyces boulardii generates novel metabolite profiles with bioactivity. *J. Agric. Food Chem.* **2011**.

11. Liu, Q.; Cao, X.; Zhuang, X.; Han, W.; Guo, W.; Xiong, J.; Zhang, X. Rice bran polysaccharides and oligosaccharides modified by Grifola frondosa fermentation: Antioxidant activities and effects on the production of NO. *Food Chem.* **2017**.

12. Whitehurst, R.J.; van Oort, M. *Enzymes in Food Technology: Second Edition*; 2009; ISBN 9781444309935.

13. Parrado, J.; Miramontes, E.; Jover, M.; Gutierrez, J.F.; Collantes de Terán, L.; Bautista, J. Preparation of a rice bran enzymatic extract with potential use as functional food. *Food Chem.* **2006**.

14. Poutanen, K. Enzymes: An important tool in the improvement of the quality of cereal foods. *Trends Food Sci. Technol.* 1997.

15. Prabhu, A.A.; Jayadeep, A. Enzymatic processing of pigmented and non pigmented rice bran on changes in oryzanol, polyphenols and antioxidant activity. *J. Food Sci. Technol.* **2015**.

16. S Vallabha, V.; Indira, T.N.; Jyothi Lakshmi, A.; Radha, C.; Tiku, P.K. Enzymatic process of rice bran: a stabilized functional food with nutraceuticals and nutrients. *J. Food Sci. Technol.* **2015**.

17. Liu, L.; Zhang, R.; Deng, Y.; Zhang, Y.; Xiao, J.; Huang, F.; Wen, W.; Zhang, M. Fermentation and complex enzyme hydrolysis enhance total phenolics and antioxidant activity of aqueous solution from rice bran pretreated by steaming with α-amylase. *Food Chem.* **2017**.

18. Dang, T.T.; Vasanthan, T. Modification of rice bran dietary fiber concentrates using enzyme and extrusion cooking. *Food Hydrocoll.* **2019**, *89*, 773–782.

19. Kim, D.; Han, G.D. High hydrostatic pressure treatment combined with enzymes increases the extractability and bioactivity of fermented rice bran. *Innov. Food Sci. Emerg. Technol.* **2012**.

20. Wen, Y.; Niu, M.; Zhang, B.; Zhao, S.; Xiong, S. Structural characteristics and functional properties of rice bran dietary fiber modified by enzymatic and enzyme-micronization treatments. *LWT - Food Sci. Technol.* **2017**.

21. Wanyo, P.; Meeso, N.; Siriamornpun, S. Effects of different treatments on the antioxidant properties and phenolic compounds of rice bran and rice husk. *Food Chem.* **2014**.

22. Fadel, A.; Plunkett, A.; Ashworth, J.; Mahmoud, A.M.; Ranneh, Y.; El Mohtadi, M.; Li, W. The effect of extrusion screw-speed on the water extractability and molecular weight distribution of arabinoxylans from defatted rice bran. *J. Food Sci. Technol.* **2018**.

23. Liu, C.; Zhang, Y.; Liu, W.; Wan, J.; Wang, W.; Wu, L.; Zuo, N.; Zhou, Y.; Yin, Z. Preparation, physicochemical and texture properties of texturized rice produce by Improved Extrusion Cooking Technology. *J. Cereal Sci.* **2011**.

24. Wang, J.; Suo, G.; De Wit, M.; Boom, R.M.; Schutyser, M.A.I. Dietary fibre enrichment from defatted rice bran by dry fractionation. *J. Food Eng.* **2016**.

25. Sairam, S.; Gopala Krishna, A.G.; Urooj, A. Physico-chemical characteristics of defatted rice bran and its utilization in a bakery product. *J. Food Sci. Technol.* **2011**.

26. Al-Okbi, S.Y.; Hussein, A.M.S.; Hamed, I.M.; Mohamed, D.A.; Helal, A.M. Chemical, rheological, sensorial and functional properties of gelatinized corn- rice bran flour composite corn flakes and tortilla chips. *J. Food Process. Preserv.* **2014**.

27. Premakumari, S.; Balasasirekha, R.; Gomathi, K.; Supriya, S.; Mohan, J.; Alagusundram, K. Development and Acceptability of Fibre Enriched Ready Mixes. *Int. J. Pure Appl. Sci. Technol. Int. J. Pure Appl. Sci. Technol* **2012**.

28. Younas, A.; Bhatti, M.S.; Ahmed, A.; Randhawa, M.A. Effect of rice bran supplementation on cookie baking quality. *Pakistan J. Agric. Sci.* **2011**.

29. Charunuch, C.; Limsangouan, N.; Prasert, W.; Wongkrajang, K. Optimization of extrusion conditions for ready-to-eat breakfast cereal enhanced with defatted rice bran. *Int. Food Res. J.* **2014**.

30. Sharif, M.K.; Butt, M.S.; Anjum, F.M.; Khan, S.H. Rice Bran: A Novel Functional Ingredient. *Crit. Rev. Food Sci. Nutr.* **2014**.

31. Henderson, A.J.; Ollila, C.A.; Kumar, A.; Borresen, E.C.; Raina, K.; Agarwal, R.; Ryan, E.P. Chemopreventive Properties of Dietary Rice Bran: Current Status and Future Prospects. *Adv. Nutr. An Int. Rev. J.* **2012**.

32. Sohail, M.; Rakha, A.; Butt, M.S.; Iqbal, M.J.; Rashid, S. Rice bran nutraceutics: A comprehensive review. *Crit. Rev. Food Sci. Nutr.* **2017**.

33. Park, H.Y.; Lee, K.W.; Choi, H.D. Rice bran constituents: immunomodulatory and therapeutic activities. *Food Funct.* **2017**.

34. Friedman, M. Rice brans, rice bran oils, and rice hulls: Composition, food and industrial uses, and bioactivities in humans, animals, and cells. *J. Agric. Food Chem.* 2013.

35. Cicero, A.F.G.; Gaddi, A. Rice bran oil and γ-oryzanol in the treatment of hyperlipoproteinaemias and other conditions. *Phyther. Res.* 2001.

36. Perez-Ternero, C.; Claro, C.; Parrado, J.; Herrera, M.D.; Alvarez de Sotomayor, M. Rice bran enzymatic extract reduces atherosclerotic plaque development and steatosis in high-fat fed ApoE−/− mice. *Nutrition* **2017**.

37. Perez-Ternero, C.; Bermudez Pulgarin, B.; Alvarez de Sotomayor, M.; Herrera, M.D. Atherosclerosis-related inflammation and oxidative stress are improved by rice bran enzymatic extract. *J. Funct. Foods* **2016**.

38. Perez-Ternero, C.; Herrera, M.D.; Laufs, U.; Alvarez de Sotomayor, M.; Werner, C. Food supplementation with rice bran enzymatic extract prevents vascular apoptosis and atherogenesis in ApoE−/− mice. *Eur. J. Nutr.* **2017**.

39. Revilla, E.; Maria, C.S.; Miramontes, E.; Bautista, J.; García-Martínez, A.; Cremades, O.; Cert, R.; Parrado, J. Nutraceutical composition, antioxidant activity and hypocholesterolemic effect of a water-soluble enzymatic extract from rice bran. *Food Res. Int.* **2009**.

40. Wilson, T.A.; Nicolosi, R.J.; Lawton, C.W.; Ausman, L.M.; Hegsted, D.M. Comparative Cholesterol Lowering Properties of Vegetable Oils: Beyond Fatty Acids. *J. Am. Coll. Nutr.* **2000**.

41. Ausman, L.M.; Rong, N.; Nicolosi, R.J. Hypocholesterolemic effect of physically refined rice bran oil: Studies of cholesterol metabolism and early atherosclerosis in hypercholesterolemic hamsters. *J. Nutr. Biochem.* **2005**.

42. Ha, T.Y.; Han, S.; Kim, S.R.; Kim, I.H.; Lee, H.Y.; Kim, H.K. Bioactive components in rice bran oil improve lipid profiles in rats fed a high-cholesterol diet. *Nutr. Res.* **2005**.

43. Accinni, R.; Rosina, M.; Bamonti, F.; Della Noce, C.; Tonini, A.; Bernacchi, F.; Campolo, J.; Caruso, R.; Novembrino, C.; Ghersi, L.; et al. Effects of combined dietary supplementation on oxidative and inflammatory status in dyslipidemic subjects. *Nutr. Metab. Cardiovasc. Dis.* **2006**.

44. Son, M.J.; Rico, C.W.; Nam, S.H.; Kang, M.Y. Effect of Oryzanol and Ferulic Acid on the Glucose Metabolism of Mice Fed with a High-Fat Diet. *J. Food Sci.* **2011**.

45. Ghatak, S.B.; Panchal, S.S. Anti-diabetic activity of oryzanol and its relationship with the anti-oxidant property. *Int. J. Diabetes Dev. Ctries.* **2012**.

46. Qureshi, A.A.; Sami, S.A.; Khan, F.A. Effects of stabilized rice bran, its soluble and fiber fractions on blood glucose levels and serum lipid parameters in humans with diabetes mellitus Types I and II. *J. Nutr. Biochem.* **2002**.

47. Justo, M.L.; Claro, C.; Vila, E.; Herrera, M.D.; Rodriguez-Rodriguez, R. Microvascular disorders in obese Zucker rats are restored by a rice bran diet. *Nutr. Metab. Cardiovasc. Dis.* **2014**.

48. Perez-Ternero, C.; Macià, A.; De Sotomayor, M.A.; Parrado, J.; Motilva, M.J.; Herrera, M.D. Bioavailability of the ferulic acid-derived phenolic compounds of a rice bran enzymatic extract and their activity against superoxide production. *Food Funct.* **2017**.

49. Horie, Y.; Nemoto, H.; Itoh, M.; Kosaka, H.; Morita, K. Fermented Brown Rice Extract Causes Apoptotic Death of Human Acute Lymphoblastic Leukemia Cells via Death Receptor Pathway. *Appl. Biochem. Biotechnol.* **2016**.

50. Kim, K.M.; Yu, K.W.; Kang, D.H.; Suh, H.J. Anti-stress and anti-fatigue effect of fermented rice bran. *Phyther. Res.* **2002**.

51. Montero, C.M.; Dodero, M.C.R.; Sanchez, D.A.G.; Barroso, C.G. Analysis of low molecular weight carbohydrates in food and beverages: a review. *Chromatographia* **2004**.

52. Zhang, S.; LI, W.; Smith, C.J.; Musa, H. Cereal-Derived Arabinoxylans as Biological Response Modifiers: Extraction, Molecular Features, and Immune-Stimulating Properties. *Crit. Rev. Food Sci. Nutr.* **2015**.

53. Voet, D.; Voet, J.G. *Biochemisty Voet*; **2014**; ISBN 9780874216561.

54. Slack, J.M.W. Molecular Biology of the Cell. In *Principles of Tissue Engineering: Fourth Edition*; **2013** ISBN 9780123983589.

55. Ooi, S.L.; McMullen, D.; Golombick, T.; Pak, S.C. Evidence-Based Review of BioBran/MGN-3 Arabinoxylan Compound as a Complementary Therapy for Conventional Cancer Treatment. *Integr. Cancer Ther.* **2018.**

56. Ghoneum, M.; Matsuura, M. Augmentation of macrophage phagocytosis by modified arabinoxylan rice bran (MGN-3/Biobran). *Int. J. Immunopathol. Pharmacol.* **2004**.

57. Ghoneum, M.; Gollapudi, S. Modified arabinoxylan rice bran (MGN-3/biobran) enhances yeast-induced apoptosis in human breast cancer cells in vitro. *Anticancer Res.* **2005**.

58. Elsaid, A.F.; Shaheen, M.; Ghoneum, M. Biobran/MGN-3, an arabinoxylan rice bran, enhances NK cell activity in geriatric subjects: A randomized, double-blind, placebo-controlled clinical trial. *Exp. Ther. Med.* **2018**.

59. Ghoneum, M.; Abedi, S. Enhancement of natural killer cell activity of aged mice by modified arabinoxylan rice bran (MGN-3/Biobran). *J. Pharm. Pharmacol.* **2004**.

60. Ghoneum, M.; Matsuura, M.; Gollapudi, S. Modified arabinoxylan rice bran (MGN-3/biobran) enhances intracellular killing of microbes by human phagocytic cells in vitro. *Int. J. Immunopathol. Pharmacol.* **2008**.

61. Ghoneum, M.; Badr El-Din, N.K.; Ali, D.A.; El-Dein, M.A. Modified arabinoxylan from rice bran, MGN-3/Biobran, sensitizes metastatic breast cancer cells to paclitaxel in Vitro. *Anticancer Res.* **2014**.

62. Gollapudi, S.; Ghoneum, M. MGN-3/Biobran, modified arabinoxylan from rice bran, sensitizes human breast cancer cells to chemotherapeutic agent, daunorubicin. *Cancer Detect. Prev.* **2008**.

63. Choi, Y.S.; Lee, J.K.; Lee, M.G.; Lee, S.G.; Jeong, H.Y.; Kang, H. Splenic T cell and intestinal IgA responses after supplementation of soluble arabinoxylan-enriched wheat bran in mice. *J. Funct. Foods* **2017**.

64. Fadel, A.; Mahmoud, A.M.; Ashworth, J.J.; Li, W.; Ng, Y.L.; Plunkett, A. Health-related effects and improving extractability of cereal arabinoxylans. *Int. J. Biol. Macromol.* **2018**.

65. Salama, H.; Medhat, E.; Shaheen, M.; Zekri, A.R.N.; Darwish, T.; Ghoneum, M. Arabinoxylan rice bran (Biobran) suppresses the viremia level in patients with chronic HCV infection: A randomized trial. *Int. J. Immunopathol. Pharmacol.* **2016**.

66. Zheng, S.; Sanada, H.; Dohi, H.; Hirai, S.; Egashira, Y. Suppressive Effect of Modified Arabinoxylan from Rice Bran (MGN-3) on D-Galactosamine-Induced IL-18 Expression and Hepatitis in Rats. *Biosci. Biotechnol. Biochem.* **2012**, *76*, 942–946.

67. Zheng, S.; Sugita, S.; Hirai, S.; Egashira, Y. Protective effect of low molecular fraction of MGN-3, a modified arabinoxylan from rice bran, on acute liver injury by inhibition of NF-κB and JNK/MAPK expression. *Int. Immunopharmacol.* **2012**.

68. Wang, L.; Zhang, H.; Zhang, X.; Chen, Z. Purification and identification of a novel heteropolysaccharide RBPS2a with anti-complementary activity from defatted rice bran. *Food Chem.* **2008**.

OBJECTIVES AND WORK PLAN

The main objective of this doctoral project was to provide new insights and valuable strategies for the valorisation of cereal milling side streams through a multidisciplinary approach.

This Ph.D. thesis was subdivided in four key study areas (**Figure 9**). Before each section a brief preface is reported in order to contextualize and justify the work carried out.

Figure 9. The four research study areas of the Ph.D. project.

The first and second sections were directed to the evaluation of the impact of cereal milling process on both biochemical contaminants (**1**) and bioactive compounds (**2**). For this reason, the study was aimed to the quantification and identification of the most widespread *Fusarium* spp. mycotoxins in different cereal

species and pearling fractions using a high-resolution mass spectrometry (HRMS) technique.

Then, the distribution of phenolic acids, betaine and choline was determined along the *Triticum* spp. milling chain, considering six different species and the corresponding by-products. The latter were important screening steps to gain information for the next activity.

The third section (**3**) was focused on the application of lactic acid fermentation to wheat bran and on the study of the overall biochemical changes produced by such treatment. In addition, micronization coupled to air-classification was used preliminary to treat rice bran with high oil content, and the lipid profile was studied for future applications.

Finally, the last part (**4**) was dedicated to the formulation of a highly consumed baked food product, such as bread, using pre-fermented wheat bran. Afterword, the bioactive properties were evaluated throughout *in vitro* cell culture assays using common and bran enriched breads for comparison.

Overall, the entire project attempted to answer the following question:

"How cereal milling by-products could be sustainably recovered and valorised?"

Limitation of the study

Taking as true the assumption which states that a total recovery of food processing residue and its value persistence is a potential sustainable improvement, a direct measure of competitiveness increment, and lower environmental impact was not possible to be performed. However, the work here presented has been carried out defining specific study design delimitations as closer as possible to the agri-food chain reality.

MAIN CONTRIBUTIONS

Considering the agri-food sector of the northern Italian country, it is possible to estimate the main impact of the present project, taking into account **the protagonists of the system and their interrelationships**. Nowadays, the cereal grains enterprises (i.e.: seed sellers, breeders, milling plants, food manufacturers, etc.) have to face several challenges resulting from climate change, new agronomic practices, stringent policies and consumer market. In this context, the continuous need to create an added value chain recovering the cereal milling by-products appears to be a **valuable strategy for maximizing the overall profits while diminishing the volume of unmanageable residues.** Following the concept of the "blue & green economy", the sustainable exploitation of different by-products from maize, wheat and rice opens doors to interesting industrial applications varying from the production of high-value raw materials for cosmetics and pharma industries, in order to promote a healthy diet through the production of nutritionally improved food products. These types of goods are **more appealing from the consumer point of view;** therefore, they could act as economy booster towards new income sources derived from the total reuse of the cereal processing materials. The approach is significantly related to the "**zero-waste" goal**, which is translatable to a creation of new market and employment spaces, and possibly forming an innovative incubator transferable to other sectors.

Open Fields s.r.l. through its technology transfer activity, helps industries and research institutes to communicate within each other and share opinions, which is the first and fundamental step for the whole agri-food system improvement.

CHAPTER I

IMPACT OF MILLING PROCESS ON SAFETY AND QUALITY TRAITS OF CEREAL GRAINS

1.1

HR-MS PROFILING AND DISTRIBUTION OF NATIVE AND MODIFIED *FUSARIUM* MYCOTOXINS IN *TRITORDEUM*, WHEAT AND BARLEY WHOLE GRAINS AND CORRESPONDING PEARLED FRACTIONS

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Preface

Emerging contaminants are always threatening the agri-food chain, at any level. The food safety related issues are coordinated at European level by policies and common recommendations. Basically, the process is constituted by the risk analysis and its corresponding components: assessment, management and communication.

"Food products intended to human consumption must be safe"

Within this concept the whole project started with a precise analysis of potential hazardous compounds present in cereal grains and their distribution within the cereal fraction. **Mycotoxins**, as explained in the **Introduction** section (pp. 23), are the most widespread bio-contaminants found in cereals. These compounds are regulated from the sampling step and the analysis to control maximum levels in food and feed is performed [1]. However, important work is continuously conducted by the European Food Safety Authority (EFSA) which provide updated information useful for all the agricultural system [2].

The study of mycotoxins in terms of classes, species and forms, and the monitoring of their distribution within cereal kernel parts could provide important information regarding the impact of processing and the overall exposure of both human and animals to these chemicals.

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[1] European Commission. Commission directive 2003/100/EC, Commission Recommendation 2006/576/EC, Commission Regulation (EC) No 401/2006, Commission Regulation (EC) No. 1881/2006, Directive 2008/98/EC.

[2] EFSA, 2014. Scientific Opinion on the risks for human and animal health related to the presence of modified forms of certain mycotoxins in food and feed. EFSA Journal 12, 3916-4023.

This is a relevant information since milling processes can increase the mycotoxins content of processing by-products [3].

Lastly, it was mandatory to determine the mycotoxins content of the whole sample set (data not showed) since they will be potentially used as raw materials for the following studies. This was carried out using an established and in-house verified multi-residual method based on a HPLC-MS/MS technique.

The following work was carried out in collaboration with the **Department of Agricultural, Forest and Food Sciences** of the **University of Torino**.

Abstract

Mycotoxins are one of the most important contaminants in cereal grains. Besides parent forms, the presence and identification of structurally modified mycotoxins is nowadays recognized as a challenging food safety-related issue and contribute to increase the human and animal exposure to these compounds. The aim of this study was to follow the distribution of *Fusarium* toxins and their main modified forms in the pearled fractions of several grain species (i.e. tritordeum, durum and bread wheat, and barley), using high-resolution mass spectrometry technique (HR-MS). A significant decreasing trend in mycotoxins concentration was observed from the outer layer to the inner kernel, along the sequential pearling process. Among modified forms, deoxynivalenol (DON) -oligoglucosides were described for the first time in naturally infected grains, while zearalenone (ZEN) -sulfate was the only ZEN-related form detected in pearling fractions. HR-MS could be confirmed as useful technique to study and characterize modified forms of mycotoxins.

Key words: modified mycotoxins, pearled fractions, tritordeum, wheat, barley, high resolution mass spectrometry.

Abbreviation used

15-Acetyl-Deoxynivalenol (15-Ac-DON), 3-Acetyl-Deoxynivalenol (3-Ac-DON), analysis of variance (ANOVA), below the limit of detection (<LOD).below the limit of quantification (<LOQ), deoxynivalenol (DON), deoxynivalenol-3-Glucoside (DON-3-Glc), dependent acquisition (DDA), electrospray source (ESI), high-resolution mass spectrometry (HR-MS), liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), nivalenol (NIV), zearalenone (ZEN), zearalenone sulphate (ZEN-Sulf).

Introduction

Whole grain cereals are an important source of bioactive compounds, micronutrients, dietary fibre and they are considered as staple food worldwide. Cereal grains are rarely consumed as whole kernel, in fact they undergo to several processes, such as the separation of the outer layers of the seed to the endosperm. These fractions are usually discarded because of their reduced sensory and technological value of the end-use products compared to those obtained from the endosperm (Zhang and Moore, 1999). However, it has been shown that bioactive compounds are mainly concentrated in the outer layers of the grain (Sovrani et al., 2012). An increasing evidence from clinical and epidemiological studies suggests that the regular consumption of wheat, as whole grain, might reduce the risk of developing chronic diseases (Bach Knudsen et al., 2017; Dykes and Rooney, 2007). Consequently, the conventional roller-milling process, which promote the removal of the outer layers of the kernel in the bran fraction, causes a great decrease in the nutritional value of the refined flour (Felizardo and Freire, 2018). To overcome the drawbacks, several grain fractionation technologies have been developed over years, to obtain flour mixes and ingredients with technologically optimized functional and nutritional attributes (Giambanelli et al., 2018; Giordano et al., 2017). Among them, sequential pearling effectively allows the separation of external bran fractions, which contain coarse fiber and are potentially subjected to contamination, from underlying fractions with potential health benefits due to their high content of bioactive compounds (Sovrani et al., 2012). These fractions can be efficiently employed as functional ingredients in bakery and particularly, as previously suggested, for bread-making (Blandino et al., 2015, 2013). On the other hand, the outer layers of the wheat kernel are mostly subjected to contamination by pesticides or natural contaminants, such as heavy metals and mycotoxins, mainly those produced by *Fusarium* spp. (Cheli et al., 2013). In particular, mycotoxins are generally found in cereal grains and overall, more than one type of mycotoxin can

be present in the same foodstuff (Freire and Sant'Ana, 2018). Deoxynivalenol (DON), belonging to the trichothecenes class and zearalenone (ZEN) are commonly accepted as the main mycotoxins occurring in wheat worldwide. These compounds gather several toxic effects, thus represent a threat for humans and animal health. Moreover, they can act as a virulence factor for Fusarium Head Blight (FHB) in cereals (Audenaert et al., 2013) making them responsible for relevant economical losses due to low yield and crop withdrawal. Besides native mycotoxins, several structurally modified forms produced in plants have been reported (Berthiller et al., 2013). Recently, EFSA has reconsidered the toxicological relevance of DON and its main modified forms, stating that a thoroughly assessment of the sum of DON, 3- and 15-Acetyl-DON (Ac-DON) and DON-3-Glucoside (DON-3-Glc) is highly recommended (Knutsen et al., 2017). These modified forms may present also different chemical properties (i.e. solubility, polarity), and arguably different toxicity. Furthermore, the combination of unknown structure, lack of analytical standard and trace concentration, their analysis using conventional techniques is challenging, but necessary as it constitutes an important information during the risk assessment process. The ability of sequential pearling to decrease the content of Fusarium mycotoxins from the outer to the inner layers of wheat, has been reported by several authors (Cheli et al., 2013; Ríos et al., 2009; Sovrani et al., 2012). However, the distribution of the modified forms of DON, DON-3Glc, 3- and 15Ac-DON, has never been tested so far.

Therefore, the aim of this study was to follow the distribution of trichothecenes, zearalenone (ZEN) and its main modified forms into sequential pearled fractions of two tritordeum (X *Tritordeum* Ascherson et Graebner), one durum wheat (*Triticum turgidum* L. subsp. *durum* (Desf.) Husnot), one bread wheat (*Triticum aestivum* L.) and one barley (*Hordeum vulgare* L.) varieties, using two different approaches. As first, a target quantification was run using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS).

Then high-resolution mass spectrometry (HRMS) was used for a full profiling of modified mycotoxins in the samples.

Materials and methods

Experimental design and raw materials

Two varieties of tritordeum (cvs. Aucan and Bulel), a durum wheat variety (cv. Saragolla), a bread wheat variety (cv. Illico) and six-row barley varietie (cv. Ketos) were cultivated side by side on the same field in northwestern of Italy (Cigliano, 45° 31' 97''N, 8°04'77''E) during the 2015-2016 growing season. The experiment was carried out in natural infection conditions, but the choice of the growing area (frequent rainfall during wheat anthesis) and of the agronomic techniques (previous crop, no fungicide application) was carried out to guarantee a medium-high level of Fusarium infection, although the adopted crop practices are commonly used by farmer in the areas. Briefly, the previous crop was maize, and the mechanical sowing was carried out on 6 November 2015, following an autumn plowing (30 cm) and disk harrowing to prepare a proper seedbed. Planting was conducted in 12 cm wide rows at a seeding rate of 450 seeds m^{-2}. A total of 140 kg N ha^{-1} was applied as a granular ammonium nitrate fertilizer, split into 60 kg N ha^{-1} at wheat tillering (GS 23), 80 kg N ha^{-1} at stem elongation (GS 32). No fungicide has been applied to control foliar and head disease. Harvesting was conducted with a combine-harvester on 21 June for the barley variety and on 4 July 2016 for the tritordeum and wheat varieties.

Pearling process

Nine fractions of kernels from each variety were obtained through incremental pearling. The pearling consisted of consecutive passages of kernels or pearled kernels in an abrasive-type grain testing mill (Model TM-05C, Satake, Tokyo, Japan). Starting from unprocessed grain samples, the kernels were initially pearled to remove 5% of the original weight, and this resulted in a first fraction (0-5% w/w). The same process was also performed to remove another seven fractions (designated fractions 5-10%, 10-15%, 15-20%, 20-25%, 25-30%, 30-35%, 35-40% w/w).

The residual 60% of the kernel (40-100% w/w) was also collected and milled by means of a laboratory centrifugal mill (Model ZM-100, Retsch, Haan, Germany) equipped with a 1-mm sieve. The same process was performed also for the unprocessed grain samples in order to obtain a wholegrain flour. Before chemical analyses, samples were ground to a fine powder (particle size < 300 µm) with a Cyclotec 1093 sample mill (Foss, Padova, Italy), and stored at -25°C until analyses were performed.

Chemicals and reagents

Analytical standards of DON (100 µg mL^{-1} in acetonitrile), DON-3-Glc (solution in acetonitrile 50.6 µg ml^{-1}), 3-Ac-ADON (50 µg mL^{-1} in acetonitrile), T-2 and HT-2 toxins (50 µg mL^{-1} in acetonitrile), nivalenol (NIV) (50 µg mL^{-1} in acetonitrile) and ZEN (100 µg mL^{-1} in acetonitrile) were purchased from Romer Labs®. HPLC-grade methanol, acetonitrile and acetic acid were purchased from Sigma-Aldrich (Taufkirchen, Germany); bidistilled water was obtained using a Milli-Q System (Millipore, Bedford, MA, USA). MS-grade formic acid from Fisher Chemical (Thermo Fisher Scientific Inc., San Jose, CA, USA) and ammonium acetate (Fluka, Chemika-Biochemika, Basil, Switzerland) were also used.

Sample preparation for LC-MS analysis

Samples were prepared according to Malachová et al., 2014 procedure, with slight modifications. Briefly, 1 g of ground cereal was stirred for 90 min at 200 strokes/min on a shaker with 4 mL of acetonitrile/water (80/20, v/v) mixture acidified with 0.1% of formic acid. An aliquot of the extract was collected and centrifuged for 10 min at 14,000 rpm at room temperature then 1 mL of supernatant was evaporated to dryness under a gentle stream of nitrogen. Finally, the residues were re-dissolved in 1 mL of water/methanol (80/20, v/v) prior to LC- MS/MS and LC-HRMS injection.

LC-MS/MS quantification of *Fusarium* mycotoxins and their modified forms

The LC-MS/MS analysis was performed on a UHPLC Dionex Ultimate 3000 instrument coupled with a triple quadrupole mass spectrometer (TSQ Vantage; Thermo Fisher Scientific Inc., San Jose, CA, USA) equipped with an electrospray source (ESI). For the chromatographic separation, a RP-C18 Kinetex EVO column 2.10×100 mm and a particle size of 2.6 µm (Phenomenex, Torrance, CA, USA) heated to 40 °C was used. 2 µL of sample extract was injected into the system; the flow rate was 0.350 mL min^{-1}. Gradient elution was performed by using 5 mM ammonium acetate in water (eluent A) and methanol (eluent B) both acidified with 0.2% acetic acid. Initial conditions were set at 2% B for 1 min, then eluent B was increased to 20% in 1 min; after an isocratic step (6 min), eluent B was increased to 90% in 9 min; after a 3 min isocratic step, the system was re-equilibrated to initial conditions for 8 min. The total run time was 28 min. MS parameters: the ESI source was operated in negative ionization mode for DON, DON-3Glc, 3Ac-DON, NIV and ZEN, and in positive ionization mode for T-2 and HT-2 toxins; spray voltage was 3,000 V, capillary temperature at 270 °C, vaporizer temperature was kept at 200 °C, sheath gas flow was set at 50 units and the auxiliary gas flow at 5 units. The S-Lens RF amplitude value and collision energies (CE) were optimized during infusion of analyte standard solutions (1 mg kg^{-1}, in methanol). Detection was performed in SRM mode, monitoring the $[M + CH_3COO]^-$ adducts for DON, NIV and modified forms, [M-H]- for ZEN and $[M+NH_4]^+$ adducts for T-2 and HT-2 toxins. The following transitions were measured: DON *m/z* 355→295 (CE = 13 eV) and *m/z* 355→265 (CE = 17 eV); DON-3Glc *m/z* 517→457 (CE = 16 eV) and *m/z* 517→427 (CE = 23 eV), 3Ac-DON *m/z* 397→337 (CE = 16 eV), m/z 397→307 (CE = 18 eV) and *m/z* 397→59 (CE = 20 eV), ZEN *m/z* 317→175 (CE = 26 eV) and *m/z* 317→131 (CE = 32 eV), HT-2 toxin *m/z* 442→263 (CE = 11 eV), *m/z* 442→215 (CE = 4 eV) and T-2 toxin *m/z* 484→215 (CE = 19 eV), *m/z* 484→185 (CE = 22 eV), NIV *m/z* 371→59 (CE = 48 eV), *m/z* 371→281 (CE = 32 eV) and *m/z* 371→311 (CE = 11 eV).

Matrix-matched calibration curves (calibration range for DON, DON-3-Glc, 3Ac-DON, NIV 100–2500 µg kg^{-1}, for ZEN, T-2 and HT-2 toxins 1-2500 µg kg^{-1},) were used for target analyte quantification. A good linearity was obtained for all the considered mycotoxins ($R^2 > 0.99$).

DON-3Glc/DON molar ratio was calculated from the values of DON and DON-3Glc by the following equation (Nakagawa et al., 2017):

$$\frac{DON3Glc}{DON}\ molar\ ratio = \frac{\frac{DON3Glc\ \left(\mu\frac{g}{kg}\right)}{MWa}}{\frac{DON\ \left(\mu\frac{g}{kg}\right)}{MWb}} * 100$$

where the *MWa* represent the molar weight of the DON-3Glc (458 Da) and *MWb* that of DON (296 Da).

HR-MS profiling of Fusarium mycotoxins and their modified forms

LC-HRMS analysis was performed on a UHPLC Dionex UltiMate 3000 instrument coupled to a Q-ExactiveTM high resolution mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with electrospray ionization (Righetti *et al*., 2017). The chromatographic separation was obtained on a Synergi 4U Hydro-RP 150 x 2.0 mm (Phenomenex, Torrance, CA, USA) heated to 30 °C. 10 µL of sample extract was injected into the system; the flow rate was 0.3 mL min^{-1}. Gradient elution was performed by using 1 mM ammonium acetate in water (eluent A) and methanol (eluent B) both acidified with 0.5% acetic acid. Initial conditions were set at 5% B followed by a linear change to 10% B in 2 min. After 2 min of isocratic step (10% B) B% increased up to 65% in 16 min. Column was then washed for 4 min with 100% B followed by a reconditioning step for 5 min using initial composition of mobile phases. The total run time was 29 min. The Q-Exactive mass analyzer was operated under negative ionization mode.

The full MS/data dependent MS/MS mode (full MS–dd-MS/MS) was set at following parameters: sheath and auxiliary gas flow rates 32 and 7 arbitrary units, respectively; spray voltage 3.3 kV; heater temperature 220 °C; capillary temperature 250 °C, and S-lens RF level 60. Following parameters were used in full MS mode: resolution 70,000 FWHM (defined for *m/z* 200; 3 Hz), scan range 100–1000 *m/z*, automatic gain control (AGC) target 3e6, maximum inject time (IT) 200 ms. Parameters for dd-MS/MS mode: intensity threshold 1e4, resolution 17,500 FWHM (defined for *m/z* 200; 12 Hz), scan range 50 – fragmented mass m/z (m/z +25), AGC target 2e5, maximum IT 50 ms, normalized collision energy (NCE) 35% with ±25% step. Only in few cases, fragmentation spectra could not be collected, due to parent ion abundance below the threshold. In this case, a tentative annotation based on accurate mass and elemental formula was performed, as already proposed (Righetti et al., 2017).

Statistical analysis

All the analyses of wheat samples (whole-grain flour, pearled fractions and residual pearled kernel) were performed in triplicate. Analysis of variance (ANOVA) was applied in order to compare the mycotoxins content in the whole-grain flours and in the different pearled fractions. The *Tukey-b*'s post-hoc test was performed for multiple comparisons. A $p<0.05$ threshold was used to reject the null hypothesis. Statistical analyses were carried out by means of SPSS for Windows, statistical package Version 25 (SPSS Inc., Chicago, Illinois).

Results and Discussion

Quantification of parent and modified *Fusarium* mycotoxins in wholegrains and pearled fractions

Due to the higher association of mycotoxins with outer layers of grains, the effect of pearling in decreasing DON content and the distribution of their modified forms into pearled fractions can be of relevance for food safety. The occurrence and distribution of main trichothecenes (NIV, DON, DON-3-Glc, 3-Ac-DON, T-2, HT-2 and ZEN) were analyzed in durum wheat cv. Saragolla (**Figure A1,** see supplementary information) as well as in two varieties of tritordeum, in bread wheat and in barley.

In terms of whole grain contamination, the overall content of DON and its modified forms was higher in durum wheat (cv. Saragolla) compared to tritordeum (cv. Aucan and cv. Bulel), bread wheat (cv. Illico) and barley (cv. Ketos) (**Table 3**).

Table 3. Trichothecenes and ZEN content in the whole grain of tritordeum, durum wheat, bread wheat and barley, expressed in μg kg^{-1} d.w. (dry weight).

Crop	Cultivar	Mycotoxins							
		DON	DON-3-Glc[1]	3-Ac-DON[1]	NIV[1]	T-2[2]	HT-2[2]	ZEN[2]	DON-3-Glc/DON *
Tritordeum	Aucan	6354±152 ab	1130±20 a	<LOD	<LOD	<LOD	<LOD	2±0 b	11.5
Tritordeum	Bulel	3209±1460 bc	1060±70 a	<LOD	<LOD	<LOD	<LOD	2±0 b	21.3
Durum wheat	Saragolla	6920±160 a	1210±20 a	387±5	859±188	3±0	11±1	58±5 a	11.3
Bread wheat	Illico	379±67 c	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	-
Barley	Ketos	241±60 c	<LOD	<LOD	<LOD	<LOD	<LOD	<LOQ	-
p-value		0.002	-					0.001	-

Results were expressed as mean ± standard deviation μg kg^{-1}. Different letters indicate a statistical difference ($p<0.05$). * expressed as molar ratio (%); [1] <LOD: <7 μg kg^{-1}; [2] <LOD: < 1 μg kg^{-1}, <LOQ: 1 μg kg^{-1}; - not determined.

The latter is easily explainable since durum wheat is known to be more susceptible to *Fusarium* contamination compared to soft wheat, and for that reason was selected for the subsequent metabolites analysis. Other *Fusarium* toxins, such as T-2, HT-2 and NIV as well as ZEN were also detected only in durum wheat. In bread wheat and barley whole grains the DON content was significantly lower than those of previous reported cereals, while its modified forms or other *Fusarium* toxins resulted lower than LOD. In fact, Illico is recognized to be a strong resistant variety of wheat grains.

The main modified form of deoxynivalenol, DON-3-Glc, was found at a concentration of 1130 µg/Kg, 1060 µg/Kg, and 1210 µg/Kg in cv. Aucan, Bulel and Saragolla, respectively, while it was < LOD in cv. Illico and cv. Ketos samples, likely in consideration of the lower accumulation of the parent form compared to the other cultivars. Considering the DON-3-Glc/DON ratio, it was found in the range 10-30%, in agreement with data reported in the literature (Berthiller et al., 2013). However, it should be noticed that DON-3-Glc/DON ratio in cereal grains can vary in relation to many factors like genotype, environmental conditions or climatic conditions, as already discussed by several authors (Berthiller et al., 2013; Cirlini et al., 2013). In addition, also high variances among the same wheat species have been found as reported in the study of (Bryła et al., 2018), in which they monitored the occurrence of these toxins along 92 polish winter wheat cultivars (growing season 2016) and the molar ratio ranged between 5 to 37 %.

Being DON the main contaminant of all the grain samples, its distribution together with the distribution of DON-3-Glc was evaluated over nine sequential pearled fractions, obtained from each cultivar considered within this study. Results are reported in **Figure 10**.

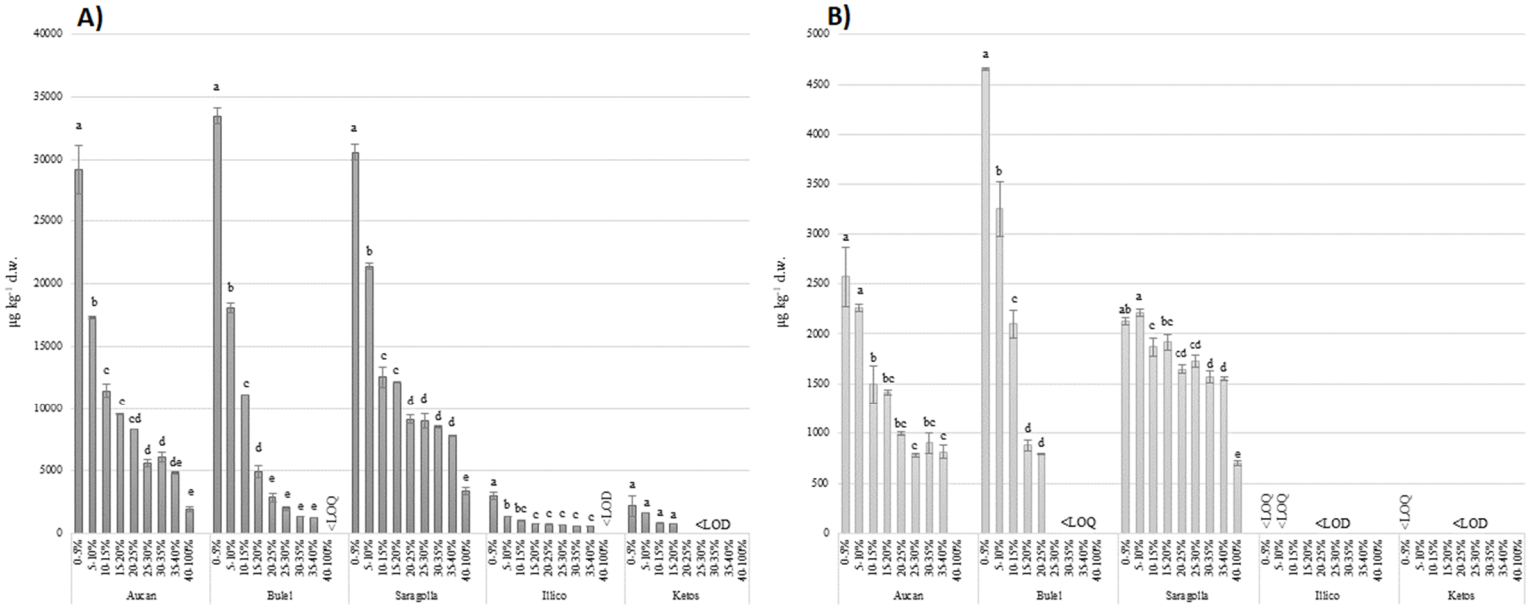

Figure 10. DON (A) and DON-3-Glc (B) concentration (μg kg^{-1} d.w.) among pearled fractions of *tritordeum* (cv. Aucan and Bulel) durum wheat (cv. Saragolla), bread wheat (cv. Illico) and barley (cv. Ketos). Different letters on top of each bar indicate a significant difference ($p<0.05$) using *Tukey-b*'s post-hoc test.

As expected, a decreasing trend of DON and DON-3-Glc was observed throughout the pearling fractions, moving from outer to inner layers. In particular, the outer fraction, mainly composed by the outer and inner pericarp (fr 0-5%) showed the higher contamination in all the considered samples.

The effectiveness of sequential pearling in decreasing mycotoxin content is clearly demonstrated by the strong decrease observed in the inner pearled kernel, representing about the 60% in weight of the initial wholegrain (**Figure 10A**). In particular, DON concentration dropped below LOQ in cv. Bulel, cv. Illico, and cv. Ketos, while it was in the range 30-40% of the DON concentration level found in the whole grain in cv. Saragolla and cv. Aucan. DON-3-Glc showed the same trend of DON (**Figure 10B**). These results are in agreement with other studies, in which the accumulation of DON is higher in the outermost fractions compared to the starchy endosperm (Šliková et al., 2010). Data obtained for DON in whole kernels and sequential pearling fraction were used for mass balance calculation. A satisfactory balance was found for all the samples, with the weighted sum of DON concentration calculated for fractions falling within the mean ± SD measured in whole kernels, as reported in **Table 4**. However, variances regarding the DON-3-Glc content in respect to its native form and the distribution trend could be also explained by the different metabolic properties of each cereal species in relationship with that of fungi. In fact, when the plant goes to the senescence period, its metabolism is almost deactivated, thus unable to produce the glucoside form of DON. Furthermore, the fungi developed in more extent on the peripheral tissues of the caryopsis, can still produce mycotoxins, as long as the moisture content during the dry-down process persists above the 20%.

Table 4. Comparison between DON concentration found in whole kernels and DON mass balance calculated from the weighted sum of DON concentration found in the pearling fractions, expressed in μg kg^{-1} d.w (dry weight).

Crop	Cultivar	Whole grain (measured)	Weighted sum of fractions (calculated)
Tritordeum	Aucan	6354 ± 152	5780
Tritordeum	Bulel	3209 ± 1460	3760
Durum wheat	Saragolla	6920 ± 160	7610
Bread wheat	Illico	379 ± 67	382
Barley	Ketos	241 ± 60	275

Regarding T-2 and HT-2 toxins content, they were detected only in whole grain and pearled fractions of durum wheat cv. Saragolla. The distribution pattern of T-2 and HT-2 toxins was similar to the one of DON, decreasing toward the inner part of the kernel (**Figure 11**). The dominating analogue was the deacetylated form (HT-2) as reported by other studies (Lindblad et al., 2013; Pascale et al., 2012).

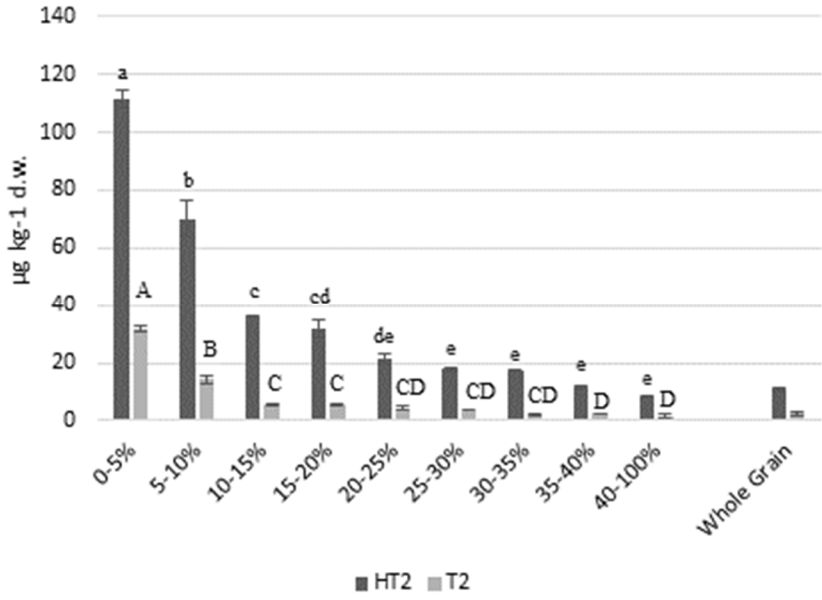

Figure 11. HT-2 and T-2 toxins concentration (µg kg^{-1} d.w.) among pearled fractions of durum wheat (cv. Saragolla.). Different letters on top of each bar indicate a significant difference ($p<0.05$) using Tukey-b's post-hoc test.

Concerning the content of DON and DON-3-Glc, a significant correlation along the pearled fractions was observed in cv. Aucan ($p = 0.0002$), cv. Bulel ($p = 0.0000$), and cv. Saragolla ($p = 0.0158$), while the low contamination found in cv. Illico and cv. Ketos did not allow any calculation. However, it should be noticed that a different toxin redistribution within the kernel fractions could be influenced by many factors. For example, the latter can be related to the fact that cereals differ for their size and shape, and consequently the progressive removal of the outer part of the grains by means of the pearling process is not homogeneous. In fact, regarding barley grains the two first pearling passages (0-5 and 5-10%) were responsible for an almost total dehulling of the kernel, while for other cereals these steps could abrade the peripheral tissues of seed. In addition, a recent study reported on the effect of rainfall and climate parameters on the distribution of mycotoxins within the kernels (Edwards et al., 2018).

In particular, DON was shown to be affected by repeated wetting and drying, causing a movement across the mill fractions. The authors demonstrated that strong rainfall could cause a large reduction of DON in the grain, predominantly from the bran fraction, resulting in a proportional increase within white flour. In this regard, it must be noticed that samples considered within this study, have been harvested in the same harvest year. This can be regarded as a main limitation, because differences in weather conditions may be reflected in a different distribution among sequential pearling fractions. This work can be regarded as a starting point for further works designed to better understand possible difference in mycotoxin distribution between wheat genotypes at different resistance level – as well as the association with specific components.

Overall, the present study clearly showed that the removal of the first two fractions (0-5% and 5-10%) could significantly reduce the content of DON and DON-3-Glc in all the considered samples. Since these two fractions correspond to the outermost layers (inner and outer pericarp ~ 12 % of kernel weight), the pearling process could be as far as comparable to the traditional milling process, even considering a very high contaminated sample.

Qualitative profiling of *Fusarium* mycotoxins in pearled fractions

To get a full picture of mycotoxin modified forms occurring in pearled fractions and to evaluate possible changes in distribution moving from the outer layer to the inner kernel, a LC-HRMS profiling was performed on durum wheat. The experiment was performed on cv. Saragolla pearled fractions in consideration of the higher amount of DON found in whole grain. The same profile in terms of modified forms was observed throughout the sequential fractions, returning 4 putative metabolites and two modified forms confirmed by analytical standard comparison (**Table 5**).

Table 5. Metabolites of DON and ZEN found in the outermost fraction (0-5%) of durum wheat cv. Saragolla. Mass deviation ppm is calculated by the values detected by full scan spectrum (resolving power 70,000 FWHM, extraction window 5 mg kg^{-1}).

Rt (min)	Formula	Detected mass (*m/z*)	Theoretic mass (*m/z*)	Ion species	Mass error (Δ ppm)	Putative metabolite
8.75	C15 H20 O6	355.1405	355.1382	$[M+H_3C_2O_2]^-$	1.9	DON
9.11	C21 H30 O11	517.1939	517.1910	$[M+H_3C_2O_2]^-$	2.4	DON-3-Glc
10.44	C27 H40 O16	589.2159	589.2138	$[M-CH_2O-H]^-$	3.6	DON-3-diGlc
10.21	C31 H44 O19	751.2677	751.2666	$[M-CH_2O-H]^-$	1.5	DON-3-triGlc
12.23	C23 H32 O12	499.1816	499.1821	$[M-H]^-$	-0.9	3-Ac-DON-Glc
14.44	C17 H22 O7	397.1516	397.1504	$[M+H_3C_2O_2]^-$	3.1	3-Ac-DON
10.58	C18 H22 O5	317.1398	317.1394	$[M-H]^-$	1.2	ZEN
9.13	C18 H22 O8 S	397.0968	397.0963	$[M-H]^-$	1.2	ZEN-Sulf

Besides DON, 3-Ac-DON and DON-3-Glc, the occurrence of di- and tri-glucoside forms of DON together with 3-Ac-DON-15-Glc were observed in all the considered fractions. In addition, ZEN and ZEN-Sulf were identified as well. According to Righetti et al., 2017, the identification process used for metabolite putative assignment starts from the extracted ion chromatogram; then the parent ion molecular formula is assigned, and theoretical and experimental isotopic pattern are compared to reduce the number of possible candidates. In the last step, the HR-MS fragmentation pattern, obtained by using data dependent acquisition (DDA), facilitate compound identification.

Oligoglycosides were annotated according to the in-source fragmentation pattern (**Figure 12**), as reported by (Zachariasova et al., 2012). The sugar moieties were bound indeed to C3 of DON, resulting in the formation of a peak at *m/z* 427.1610 [M-CH_2O-H]-. It is worth of notice that this is the first study showing the occurrence of DON-oligoglycosides in grains. These forms have been reported before as resulting from the malting process in brewing, as the effect of enzymatic release from cell wall polysaccharides (Maul et al., 2012).

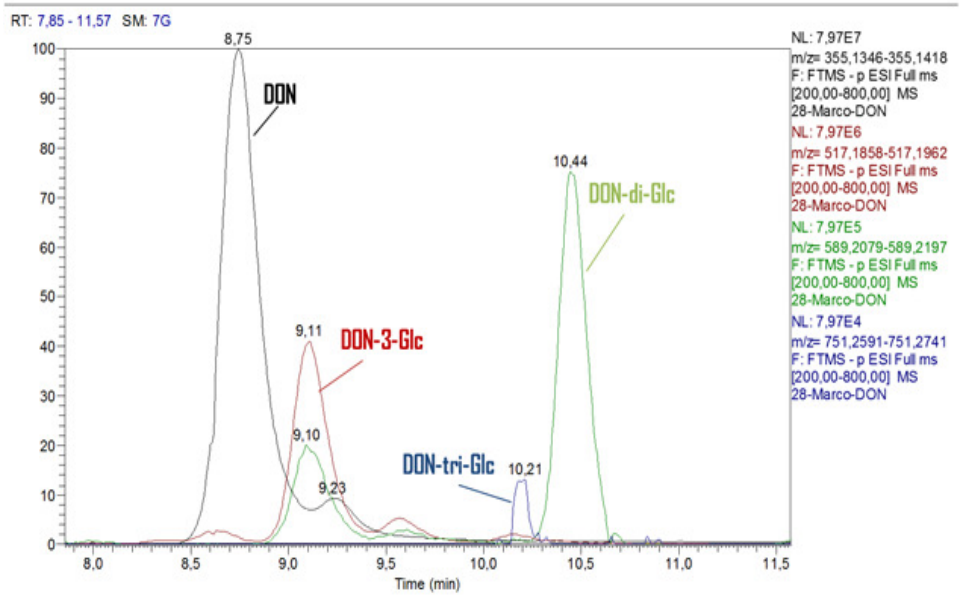

Figure 12. Extracted ion chromatogram (EIC) of DON and DON oligoglycosides. Due to low abundance, EIC intensities of DON-3-Glc, DON-3di-Glc and DON-3tri-Glc were multiplied by a factor of 10, 100 and 1000 respectively.

Besides DON-oligoglycosides, one of the acetylated forms of DON (3-Ac-DON) was detected in the full scan mass spectrum (*m/z* 397.1504) (**Figure 13**). The acetylation of DON can take place on two sites of the backbone; therefore, two isomeric forms might be expected. Nevertheless, as reported by (Schmeitzl et al., 2015), the fragment ion at *m/z* 173.0462 is characteristic for the 3-Ac-DON. In our study only the 3-Ac-DON isomer was found and confirmed by comparison with analytical standard.

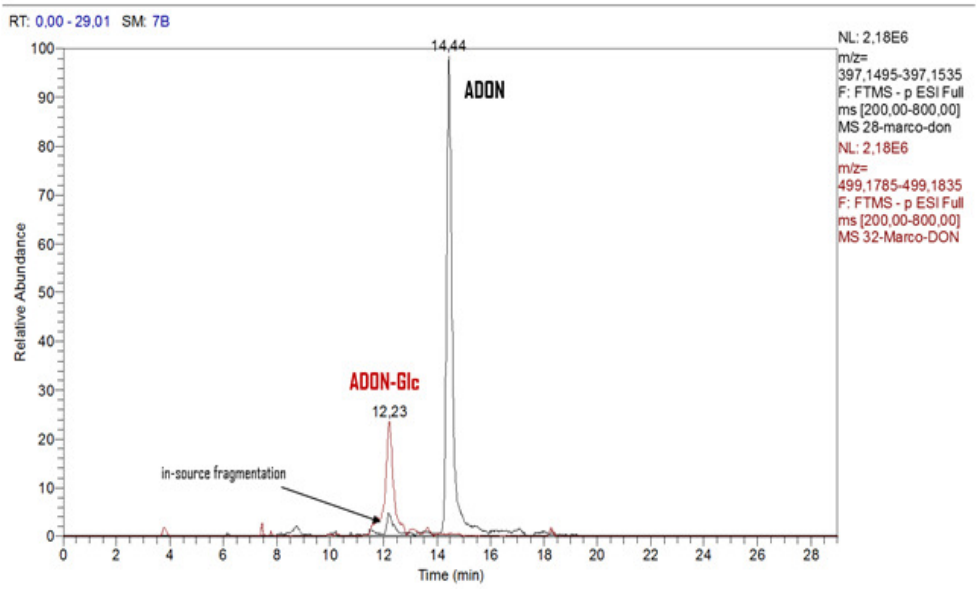

Figure 13. EIC of 3-Ac-DON (*m/z* 397.1504) and 3-Ac-DON-Glc (*m/z* 499.1821), in-source fragmentation and loss of glucose from 3-Ac-DON-Glc. Due to low abundance, EIC intensities of 3-Ac-DON-Glc were multiplied by a factor of 10.

Together with 3-Ac-DON, its glycosylated form was found in kernel fractions, where the 3Ac-DON contamination was detected. Due to its low intensity no HR-MS/MS spectrum was obtained. However, it was possible to putatively confirm the 3-Ac-DON-Glc identity by the low mass error (-0.9 Δ ppm) (**Table 5**), the isotopic pattern and the retention time, anticipated in respect to its aglycone (3-Ac-DON). In addition, also the in-source fragmentation was reported, in which the breakage of the ether bond released the 3-Ac-DON moiety. Moreover, no molecular ion (*m/z* 397.1504) was found in both standard reference and sample (**Figure A2,** see supplementary information). This means that even with low collision energy (CE, 10v) the $[M+H_3C_2O_2]^-$ it was completely fragmented.

Among sulfated forms, DON-Sulf was not observed in wheat pearling fractions, in agreement with studies reporting that sulfation is a minor biotransformation route in plants for DON (Knutsen et al., 2017).

On the other hand, ZEN-Sulf was annotated together with ZEN, on the basis of its molecular ion $[M-H]^-$ at *m/z* 397.0952, and consistent fragmentation (**Figure A3,** see supplementary information).

In terms of concentration of the modified forms of mycotoxins, their abundance was calculated as the total peak areas of the mycotoxins detected and the peak area of each modified form, thus expressed as percentage (%). For example, in first pearled fraction (0.5%) the di-glycoside of DON accounted for 0.685 %, while a 0.047 % for the DON-3-triGlc and 0.120 % for 3-Ac-DON-Glc. In the rest of pearled fractions, the relative abundance ranged between 0.317-0.012, 0.038-0.06 and 0.090-0.052 %, for DON-3-diGlc, DON-3-triGlc and 3-Ac-DON-Glc, respectively. Moreover, for the ZEN-S the relative abundance in fraction 0-5 %, in respect to its native form, was 15.527 % and ranged between 20.43-9.068 % in the other seed fractions. Structural modification of the native form of mycotoxins, could be interpreted as a resistence mechanism of the plant (Buerstmayr and Lemmens, 2015), in which the conjugation of the DON toxin to a sugar or/and sulphates increases the polarity of the molecule that can then be stored in cell vacuole (Berthiller et al., 2005).

More studies have to be conducted in order to increase the information regarding the toxicity of modified mycotoxins. Nevertheless, they are extremely important and must be taken into account in food safety areas since the native, and toxic, form might be released during digestion (Dall'Erta et al., 2013). Although the occurrence of these compounds is usually lower than the parent forms, they should be monitored in food chain otherwise this could lead to an under estimation of the real mycotoxin human and animal intake, as suggested by the recent EFSA opinion (EFSA, 2016). Taken altogether, profiling data reported within this study confirm the occurrence of a complex mixture of parent and modified forms of Fusarium mycotoxins in grains and although the modified mycotoxin forms represent a very little in terms of concentration they could lead to a huge problem for human and animal safety.

Conclusion

In conclusion, results obtained in this work increase the knowledge on the distribution of trichothecenes and zearalenone among the cereal grain tissues. DON was the mycotoxins found in higher concentration in all the cereal species and in the corresponding pearled fractions. Among them, the peripheral layers were the most contaminated (from 0-15 % of total kernel weight), underlying the importance of considering mycotoxin contamination when milling by-products are used in food formulation, with the final aim to increase the nutritional value of the products. The sequential pearling process showed a good potential for the mitigation of mycotoxin concentration in the endosperm, through the controlled removal of the outer layers. Considering the genetic susceptibility to fungi contamination of the wheat varieties taken under study, the pearling process achieve a 60 % of DON reduction and total absence of modified forms in inner part of cereal grains. Finally, the HR-MS analysis could elucidate clearly few of nowadays non-regulated modified forms of mycotoxins, laying the groundwork for future studies focused on the in-plant metabolism or/and studies regarding the biological activity of these compounds in human or animal organisms.

Author contribution

CD, MB, GG and MS designed the study. DG, VS and MB provided the sample set and performed the pearling process. MS and LR performed the analytical analysis. MS drafted the paper. All the authors contributed to the data interpretation, correction and critical review of the manuscript.

ACKNOWLEDGMENT

The authors kindly acknowledge Dr. Michele Suman and Mr. Dante Catellani, Barilla S.p.A., for the technical support in HR-MS analysis.

Supplementary Information

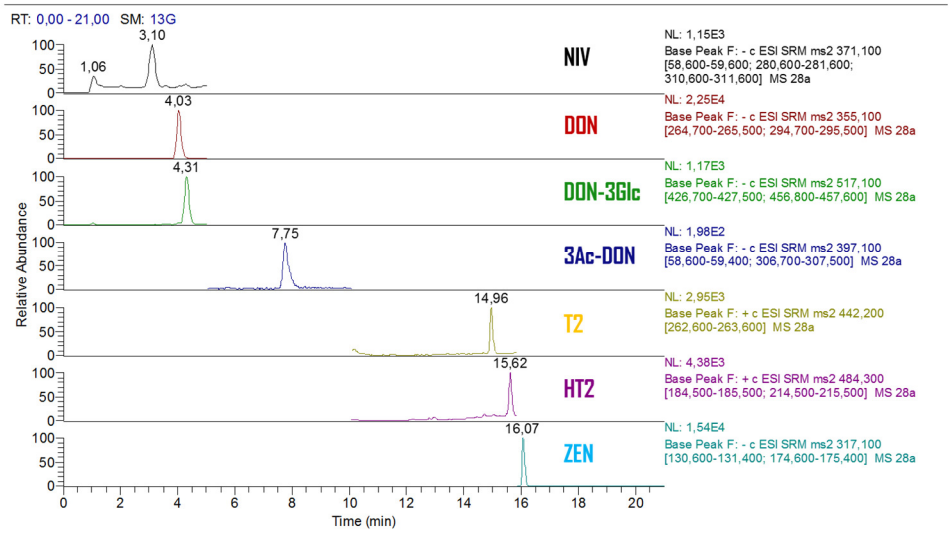

Figure A1. SRM (Selected Reaction Monitoring) of the monitored toxins in 0-5% pearled fraction of durum wheat cv. Saragolla.

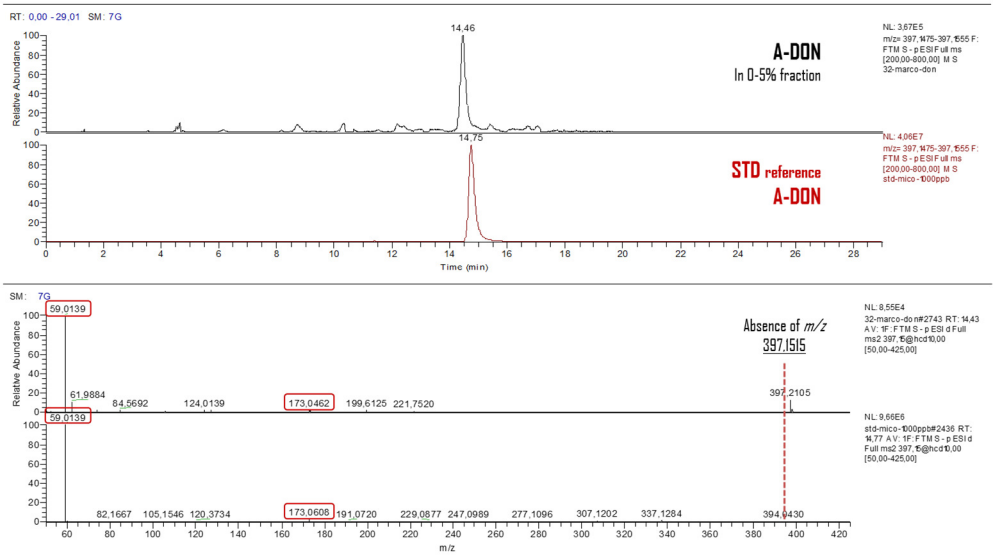

Figure A2. EIC of ADON in sample and in standard reference at 1000 µg kg^{-1}. Below chromatograms are reported the mass spectra, emphasizing the absence of the molecular ion and the characteristic fragmentation pattern of 3Ac-DON (red circles).

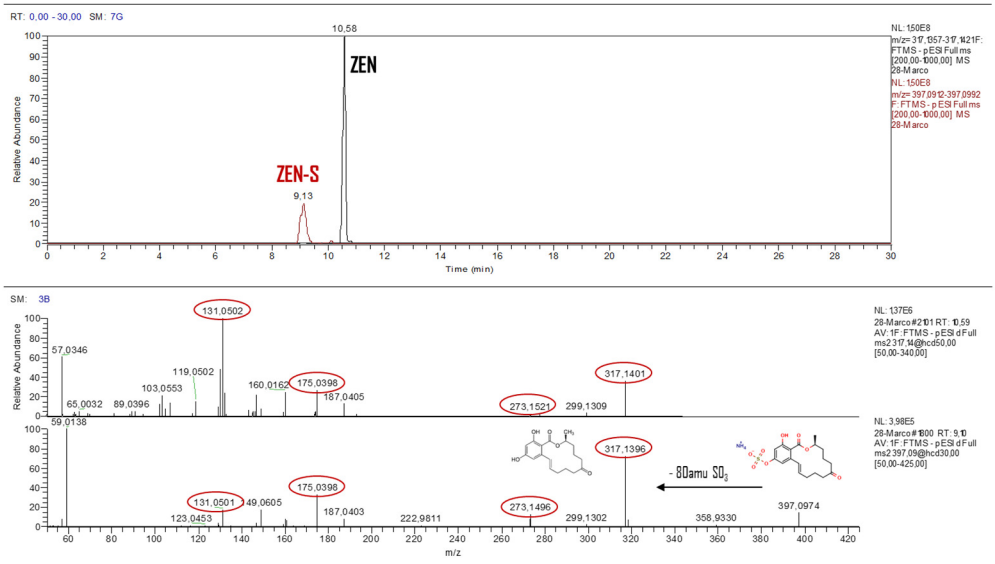

Figure A3. EIC of ZEN (*m/z* 317.1389) and ZEN-Sulphate (*m/z* 397.0952) and correspondent LC-HRMS/MS spectra. The loss of SO_3 from the ZEN molecule is clearly appreciable in the lower mass spectra, in which the ZEN molecular ion is generated.

References

Audenaert, K., Vanheule, A., Höfte, M., Haesaert, G., 2013. Deoxynivalenol: A major player in the multifaceted response of Fusarium to its environment. Toxins (Basel). https://doi.org/10.3390/toxins6010001

Bach Knudsen, K.E., Nørskov, N.P., Bolvig, A.K., Hedemann, M.S., Lærke, H.N., 2017. Dietary fibers and associated phytochemicals in cereals. Mol. Nutr. Food Res. https://doi.org/10.1002/mnfr.201600518

Berthiller, F., Crews, C., Dall'Asta, C., De Saeger, S., Haesaert, G., Karlovsky, P., P. Oswald, Isabelle Seefelder, W., Speijers, G., Stroka, J., 2013. Masked mycotoxins: A review.". Mol. Nutr. Food Res. 165–186.

Berthiller, F., Dall'Asta, C., Schuhmacher, R., Lemmens, M., Adam, G., Krska, A.R., 2005. Masked mycotoxins: Determination of a deoxynivalenol glucoside in artificially and naturally contaminated wheat by liquid chromatography-tandem mass spectrometry. J. Agric. Food Chem. https://doi.org/10.1021/jf047798g

Blandino, M., Locatelli, M., Gazzola, A., Coïsson, J.D., Giacosa, S., Travaglia, F., Bordiga, M., Reyneri, A., Rolle, L., Arlorio, M., 2015. Hull-less barley pearling fractions: Nutritional properties and their effect on the functional and technological quality in bread-making. J. Cereal Sci. https://doi.org/10.1016/j.jcs.2015.06.004

Blandino, M., Sovrani, V., Marinaccio, F., Reyneri, A., Rolle, L., Giacosa, S., Locatelli, M., Bordiga, M., Travaglia, F., Coïsson, J.D., Arlorio, M., 2013. Nutritional and technological quality of bread enriched with an intermediated pearled wheat fraction. Food Chem. https://doi.org/10.1016/j.foodchem.2013.04.122

Bryła, M., Ksieniewicz-Wozniak, E., Agnieszka, W., Szymczyk, K., Jedrzejczak, R., 2018. Natural occurrence of nivalenol, deoxynivalenol, and deoxynivalenol-3-glucoside in Polish winter wheat. Toxins (Basel). 10(2).

Buerstmayr, H., Lemmens, M., 2015. Breeding healthy cereals: genetic improvement of *Fusarium* resistance and consequences for mycotoxins. World Mycotoxin J. https://doi.org/10.3920/WMJ2015.1889

Cheli, F., Pinotti, L., Rossi, L., Dell'Orto, V., 2013. Effect of milling procedures on mycotoxin distribution in wheat fractions: A review. LWT - Food Sci. Technol. https://doi.org/10.1016/j.lwt.2013.05.040

Cirlini, M., Generotti, S., Dall'Erta, A., Lancioni, P., Ferrazzano, G., Massi, A., Galaverna, G., Dall'Asta, C., 2013. Durum wheat (Triticum Durum Desf.) lines show different abilities to form masked mycotoxins under greenhouse conditions. Toxins (Basel). https://doi.org/10.3390/toxins6010081

Dall'Erta, A., Cirlini, M., Dall'Asta, M., Del Rio, D., Galaverna, G., Dall'Asta, C., 2013. Masked mycotoxins are efficiently hydrolyzed by human colonic microbiota releasing their aglycones. Chem. Res. Toxicol. https://doi.org/10.1021/tx300438c

Dykes, L., Rooney, L.W., 2007. Phenolic compounds in cereal grains and their health benefits. Cereal Foods World. https://doi.org/10.1094/CFW-52-3-0105

Edwards, S.G., Kharbikar, L.L., Dickin, E.T., MacDonald, S., Scudamore, K.A., 2018. Impact of pre-harvest rainfall on the distribution of fusarium mycotoxins in wheat mill fractions. Food Control. https://doi.org/10.1016/j.foodcont.2018.02.009

EFSA Panel on Contaminants in the Food Chain (CONTAM). 2016. Scientific Opinion on the risks for human and animal health related to the presence of modified forms of certain mycotoxins in food and feed. EFSA J. https://doi.org/10.2903/j.efsa.2014.3916

Felizardo, M.P., Freire, J.T., 2018. Characterization of barley grains in different levels of pearling process. J. Food Eng. https://doi.org/10.1016/j.jfoodeng.2018.03.017

Freire, L., Sant'Ana, A.S., 2018. Modified mycotoxins: An updated review on their formation, detection, occurrence, and toxic effects. Food Chem. Toxicol. https://doi.org/10.1016/j.fct.2017.11.021

Giambanelli, E., Ferioli, F., D'Antuono, L.F., 2018. Retention of alkylresorcinols, antioxidant activity and fatty acids following traditional hulled wheat processing. J. Cereal Sci. https://doi.org/10.1016/j.jcs.2017.10.010

Giordano, D., Locatelli, M., Travaglia, F., Bordiga, M., Reyneri, A., Coïsson, J.D., Blandino, M., 2017. Bioactive compound and antioxidant activity distribution in roller-milled and pearled fractions of conventional and pigmented wheat varieties. Food Chem. https://doi.org/10.1016/j.foodchem.2017.04.065

Knutsen, H., Barregård, L., Bignami, M., Brüschweiler, B., Ceccatelli, S., Cottrill, B., Dinovi, M., Edler, L., Grasl-Kraupp, B., Hogstrand, C., Hoogenboom, L. (Ron), Nebbia, C.S., Oswald, I., Petersen, A., Rose, M., Roudot, A., Schwerdtle, T., Vleminckx, C., Vollmer, G., Wallace, H., Dall'Asta, C., Gutleb, A., Metzler, M., Oswald, I., Parent-Massin, D., Binaglia, M., Steinkellner, H., Alexander, J., 2017. Appropriateness to set a group health based guidance value for T2 and HT2 toxin and its modified forms. EFSA J. https://doi.org/10.2903/j.efsa.2017.4655

Lindblad, M., Gidlund, A., Sulyok, M., Börjesson, T., Krska, R., Olsen, M., Fredlund, E., 2013. Deoxynivalenol and other selected fusarium toxins in swedish wheat - occurrence and correlation to specific fusarium species. Int. J. Food Microbiol. https://doi.org/10.1016/j.ijfoodmicro.2013.07.002

Malachová, A., Sulyok, M., Beltrán, E., Berthiller, F., Krska, R., 2014. Optimization and validation of a quantitative liquid chromatography-tandem mass spectrometric method covering 295 bacterial and fungal metabolites including all regulated mycotoxins in four model food matrices. J. Chromatogr. A. https://doi.org/10.1016/j.chroma.2014.08.037

Maul, R., Müller, C., Rieß, S., Koch, M., Methner, F.J., Irene, N., 2012. Germination induces the glucosylation of the Fusarium mycotoxin deoxynivalenol in various grains. Food Chem. https://doi.org/10.1016/j.foodchem.2011.08.077

Nakagawa, H., He, X., Matsuo, Y., Singh, P.K., Kushiro, M., 2017. Analysis of the masked metabolite of deoxynivalenol and fusarium resistance in CIMMYT wheat germplasm. Toxins (Basel). https://doi.org/10.3390/toxins9080238

Pascale, M., Panzarini, G., Visconti, A., 2012. Determination of HT-2 and T-2 toxins in oats and wheat by ultra-performance liquid chromatography with photodiode array detection. Talanta. https://doi.org/10.1016/j.talanta.2011.12.017

Righetti, L., Rolli, E., Galaverna, G., Suman, M., Bruni, R., Dall'Asta, C., 2017. Plant organ cultures as masked mycotoxin biofactories: Deciphering the fate of zearalenone in micropropagated durum wheat roots and leaves. PLoS One. https://doi.org/10.1371/journal.pone.0187247

Ríos, G., Pinson-Gadais, L., Abecassis, J., Zakhia-Rozis, N., Lullien-Pellerin, V., 2009. Assessment of dehulling efficiency to reduce deoxynivalenol and Fusarium level in durum wheat grains. J. Cereal Sci. https://doi.org/10.1016/j.jcs.2009.01.003

Schmeitzl, C., Warth, B., Fruhmann, P., Michlmayr, H., Malachová, A., Berthiller, F., Schuhmacher, R., Krska, R., Adam, G., 2015. The metabolic fate of deoxynivalenol and its acetylated derivatives in a wheat suspension culture: Identification and detection of DON-15-O-glucoside, 15-acetyl-DON-3-O-glucoside and 15-acetyl-DON-3-sulfate. Toxins (Basel). https://doi.org/10.3390/toxins7083112

Šliková, S., Šrobárová, A., Šudyová, V., Polišenská, I., Gregová, E., Mihálik, D., 2010. Response of oat cultivars to Fusarium infection with a view to their suitability for food use. Biologia (Bratisl). https://doi.org/10.2478/s11756-010-0055-1

Sovrani, V., Blandino, M., Scarpino, V., Reyneri, A., Coïsson, J.D., Travaglia, F., Locatelli, M., Bordiga, M., Montella, R., Arlorio, M., 2012. Bioactive compound content, antioxidant activity, deoxynivalenol and heavy metal contamination of pearled wheat fractions. Food Chem. https://doi.org/10.1016/j.foodchem.2012.04.045

Zachariasova, M., Vaclavikova, M., Lacina, O., Vaclavik, L., Hajslova, J., 2012. Deoxynivalenol oligoglycosides: New "masked" fusarium toxins occurring in malt, beer, and breadstuff, in: Journal of Agricultural and Food Chemistry. https://doi.org/10.1021/jf302069z

Zhang, D., Moore, W., 1999. Wheat bran particle size effects on bread baking performance and quality. J. Sci. Food Agric. https://doi.org/10.1002/(SICI)1097-0010(19990501)79:6<805::AID-JSFA285>3.0.CO;2-E

1.2

The impact of processing on the phenolic acids, free betaine and choline in *Triticum* spp. L. whole grains and milling by-products.

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Preface

In this second part of the first Chapter, the study was focused on the distribution and quantification of important bioactive compounds named phenolic acids and methyl donor substances (betaine and choline) in industrially produced milling by-products. These compounds are nowadays object of study, in particular for their beneficial health-related properties. In fact, besides nutrient content, the consumption of whole cereal grains has been recognised as a factor that decreases the risk of overweight, coronary diseases, etc. [1]. These resulted in a higher disposition of governments and organizations to gather the knowledge on cereal grains nutrients for guidelines establishments [2]. Moreover, it is also important to increase the knowledge of the anatomical distribution of specific substances within cereal kernel in order to help breeding programmes and improving food processes.

In addition, this step was useful for an initial screening aimed to determine the potential use of by-products in the next pre-treatment phase (**Chapter II, sections 2.1 and 2.2**).

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[1] Jonnalagadda, S. S., Harnack, L., Hai Liu, R., McKeown, N., Seal, C., Liu, S., & Fahey, G. C. (2011). Putting the whole grain puzzle together: health benefits associated with whole grains—summary of American Society for Nutrition 2010 Satellite Symposium. The Journal of nutrition, 141(5), 1011S-1022S.

[2] Folloni, S., & Ranieri, R. (2013). Whole grain products in (Southern) Europe: Consumer trends and technological implications. Proceedings Whole Grains Summit 2012.

Abstract

Wheat (*Triticum* spp. L.) is considered one of the essential source of nutrients and bioactive compounds with recognized beneficial effects. Wheat undergoes several processes with the final aim of separating the endosperm from the outer layers, usually discarded. In this study, free and bound phenolic acids (PAs) profile, betaine and choline contents were quantified in six different wheat species (durum and bread wheat, turanicum wheat, einkorn, emmer and spelt), the corresponding milling by-products (bran, middlings, aleurone and I, II and III steps of debranning) and flour/semolina, using UHPLC-MS/MS methods. The bound form of phenolics was the component present in higher concentration (80% of the total, in average) and ferulic acid was the most abundant compounds, representing between 67-73 % of total PAs. Among the species, bread wheat grain totalized the highest content of total PAs (1209.31 ± 7.3 μg g^{-1} d.w.). Betaine and choline are abundantly present in wheat species. In general, the highest content of bioactive compounds was found in bran (3 times higher than whole grains), emphasizing the good nutritional profile of these by-products. The milling process leads to a severe reduction of phenolic acids and methyl-donors in the end-products.

Key words: wheat milling, by-products, whole grains, *Triticum* spp., phenolic acids, betaine, choline.

Abbreviation used

<LOQ, below the limit of quantification; 4-HB, 4-hydroxybenzoic acid; Caff, caffeic acid; c-Fer, cis-Ferulic acid; Dif, diferulates; d.w., dry weight; ESI, electrospray ionization; HILIC, hydrophilic interaction liquid chromatography; UHPLC, ultra-high performance liquid chromatography; MS, mass spectrometry; MRM, multiple reaction monitoring; PAs, phenolic acids; p-C, para-Coumaric acid; SD, standard deviation.; Sin, sinapic acid; t-Fer, trans-Ferulic acid.

Introduction

Wheat is one of the most important crops in the world. Wheat cereals belong to the Triticum spp. genus which comprehend several species, *Triticum turgidum* subsp. *durum Desf*. (known as durum wheat) and *Triticum aestivum* L. (known as bread wheat) are the most widely known, mainly because of their end use to produce many commodities such as pasta, bread and baked products (Shewry et al., 2013). In addition to these species, today there is a growing interest on the so-called "ancient" wheat, which are apparently more tolerant to abiotic and biotic stresses, like drought, pests, cold, and heat (Arzani & Ashraf, 2017). Moreover, greater health related properties than the classic bread and durum wheat have been reported for these wheat species (Arzani & Ashraf, 2017; Longin et al., 2016).

The so-called "ancient" wheat include *T. turgidum* subsp. *turanicum*, *T. monococcum* (einkorn), *T. turgidum* subsp. *dicoccum* (emmer) and *T. aestivum* subsp. *spelta* (spelt). Genetically, these species embrace all levels of ploidy. In fact, durum wheat, *turanicum* and emmer are tetraploids (2n=4x=28; genomes AABB), einkorn is diploid (2n=2x=14; genome AA) and bread wheat and spelt are hexaploids (2n=6x=42; genomes AABBDD) (Arzani, 2011). In terms of morphology, wheat grain is mainly structured in three parts, the endosperm (81-84%), that plays a role as storage of energy (starch granules), the bran (14-16%), which is composed by the outer layers protecting the grain, and the germ (2-3%), in which the genetic material is enclosed. However, for their suitability as ingredient in food preparation, the whole grain undergoes several processes generally defined as milling.

During this procedure a large amount of by products are generated, mainly composed by the most outer layer of the seeds. It has been determined that the by-products stream account for about 23-27% of the milling output (Serna-Saldivar, 2012). Nowadays these by-products are mainly directed to the feed industry, nevertheless they preserve their nutritional quality.

In fact, several recent studies have stressed the role of cereal grain consumption against cardiovascular diseases (Katcher et al., 2008), colorectal cancer and other health issues (Schatzkin et al., 2007). The beneficial properties are mainly associated with the occurrence of bioactive compounds and fibres. These molecules represent the product of the plant specialized metabolism, since it is a form of adaptation to specific ecological situation (Pichersky & Lewinsohn, 2011).

Among bioactive compounds are the phenolic acids (PAs), from either hydroxybenzoic (i.e.: p-hydroxybenzoic acid, vanillic acid and syringic acid) and hydroxycinnamic (i.e.: p-coumaric acid and ferulic acid) acid classes, recognized for their antioxidant activity and protective effects towards the oxidation processes. In plants, mainly two fractions of these compounds are present, a bound (most abundant) strictly bonded to polysaccharides that compose the cell wall, and a free component (Liyana-Pathirana & Shahidi, 2007) mainly present in the endosperm. Furthermore, wheat cereal products are also important sources of other non-essential nutrients such as betaine and its precursor, choline. They are chemically very similar, and they have been reported to exert a wide range of beneficial effects in humans (Craig, 2004). Betaine plays an important role in the conversion and detoxification of homocysteine to methionine in human liver and kidneys, acting as methyl group donor (De Zwart et al., 2003; Ross, Zangger, & Guiraud, 2014).

Likewise, choline is well metabolized in humans and converted in acetylcholine and phosphatidylcholine, which are important for the normal functions of cells (Zeisel, 2006). These compounds are mainly concentrated in the most external layers of the seed, such as the bran, germ and mostly in the aleurone. This means that highly refined products are more likely to be deficient in bioactive compounds (Andersson et al., 2013). However, numerous studies reported a high variance in terms of bioactive compounds concentration, either in whole grain and by-products (Abdel-Aal et al., 2001; Li, Shewry, & Ward, 2008). This is mainly due to the different wheat genotype, growing location, environment, and the interaction between these factors (Laddomada et al., 2017; Yu, Haley, Perret, & Harris, 2004).

In fact, as reported by Fernandez-Orozco, Li, Harflett, Shewry, & Ward, 2010, certain genotypes were more resistant to harsh environmental conditions affecting mostly the free (soluble) phenolic component.

Therefore, in order to increase the knowledge about the occurrence of bioactive compounds, the main objectives of this paper were (1) to determine the concentration of principal phenolic acids, free betaine and choline in six different Triticum species and (2) to monitor their distribution within the major fractions of the caryopsis produced after an industrial-scale milling process, using a UHPLC-MS/MS analytical method.

Material and Methods

Raw materials

All samples, whole grains and the corresponding milling by-products, were produced in industrial-scale durum and bread wheat mills. All durum wheat mills are equipped with the three-step debranning technology (Delfino&Giancaspro, Italy) that, in one case, is also combined to air-classification by a SeparMicroSystems (Italy) turbo-separator. For each cereal species different types of industrial-scale by-products were produced:

- *Triticum turgidum* subsp. *durum* Desf.: whole grain (WG), semolina (S), bran (B), fine bran (FB), middlings (M), aleurone (A), I, II and III steps of debranning (I°, II° and III° respectively);
- *T. aestivum* L.: whole grain (SWG), refined flour (F), bran (SB) and middlings (SM);
- *T. turgidum* subsp. *turanicum*: whole grain (TWG), semolina (TF), bran (TB) and middlings (TM);
- *T. monococcum* L.: whole grain (MWG), wholemeal flour (WMF) and middlings (MM);
- *T. turgidum* subsp. *dicoccum*: whole grain (DWG), flour (DF), bran (DB) and middlings (DM);
- *T. aestivum* subsp. *spelta*: whole grain (SpWG), refined flour (SpF), bran (SpB) and middlings (SpM).

On arrival, the whole grain samples were ground with an IKA a11 basic lab mill (IKA Mills) to particle size <200 mm, vacuum-packed in polyethylene bags and kept at -20°C prior to analysis. The proximate composition is reported as Supplementary Information (**Table 1S**).

Production of by-products. The wheat lots came from the 2015-2016 crop and were a commercial blend of different varieties. Durum wheat commercial lots were from either Italy (Marche, Emilia Romagna and Apulia regions) or the US (Northern Plains, North Dakota, ND) and collected from both Italian and American industrial milling plants. Commercial lots of all the other Triticum species were from Italy. Sampling: For each fraction, five sub-samples of the same lot were collected at different times and combined into one. A brief description of the milling by-products and the flow charts for the different wheat species are provided in **Table 6** and **Figure 1AS, BS and CS**, respectively (see supplementary information).

Table 6. Industrial milling by-products.

Fraction	Description	Average diameter (µm)	Median diameter (µm)	Amplitude distribution (Span)
Whole grain	Whole kernels coming from different commercial lots	-	-	-
Semolina/refined flour	Semolina is the main product of durum wheat milling while flour is the main product of wheat, einkorn, emmer, spelt and *turanicum* milling. Both semolina and flour are from the endosperm: vitreous endosperm is for durum wheat and floury endosperm is for the other species. Average particle sizes are 250 µm for semolina and 100 µm for refined flour respectively	-	-	-
Bran	Bran separated during the milling phase with particle size ranging between 0.8-1.2 mm	1050	950	1.5
Middlings	Fine bran particles and fine endosperm particles with some bran still attached	400	390	1.5
Aleurone	Fraction obtained by air-classification of the debranning fractions and containing a significant amount of aleurone layer	-	-	-
I, II and III steps of debranning	Fine bran fractions obtained through the first, second or third debranning steps having different particles sizes	680 (I), 450 (II), 310 (III)	530 (I), 370 (II), 220 (III)	2.2 (I), 2.4 (II), 3.0 (III)

Proximate composition

The analyses of moisture content (ISTISAN 1996/34, met. B), total nitrogen (ISTISAN 1996/34, conversion factor: 5.70), crude fat content (ISTISAN 1996/34, met. A), ash content (ISTISAN 1996/34) and total dietary fiber (AOAC 985.29:1997) were carried out by an external accredited laboratory of food analysis, which follows the official analytical methods used in food chemical control (Onori, Orefice & Stacchini, 1996). Then, the carbohydrate content was calculated by difference.

Chemicals

HPLC-grade acetonitrile (>99.9%), ethyl acetate (>99.8%), formic acid (>95.0%), acetic acid, hydrochloric acid (HCl, 37.0%), methanol (>99.9%), sodium hydroxide (NaOH, >98.0%), phenolic acid standards (caffeic acid >98%, p-hydroxybenzoic acid >99%, p-coumaric acid >98%, sinapic acid >98% and trans-ferulic acid >99%) betaine solution (0.1 M) and choline chloride (>99%) were purchased from Sigma-Aldrich (St. Louis, Missouri, US). The cis-ferulic acid was obtained by complete conversion of a trans-ferulic acid solution under UV light.

Extraction and analysis of free and bound phenolic acids

Phenolic acids were extracted and analysed according to Righetti *et al*., (2019). For the free phenolic compounds extraction, 1 g of finely ground sample was added to 4 mL methanol/water (70/30, v/v) mixture and put on a shaker at 200 strokes/min for 10 min at 25 °C, then was centrifuged for 10 min at 4000 rpm. After that, 1 mL of the supernatant was collected and brought to dryness under a gentle Nitrogen flow. The residue was dissolved with 200 µL of acidified water (0.2% of formic acid) before the analyses. While, the bound phenolic fraction was directly extracted from the residue generated from the previous procedure using 20 mL of NaOH 2N at room temperature for 1 h. After the hydrolysis, the pH was corrected to 3 by the addition of HCl 6N.

The bound phenolic fraction was then recovered with 10 mL of ethyl acetate, shaking samples for 10 minutes at 200 strokes/min. After a centrifugation step at 4000 rpm for 10 min, the supernatant was separated and using a gentle N flux was taken to dryness. The residue was recovered with 500 µL of 0.2% of formic acid water. For the separation of the analytes, a RP-C18 SunShell column (2.6 µm, 100 x 2.10 mm; Chroma Nik Technologies, Osaka, Japan) was used applying as eluents 0.1 % formic acid bi-distilled water and methanol (phase A and B, respectively). The gradient for the elution began from 2% of B with a 1 min isocratic step, then increased to reach 30% in 13 min; afterwards B was increased to 80% when reached 20 min and, after a rapid 2 min step, were restored the initial conditions for an overall run time was 30 minutes. Column temperature was set at 35°C, flow was maintained at 0.3 mL min^{-1}. For each sample, 2 µL were injected. Analytes were monitored in negative ionization mode using spray voltage at 3500 V, capillary temperature at 270 °C, vaporizer temperature at 200 °C, sheath gas flow at 50 units and, finally, auxiliary gas flow at 5 units. S-Lens RF amplitude and Collision Energy (CE) parameters were obtained by tuning methanolic solutions of the molecules analysed (1 mg L^{-1}). SRM modality was used as detection method, applying the following *m/z* transitions: 4-hydroxybenzoic acid (4-HB) *m/z* 137 → 93 (CE = 34 eV), *m/z* 137 → 65 (CE = 17 eV); p-coumaric acid (p-C) *m/z* 163 → 119 (CE = 14 eV), *m/z* 163 → 93 (CE = 34 eV); caffeic acid (Caff) *m/z* 179 → 135 (CE = 19 eV), *m/z* 179 → 107 (CE = 24 eV); ferulic acid (t-Fer) *m/z* 193 → 178 (CE = 18 eV), *m/z* 193 → 134 (CE = 14 eV), *m/z* 193 → 134 (CE = 19 eV); sinapic acid (Sin) *m/z* 223 → 208 (CE = 15 eV), *m/z* 223 → 164 (CE = 18 eV), *m/z* 223 → 120 (CE = 34 eV). Dimeric ferulic (Dif) acids with respective $[M-H]^-$ value of *m/z* 385 were analysed in full scan MS^2 mode and quantified as ferulic acid equivalents. The quantification was performed following two different calibration sets prepared in the solvent above described (0.05 - 5 µg gr^{-1}; 5 - 100 µg gr^{-1}).

Extraction and analysis of free betaine and choline

The extraction of betaine and choline from cereal samples was performed following the procedure proposed by Bruce, Guy, Rezzi, & Ross, (2010) with some modifications. Briefly, 1 gram of fine grounded sample was extracted with 15 mL of a 50% methanol/water solution. As betaine and choline may not be fully released from the sample matrix, samples were extracted sequentially six times, and extracts were pooled. Samples were then vortexed for 2 min in a vortex mixer and then centrifuged (10 min, 4 °C, 4000 rpm). The supernatant was then transferred to LC-MS vials and opportunely diluted for analysis. For the separation of the analytes, a HILIC XBridge BEH column (2.5µm, 150x3 mm; Waters, Massachusetts, USA) was used applying as eluents acetonitrile (phase A), bi-distilled water (phase B) both acidified with formic acid (0.2%) and 20 mM ammonium formate 1% formic acid (phase C).

The elution gradient started from 1% of B, 10% of C and 89% of A and, after an initial isocratic step of 1 min, B increased at 10% in 3 min; then at 5 min the percentage of B was further increased at 63% and, after a flashing step of 2 minutes, the initial conditions were restored. The total run time was 13 minutes, the column temperature was set at 35°C while the flow was maintained at 0.4 mL min^{-1}. For each sample, 3 µL were injected. Betaine and choline were monitored in positive ionization mode (spray voltage = 4000V), with the capillary and vaporizer temperature at 325 °C, the sheath gas flow was set at 50 units and the auxiliary gas flow at 5 units; the other parameters such as S-Lens RF amplitude and Collision Energy (CE) values were obtained and set by tuning methanolic solutions of each considered molecule (1 µg mL-1) as described for the phenolic acids content. Detection was carried out using SRM modality, using the following transitions: betaine *m/z* 118 → 42 (CE = 32 eV), *m/z* 118 →58 (CE = 24 eV), *m/z* 118 →59 (CE = 19 eV); Choline *m/z* 104 → 58 (CE = 33 eV), *m/z* 104 → 60 (CE = 25 eV). For quantification, a calibration set was prepared for betaine and choline (10-1000 µg g^{-1}) using commercial standards.

Statistical analyses

Sample extraction and analysis was performed in triplicate. Differences in the content of bioactive compounds between cereal species and among milling by-products were determined using one-way analysis of variance ANOVA with the *Tukey-b*'s post-hoc test. Correlation between total, free and bound phenolic acids was performed following the Pearson correlation test. Results were considered significant at $p < 0.05$ (reject the null hypothesis). All statistical analyses were performed using IBM SPSS statistics 21 software.

Results and Discussion

Whole grains and flours/semolina phenolic acids content

The phenolic acids profile of whole grain *Triticum* spp. differs significantly between species, as reported in **Table 7** and **8**. Regarding the soluble free component, the predominant PAs were t-Fer and Sin, as already reported by (Moore et al., 2005). Free t-Fer acid was the most abundant compound in all species (**Table 8**), except for *T. turanicum*, for which sinapic acid was found in higher concentration. 4-HB, p-C and caffeic acids were found as the lowest PAs in whole grains. Although p-C was quantified in durum and bread wheat, in the other whole grains it was below the limit of quantification (0.05 μg g^{-1}). In addition, in einkorn, emmer and spelt the free caffeic acid content was significantly higher than in durum, bread and *turanicum* wheats. Moreover, when the phenolic acids content of semolina and flours was compared to the respective whole grain, a not negligible difference was noticed. In fact, in durum, bread and *turanicum* wheat flours only the free form of caffeic acid was quantified. While in einkorn, emmer and spelt flours also t-Fer and Sin acids were found. The bioactive compound loss is mainly attributable to the milling process resulting in a separation of the outer layers from the starchy endosperm. Besides, the contribution of the soluble PAs component differs for each compound and for each wheat species. For example, the free form of 4-HB accounts between 4.5-11.7 %, 0.7-3.6 % for *p*-C, 18.8-73.7 % for Caff, 0.3-0.5 % and 1.8-21.5 % for t-Fer and Sin, respectively. These results are in accordance with other studies (Mpofu, Sapirstein, & Beta, 2006; Okarter, Liu, Sorrells, & Liu, 2010). The presence of a high free phenolic content is important from the nutritional point of view. In fact, in this form they can be easily absorbed in the human intestine exerting their beneficial functions.

Table 7. Free and bound phenolic acids content (μg g $^{-1}$ d.w.) of different whole grain Triticum species and corresponding milling by-products.

Wheat species	Milling product	4-HB		p-C		Caff		Sin	
		Free	Bound	Free	Bound	Free	Bound	Free	Bound
Durum wheat	WG	0.14±0.07d	1.45±0.25d	0.14±0.02cde	6.51±+0.60c	0.12±0.00e	0.46±0.08cd	0.66±0.48c	24.91±2.93de
	S	<LOQ	<LOQ	0.06±0.01e	0.18±0.26cd	0.13±0.00e	<LOQ	<LOQ	4.12±0.57e
	B	0.61±0.05b	5.81±0.47ab	0.22±0.04cd	13.50±0.09ab	0.25±0.00cd	1.45±0.02ab	7.06±0.14b	75.86±4.28b
	FB	0.25±0.03d	4.22±0.42abc	0.11±0.03de	9.85±0.34abc	0.20±0.06de	1.13±0.11abc	2.37±1.34de	62.71±2.87bc
	M	0.40±0.05bc	3.22±0.94c	0.18±0.02cde	7.90±4.33bc	0.28±0.01c	1.00±0.37bc	3.74±0.17cd	58.55±9.71bc
	A	0.46±0.23bc	5.91±0.96a	0.36±0.07ab	14.52±2.44a	0.18±0.01e	1.82±0.41a	4.91±1.29bcd	120.29±25.60a
	I	1.43±0.05a	3.98±0.43abc	0.47±0.01a	9.54±0.87abc	0.43±0.01a	0.95±0.06bc	11.19±0.29a	35.60±1.99cde
	II	1.43±0.10a	4.95±0.27abc	0.27±0.01bc	10.53±0.07abc	0.36±0.01b	1.46±0.10ab	6.42±0.85bc	63.42±3.55bc
	III	0.46±0.01bc	3.86±0.23bc	0.20±0.03cd	10.44±0.88abc	0.28±0.00c	1.30±0.16ab	3.56±0.13cd	53.88±1.66bcd
Bread wheat	BWG	0.11±0.05b	2.30±0.07b	0.07±0.01	5.02±0.44b	0.31±0.06b	1.22±0.03b	1.19±0.08c	40.29±3.24ba
	F	<LOQ	0.30±0.04b	<LOQ	<LOQ	0.14±0.01b	<LOQ	<LOQ	2.38±0.25b
	BB	0.48±0.19a	11.73±3.69a	0.08±0.02	16.91±5.34a	0.78±0.14a	4.92±1.4a	7.93±1.42a	123.77±49.82a
	BM	0.19±0.04b	5.02±0.12b	0.09±0.01	9.12±0.35b	0.39±0.00b	0.87±0.10b	3.35±0.37b	84.96±1.94ba

(Table 7 continued)		4-HB		p-C		Caff		Sin	
Wheat species	**Milling product**	**Free**	**Bound**	**Free**	**Bound**	**Free**	**Bound**	**Bound**	**Free**
Turanicum	TWG	0.10±0.02b	1.48±0.29c	0.08±0.01	4.44±0.06c	0.12±0.00	0.53±0.05b	3.71±0.92b	13.42±1.16b
	TF	<LOQ	0.63±0.00c	<LOQ	1.15±0.27d	0.12±0.00	0.16±0.01c	<LOQ	4.78±0.86b
	TB	0.63±0.25a	10.87±0.39b	0.19±0.09	20.78±0.34b	0.14±0.01	3.16±0.05a	6.08±1.23a	59.70±1.57a
	TM	0.41±0.10b	12.70±0.46a	0.16±0.05	25.71±1.39a	0.14±0.00	3.07±0.13a	2.99±0.41b	68.36±6.65a
Einkorn	MWG	0.18±0.07	1.52±0.56b	0.06±0.01	4.65±2.03b	1.07±0.12	0.38±0.13b	0.54±0.23b	29.01±9.89b
	WMF	0.29±0.12	1.41±0.07b	0.09±0.01	5.42±0.27b	1.22±0.09	0.58±0.00b	1.12±0.16b	29.77±0.30b
	MM	0.51±0.17	5.84±0.32a	0.16±0.09	19.47±0.06a	1.70±0.73	5.33±0.07a	1.56±0.85a	116.11±0.45a
Emmer	DWG	0.11±0.03	2.28±0.06c	0.07±0.00	11.12±0.64c	0.35±0.01	1.10±0.13c	1.14±0.02	29.49±7.91c
	DF	<LOQ	1.26±0.09d	<LOQ	3.23±0.14d	0.26±0.02	0.70±0.05c	0.36±0.03	21.92±0.67c
	DB	0.27±0.06	14.84±0.10a	0.09±0.00	43.05±1.63a	0.45±0.01	4.27±0.30a	2.05±0.56	169.28±5.95a
	DM	0.25±0.17	7.63±0.29b	0.08±0.03	17.26±0.9b6	0.51±0.22	2.90±0.09b	1.45±0.79	140.41±1.61b
Spelt	SpWG	0.09±0.03c	1.55±0.01c	0.05±0.00	5.15±0.47c	0.44±0.00c	0.82±0.24c	0.84±0.01c	22.80±1.67c
	SpF	<LOQ	<LOQ	<LOQ	<LOQ	0.16±0.02d	<LOQ	<LOQ	3.82±0.12d
	SpB	0.73±0.21a	9.14±0.28a	0.07±0.02	21.04±0.51a	1.02±0.06a	7.54±0.53a	3.19±0.36a	115.42±3.08a
	SpM	0.49±0.15b	4.09±0.11b	0.10±0.00	10.55±0.14b	0.93±0.02b	5.02±0.22b	2.38±0.03b	75.00±2.17b

4-HB: 4-hydroxybenzoic acid; p-C: p-Coumaric acid; Caff: caffeic acid; Sin: sinapic acid. Results are expressed as μg gr^{-1} d.w. Different letters in the same column indicate a significant difference ($p<0.05$) using Tukey-b's post-hoc test. <LOQ: 0.05 μg g^{-1} d.w.

The bound and conjugated phenolic acids are the components present in higher concentration in cereals. These compounds are strictly linked to the fibrous elements of the seed, like lignins, cellulose, arabinoxylans and sometimes to sugar moieties. As for the free component, the most abundant PAs found in whole grains were t-Fer and Sin, while the least concentrated ones were p-C, 4-HB and caffeic acids. Interestingly, among the secondary phenolic acids, p-C was the most abundant in whole grains, in particular in emmer, indicating that this PA in cereals is mainly found in the bound form. Likewise, the t-Fer is mostly present in its matrix-linked form, accounting between 99.5-99.7 % of the total. These results were also reported in the study of Nicoletti et al., (2013). On the contrary, the bound form of caffeic acid in einkorn accounts only for the 26.3 %, indicative of a high free content of this compound in einkorn. Furthermore, the isomer of t-Fer, *cis*-Ferulic acids (c-Fer) could be found in cereals. Arguably, the c-Fer is the isomer found in the lowest concentration, nevertheless it was highly present in almost all the whole grain species except for the *spelta* wheat, in which it was found as <LOQ, as reported in **Table 8**. The highest content of this compound was found in the bread wheat whole grain. In addition, the dimers (Dif) of t-Fer, composed by two moieties of ferulic acid with *m/z* 385 (**Figure 14**), can be also found in cereals. Here it was highly concentrated in durum, bread and *turanicum* whole grains, and significantly less abundant in einkorn, emmer and spelt whole grains. Moreover, a remarkable content of diferulates was also found in flours. These compounds are strictly linked to the cell wall polysaccharides and the 5-5- and 5-8-dehydrodiferulate esters are the mostly reported (Li et al., 2008). Shibuya, (1984) reported the correlation between the occurrence of this compounds in the starchy endosperm and the presence of high carbohydrates content.

Table 8. Content (μg g $^{-1}$ d.w.) of the different ferulic acid forms in *Triticum* spp. whole grains and corresponding milling by-products.

Wheat species	Milling product	t-Fer		c-Fer	Dif
		Free	Bound		
Durum wheat	WG	1.72±0.01d	369.70±67.3bc	91.56±1.84cd	314.07±65.26c
	S	<LOQ	45.37±2.33c	<LOQ	<LOQ
	B	6.74±0.41b	1093.59±63.15a	235.83±8.44bc	564.46±19.66ab
	FB	2.19±1.28d	759.44±3.85ab	214.18±0.76bc	394.43±26.64bcd
	M	3.38±0.45cd	722.59±312.56ab	225.46±94.68bc	441.26±1.82bcd
	A	2.92±0.38d	1094.20±249.70a	430.18±94.91a	665.89±138.49a
	I	12.45±0.38a	623.29±33.98ab	201.25±16.58bc	342.02±27.61cd
	II	4.94±0.06c	965.59±5.62a	289.83±15.54b	554.38±32.05ab
	III	2.42±0.29d	821.00±3.01ab	268.85±14.26b	522.86±29.44abc
Bread wheat	BWG	1.94±0.65b	564.15±0.88c	253.89±11.93b	338.80±0.06a
	F	<LOQ	19.00±1.53d	16.73±1.79c	34.04±1.99b
	BB	9.52±1.56a	1330.79±7.93a	445.95±1.87a	533.25±172.83a
	BM	3.07±0.74b	1148.41±63.76b	273.97±40.49b	616.47±34.32a
Turanicum	TWG	1.02±0.29c	208.33±15.05c	46.44±0.47c	345.28±0.62c
	TF	<LOQ	66.40±3.03d	29.62±0.62d	104.82±14.72d
	TB	6.80±1.82a	1571.14±59.63a	352.18±28.27b	822.67±56.58a
	TM	3.84±0.60b	1331.04±74.22b	442.59±10.05a	659.14±9.77b

(Table 8 continued)		t-Fer		c-Fer	Dif
Wheat species	**Milling product**	**Free**	**Bound**		
Einkorn	MWG	1.08±0.35	336.37±1.2b	88.07±1.21c	133.43±52.28b
	WMF	0.80±0.13	219.67±3.36c	117.49±3.92b	119.03±46.97b
	MM	1.34±0.32	1239.70±10.94a	421.75±1.61a	325.64±14.32a
Emmer	DWG	1.19±0.08b	310.66±3.28c	64.25±10.52c	113.44±18.92c
	DF	0.69±0.00b	176.90±8.67d	37.32±0.01d	161.37±2.6b
	DB	2.90±0.24a	1894.73±0.26a	338.00±34.87a	485.13±2.69a
	DM	1.90±0.73ab	1340.13±4.94b	188.03±7.21b	500.91±0.10a
Spelt	SpWG	1.27±0.28c	300.64±36.32c	21.26±1.92c	234.94±22.34b
	SpF	0.60±0.01d	32.39±3.69d	<LOQ	43.83±1.63c
	SpB	4.73±0.13a	1696.15±33.59a	230.24±16.79a	580.57±64.49a
	SpM	3.31±0.01b	1033.24±28.57b	50.48±14.69b	517.23±11.02a

t-Fer: *trans*-ferulic acid; *c*-Fer: *cis*-ferulic acid; Dif: diferulic acid. Results are expressed a µg/gr d.w. Different letters in the same column indicate a significant difference ($p<0.05$) using *Tukey-b's* post-hoc test. <LOQ: 0.05 µg g^{-1} d.w.

Moreover, the differences in phenolic content found in the end-products, flours and semolina, were more pronounced, stressing the effect of the processing. In fact, very low quantity of PAs was found in flours and semolina, due to the high refining degree. Instead, wholemeal flour produced from einkorn showed no differences in terms of PAs content with respect to the whole grains. The latter remarks the fact that the most of bioactive components were found in the outermost layers of the kernel (Siebenhandl et al., 2007). It is worth to mention that of from one side the milling process improves the technological and sensorial quality of cereals, from the other side beneficial compounds are lost.

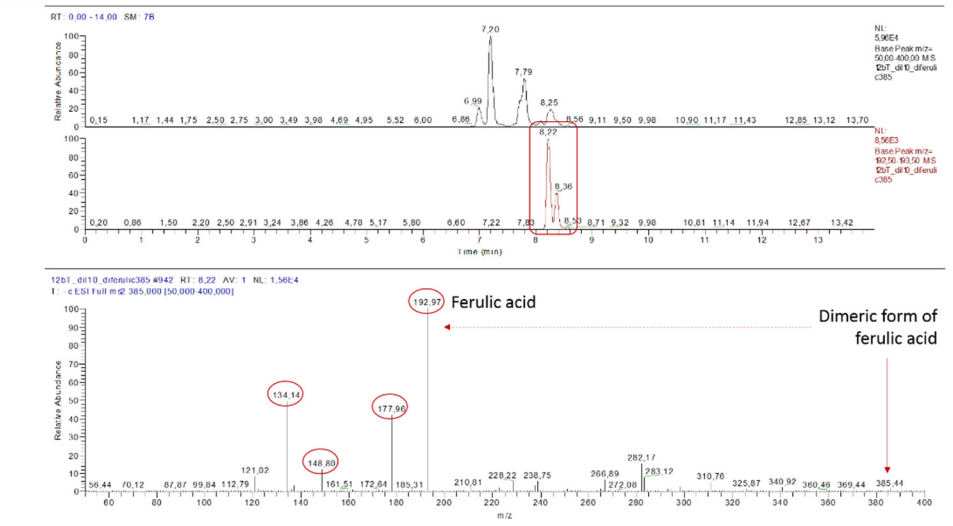


Figure 14. Chromatogram and mass spectrum of the full scan analysis of the ion m/z 385. The EIC (extracted ion chromatogram) of ion *m/z* 193 (ferulic acid) is represented in red. The ion fragments surrounded by red circles represent the characteristic fragmentation pattern of the ferulic acid.

The total phenolic acid content (TPA) is represented by the sum of the free and bound form of these compounds. This last component gave the highest contribution to this parameter. In fact, a positive correlation was found between the TPA and both the bound (r=0.57) and free (r=0.56) components of PAs.

However, significant differences in TPA between whole grains were found (**Figure 15**). The highest TPA was obtained by the bread wheat (1209.31±7.3 μg g^{-1} d.w.), followed by the durum wheat (811.4±137.7 μg g^{-1} d.w). Nevertheless, no significant difference was found between *turanicum* (608.1±13.5 μg g^{-1} d.w), einkorn (596.4.0±64.6 μg g^{-1} d.w), emmer (508.3±24.4 μg g^{-1} d.w) and spelt (568.6±60.4 μg g^{-1} d.w). These findings are consistent with the studies conducted by Brandolini, Castoldi, Plizzari, & Hidalgo, (2013), in which they performed a two-year evaluation of phenolic acids composition, total polyphenols content and antioxidant activity on einkorn, emmer, durum and bread wheat. Since ferulic acid is the most common phenolic acid found in cereals (Okarter et al., 2010), its total content in whole grains was similar and ranged between 89.5 % (emmer) to 93 % (bread wheat), as reported by Brandolini et al., (2013). Besides, the effect of the milling process on the total amount of the PAs were calculated. The sequential removing of the bran fraction caused an important decrease of these bioactive compounds in the flour and semolina samples. For example, in durum wheat semolina and bread wheat flour a 94 % loss of total PAs was detected. Likewise, the same trend was seen in *turanicum* semolina (62%) and spelt flour (82%); however, the milling effect was less marked for emmer flour (11%). This high variance could be due to many variables, such as the seed morphology and shape, but also the technology used to separate the external tissues from the endosperm (Beta, Nam, Dexter, & Sapirstein, 2005). However, when part of these outer layers, richer in PAs, are preserved in the flour, this decrease is negligible. For instance, the PAs loss in einkorn wholemeal flour was the 2 %.

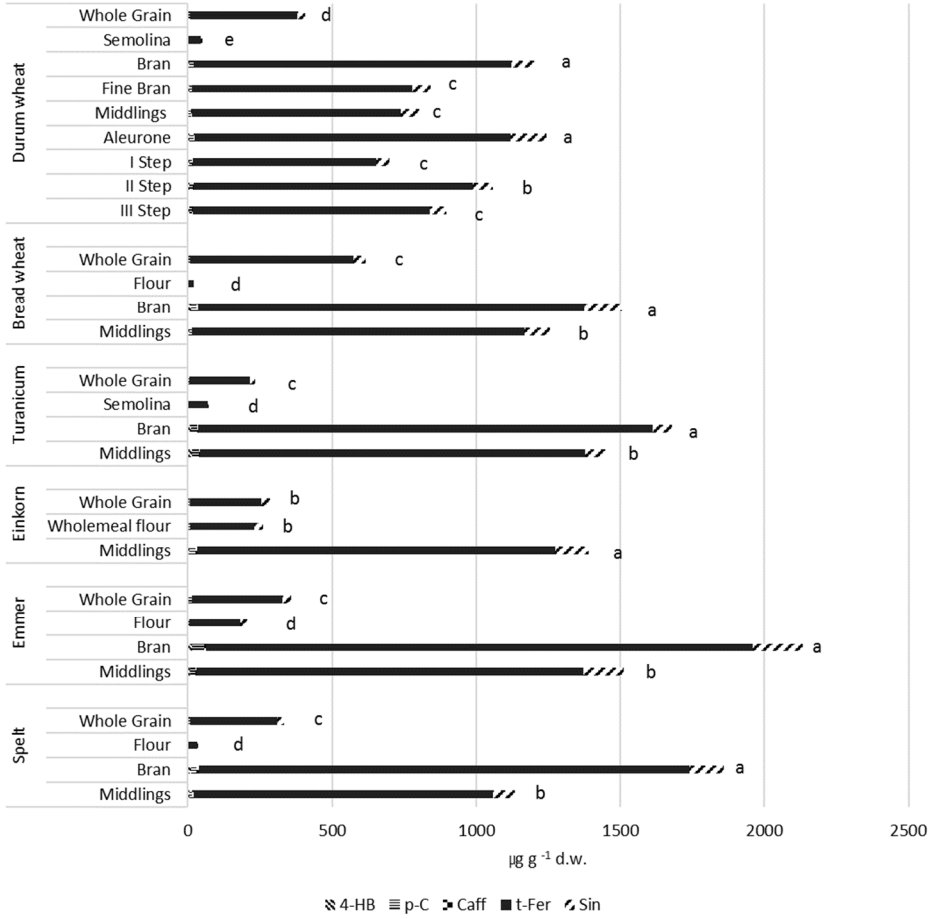


Figure 15. Total phenolic acids (TPA) content of *Triticum* whole grains and milling by-products. Different letters on top of bars indicate a significant difference ($p < 0.05$) using Tukey-b's post-hoc test. The contribution of the different phenolic acids on the total content is also showed.

Milling by-products phenolic acids content

Cereal milling by-product streams can differ in function of the seed shape and morphology and in relation to the milling technology used. In general, bran and middlings are produced during the kernel fractionation. The most abundant PAs were the t-Fer and Sin whereas 4-HB, Caff and p-C were the least abundant.

Nevertheless, differences among wheat species were found as reported in **Table 7** and **8**. Free t-Fer and Sin were found in higher amount in bran than the

middlings by-product. In particular, they were more abundant in bread wheat bran and, in order, in *turanicum*, durum, spelt, emmer and einkorn bran. 4-HB was more concentrated in bran fractions and p-C was less variable between by-products. Interestingly this p-C was quantified below the limit of quantification (calculated as the slope of calibration curve divided by the standard deviation of response, multiplied by ten, as suggested by the ICH-International Conference on Harmonisation) in bran and middlings of bread wheat and in spelt bran. In addition, the highest amount of caffeic acid was found in spelt bran. Regarding the durum wheat fractions, since different milling process is commonly applied, also different by-product side streams are produced. In our case, aleurone tissue, I, II and III steps of debranning were obtained from the milling processing of whole durum wheat grain. Overall, the I and II steps of debranning were the fractions in which free phenolic acids were more abundant, followed by the aleurone layer. However, the percentage in terms of weight of these fractions in respect to the whole is very low (around 10 % as sum of the three steps), thus also the contribution to the total phenolic acids content is negligible. Beside this, the *c*-Fer and diferulates were most abundant in the aleurone fraction. This cereal grain component is nowadays largely used for the production of wheat bread since it could confer a better sensorial quality (Bagdi et al., 2016) and an increased bioavailability (Bresciani et al., 2016)of bioactive compounds in respect to the wheat bran addition.

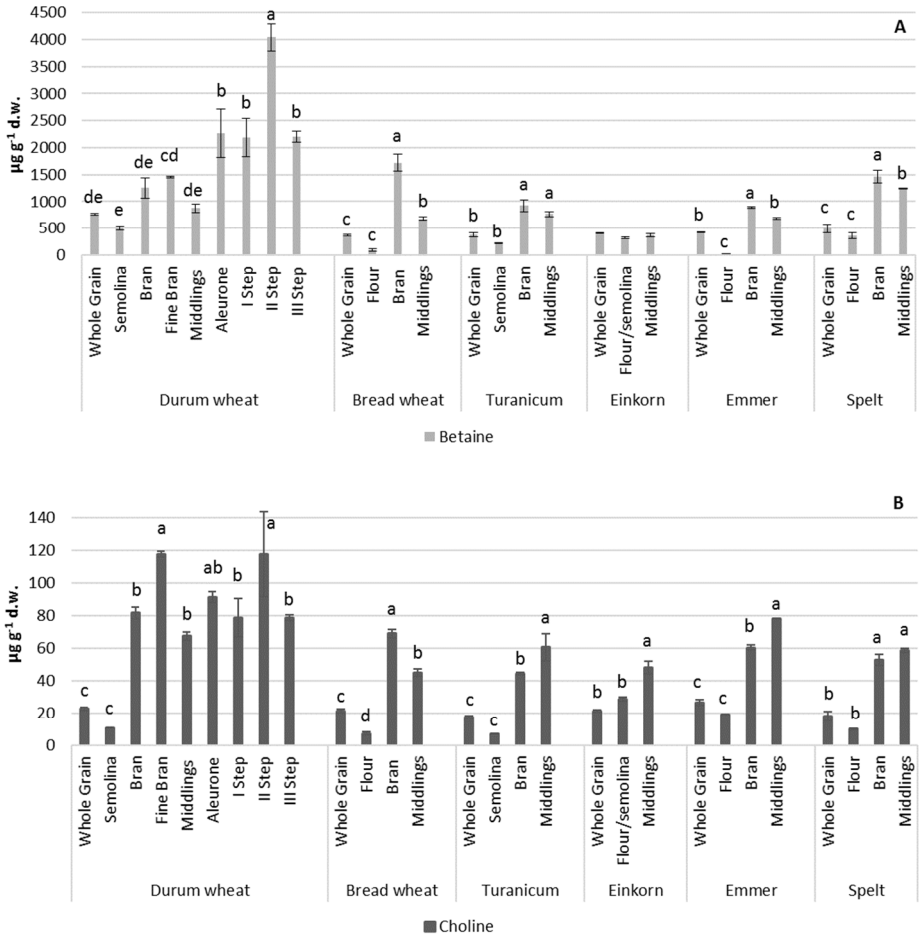


Figure 16. The free betaine (A) and choline (B) content in whole grain and milling fractions of wheat species. The results are expressed on a d.w. basis. Different letters on the top of the bars indicate a significant difference ($p<0.05$) using the *Tukey-b's* post hoc test.

Bran, middlings and other cereal milling by-products are mainly composed by several layers of fibrous material as reported in **Table S1** (see supplementary information). Therefore, a higher contribution of the bound phenolic component was expected. The general composition was similar to the whole grain, represented by the major PAs as t-Fer, its dimers, c-Fer and Sin, and minorities as p-C, 4-HB and caffeic acids. As for whole grains, the p-C acid was the most abundant among the secondary PAs, found in highest concentration in emmer bran.

Since the ferulic acid is the most abundant PA found in cereals, the sum of *cis* and *trans* isomers accounts between 67-73 % of total PAs, in accordance to Gallardo, Jiménez, & García-Conesa,(2006). However, the bound form of this PA was preponderant, ranging between 97-99 %. In fact, the bound and insoluble phenolic compounds are mainly found linked by means of ester or ether bonds to the five carbon atoms sugars of arabinoxylans, as described by Smith & Hartley, (1983). The highest content of t-Fer was found in emmer bran, followed by spelt bran and *turanicum* bran. The dimeric form of ferulic acid was also found in milling fractions, in particular the highest value was measured in *turanicum* bran where it was present entirely in the bound form. Among by-products, the highest concentration of bound PAs was found in the bran fraction. Regarding durum wheat by-products, aleurone showed the highest content of all bound phenolic acids, indicating that these compounds are strictly connected to the polysaccharides of the cereal grain cell wall. Overall, I, II and III steps of debranning bound PAs content did not significantly differ from the aleurone layer. In fact, since the debranning process starts from the outer to the inner part of the kernels, possibly a small portion of aleurone would be present which can influence the latter composition.

The total phenolic acids content in wheat milling by-products progressively increased from the inner (endosperm) to the outer layers (bran and germ) of the seed, as depicted in **Figure 15**. The TPA content differs significantly between cereal species and the highest amount was found in emmer (2955,07±25,9 µg g^{-1} d.w, bran) and *turanicum* wheat (2550,15±66,47 µg g^{-1} d.w, middlings). The by-products commonly called “bran” is composed by several layers including the aleurone, testa and pericarps, as shown in **Figure 5**. They could differ between species in terms of thickness and composition and could represent a good source of bioactive compounds (Hidalgo & Brandolini, 2008; Serna-Saldivar, 2012). These compounds could play an important role in terms of plant physiology. In fact, the accumulation of specialized metabolites is a recognized strategy for plant protection against pathogens or/and biotic/abiotic stimulus (Agrios, 2005).

Furthermore, a very high concentration of phenolic compounds was seen in bran and middling samples. These compounds could play an important role in terms of plant physiology. In fact, the accumulation of secondary metabolites is a recognized strategy for plant protection against pathogens or/and biotic/abiotic stimulus (Agrios, 2005). The latter is probably the reason why phenolic acids are more concentrated in the outer layers of the seed, which constitute the first barrier between the core and the environment. In addition, each by-products account between 12-14 % of the total weight of the grain, nevertheless they are used in large amount as ingredients or supplements. For example, the concentration factor, in percentage, ranged between 206-607 % in bran samples, and 177-452 % in middling samples. Nevertheless, the seed size plays an important role during the milling process. In fact, in function of the dimension and the shape of the kernel, the portion removed during the processing could be different. In this way, grains that are bigger have a higher portion of endosperm, on the contrary in smaller grains like einkorn, the bran fraction will account for more weight (Brandolini & Hidalgo, 2011). In this way, a higher concentration factor should not be intended as a higher phenolic content, which has to be related to the % (w/w) of tissue removed from the kernel.

Free betaine and choline content

Whole grain and flour/semolina

Methyl donor compounds, like betaine and choline, play important role in human diet and, probably wheat is the most important dietary source for human (Likes, Madl, Zeisel, & Craig, 2007). **Figure 16** shows the free betaine (A) and choline (B) content of whole grains and corresponding milling by-products. The highest content of betaine was found in durum wheat whole grain (757.13±15.96 µg g^{-1} d.w), while the highest choline content in emmer whole grain (26.47±1.93µg g^{-1} d.w). Flour and semolina samples had the lowest betaine and choline content, meaning a detrimental effect caused by the sequential removing of the seed outer layers.

For example, the betaine and choline concentration in spelt flour (360,61±53.78 and 9.94±0.73 μg g^{-1} d.w, respectively), corresponds to a loss of 27 and 45 %, respectively, as compared to the whole grain. Overall, the pauperization effect caused by the milling determined a decreased betaine and choline content, which ranged between 23-94 % and 28-67 %, respectively. These results are in agreement with those reported by Likes, Madl, Zeisel, & Craig, (2007). However, the variability in betaine and choline content can differ in respect to cereal genotypes and in function of the growing conditions (Corol et al., 2012). In addition, as discussed by Burg, Ferraris, & Dmitrieva, (2007) and Ross et al., (2014), betaine acts as an osmolyte in plants, hence its occurrence is related to the osmotic stresses and proper cell volume regulations. In this study the betaine content of bread wheat was 367.56±17.88 μg g^{-1} d.w. Similar values for bread wheat whole grain were found by Kojić et al., (2017), who studied the betaine levels in cereals pseudocereals and their products. However, the betaine content in spelt grain (495.82±84.03 μg g^{-1} d.w) was lower than the level found in that study. Furthermore, *turanicum*, einkorn and emmer free betaine content was 378.61±32.69, 415.17±0.86 and 427.91±6.34 μg g^{-1} d.w, respectively. Choline content was lower and ranged between 17.37±1.16 (*turanicum*) 22.55±0.89 μg g^{-1} d.w (durum wheat). These findings are slightly lower than the results reported by Ross et al., (2014). Nevertheless, the high results variability could be due to the different extraction and analysis methods. However, in this study, the importance of the sequential extraction of the matrix is underlined as explained by Bruce et al., (2010) and Hefni, McEntyre, Lever, & Slow, (2016).

Milling by-products

The determination of specialized metabolites of different cereal fractions obtained by technological process can be useful for the evaluation of nutrients distribution and availability and also can provide information about the degree of the milling step.

Between the two methyl-donor compounds, betaine occurs in higher concentration than choline. The content of the free betaine in cereal milling by-products was higher in bran, as already reported in the study conducted by Likes et al., (2007), Graham, Hollis, Migaud, & Browne, (2009), Ross et al., (2014) and Kojić et al.,(2017).

The highest content of free betaine was found in bread wheat bran (1720,05±160.94 μg g^{-1} d.w) while choline was the highest in durum wheat bran (81.71±3.70 μg g^{-1} d.w) and emmer middlings (78.2±0.20 μg g^{-1} d.w). In addition, also spelt bran appears to be a good source of betaine (1464.12±115.78 μg g^{-1} d.w). These findings are slightly lower than those reported by Bruce et al., (2010), however they fall in the range proposed by Patterson et al., (2008). Nevertheless, they were very different to the results found by Zeisel, (2006) and Filipčev, Kojić, Krulj, Bodroža-Solarov, & Ilić, (2018). The latter could be related to the fact that different methods were employed, although more studies must clarify this high variability. In addition, it is possible that an important portion of these compounds is present in bound or linked form in cereals. Interestingly, the choline content in middlings of ancient wheat species appears to be richer than in the bran fraction, while this trend was opposite in durum and bread wheats. In relation to the durum wheat milling process, the II step of debranning was found to be significantly richer in betaine and choline content, confirming that methyl-donors are mostly present in the outer layer of cereal grain.

Conclusion

In conclusion, a wide variability in terms of bioactive compounds was found among the *Triticum* species and by-products. Regarding phenolic acids, the soluble component was highly lower than the bound and insoluble component, representing in average ~ 80 % of the total phenolic acids. Among them, ferulic acid was the predominant, especially in the outer layers (bran and middlings) where it was found strictly bound to the matrix (~ 80 %). In addition, also ferulic acid *cis* isomer and its dimeric form were found in high amount. Bread wheat had the highest total phenolic acids content, nevertheless when by-products were compared, emmer bran was the highest one; this fact underlines the importance of the shape, dimension and processing variables. In relation to the free betaine and choline content, in this study we confirmed the relevance of cereal grains and by-products as good sources of these compounds. The distribution trend reflects the one of phenolic compounds, decreasing toward the inner part of the seed. Overall, the milling process extremely reduced the content of bioactive compounds, meaning that the end-products were poor in these substances. However, the re-integration of the outer layers to produce a wholemeal flour allowed to overcome the problem, as seen for einkorn wholemeal flour. Finally, information regarding the occurrence of these type of compounds in *Triticum* flours and by-products could be useful to the determination of accurate levels of intake.

Authors contribution

MS, CD and GG designed the study. RR and SF gathered the sample collection and followed the milling process. MS performed the analysis. MS, CD and GG interpreted the results. MS drafted the paper. All the authors contributed to the critical review of the manuscript.

Supplementary Information

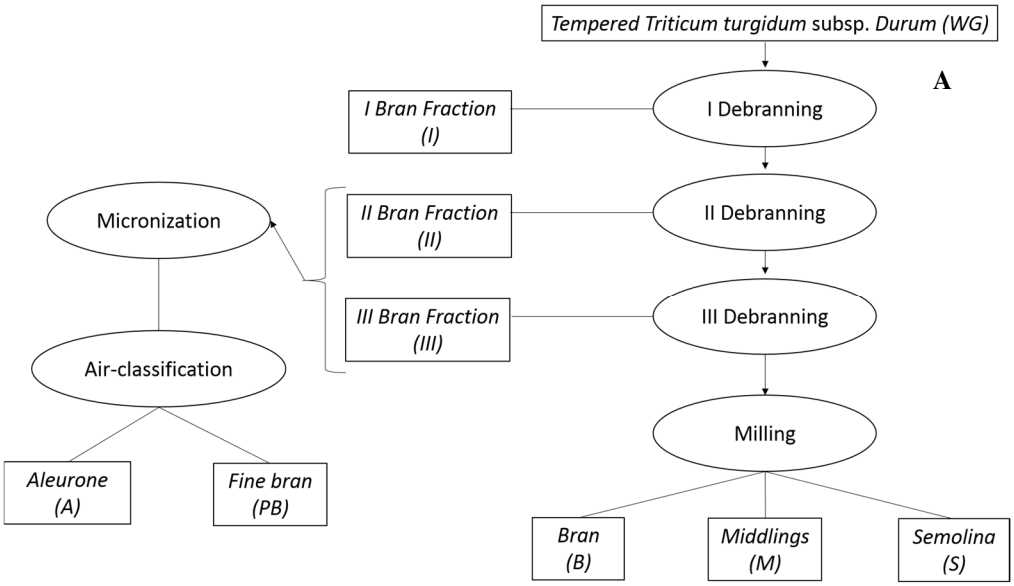
Table 1S. Proximate composition of the whole grains and milling products of wheat species.

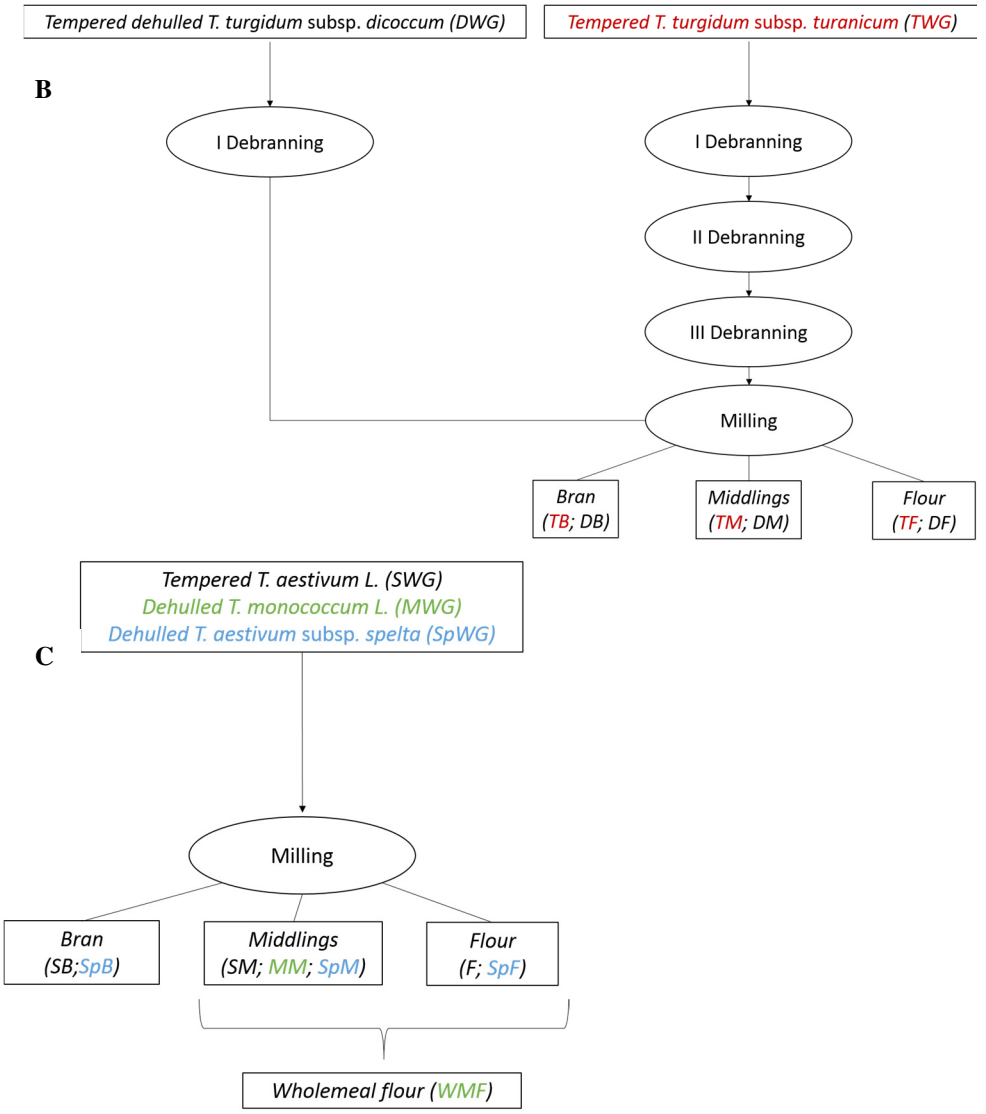
Wheat species	Milling product	Moisture	Ash	Crude Fat	Proteins	Carbohydrate*	TDF
Durum wheat	Whole grain	14.8±0.2	1.63±0.0	1.2±0.2	10.3±1.0	72.0	16.3±1.6
	Semolina	12.7±0.2	0.8±0.0	1.6±0.1	9.6±1.1	75.3	4.7±0.5
	Bran	11.3±0.2	4.4±0.0	3.3±0.3	12.6±1.2	68.4	40.1±3.1
	Fine Bran	10.2±0.2	4.4±0.0	3.1±0.2	13.5±1.0	68.8	25.5±2.1
	Middling	9.8±0.2	3.9±0.0	3.4±0.2	12.8±1.0	70.1	31.2±1.8
	Aleurone	10.1±0.2	5.3±0.0	5.5±0.2	17.1±1.2	62.0	33.4±2.1
	I Debr	15.8±0.2	4.4±0.0	0.6±0.2	11.1±1.1	68.1	49.4±3.5
	II Debr	13.9±0.2	4.8±0.0	3.2±0.2	14.4±1.2	63.6	40.2±2.6
	III Debr	13.7±0.2	4.7±0.0	2.5±0.2	14.7±1.1	64.3	31.5±1.9
Bread wheat	Whole grain	14.4±0.2	1.63±0.0	2.5±0.2	14.2±1.1	67.3	15.5±1.6
	Flour	13.0±0.2	0.51±0.0	1.1±0.1	13.8±1.1	71.7	4.0±0.4
	Bran	12.7±0.2	6.87±0.0	3±0.2	16.3±1.2	61.2	45.2±4.5
	Middling	11.3±0.2	2.78±0.0	4.1±0.2	15.3±1.2	66.5	20.9±2.1

(Table 1S continued)	Milling product	Moisture	Ash	Crude Fat	Proteins	Carbohydrate*	TDF
Turanicum	Whole grain	11.8±0.2	1.7±0.0	1.7±0.2	13.2±1.0	71.6	13.0±1.3
	Semolina	11.4±0.2	0.8±0.0	0.9±0.1	12.5±1.0	74.4	4.8±0.5
	Bran	9.1±0.2	4.5±0.0	3.3±0.2	14.5±1.1	68.6	40.7±1.0
	Middling	9.0±0.2	5.0±0.0	3.2±0.2	15.2±1.2	67.5	37.2±3.7
Einkorn	Whole grain	9±0.2	1.8±0.0	3.3±0.2	10.9±0.9	75.0	10.0±1.0
	WM flour	8.8±0.2	1.8±0.1	3.3±0.2	10.9±0.9	75.2	9.9±1.0
	Middling	7.5±0.2	6.2±0.0	6.0±0.3	15.5±1.2	64.8	25.7±2.5
Emmer	Whole grain	6.4±0.2	1.9±0.0	2.5±0.2	11.1±0.9	78.1	12.6±1.3
	Flour	5.9±0.2	1.4±0.0	2.0±0.2	10.8±0.9	80.0	7.1±0.7
	Bran	6.5±0.2	6.6±0.0	6.0±0.3	14.0±1.1	66.9	33.9±3.4
	Middling	5.2±0.2	5.4±0.0	6.4±0.3	14.7±1.1	68.4	23.3±2.3
Spelt	Whole grain	9.5±0.2	1.9±0.0	2.7±0.2	11.7±0.9	74.2	13.9±1.4
	Flour	8.5±0.2	0.8±0.0	1.8±0.2	10.6±0.9	78.3	4.8±0.5
	Bran	8.3±0.2	8.0±0.0	5.9±0.3	15.3±1.2	62.5	39.9±4.0
	Middling	7.5±0.2	4.9±0.0	7.0±0.3	16.4±1.3	64.1	27.4±2.7

*calculated by difference, TDF, total dietary fibre; The results are expressed as average ± MU (measurement uncertainty) (%) on a d.w. basis.

Figure 1S: Flow charts of *Triticum turgidum* subsp. *durum* Desf. (**A**), *T. turgidum* subsp. *turanicum*, *T. turgidum* subsp. *dicoccum* (**B**) and *T. aestivum* L, *T. aestivum* subsp. *spelta* and *T. monococcum* L (**C**) milling process.




B
Tempered dehulled T. turgidum subsp. dicoccum (DWG)
Tempered T. turgidum subsp. turanicum (TWG)
I Debranning
I Debranning
II Debranning
III Debranning
Milling
Bran
(TB; DB)
Middlings
(TM; DM)
Flour
(TF; DF)
C
Tempered T. aestivum L. (SWG)
Dehulled T. monococcum L. (MWG)
Dehulled T. aestivum subsp. spelta (SpWG)
Milling
Bran
(SB;SpB)
Middlings
(SM; MM; SpM)
Flour
(F; SpF)
Wholemeal flour (WMF)

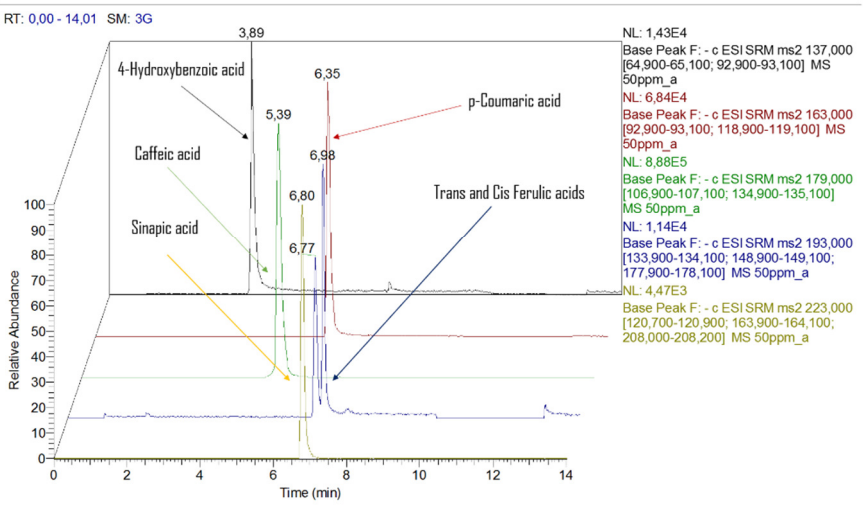


Figure 2S. Chromatograms of the monitored phenolic acids (standard reference at 50 µg g $^{-1}$).

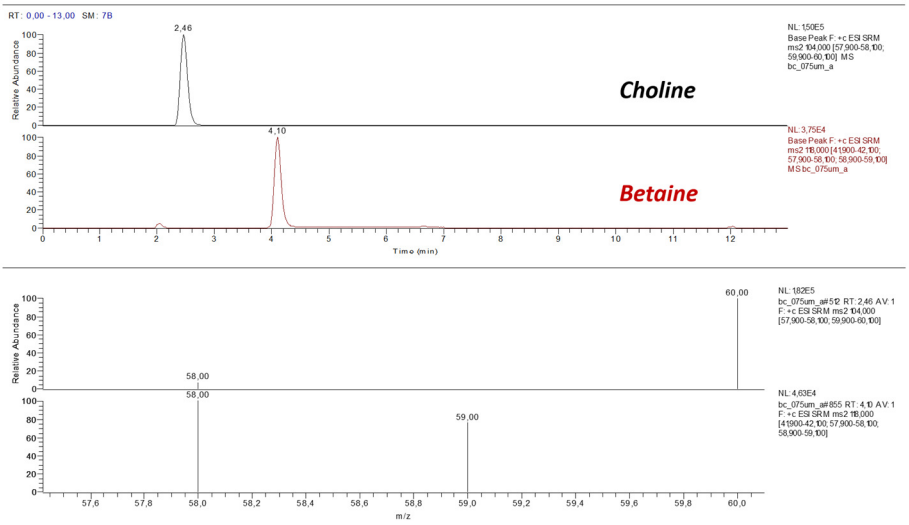


Figure 3S. Chromatogram and mass spectra of betaine and choline standard reference working solution.

References

Abdel-Aal, E. S. M., Hucl, P., Sosulski, F. W., Graf, R., Gillott, C., & Pietrzak, L. (2001). Screening spring wheat for midge resistance in relation to ferulic acid content. *Journal of Agricultural and Food Chemistry*. https://doi.org/10.1021/jf010027h

Agrios, G. N. (2005). Plant pathology. In *Molecular Plant Pathologyathology*. https://doi.org/10.1111/mpp.12135

Andersson, A. A. M., Andersson, R., Piironen, V., Lampi, A. M., Nyström, L., Boros, D., … Åman, P. (2013). Contents of dietary fibre components and their relation to associated bioactive components in whole grain wheat samples from the HEALTHGRAIN diversity screen. *Food Chemistry*. https://doi.org/10.1016/j.foodchem.2012.09.074

Arzani, A. (2011). Emmer (Triticum Turgidum Spp. Dicoccum) Flour and Breads. In *Flour and Breads and their Fortification in Health and Disease Prevention*. https://doi.org/10.1016/B978-0-12-380886-8.10007-8

Arzani, A., & Ashraf, M. (2017). Cultivated Ancient Wheats (Triticum spp.): A Potential Source of Health-Beneficial Food Products. *Comprehensive Reviews in Food Science and Food Safety*. https://doi.org/10.1111/1541-4337.12262

Bagdi, A., Tóth, B., Lorincz, R., Szendi, S., Gere, A., Kókai, Z., … Tömösközi, S. (2016). Effect of aleurone-rich flour on composition, baking, textural, and sensory properties of bread. *LWT - Food Science and Technology*. https://doi.org/10.1016/j.lwt.2015.08.073

Beta, T., Nam, S., Dexter, J. E., & Sapirstein, H. D. (2005). Phenolic content and antioxidant activity of pearled wheat and roller-milled fractions. *Cereal Chemistry*. https://doi.org/10.1094/CC-82-0390

Brandolini, A., Castoldi, P., Plizzari, L., & Hidalgo, A. (2013). Phenolic acids composition, total polyphenols content and antioxidant activity of Triticum

monococcum, Triticum turgidum and Triticum aestivum: A two-years evaluation. *Journal of Cereal Science*. https://doi.org/10.1016/j.jcs.2013.03.011

Brandolini, A., & Hidalgo, A. (2011). Einkorn (Triticum Monococcum) Flour and Bread. In *Flour and Breads and their Fortification in Health and Disease Prevention*. https://doi.org/10.1016/B978-0-12-380886-8.10008-X

Bresciani, L., Scazzina, F., Leonardi, R., Dall'Aglio, E., Newell, M., Dall'Asta, M., … Del Rio, D. (2016). Bioavailability and metabolism of phenolic compounds from wholegrain wheat and aleurone-rich wheat bread. *Molecular Nutrition and Food Research*. https://doi.org/10.1002/mnfr.201600238

Bruce, S. J., Guy, P. A., Rezzi, S., & Ross, A. B. (2010). Quantitative measurement of betaine and free choline in plasma, cereals and cereal products by isotope dilution LC-MS/MS. *Journal of Agricultural and Food Chemistry*. https://doi.org/10.1021/jf903930k

Burg, M. B., Ferraris, J. D., & Dmitrieva, N. I. (2007). Cellular Response to Hyperosmotic Stresses. *Physiological Reviews*. https://doi.org/10.1152/physrev.00056.2006

Corol, D. I., Ravel, C., Raksegi, M., Bedo, Z., Charmet, G., Beale, M. H., … Ward, J. L. (2012). Effects of genotype and environment on the contents of betaine, choline, and trigonelline in cereal grains. *Journal of Agricultural and Food Chemistry*. https://doi.org/10.1021/jf3008794

Craig, S. A. S. (2004). Betaine in human nutrition. *American Journal of Clinical Nutrition*.

De Zwart, F. J., Slow, S., Payne, R. J., Lever, M., George, P. M., Gerrard, J. A., & Chambers, S. T. (2003). Glycine betaine and glycine betaine analogues in common foods. *Food Chemistry*. https://doi.org/10.1016/S0308-8146(03)00063-3

Fernandez-Orozco, R., Li, L., Harflett, C., Shewry, P. R., & Ward, J. L. (2010). Effects of environment and genotype on phenolic acids in wheat in the HEALTHGRAIN diversity screen. *Journal of Agricultural and Food Chemistry*. https://doi.org/10.1021/jf102017s

Filipčev, B., Kojić, J., Krulj, J., Bodroža-Solarov, M., & Ilić, N. (2018). Betaine in Cereal Grains and Grain-Based Products. *Foods*, *7*(4), 49. https://doi.org/10.3390/foods7040049

Gallardo, C., Jiménez, L., & García-Conesa, M. T. (2006). Hydroxycinnamic acid composition and in vitro antioxidant activity of selected grain fractions. *Food Chemistry*. https://doi.org/10.1016/j.foodchem.2005.07.053

Graham, S. F., Hollis, J. H., Migaud, M., & Browne, R. A. (2009). Analysis of betaine and choline contents of aleurone, bran, and flour fractions of wheat (triticum aestivum L.) using H nuclear magnetic resonance (NMR) spectroscopy. *Journal of Agricultural and Food Chemistry*. https://doi.org/10.1021/jf802885m

Hefni, M., McEntyre, C., Lever, M., & Slow, S. (2016). Validation of HPLC-UV methods for the quantification of betaine in foods by comparison with LC-MS. *Food Analytical Methods*. https://doi.org/10.1007/s12161-015-0195-6

Hidalgo, A., & Brandolini, A. (2008). Protein, ash, lutein and tocols distribution in einkorn (Triticum monococcum L. subsp. monococcum) seed fractions. *Food Chemistry*. https://doi.org/10.1016/j.foodchem.2007.08.009

Katcher, H. I., Legro, R. S., Kunselman, A. R., Gillies, P. J., Demers, L. M., Bagshaw, D. M., & Kris-Etherton, P. M. (2008). The effects of a whole grain-enriched hypocaloric diet on cardiovascular disease risk factors in men and women with metabolic syndrome. *American Journal of Clinical Nutrition*.

Kojić, J., Krulj, J., Ilić, N., Lončar, E., Pezo, L., Mandić, A., & Bodroža Solarov, M. (2017). Analysis of betaine levels in cereals, pseudocereals and their products. *Journal of Functional Foods*. https://doi.org/10.1016/j.jff.2017.07.052

Laddomada, B., Durante, M., Mangini, G., D'Amico, L., Lenucci, M. S., Simeone, R., … Blanco, A. (2017). Genetic variation for phenolic acids concentration and composition in a tetraploid wheat (Triticum turgidum L.) collection. *Genetic Resources and Crop Evolution*. https://doi.org/10.1007/s10722-016-0386-z

Li, L., Shewry, P. R., & Ward, J. L. (2008). Phenolic acids in wheat varieties in the healthgrain diversity screen. *Journal of Agricultural and Food Chemistry*. https://doi.org/10.1021/jf801069s

Likes, R., Madl, R. L., Zeisel, S. H., & Craig, S. A. S. (2007). The betaine and choline content of a whole wheat flour compared to other mill streams. *Journal of Cereal Science*. https://doi.org/10.1016/j.jcs.2006.11.002

Liyana-Pathirana, C. M., & Shahidi, F. (2007). The antioxidant potential of milling fractions from breadwheat and durum. *Journal of Cereal Science*. https://doi.org/10.1016/j.jcs.2006.08.007

Longin, C. F. H., Ziegler, J., Schweiggert, R., Koehler, P., Carle, R., & Würschum, T. (2016). Comparative study of hulled (einkorn, emmer, and spelt) and naked wheats (durum and bread wheat): Agronomic performance and quality traits. *Crop Science*. https://doi.org/10.2135/cropsci2015.04.0242

Moore, J., Hao, Z., Zhou, K., Luther, M., Costa, J., & Yu, L. (2005). Carotenoid, tocopherol, phenolic acid, and antioxidant properties of Maryland-grown soft wheat. *Journal of Agricultural and Food Chemistry*. https://doi.org/10.1021/jf050481b

Mpofu, A., Sapirstein, H. D., & Beta, T. (2006). Genotype and environmental variation in phenolic content, phenolic acid composition, and antioxidant activity of hard spring wheat. *Journal of Agricultural and Food Chemistry*. https://doi.org/10.1021/jf052683d

Nicoletti, I., Martini, D., De Rossi, A., Taddei, F., D'Egidio, M. G., & Corradini, D. (2013). Identification and quantification of soluble free, soluble conjugated, and insoluble bound phenolic acids in durum wheat (triticum turgidum L. var. durum) and derived products by RP-HPLC on a semimicro separation scale. *Journal of Agricultural and Food Chemistry*. https://doi.org/10.1021/jf403568c

Okarter, N., Liu, C. S., Sorrells, M. E., & Liu, R. H. (2010). Phytochemical content and antioxidant activity of six diverse varieties of whole wheat. *Food Chemistry*. https://doi.org/10.1016/j.foodchem.2009.06.021

Patterson, K. Y., Bhagwat, S. A., Williams, J. R., Howe, J. C., Holden, J. M., Zeisel, S. H., … Mar, M.-H. (2008). USDA Database for the Choline Content of Common Foods. In *Nutrient Data Laboratory*. https://doi.org/10.15482/USDA.ADC/1178141

Pichersky, E., & Lewinsohn, E. (2011). Convergent Evolution in Plant Specialized Metabolism. *Annual Review of Plant Biology*. https://doi.org/10.1146/annurev-arplant-042110-103814

Righetti, L., Cirlini, M., Folloni, S., Ranieri, R., Galaverna, G., Bertuzzi, T., ... & Giorni, P. (2019). 5-n-alkylresorcinols but not hydroxycinnamic acids are directly related to a lower accumulation of deoxynivalenol and its glucoside in Triticum spp. Genotypes with different ploidity levels. *Journal of cereal science*, *85*, 214-220. doi.org/10.1016/j.jcs.2018.11.011

Ross, A. B., Zangger, A., & Guiraud, S. P. (2014). Cereal foods are the major source of betaine in the Western diet - Analysis of betaine and free choline in cereal foods and updated assessments of betaine intake. *Food Chemistry*. https://doi.org/10.1016/j.foodchem.2013.08.122

Schatzkin, A., Mouw, T., Park, Y., Subar, A. F., Kipnis, V., Hollenbeck, A., … Thompson, F. E. (2007). Dietary fiber and whole-grain consumption in relation to colorectal cancer in the NIH-AARP Diet and Health Study. *American Journal of Clinical Nutrition*.

Serna-Saldivar, S. O. (2012). Cereal Grains: Laboratory Reference and Procedures Manual. In *Cereal Grains: Laboratory Reference and Procedures Manual*. https://doi.org/10.1111/j.1365-2354.2006.00718.x

Shewry, P. R., Hawkesford, M. J., Piironen, V., Lampi, A. M., Gebruers, K., Boros, D., … Ward, J. L. (2013). Natural variation in grain composition of wheat and related cereals. *Journal of Agricultural and Food Chemistry*. https://doi.org/10.1021/jf3054092

Shibuya, N. (1984). Phenolic acids and their carbohydrate esters in rice endosperm cell walls. *Phytochemistry*. https://doi.org/10.1016/S0031-9422(00)80526-9

Siebenhandl, S., Grausgruber, H., Pellegrini, N., Del Rio, D., Fogliano, V., Pernice, R., & Berghofer, E. (2007). Phytochemical profile of main antioxidants in different fractions of purple and blue wheat, and black barley. *Journal of Agricultural and Food Chemistry*. https://doi.org/10.1021/jf072021j

Smith, M. M., & Hartley, R. D. (1983). Occurrence and nature of ferulic acid substitution of cell-wall polysaccharides in graminaceous plants. *Carbohydrate Research*. https://doi.org/10.1016/0008-6215(83)88036-7

Verma, B., Hucl, P., & Chibbar, R. N. (2009). Phenolic acid composition and antioxidant capacity of acid and alkali hydrolysed wheat bran fractions. *Food Chemistry*. https://doi.org/10.1016/j.foodchem.2009.03.060

Yu, L., Haley, S., Perret, J., & Harris, M. (2004). Comparison of wheat flours grown at different locations for their antioxidant properties. *Food Chemistry*. https://doi.org/10.1016/j.foodchem.2003.08.037

Zeisel, S. H. (2006). Choline: Critical Role During Fetal Development and Dietary Requirements in Adults. *Annual Review of Nutrition*. https://doi.org/10.1146/annurev.nutr.26.061505.111156

CHAPTER II

INNOVATIVE STRATEGIES FOR THE CEREAL MILLING BY-PRODUCTS VALORISATION

2.1

Solid state lactic acid fermentation: a strategy to improve wheat bran functionality

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Preface

After the comprehensive characterization of the available industrial milling by-products carried out during the previous studies, the next task was to apply innovative technologies to these processing residues. As explained in the **Introduction** section, the lactic acid fermentation in solid state is an "old but new" technique which nowadays is used for the overall agro-industrial wastes amelioration [1,2].

This methodology can lead to multiple favorable results. In fact, microorganisms can use food-waste matrices as substrate for growth to produce high-value compounds (i.e. aroma compounds, lactic acid, antifungal compounds) for the next separation and isolation. In this specific case, the waste is not recovered but only partially exploited, generating an exhaust reside which must be managed; hence the value chain and sustainability may be still improved. Moreover, as used in this project, they can deeply modify the characteristics of the fermented matrix in order to use it as whole novel ingredient for future formulations.

The methodology whereby this process can be performed is also different. The most used approach intends to identify and isolate the different micro-ecology of the food matrix in order to use the already present bacteria for the fermentation procedure. Differently, it is possible to use selected microorganism with known metabolic characteristics in order to broader the spectrum of potential modification. In fact, it is possible that several microorganisms, especially lactic acid bacteria (LAB), have matrix-dependent activities since their growth depends on the environmental conditions and the substances composing the substrate.

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[1] Rollan, G. C., Gerez, C. L., & LeBlanc, J. G. (2019). Lactic Fermentation as a Strategy to Improve the Nutritional and Functional Values of Pseudocereals. *Frontiers in Nutrition*, *6*, 98.
[2] Verni, M., Rizzello, C. G., & Coda, R. (2019). Fermentation biotechnology applied to cereal industry by-products: nutritional and functional insights. *Frontiers in nutrition*, 6.

As an example, in the following study, a strain isolated from dairy process has been selected for the fermentation, supported by several preliminary results indicating a different biosynthetic pathway in respect to LAB isolated from vegetable origin materials [3].

[3] Ricci, A., Cirlini, M., Maoloni, A., Del Rio, D., Calani, L., Bernini, V., ... & Lazzi, C. (2019). Use of Dairy and Plant-Derived Lactobacilli as Starters for Cherry Juice Fermentation. Nutrients, 11(2), 213.

Abstract

Wheat bran, a by-product produced in huge amount during cereal milling, is today largely unexploited because of its poor suitability as food ingredient. Solid-state fermentation (SSF) using a *Lactobacillus rhamnosus strain* was applied to wheat bran and its influence on bioactive compounds (free and bound phenolic acids) and their antioxidant activity were evaluated. Moreover, the phytic acid (PAc) degradation and arabinoxylans (WEAX) solubilization properties were studied: the SSF treatment resulted in an almost 37 % decrement and three times increment of PAc and WEAX, respectively. Finally, in order to get the bigger picture, microbial metabolites and the volatile profile of fermented wheat bran were characterized, showing amino acids and lipids metabolites and a complex aroma profile. Overall, lactic acid fermentation can be considered a valuable pre-treatment for the valorisation of cereal by-products.

Key words: Lactic acid bacteria (LAB), solid state fermentation, bioprocessing, wheat by-products, fermentation metabolites, nutritional improvement.

Abbreviation Used

<LOQ, below the limit of quantification; 4-HB, 4-hydroxybenzoic acid; ABTS, 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); AOA, anti-oxidant activity; AWB, autoclaved wheat bran; Caff, caffeic acid; CID, collision-induced dissociation; d.w., dry weight; Dif, diferulates; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EI, electronic impact; EIC, extracted ion chromatogram; ESI, electrospray ionization; FRAP, ferric reducing antioxidant power; FWB24, fermented wheat bran at 24 hours; FWB48, fermented wheat bran at 48 hours; GAE, gallic acid equivalent; GC-HS-SPME-MS, gas chromatography head space solid phase micro extraction mass spectrometry; LAB, lactic acid bacteria; MS, mass spectrometry; PA, phenolic acid; PAc, phytic acid; *p*-C, *para*-Coumaric acid; PCA, plate count agar; SD, standard deviation.; Sin, sinapic acid; SRM, single reaction monitoring; TBC, total microbial count; TEAC, trolox equivalent antioxidant capacity; t-Fer, *trans*-Ferulic acid; TPC, total phenolic content; TSC, total spore count; UHPLC, ultra-high performance liquid chromatography; UV, ultraviolet; Vis, visible; WB wheat bran; WEAX, water extractable arabinoxylan.

Introduction

Wheat (*Triticum* spp.) is one of the most cultivated crops worldwide, and it is considered a staple food in both developed and developing countries. Before consumption, wheat cereal grains pass through numerous processes for the production of bread, pasta, and baked goods in general. These processes generate a huge amount of residue side-streams (Sozer, Nordlund, Ercili-Cura, & Poutanen, 2017), mainly used as ingredients in feed formulation and rarely directed to human nutrition.

Despite that, nowadays bran and other cereal by-products are commonly used to increase the nutritional quality of foodstuffs, such as high-fiber bread or biscuits and whole grain pasta (Coda, Katina, & Rizzello, 2015). In fact, it is widely recognized that the most important macronutrients (protein, lipids and dietary fiber), micronutrients (vitamins and minerals) and bioactive compounds (polyphenols) are concentrated in seed outermost tissues (Hemdane et al., 2016). On the other hand, also undesired compounds occur in these fractions, such as phytic acid (inositol polyphosphate) and tannins, which are recognised anti-nutritive compounds (Kumar, Sinha, Makkar, & Becker, 2010). Moreover, it is worth noting that cereal bran or pericarp included as ingredients in a baked product often adversely affect the taste and flavour quality perceived by consumers. In particular, wheat bran confers a browner colour, an astringent and bitter taste and a poor consistency and texture to the final product (Heiniö et al., 2016).

In addition, the poor technological properties of wheat bran, characterized by a low water binding capacity, low gas holding capacity and poor viscosity of dough (Hemdane et al., 2016), negatively influences the manufacturing process. For all these reasons, nowadays many innovative technologies are being studied and applied as pre-treatments for the improvement of nutritional and sensorial characteristics of wheat bran.

Among them, the effects of lactic acid fermentation on the rheology (Messia et al., 2016) and, in a minor extent, on the nutritional value of bran-added products have been studied (Coda et al., 2015).

This technique has shown several positive effects such as the increase of the content and of the bioavailability of bioactive compounds (polyphenols), the release of arabinoxylans in their water-soluble form, the degradation of antinutritive compounds and the modification of sensorial properties (Coda, Rizzello, Curiel, Poutanen, & Katina, 2014; Filannino, Di Cagno, & Gobbetti, 2018). In this work, the ability of a dairy strain LAB to modify the overall characteristics of wheat bran was studied.

Material and Methods

Raw materials and chemicals

Wheat bran (WB) of *Triticum turgidum* subsp. *turanicum* whole grain (moisture 9.09 g/100g, ash 4.51 g/100g, protein 14.53 g/100 g, carbohydrates 68.57 g/100 g, lipids 3.3 g/100 g and total dietary fibre 40.7 g/100 g, average particle size 1 mm) was provided by durum wheat local industrial mills. Commercial lots of whole grain cereal were from Italy and came from the 2015-2016 crop year. Sampling for bran fraction was carried out by five sub-samples of the same lot collected at different times and combined into one during the milling process. HPLC-grade acetonitrile (>99.9%), ethyl acetate (>99.8%), formic acid (>95.0%), acetic acid, hydrochloric acid (HCl, 37.0%), methanol (>99.9%), sodium hydroxide (NaOH, >98.0%), phenolic acid standards (caffeic acid >98%, 4-hydroxybenzoic acid >99%, *p*-coumaric acid >98%, sinapic acid >98% and trans-ferulic acid >99%), chloridric acid (37 %), potassium persulfate (99,9%), iron (III) chloride, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (97 %), gallic acid (>98%), Folin & Ciocalteu's phenol reagent (2 N), 2,2-diphenyl-1-picrylhydrazyl, 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ, >98%), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt were all purchased from Sigma-Aldrich (St. Louis, Missouri, US).

Fermentation process

Lactobacillus rhamnosus 1473, a facultative hetero-fermentative strain isolated from Parmigiano Reggiano cheese (collection of Food and Drug Department, University of Parma, Italy) was singly used as starter for fermentation. The strain preparation and starter inoculum were prepared as described by Ricci et al., 2018. WB was sterilized and water was added (75%, AWB). *L. rhamnosus* 1473 was inoculated into AWB in order to reach 7 Log CFU mL^{-1} and incubated at 37 °C for 24 h (FWB24) and 48 h (FWB48).

Fermentation experiments were carried out in triplicate. Wheat bran samples without starter were incubated at 37 °C for 24 and 48 h and used as controls. Non-fermented sterilized wheat bran was also included in the sample set. Samples were lyophilized, accurately minced and stored at -80°C until extraction and analyses. The microbial count (TBC) was performed on WB, AWB and FWB48 on MRS agar (Oxoid, Milan, Italy) incubating at 37 °C for 48 h. The pH of WB, AWB, FWB24 and FWB48 samples was measured by pH meter (Mettler Toledo, Switzerland). Microbial counts and pH measurement were performed in triplicate.

Phenolic compounds profiling

Sample preparation for free and total phenolic compounds

Free and bound phenolic compounds were extracted from WB, AWB, FWB24 and FWB48. The extraction of free phenolic compounds was performed according to Verma et al., 2009, considering both the bound and free phenolic acid fractions. The extracts were also used for the UHPLC-MS/MS analysis and other assays.

UHPLC-ESI-MS/MS profiling

The UHPLC-ESI-MS/MS analysis was performed on WB, AWB, FWB24 and FWB48 using an UHPLC Dionex Ultimate 3000 instrument coupled with a triple quadrupole mass spectrometer (TSQ Vantage; Thermo Fisher Scientific Inc., San Jose, CA, USA) equipped with an electrospray source (ESI), following the procedure of Verma et al., 2009. Detection was carried out by Selected Reaction Monitoring (SRM), using the transitions reported in **Table S1** (see supplementary information)**.** Dimeric forms of ferulic acid with $[M-H]^-$ value of *m/z* 385 were analysed in full scan MS^2 mode and quantified as ferulic acid equivalents (sum in µg of ferulic acid equivalent per g^{-1}). For quantification, two different calibration sets were prepared using acidified water as solvent (0.2% of formic acid):

one with a calibration range of 0.05-5 µg g^{-1} and one in the range of 5-100 µg g^{-1} for free and bound phenolic compounds respectively, obtaining a good linearity (R^2 > 0.99) for both calibration ranges.

Total phenolic content (TPC)

Free and bound total phenolic content (TPC) of WB, AWB, FWB24 and FWB48 was analysed by the Folin–Ciocalteu's method according to Singleton, Orthofer, & Lamuela-Raventós, 1998. Calibration curve was prepared using gallic acid as reference compound (100-1000 mg Kg^{-1}) and results were expressed as mg of gallic acid equivalents (GAE) per Kg on dry weight basis.

Determination of the antioxidant activity (AOA) using DPPH, FRAP and ABTS assays

The antioxidant activity of WB, AWB, FWB24 and FWB48 free and bound phenolic extracts were evaluated by the DPPH radical scavenging activity assay (Brand-Williams, Cuvelier, & Berset, 1995), by the FRAP assay (Pulido, Bravo, & Saura-Calixto, 2000) and by the ABTS+ radical cation scavenging assay (Re et al., 1999). The % inhibition was calculated from the regression equation prepared using Trolox (0.1-1 mM) as reference standard and results were expressed as mmol Trolox equivalent (TEAC) g^{-1} dry weight.

Quantification of phytic acid (PA)

Phytic acid contents of WB, AWB, FWB24 and FWB48 were determined spectrophotometrically using Megazyme test kit KPHYT 05/07 (Megazyme International Ireland Limited, Bray, Ireland). Results were expressed as g of phytic acid per 100 g^{-1} dry weight.

Quantification of water extractable arabinoxylans (WEAX)

The WEAX of WB, AWB, FWB24 and FWB48 was determined according to Kiszonas et al., 2012.

0.4 g of samples were extracted with 20 mL of distilled water at room temperature under constant agitation. Extracts were centrifuged at 4000 rpm for 10 min at room temperature. Then, 100 µL of supernatant, 100 µL of distilled water and 2 mL of daily prepared working solution, composed by 1 g / 5 mL ethanol of phloroglucinol, 2 mL of hydrochloric acid, 110 mL of glacial acetic acid and 1 mL of a 17.5 g L^{-1} glucose in water solution, were added into stoppered glass tubes (12 mL, 16 x 100 mm). The tubes were then placed for 25 min in a water bath at 100 °C and successively cooled in ice. The absorbance was measured at 552 nm and 510 nm sequentially, using an UV-Vis spectrophotometer. D-(+)-Xylose was used as standard for the calibration curve (0.05-30 mg Kg^{-1}). Finally, the WEAX content was calculated subtracting the absorbance value at 510 nm, which corresponds to hexose interferences, from the absorbance value at 552 nm and the obtained value was compared with the regression equation.

Fermentation metabolites analysis with UPLC-ESI-LTQ/MS

The aqueous methanolic (3/7, v/v) extracts derived from WB, AWB, FWB24 and FWB48 were analysed using an Accela UHPLC 1250 equipped with a linear ion trap-mass spectrometer (MS) (LTQ XL, Thermo Fisher Scientific Inc., San Jose, CA, USA) attached to a heated electrospray ionization probe (H-ESI-II; Thermo Fisher Scientific Inc., San Jose, CA, USA), using the protocol described by Ricci, Cirlini, Maoloni, et al., 2019.

Volatile profile HS-SPME-GC-MS

Volatile profiles of WB, AWB, FWB24 and FWB48 were carried out using head space solid-phase micro extraction (HS-SPME) and analysed by a gas chromatograph (Thermo Scientific Trace 1300 gas chromatograph)

coupled to a Thermo Scientific ISQ single quadrupole mass spectrometer equipped with an electronic impact (EI) source, according to Dall'Asta et al., 2011. Mass spectra of wheat bran samples were used for the identification of the main volatile compounds, using the NIST 14 library of mass spectra. The semi-quantification of all detected gas-chromatographic peaks was carried out using toluene as internal standard.

Statistical analysis

One-way ANOVA was used to compare the different results obtained for WB, AWB, and FWB24 and FWB48. Results obtained from three fermentation replicates (n=3) and three experimental replicates (n=3) were analysed using *Tukey-b's* post-hoc test (significance level <alpha>=0.05). Statistical analyses were carried out using SPSS Statistics 21.0 software (SPSS Inc., Chicago, IL). Moreover, *Pearson correlation* analysis was performed to measure the relationship between Folin-Ciocalteu's assay, and the antioxidant activity tests.

Results and Discussion

L. rhamnosus 1473 growth and pH assessment

Wheat bran fermentation is poorly reported in the literature and not extensively investigated. In particular, the employment of lactic acid bacteria is scarcely explored and only few studies were available (Arte et al., 2015; Messia et al., 2016; Prückler et al., 2015), and it is worthy of note that *L. rhamnosus* fermentation was never reported before. In this study, wheat bran microbial contamination was examined before fermentation, resulting in a total microbial count of ca. 5 Log CFU/g^{-1}. Therefore, a sterilization step was necessary to eliminate the endogenous microflora and to accurately evaluate the metabolic properties of *L. rhamnosus 1473*. Its growth ability was monitored after 48h revealing the increase in microbial cells number (10.42±0.10 Log CFU/g^{-1}) from the original inoculum (ca. 7 Log CFU/g^{-1}). A significant pH decrease (from 6.53±0.22 to 4.70±0.10) was also observed (**Table 9**).

Table 9. pH, total microbial count (TBC) and total spore count (TSC) of native wheat bran (WB), autoclaved wheat bran (AWB) and wheat bran after 24 (FWB24) and 48 (FWB48) hours of fermentation.

Sample	***pH***	***TBC***	***TSC***
		Log CFU g^{-1}	
WB	6.41±0.06b	5.32±0.14	2.56±0.66
AWB	6.53±0.22b	7.78±0.22[a]	<Log $^{-1}$
FWB24	4.67±0.08a	-	-
FWB48	4.70±0.10a	10.42±0.10	<Log $^{-1}$

Results are reported as mean of three fermentation replicates and three experimental replicates ± standard deviation (n=9). Different letters mean a significant difference (<alpha>=0.05) between samples, following the *Tukey b*'s post-hoc test. – not measured. NF not found. [a] after the inoculum.

Free, bound and total phenolic content and antioxidant activity

Results regarding the total phenolic content and the antioxidant activity of the free and bound extracts are reported in **Table 10.** Arguably, it was observed that free TPC decreased after autoclaving, but interestingly it increased during the fermentation process, with no significant difference between 24 and 48 hours. This phenomenon could be explained considering that phenolic compounds that are soluble in the matrix are also more sensitive to high temperature and can be degraded during the thermal treatment. On the other hand, the release of such compounds by the action of fermentation could occur. On the contrary, an opposite behaviour was observed for bound components. In fact, they increased after the thermal treatment and then decreased during the fermentation.

This is possibly due to the neoformation of Maillard reaction's related compounds during the sterilization process, such as complex polyphenols (Ragaee, Seetharaman, & Abdel-Aal, 2014). Since both processing (autoclaving and fermentation) modified the matrix composition by the solubilization and deconjugation of bound phenolic compounds, the ratio between total free and bound polyphenols was calculated (**Table 10**). Despite total phenolic content did not increase significantly during the fermentation in comparison to WB, the F/B ratio was higher in bran fermented for 24 and 48 hours. This means that *L. rhamnosus* metabolised the conjugated phenolic compounds, thus breaking the linkage between them and the cell-wall polysaccharides. These results are consistent with those previously reported by Zhao et al., 2017. Regarding the antioxidant activity (AOA), measured with the three different assays, significant differences were observed between the samples. In the case of DPPH, the soluble antioxidant compounds decreased after the thermal treatment, while for ABTS and FRAP test no differences were found. Furthermore, a good positive correlation was found between total AOA measured with DPPH and ABTS tests and TPC method (r: 0.97; r: 0.22, $p<0.05$, respectively), while a negative correlation was found for the FRAP assay (r: -0.80, $p<0.05$).

This means that antioxidant activity is mainly due to phenolic compounds, although a minor contribution could be also due to other molecules which could have antioxidant potential such as peptides and amino acids, or also to newly formed/released bioactive compounds produced by the LAB metabolism. Finally, the F/B ratio (sum of free to sum of bound antioxidant activity assays ratio) of antioxidant activity also increased after fermentation, indicating an increased content of free and soluble antioxidant compounds. These compounds could exert a positive protection effect against the lipid oxidative process, known to be a cause of poor sensorial quality of finished food products (Calligaris, Manzocco, Anese, & Nicoli, 2016). Overall, the total AOA and TPC reported in this study are in line with other investigations (Nordlund, Katina, Aura, & Poutanen, 2013; Zhao et al., 2017).

Table 10. Changes in total phenolic content (TPC), overall antioxidant activity (DPPH, ABTS and ferric reducing ability of plasma (FRAP)), phytic acid (PA) and water extractable arabinoxylans (WEAX) of wheat bran (WB), autoclaved wheat bran (AWB) and wheat bran after 24 (FWB24) and 48 (FWB48) hours of fermentation.

Sample	*DPPH*			*ABTS*			*FRAP*			*F/B* [b]	*PA*	*WEAX*
	mm TEAC g^{-1}										g 100 gr^{-1}	mg g^{-1}
	Free	Bound	Tot	Free	Bound	Tot	Free	Bound	Tot			
WB	3.6±0.1b	17.7±0.2a	20.0±0.2	10.2±0.2b	40.5±1.2a	50.7±1.1	11.0±0.4c	34.5±0.4a	48.9±0.6	0.27	2.7±0.2a	12.6±0.1d
AWB	2.2±0.0c	17.9±1.0a	21.5±0.6	10.6±0.2b	38.6±2.3a	49.2±2.4	11.6±0.8b	36.3±0.3a	45.5±0.3	0.26	2.6±0.0a	14.7±0.2c
FWB24	3.5±0.3b	15.2±2.1a	18.7±2.2	10.7±0.2b	40.2±2.7a	50.9±3.5	12.6±0.4b	33.6±6.5a	49.6±5.7	0.30	2.3±0.2b	22.7±2.9b
FWB48	4.0±0.2a	13.9±1.1b	17.8±1.1	12.0±0.8a	33.9±3.7b	45.9±1.8	19.2±3.8a	29.0±3.6a	48.1±1.0	0.46	1.7±0.1c	32.4±2.8a

Sample	*TPC*			*F/B* [a]
	mg GAE Kg^{-1}			
	Free	Bound	Total	
WB	1174.9±184.7b	2451.2±109.4b	4247.0±200.6b	0.48
AWB	1043.5±0.7c	3203.5±0.0a	4599.6±90.4a	0.33
FWB24	1447.2±178.1a	2343.8±315.0b	3791.1±241.8b	0.62
FWB48	1553.3±70.4a	2271.1±374.1b	3824.3±395.0b	0.68

Results are represented as mean of three fermentation replicates and three experimental replicates ± standard deviation (n=9). Data with different letters in the same column are significantly different (<alpha>=0.05), following the *Tukey b*'s post-hoc test. [a] F/B: sum of free to sum of bound ratio. [b] sum of free antioxidant activity (AOA) assays to sum of bound AOA assays ratio.

Phytic acid degradation

Although rich in bioactive compounds, wheat bran and external layers of cereal grains in general have also high amount of phytic acid and phytates, which are recognised as anti-nutritive molecules negatively affecting the dietary bioavailability of important minerals such as Ca^{2+}, Mg^{2+}, Fe^{2+}, and Zn^{2+}, and amino acids (Carrizo et al., 2016). Consequently, from the nutritional point of view, phytate degradation is desirable to improve mineral bioavailability.

Results are showed in **Table 10**. The sterilization step did not significantly modify the content of phytic acid in WB. On the contrary, wheat bran fermentation decreased the phytic acid content to 36,4% in comparison to WB, in agreement with results reported by Zhao et al., 2017. The hydrolysis of phytic acid is generally carried out by phytase and phosphatase enzymes that can be found in microorganisms or food matrix. Being the endogenous phytases present in wheat bran probably inactivated during the thermal treatment, degradation of phytic acid is probably due to a phytate-degrading activity expressed in *Lactobacillus rhamnosus 1473*, as already reported in strains of the same species (Carrizo et al., 2016).

Arabinoxylans solubilisation

Arabinoxylans are important compounds that characterize the structure of vegetable cells, in particular those of cereals. They are present in both water-soluble and insoluble forms, and the former has recognised positive effects on the bread dough rheology (Courtin & Delcour, 2002). After thermal step and fermentation, the WEAX content of wheat bran increased significantly (**Table 10**). Sterilization induced a significant solubilization of these compounds, but LAB enhances WEAX content almost three times compared to WB. Specific enzymes, such as endoxylanases, can hydrolyse the backbone of high molecular weight arabinoxylans. These results are in agreement with those reported by Zhao et al., 2017.

Free, bound and total phenolic acids profile

Phenolic acids are the most abundant bioactive compounds present in wheat bran, and more in general in cereal grains. They can occur in soluble or insoluble forms. Thermal processing and fermentation of wheat bran significantly modified the composition of this matrix as shown in **Table 11**. Overall, a decreasing of the free phenolic acids was measured after the sterilization step, while a slight increase of the insoluble component was obtained. However, the free phenolic acids content significantly increased when wheat bran was submitted to lactic acid fermentation, albeit no difference was found between the 24 and 48 hours of treatment. Nutritional improvement is not only related to the increased content of potentially bioactive compound but is determined also by their bioaccessibility. Thus, soluble compounds are more likely to be absorbable in the human gastrointestinal tract and to be able to exert their beneficial functions (Mateo Anson et al., 2011). Several enzymes could be responsible for the solubilization of phenolic acids, such as endoxylanases, xylosidases, arabinofurosidases and ferulic acid esterases, especially related to fermentation processes (Faulds, Mandalari, LoCurto, Bisignano, & Waldron, 2004). This can be underlined by the F/B ratio (sum of free PAs to sum of bound PAs ratio), with a three-fold increase after lactic acid fermentation. Interestingly, among phenolic acids, a relatively high content of caffeic acid was found in fermented wheat bran, indicating that some metabolic activity of microorganism occurred. Indeed, previous studies pointed out that *Lactobacillus* spp. can produce caffeic acid starting from chlorogenic acids, which is present in wheat (Žilić et al., 2011), by hydrolysis, even if the metabolism of phenolics is LAB-specific (Filannino, Bai, Di Cagno, Gobbetti, & Ga¨nzle, 2015).

In addition, being caffeic acid a strong inhibitor of lipid peroxidation, as reported by the study of Khennouf et al., 2003, this is very important since wheat bran is a matrix particularly sensitive to the lipid oxidation. Moreover, the bound PAs component significantly diminished during fermentation, in particular the *p*-C, t-Fer and Sin acids.

This is possibly correlated to the metabolic properties of *L. rhamnosus* 1473, which can convert these phenolic compounds to other microbial metabolites such as dihydroferulic acid or dihydrosinapic acid. Indeed, Filannino et al., 2015 demonstrated that strains belonging to *Lactobacillus* species can use hydroxycinnamic acids as external acceptor of electrons, thus exploiting an energy advantage. These modified forms have different absorption pathway and even an increased bioactivity compared to their parent form (Gobbetti et al., 2018). Moreover, the dimeric form of ferulic acid, Dif, was also detected at relevant concentration, although no significant differences were found among fermented and non-fermented wheat bran.

Table 11. Free and bound phenolic acids (PAs) content in wheat bran (WB), autoclaved wheat bran (AWB) and wheat bran after 24 (FWB24) and 48 (FWB48) hours of fermentation.

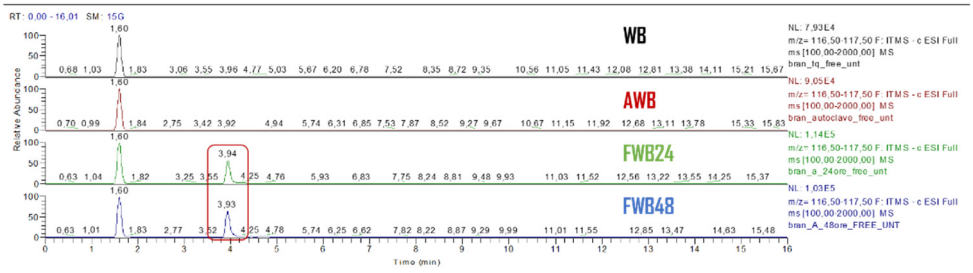
Sample	*4-HB*		*p-C*		*Caff*		*t-Fer*		*Sin*		*Dif*	*F/B* [a]
	Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound		
	μg g^{-1} d.w.											
WB	2.4±0.2b	4.7±1.3a	1.3±0.1b	45.2±1.6c	<LOQ	<LOQ	31.0±1.4b	3643.5±63.3a	7.1±1.1b	111.2±14.3a	2324.7±38.3a	1,04
AWB	1.4±0.5c	6.0±0.8a	0.8±0.4c	60.2±4.2a	<LOQ	<LOQ	12.7±4.7c	3870.4±245.2a	5.8±1.2c	135.1±16.3a	2364.7±76.5a	0,54
FWB24	3.8±0.7a	5.8±1.2a	2.6±0.3a	59.1±8.1b	12.6±1.3a	0.9±0.1a	50.6±5.9a	3786.0±562.3a	12.7±1.8a	81.6±13.8c	2489.0±339.9a	2,10
FWB48	4.0±0.6a	5.8±1.1a	2.8±0.2a	38.3±3.6d	15.0±1.3a	0.8±0.2a	47.9±3.8a	2922.3±281.0b	12.3±2.6a	57.4±9.9d	2394.7±458.1a	2,71

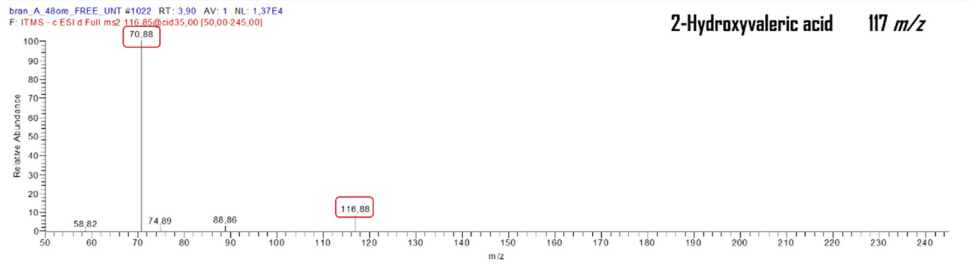
Results are represented as mean of three fermentation replicates and two experimental replicates (n=6). Different letters in the same column mean a significant difference ($p<0.05$) between samples, following the *Tukey b*'s post-hoc test. <LOQ 0.05 μg g^{-1}. 4-HB: 4-hydroxybenzoic acid p-C: para-Coumaric, Caff: caffeic acid, t-Fer: trans-Ferulic acid, Dif: diferulates; acid; [a] F/B: sum of free PAs to sum of bound PAs ratio.

Fermentation metabolites

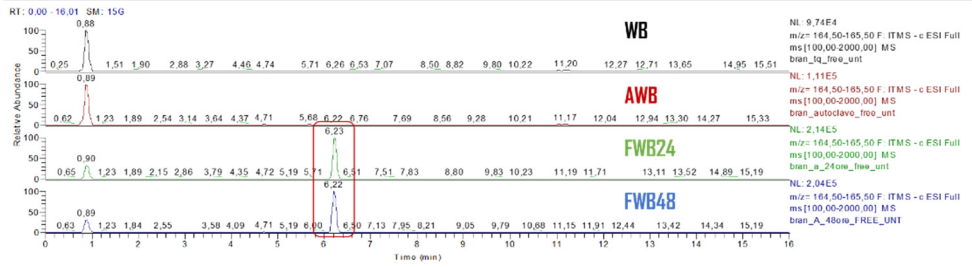
Fermented and raw wheat bran were also analysed using an LC-MS untargeted approach, with the aim to discover newly formed metabolites deriving from lactic acid fermentation. In **Table 12** the mass spectral characteristics of the putative fermentation metabolites found in fermented wheat bran and not in untreated wheat bran are reported. These compounds mainly derive from amino acids and fatty acids degradation. For example, 3-phenyllactic (**Figure 17B**) acid probably derives from the conversion of the amino acid phenylalanine into phenylpyruvic acid via transamination and successive degradation by specific enzymes (hydroxyl acid dehydrogenase) (Valerio, Di Biase, Lattanzio, & Lavermicocca, 2016). Consequently, as reported by other authors, 3-hydroxyphenyllactic (**Figure 17C**) acid could be a degradation metabolite of tyrosine, largely occurring in wheat cereal (Ricci, Cirlini, Calani, et al., 2019). Furthermore, other amino acids present in wheat bran can also be transformed by LAB metabolic pathways. In fact, 2-hydroxyvaleric (**Figure 17A**) acid can originate from valine, leucine and/or isoleucine and indole-3-lactic acid from tryptophan (Koistinen et al., 2018).

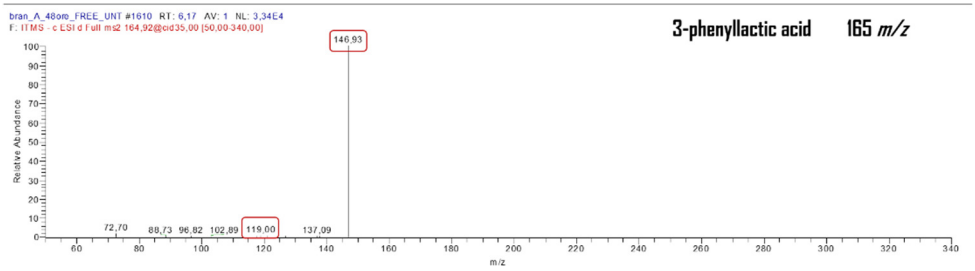
A


WB
AWB
FWB24
FWB48
Relative Abundance
Time (min)


2-Hydroxyvaleric acid
117 m/z
Relative Abundance
m/z

B


WB
AWB
FWB24
FWB48
Relative Abundance
Time (min)


3-phenyllactic acid
165 m/z
Relative Abundance
m/z

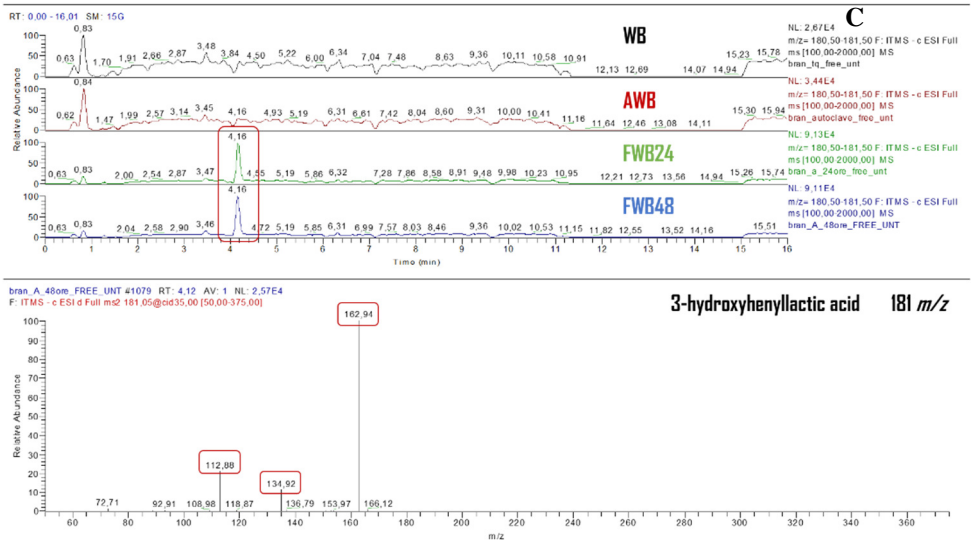


Figure 17. Extracted ion chromatogram (EIC) of 2-Hydroxyvaleric (A), 3-Phenyllactic (B) 3-Hydroxyphenyllactic acids (C) and corresponding mass spectra, found in wheat bran after 24 (FWB24) and 48 (FWB48) hours of fermentation. Specific fragments are surrounded by red circles. The figures also show the chromatograms wheat bran (WB), autoclaved wheat bran (AWB).

Nowadays, these carboxylic acids are receiving attention due to their protective properties against pathogenic bacteria (Kim & Oh, 2013), fungi (Valerio et al., 2016) and also for their anti-mycotoxigenic features (Guimarães, Santiago, Teixeira, Venâncio, & Abrunhosa, 2018). In addition, amino acids are important precursor of several flavour such as aldehydes and alcohols that characterize sourdough fermentation and baked products (Corsetti & Settanni, 2007). Also fatty acids can be metabolised by LAB (Kim & Oh, 2013). Indeed, *L. rhamnosus* 1473 appears to be able to convert fatty acids (FA) in their hydroxylated form, with one or more hydroxyl groups in different position of the hydrocarbon chain. Wheat bran has a relative high content of lipid and it is characterized by a complex mixture of triglycerides and free fatty acids. These are mainly constituted by mono- and polyunsaturated FA, such as oleic and linoleic acids.

Lipid oxidation metabolism is governed by specific endogenous enzymes from both vegetable and bacterial origin. Also this class of compounds represents an interesting innovation point mainly because of their health-related (Moreno, 2009), anti-fungal and technological (Metzger & Bornscheuer, 2006). It is also important to mention that these compounds could contribute to the sensorial and nutritional properties of wheat bran.

Table 12. Mass spectral characteristics of compounds detected in fermented wheat bran.

Putative compound	$[M-H]^-$ (*m/z*)	Rt (min)	MS^2	Compound class	Reference
2-Hydroxyvaleric acid	117	3.9	71, 117	Amino acid degradation	(Kang, Price, Ashton, Tapsell, & Johnson, 2016; Koistinen et al., 2018)
3-Hydroxyphenyllactic acid	181	4.13	135, 163, 113		
3-Phenyllactic acid	165	6.2	119, 147		
Indole-3-lactic acid	204	6.55	158, 116, 142, 128		
Tetrahydroxy octadecenoic acid	345	9.04	327, 309	Fatty acid hydroxylation	
Trihydroxy octadecadienoic	327	9.13	309, 291, 239		
Trihydroxy octadecenoic acid	329	10.59	311, 293, 275, 211, 201, 171		
Dihydroxy-octadecadienoic acid	313	10.71	293, 275		
Dihydroxy-octadecenoic acid	313	10.83	295, 277, 183		

Identified based on MS^n data and retention time and their comparison with MS^n and data from reference sources. Tentatively identified based on MS^n and retention time and other literature evidence.

Volatile profile of fermented wheat bran

In **Table 13** the main volatile compounds detected in wheat bran samples by GC-HS-SPME-MS analysis are reported. A total of 47 compounds were identified, belonging to different classes: alcohols, aldehydes, ketones, carboxylic acids, furan derivatives and esters. Arguably, some compounds increased or decreased in terms of concentration, due to the *L. rhamnosus* 1473 metabolism. Alcohols were the most abundant compounds, both in terms of concentration and identified molecules (**Table S2**). These results are in agreement with the study by Ricci et al., 2018, in which the same strain was used to ferment elderberry juice. In the present study ethanol and ethyl acetate were not found in fermented wheat bran, probably because other reactions that use these molecules as precursor were involved. Certain aldehydes were found after autoclaving such as 5-ethylcyclopentene-1-carbaldehyde, benzaldehyde and 2,4-dimethylbenzaldehyde and were still present after fermentation. Then, furan derivatives, characteristic of bread aroma (Zhou & Therdthai, 2012), were also identified in AWB. Other molecules such as nonanal could be formed by lipoxygenase activity (Zhou & Therdthai, 2012). Globally, fermented wheat bran showed completely different aroma notes in comparison with unfermented bran: this is particularly important from the consumer point of view, leading to an improved acceptability of the sensorial quality of the product.

Table 13. Volatile compounds, their relative abundance and corresponding odour perception according to GC–MS analysis of wheat bran (WB), autoclaved wheat bran (AWB) and wheat bran after 24 (FWB24) and 48 (FWB48) hours of fermentation.

Class	*Compound*	*Odour perception* [a]	*WB*	*AWB*	*FWB24*	*FWB48*
			Relative Abundance [b]			
Alcohols	Ethanol	Strong, alcohol	+	+	-	-
	Isoamyl alcohol	Pungent, fusel	+	+	-	-
	1-Pentanol	Pungent, fusel	+	+	-	NF
	2-Heptanol	Fruity	-	-	+	+
	1-Hexanol	Green grass	+	+	-	-
	4-Methylcyclohexanol	Woody	+	+	NF	NF
	1-Octen-3-ol	Fruity	-	-	+	+
	1-Heptanol	Solvent	+	+	NF	NF
	2,3-Butanediol	Butter cream	+	+	-	-
	1-Octanol	Waxy	+	NF	NF	NF
	2-Octen-1-ol	Fatty	+	NF	NF	NF
	1-nonanol	Floral	+	NF	NF	NF
	3-Nonen-1-ol	Fatty	+	NF	NF	NF
	2-Nonen-1-ol	Fatty	+	NF	NF	NF
	Phenethyl alcohol	Fruity	+	+	-	NF
	Deca-2,4-dien-1-ol	Fatty	+	NF	-	NF

(Table 5 continued)	*Compound*	*Odour perception* [a]	*WB*	*AWB*	*FWB24*	*FWB48*
Alcohols	Cyclohexanol	Camphor menthol	NF	NF	+	+
	1-Penten-3-ol	Fruity	NF	NF	+	+
	Cyclohexanol, 2 methyl 5	Fruity	NF	NF	+	+
	4-Ethylphenol	Smoky	+	+	NF	NF
	1-Nonen-4-ol	Sweety	NF	NF	NF	+
	4,4,6-Trimethylcyclohex-2-en-1-ol	Floral, balsamic	NF	NF	NF	+
Ketones	Acetoin	Sweet cream	+	+	NF	NF
	2-Heptanone	Cheesy	+	+	-	-
	3-Octanone	Green grass	+	+	-	-
	2-Octanone	Milky	+	+	-	-
	2-Nonanone	Fruity	+	+	-	NF
	6-Methyl-5-hepten-2-one	Fatty, green	+	-	NF	NF
	Camphor	Grass, woody	+	+	NF	NF
	5-Pentyloxolan-2-one	Floral	-	-	+	+
	3-Ethylcyclopentan-1-one	Vegetal, natural	NF	NF	+	+
	3-Octen-2-one	Melon	NF	NF	+	+
	2-Decanone	Floral	NF	NF	+	+
	2(3H)-Furanone	Grass	NF	NF	+	+

(Table 5 continued)	*Compound*	*Odour perception* [a]	*WB*	*AWB*	*FWB24*	*FWB48*
Aldehyde	trans-2-Octenal	Fatty	+	+	-	-
	5-Ethylcyclopentene-1-carbaldehyde	Fruity	NF	+	+	+
	Benzaldehyde	Fruity	NF	+	+	+
	2,4-Dimethylbenzaldehyde	Floral	NF	+	+	NF
Carboxylic acids	Pentanoic acid	Cheesy	+	+	-	-
	Octanoic acid	Cheesy	+	+	-	-
	Hexanoic acid	Cheesy	+	+	-	-
	Heptanoic acid	Cheesy	+	+	NF	NF
Furan derivatives	2-Ethylfuran	Solvent, pungent	NF	+	+	+
	2-Butylfuran	Fruity	NF	+	+	+
	cis-2-(2-Pentenyl) furan	Natural, floral	NF	+	+	NF
	2-(2-Pentenyl) furan	Fruity	NF	+	+	+
Esters	Ethyl Acetate	Fruity	+	+	NF	NF
	Acetic acid	Fruity	+	+	-	-

+, found in higher concentration; -, found in lower concentration; NF, not found. [a] Based on data reported in literature and information found at: http://www.thegoodscentscompany.com/, [b], calculated on the basis of internal standard semi-quantification (see **Table S2 in supplementary material**).

Conclusion

In conclusion, the aim of the present work was to give a complete overview on the ability of SSF, using lactic acid bacteria, to convert a low value matrix in a high functional food ingredient. To the best of our knowledge this is the first study based on *L. rhamnosus* species wheat bran fermentation. In addition, differently from the currently available literature, free and bound phenolic components and antioxidant activity of fermented wheat bran were analysed. This bioprocess effectively improved the composition of wheat bran, resulting in an improved nutritional profile and complex structure modification. Phytic acids decreased almost three times while the soluble arabinoxylans triplicate their concentration. More important, beside the TPC slightly decrease, free components increased significantly after fermentation enhancing the soluble AOA of wheat bran. Then, microbial metabolites, deriving from amino acids and lipid metabolism, were identified in fermented wheat bran. These molecules are nowadays receiving great attention due to their multipurpose properties. Volatile profile was also evaluated, stressing the complexity of the aroma compounds created during fermentation. On the base of these results, lactic acid fermentation could be confirmed as an interesting innovative pre-treatment of wheat bran, capable to potentially enhance its health and sensorial properties.

Authors contribution

C.D., G.G., C.L. and M.S. conceived and designed the experiments. M.S. performed all the experiments and analysed the data. M.S., A.R. and L.B. interpreted the results. M.S., C.D., G.G. and C.L. drafted the paper. All the authors contributed to the critical review and revision of the manuscript.

Acknowledgment

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Supporting information

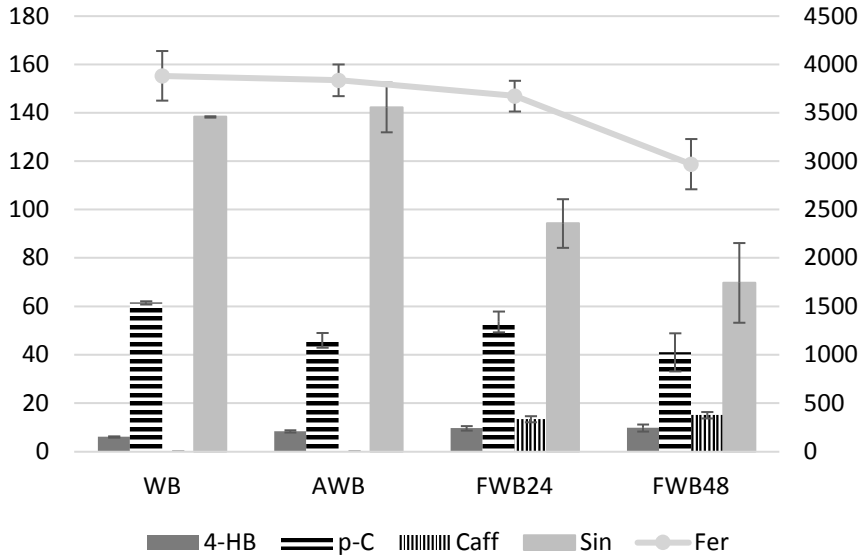

Figure S1. Total phenolic acids content in samples. The left y axis refers to secondary phenolic acids (4-HB, p-C, Caff and Sin) while the right y axis refers to most abundant phenolic acid (t-Fer). Results were expressed as mean µg g^{-1} dry weight. Different letters on top of each bars mean a significant difference ($p<0.05$) between samples, following the *Tukey b*'s post-hoc test.

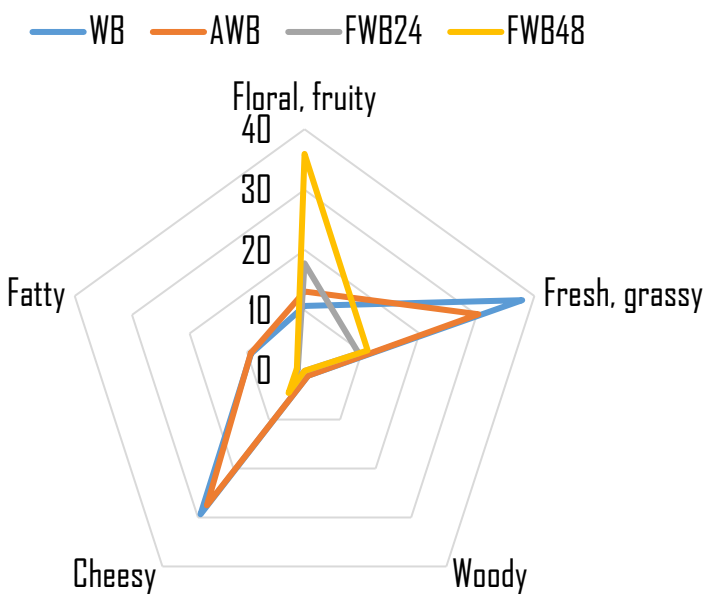

Figure S2. Overall flavour perceptions of fermented and non-fermented wheat bran in relation to the compounds and its flavour note discovered with HS-SPME-GC-MS analysis.

Table S1. Mass spectrometry characteristics of phenolic acids analysis.

Compound	Monitored ion	Transition *m/z* (CE)
4-hydroxybenzoic acid	[M-H]	137→ 93 (34)
		137→ 65 (17)
***p*-coumaric acid**		163 → 119 (14)
		163 → 93 (34)
Caffeic acid		179 → 135 (19)
		179 → 107 (24)
Ferulic acid		193 → 178 (18)
		193 → 134 (14)
Sinapic acid		223 → 208 (18)
		223 → 164 (34)

CE, Collision Energy expressed as eV.

Table S2. Concentration (μg/ml) of volatile compounds identified in samples.

Alcohols	**WB**	**AWB**	**FWB24**	**FWB48**
Ethanol	8,41 ± 1,39b	7,91 ± 1,01b	0,15 ± 0,08a	0,10 ± 0,03a
Isoamyl alcohol	1,47 ± 0,18a	1,73 ± 0,45a	0.93 ± 0,10b	0.81 ± 0,15b
1-Pentanol	3,86 ± 0,59a	4,01 ± 0,95a	0,08 ± 0,00b	-
2-Heptanol	0,35 ± 0,07b	0,48 ± 0,12b	9,10 ± 0,85a	10,06 ± 2,21a
1-Hexanol	36,49 ± 7,41a	30,31 ± 4,12a	-	-
4-Methylcyclohexanol	1,73 ± 0,13a	1,83 ± 0,53a	-	-
1-Octen-3-ol	0,52 ± 0,05b	0,53 ± 0,17b	4,05 ± 0,78a	3.86 ± 0,47a
1-Heptanol	2,88 ± 0,69a	2,15 ± 0,88a	-	-
2,3-Butanediol	18,91 ± 3,17b	16,47 ± 2,89b	0,62 ± 0,13a	0,67 ± 0,21a
1-Octanol	3,65 ± 0,41	-	-	-
2-Octen-1-ol	1,12 ± 0,15	-	-	-
1-Nonanol	0,86 ± 0,14	-	-	-
3-Nonen-1-ol	0,45 ± 0,08	-	-	-
2-Nonen-1-ol	0,78 ± 0,06	-	-	-
Phenethyl alcohol	0,52 ± 0,13	-	-	-
Deca-2,4-dien-1-ol	0,54 ± 0,05b	-	0,07 ± 0,05a	-
Cyclohexanol	-	-	0,05 ± 0,01a	0,06 ± 0,01a

(Table S2 continued)	**WB**	**AWB**	**FWB24**	**FWB48**
1-Penten-3-ol	-	-	0,19 ± 0,03b	0,10 ± 0,05a
Cyclohexanol, 2 methyl 5	-	-	0,10 ± 0,01a	0,52 ± 0,56b
4-Ethylphenol	0,52± 0,13b	0,48± 0,19b	-	-
1-Nonen-4-ol	-	-	-	0,25 ± 0,13b
4,4,6-Trimethylcyclohex-2-en-1-ol				0,27 ± 0,10
Ketones	-	-	-	-
Acetoin	1,26 ± 0,63a	1,13 ± 0,28a	-	-
2-Heptanone	4,49 ± 1,03b	4,12 ± 0.98b	2,15 ± 0,17a	2,22 ± 0,26a
3-Octanone	0,91 ± 0,11b	0,87 ± 0,17b	0,09 ± 0,03a	0,11 ± 0,05a
2-Octanone	1,93 ± 0,44b	1,78 ± 0,91b	0,54 ± 0,04a	0,66 ± 0,25a
2-Nonanone	1,05 ± 0,02b	0,82 ± 0,15b	0,40 ± 0,11a	-
6-Methyl-5-hepten-2-one	0,15 ± 0,06	0,08 ± 0,04	-	-
Camphor	0,48 ± 0,10a	0,41 ± 0,17a	-	-
5-Pentyloxolan-2-one	0,36 ± 0,10a	0,46 ± 0,08a	0,74 ± 0,07b	0,78 ± 0,11b
3-Ethylcyclopentan-1-one	-	-	0,27 ± 0,09a	0,29 ± 0,08a
3-Octen-2-one	-	-	0,36 ± 0,03a	0,35 ± 0,10a
2-Decanone	-	-	0,16 ± 0,10a	0,17 ± 0,07a
2(3H) -Furanone	-	-	0,24 ± 0,04a	0,79 ± 0,01a

(Table S2 continued)	WB	AWB	FWB24	FWB48
Aldehydes				
trans-2-Octenal	2,50 ± 0,18c	2,22 ± 0,31c	1,27 ± 0,10b	0,38 ± 0,45a
5-Ethylcyclopentene-1-carbaldehyde	-	1,61 ± 0,17a	1,53 ± 0,07a	1,46 ± 0,38a
Benzaldehyde	-	0,86 ± 0,09a	0,68 ± 0,12a	0,80 ± 0,38a
2,4-Dimethylbenzaldehyde	-	0,36 ± 0,14a	0,22 ± 0,17a	-
Carboxylic acids				
Pentanoic acid	0,30 ± 0,01b	0,28 ± 0,08b	0,11 ± 0,05a	0,18 ± 0,03a
Octanoic acid	2,28 ± 0,09b	2,01 ± 0,17b	0,51 ± 0,22a	0,75 ± 0,02a
Hexanoic acid	2,68 ± 0,16b	2,31 ± 0,26b	0,33 ± 0,14a	0,24 ± 0,14a
Heptanoic acid	0,70 ± 0,07a	0,61 ± 0,17a	-	-
Furan derivatives				
2-Ethylfuran	-	0,41 ± 0,11a-	0,32 ± 0,15a	0,38 ± 0,14a
2-Butylfuran	-	0,61 ± 0,15a	0,46 ± 0,22a	0, 58 ± 0,17a
cis-2-(2-Pentenyl) furan	-	0,32 ± 0,05a	0,39 ± 0,06a	-
2-(2-Pentenyl) furan	-	-	12,98 ± 18,31a	30,62 ± 13,65b

(Table S2 continued)	**WB**	**AWB**	**FWB24**	**FWB48**
Esters				
Ethyl Acetate	1,07 ± 0,05a	0.93 ± 0,17a	-	-
Acetic acid	1,42 ± 0,23b	1,28 ± 0,31b	0,18 ± 0,11a	0,29 ± 0,12a

Results are represented as mean of three biological replicates (*n=3*) and three experiment replicates (*n=2*). Different letters mean a significant difference ($p<0.05$) between samples, following the *Tukey b*'s post-hoc test. -: not found. WB: wheat bran. AWB: autoclaved wheat bran. FWB24: Fermented wheat bran after 24 hours. FWB48: Fermented wheat bran after 48 hours.

References

Arte, E., Rizzello, C. G., Verni, M., Nordlund, E., Katina, K., & Coda, R. (2015). Impact of Enzymatic and Microbial Bioprocessing on Protein Modification and Nutritional Properties of Wheat Bran. *Journal of Agricultural and Food Chemistry*. https://doi.org/10.1021/acs.jafc.5b03495

Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT - Food Science and Technology*. https://doi.org/10.1016/S0023-6438(95)80008-5

Calligaris, S., Manzocco, L., Anese, M., & Nicoli, M. C. (2016). Shelf-life Assessment of Food Undergoing Oxidation–A Review. *Critical Reviews in Food Science and Nutrition*. https://doi.org/10.1080/10408398.2013.807222

Carrizo, S. L., Montes de Oca, C. E., Laiño, J. E., Suarez, N. E., Vignolo, G., LeBlanc, J. G., & Rollán, G. (2016). Ancestral Andean grain quinoa as source of lactic acid bacteria capable to degrade phytate and produce B-group vitamins. *Food Research International*. https://doi.org/10.1016/j.foodres.2016.08.013

Coda, R., Katina, K., & Rizzello, C. G. (2015). Bran bioprocessing for enhanced functional properties. *Current Opinion in Food Science*. https://doi.org/10.1016/j.cofs.2014.11.007

Coda, R., Rizzello, C. G., Curiel, J. A., Poutanen, K., & Katina, K. (2014). Effect of bioprocessing and particle size on the nutritional properties of wheat bran fractions. *Innovative Food Science and Emerging Technologies*. https://doi.org/10.1016/j.ifset.2013.11.012

Corsetti, A., & Settanni, L. (2007). Lactobacilli in sourdough fermentation. *Food Research International*. https://doi.org/10.1016/j.foodres.2006.11.001

Courtin, C. M., & Delcour, J. A. (2002). Arabinoxylans and endoxylanases in wheat flour bread-making. *Journal of Cereal Science*. https://doi.org/10.1006/jcrs.2001.0433

Dall'Asta, C., Cirlini, M., Morini, E., & Galaverna, G. (2011). Brand-dependent volatile fingerprinting of Italian wines from Valpolicella. *Journal of Chromatography A*. https://doi.org/10.1016/j.chroma.2011.08.042

Faulds, C. B., Mandalari, G., LoCurto, R., Bisignano, G., & Waldron, K. W. (2004). Arabinoxylan and mono- and dimeric ferulic acid release from brewer's grain and wheat bran by feruloyl esterases and glycosyl hydrolases from Humicola insolens. *Applied Microbiology and Biotechnology*. https://doi.org/10.1007/s00253-003-1520-3

Filannino, P., Bai, Y., Di Cagno, R., Gobbetti, M., & Ga¨nzle, M. G. (2015). Metabolism of phenolic compounds by Lactobacillus spp. during fermentation of cherry juice and broccoli puree. *Food Microbiology*. https://doi.org/10.1016/j.fm.2014.08.018

Filannino, P., Di Cagno, R., & Gobbetti, M. (2018). Metabolic and functional paths of lactic acid bacteria in plant foods: get out of the labyrinth. *Current Opinion in Biotechnology*. https://doi.org/10.1016/j.copbio.2017.07.016

Gobbetti, M., Angelis, M. De, Di Cagno, R., Calasso, M., Archetti, G., & Rizzello, C. G. (2018). Novel insights on the functional/nutritional features of the sourdough fermentation. *International Journal of Food Microbiology*.

Guimarães, A., Santiago, A., Teixeira, J. A., Venâncio, A., & Abrunhosa, L. (2018). Anti-aflatoxigenic effect of organic acids produced by Lactobacillus plantarum. *International Journal of Food Microbiology*. https://doi.org/10.1016/j.ijfoodmicro.2017.10.025

Heiniö, R. L., Noort, M. W. J., Katina, K., Alam, S. A., Sozer, N., de Kock, H. L., … Poutanen, K. (2016). Sensory characteristics of wholegrain and bran-rich cereal foods - A review. *Trends in Food Science and Technology*. https://doi.org/10.1016/j.tifs.2015.11.002

Hemdane, S., Jacobs, P. J., Dornez, E., Verspreet, J., Delcour, J. A., & Courtin, C. M. (2016). Wheat (Triticum aestivum L.) Bran in Bread Making: A Critical Review.

Comprehensive Reviews in Food Science and Food Safety. https://doi.org/10.1111/1541-4337.12176

Kang, J., Price, W. E., Ashton, J., Tapsell, L. C., & Johnson, S. (2016). Identification and characterization of phenolic compounds in hydromethanolic extracts of sorghum wholegrains by LC-ESI-MSn. *Food Chemistry*. https://doi.org/10.1016/j.foodchem.2016.05.052

Khennouf, S., Benabdallah, H., Gharzouli, K., Amira, S., Ito, H., Kim, T. H., … Gharzouli, A. (2003). Effect of tannins from Quercus suber and Quercus coccifera leaves on ethanol-induced gastric lesions in mice. *Journal of Agricultural and Food Chemistry*. https://doi.org/10.1021/jf020808y

Kim, K. R., & Oh, D. K. (2013). Production of hydroxy fatty acids by microbial fatty acid-hydroxylation enzymes. *Biotechnology Advances*. https://doi.org/10.1016/j.biotechadv.2013.07.004

Kiszonas, A. M., Courtin, C. M., & Morris, C. F. (2012). A critical assessment of the quantification of wheat grain arabinoxylans using a phloroglucinol colorimetric assay. *Cereal Chemistry*. https://doi.org/10.1094/CCHEM-02-12-0016-R

Koistinen, V. M., Mattila, O., Katina, K., Poutanen, K., Aura, A. M., & Hanhineva, K. (2018). Metabolic profiling of sourdough fermented wheat and rye bread. *Scientific Reports*. https://doi.org/10.1038/s41598-018-24149-w

Kumar, V., Sinha, A. K., Makkar, H. P. S., & Becker, K. (2010). Dietary roles of phytate and phytase in human nutrition: A review. *Food Chemistry*. https://doi.org/10.1016/j.foodchem.2009.11.052

Mateo Anson, N., Aura, A.-M., Selinheimo, E., Mattila, I., Poutanen, K., van den Berg, R., … Haenen, G. R. M. M. (2011). Bioprocessing of Wheat Bran in Whole Wheat Bread Increases the Bioavailability of Phenolic Acids in Men and Exerts Antiinflammatory Effects ex Vivo. *Journal of Nutrition*. https://doi.org/10.3945/jn.110.127720

Messia, M. C., Reale, A., Maiuro, L., Candigliota, T., Sorrentino, E., & Marconi, E. (2016). Effects of pre-fermented wheat bran on dough and bread characteristics. *Journal of Cereal Science*. https://doi.org/10.1016/j.jcs.2016.03.004

Metzger, J. O., & Bornscheuer, U. (2006). Lipids as renewable resources: Current state of chemical and biotechnological conversion and diversification. *Applied Microbiology and Biotechnology*. https://doi.org/10.1007/s00253-006-0335-4

Moreno, J. J. (2009). New aspects of the role of hydroxyeicosatetraenoic acids in cell growth and cancer development. *Biochemical Pharmacology*. https://doi.org/10.1016/j.bcp.2008.07.033

Nordlund, E., Katina, K., Aura, A. M., & Poutanen, K. (2013). Changes in bran structure by bioprocessing with enzymes and yeast modifies the invitro digestibility and fermentability of bran protein and dietary fibre complex. *Journal of Cereal Science*. https://doi.org/10.1016/j.jcs.2013.05.006

Prückler, M., Lorenz, C., Endo, A., Kraler, M., Dürrschmid, K., Hendriks, K., … Michlmayr, H. (2015). Comparison of homo- and heterofermentative lactic acid bacteria for implementation of fermented wheat bran in bread. *Food Microbiology*. https://doi.org/10.1016/j.fm.2015.02.014

Pulido, R., Bravo, L., & Saura-Calixto, F. (2000). Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. *Journal of Agricultural and Food Chemistry*. https://doi.org/10.1021/jf9913458

Ragaee, S., Seetharaman, K., & Abdel-Aal, E. S. M. (2014). The Impact of Milling and Thermal Processing on Phenolic Compounds in Cereal Grains. *Critical Reviews in Food Science and Nutrition*. https://doi.org/10.1080/10408398.2011.610906

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*. https://doi.org/10.1016/S0891-5849(98)00315-3

Ricci, A., Cirlini, M., Calani, L., Bernini, V., Neviani, E., Del Rio, D., … Lazzi, C. (2019). In vitro metabolism of elderberry juice polyphenols by lactic acid bacteria. *Food Chemistry*, *276*, 692–699. https://doi.org/10.1016/j.foodchem.2018.10.046

Ricci, A., Cirlini, M., Levante, A., Dall'Asta, C., Galaverna, G., & Lazzi, C. (2018). Volatile profile of elderberry juice: Effect of lactic acid fermentation using L. plantarum, L. rhamnosus and L. casei strains. *Food Research International*. https://doi.org/10.1016/j.foodres.2017.11.042

Ricci, A., Cirlini, M., Maoloni, A., Del Rio, D., Calani, L., Bernini, V., … Lazzi, C. (2019). Use of Dairy and Plant-Derived Lactobacilli as Starters for Cherry Juice Fermentation. *Nutrients*, *11*(2), 213. https://doi.org/10.3390/nu11020213

Singleton, V. L., Orthofer, R., & Lamuela-Raventós, R. M. (1998). Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. *Methods in Enzymology*. https://doi.org/10.1016/S0076-6879(99)99017-1

Sozer, N., Nordlund, E., Ercili-Cura, D., & Poutanen, K. (2017). Cereal side-streams as alternative protein sources. *Cereal Foods World*. https://doi.org/10.1094/CFW-62-4-0132

Valerio, F., Di Biase, M., Lattanzio, V. M. T., & Lavermicocca, P. (2016). Improvement of the antifungal activity of lactic acid bacteria by addition to the growth medium of phenylpyruvic acid, a precursor of phenyllactic acid. *International Journal of Food Microbiology*. https://doi.org/10.1016/j.ijfoodmicro.2016.01.011

Verma, B., Hucl, P., & Chibbar, R. N. (2009). Phenolic acid composition and antioxidant capacity of acid and alkali hydrolysed wheat bran fractions. *Food Chemistry*. https://doi.org/10.1016/j.foodchem.2009.03.060

Zhao, H. M., Guo, X. N., & Zhu, K. X. (2017). Impact of solid state fermentation on nutritional, physical and flavor properties of wheat bran. *Food Chemistry*. https://doi.org/10.1016/j.foodchem.2016.08.062

Zhou, W., & Therdthai, N. (2012). Fermented bread. In *Handbook of Plant-Based Fermented Food and Beverage Technology, Second Edition*. https://doi.org/10.1201/b12055

Žilić, S., Hadži-Tašković Šukalović, V., Dodig, D., Maksimović, V., Maksimović, M., & Basić, Z. (2011). Antioxidant activity of small grain cereals caused by phenolics and lipid soluble antioxidants. *Journal of Cereal Science*. https://doi.org/10.1016/j.jcs.2011.08.006

2.2

Impact of air-classification, with and without micronization, on the lipid component of rice bran (*Oryza sativa* L.): a focus on mono-, di- and triacylglycerols.

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Preface

Besides bio-processing, several other techniques based on different principles can be used to treat cereal milling by-products for their subsequent amelioration. The so-called dry technologies exploit the action of pressurized air for the differentiation of multiple fractions of a same by-product. Arguably these types of processes are highly used for low moisture and pulverulent materials, such as cereal by-products. The high advantage of using air-classification methods lies on their low input requirements and procedure times, in fact no addition or removal of solvent and water, nor heat treatment are required. Additionally, the by-products nature would not be compromised.

In this way, rice bran is a very suitable material, due to its chemical and physical properties. In fact, these technologies have been already applied to this by-product for the production of high protein and soluble rice bran fractions (see **Table 1 Introduction**, pp. 50 and 64). However, as most of cereals bran, it has a high content of lipid compounds (9-15 %). Among this classes, there are substances which can exert emulsifying properties in food formulations, named mono- and di-glycerides of fatty acids, widely used as additive in food manufacturing.

Therefore, starting from these assumptions, in this preliminary study, the efforts were focused on the most comprehensive lipid molecules profiling of air classified rice bran, with respect to the non-treated by-product, in order to use the best conditions for the future trial studies.

Abstract

Rice (Oryza sativa) bran is an important by-product produced during cereal milling process, rich in several valuable compounds, such as lipids. Moreover, considering the valorisation potential of these material, the application of innovative and low-impact techniques can improve the overall quality of by-products for their future exploitation in the food manufacturing. In this study, the impact of air-classification and micronization (fine grinding) of rice bran on the lipid components was studied. These treatments allowed to obtain bran fractions, with different granulometry, from coarse to very fine. The total crude fat content was significantly higher in fine air-classified rice bran fractions. Besides, polyunsaturated triacylglycerols (TAG) were the most abundant compounds and monoacylglycerols concentration increased from ~15 % in rice bran to ~22 % in fine fraction. Considering the relevant emulsifying properties of these compounds, this fraction could be used as functional ingredient for the quality improvement of cereal-based products, without modifying the valuable fatty acid profile.

Key words: air classification, micronization, rice bran oil by-products, mono- di-triacylglycerols, food emulsifier, lipid profile.

Abbreviations used

<LOQ, below the limit of quantification; A, Arachidin; C, Coarse; CE, collision energy; d.w., dry weight; DAG, diacylglycerol; EI, electron impact; ESI, electrospray ionization; F, Fine; FA, fatty acid; Ff, ultra-fine GC-MS, gas chromatography coupled to mass spectrometry; HPLC, high performance liquid chromatography; IPA, 2-propanol; L, Linolein; Ln, Linolenin; MAG, monoacylglycerol; MeOH, methanol; MRB, micronized rice bran; MRBc, coarse fraction of micronized and air-separated rice bran; MRBc1, coarse fraction of micronized and air-separated rice bran with different operating conditions; MRBf, fine fraction of micronized and air-separated rice bran; MRBf1, fine fraction of micronized and air-separated rice bran with different operating conditions; MRBFf, ultra-fine fraction of micronized and air-separated rice bran with different operating conditions; MRM, multiple reaction monitoring; MS, mass spectrometry; MUFA, monounsaturated fatty acid; O, Olein; P, Palmitin; Po, Palmitolein; PUFA, polyunsaturated fatty acid; RB, rice bran; RBc, coarse fraction of air-separated rice bran; RBc1, coarse fraction of air-separated rice bran with different operating conditions; RBf, fine fraction of air-separated rice bran; RBf1, fine fraction of air-separated rice bran with different operating conditions; RBFf, ultra-fine fraction of air-separated rice bran with different operating conditions; S, Stearin; SD, standard deviation; SFA, saturated fatty acid; TAG, triacylglycerol.

Introduction

The management of agricultural processing side streams is nowadays a huge challenge which involves several different food chains. Cereal crops are staple food worldwide and many products are obtained by milling their caryopsis (flour, bran, etc.) for the production of bakery goods, pasta, etc. Among cereals, rice is the third most cultivated crop worldwide, but it is highly consumed as refined kernel: in this case, the outer layers, composed by bran (aleurone, testa, inner and outer pericarps) and seed germ are usually discarded. Since their poor suitability in food manufactures, these by-products are generally directed to the feed industry, losing their potential in terms of nutrients profile (García-Lara, 2010; Pfaltzgraff *et al.*, 2013). Rice bran and germ fractions account about 12-15% of the seed total weight, which is directly translatable to about 65 millions of tons per year (worldwide) of material difficult to manage, with a low added value (Kahlon, 2009). Thanks to the high fat content of rice bran (~ 15 %, average, Kahlon, 2009), the oil extraction is one of the most spread practice for the exploitation of such by-product, although lipid peroxidation promoted by the endogenous enzymes of rice seeds is the toughest obstacle to overcome (Sairam, Gopala Krishna and Urooj, 2011). Nevertheless, the lipid components are attracting attention for their possible interesting application in the food industry, for the production of cosmetics and for the preparation of dietary supplements (Metzger and Bornscheuer, 2006). Indeed, besides lipophilic bioactive compounds, there are other classes of interesting molecules such as the emulsifiers. Today the most widespread additive used in food manufacture is a mix of mono- and di-glycerides of fatty acids (E-471), a key ingredient of several food preparations and in gluten-free formulas (Orthoefer, 2008). However, the by-product valorisation through a selective compounds class recovery is not an approach sufficiently sustainable in a zero-waste context.

Therefore, several strategies based on bioprocessing (Coda, Katina and Rizzello, 2015) and physical treatments (Wang *et al.*, 2016)

are today applied in order to valorise and recover agro-industrial by-products, with the final aim to foster the circular economy. Air classification and particle size reduction are innovative techniques based on physical principles, currently applied in food industry to enhance certain properties of powdery material (Coda *et al.*, 2014; Schutyser *et al.*, 2015), or to increase some valuable food components (Pelgrom *et al.*, 2013; Zhang *et al.*, 2019) and also used in combination for a bioactivity improvement of fruits (Puupponen-Pimiä *et al.*, 2016). Their mainly positive aspects are that the addition or removal of water or chemicals in not required, then the native functionality of the matrix components is not altered and finally the uniformity of the particle size leads to an improved control of particle properties, such as dispersion or suspension (Hemery *et al.*, 2011). In addition, many researchers reported the effect of these treatments on the technological improvement of different flours, including the enrichment of bioactive compounds (Bottega *et al.*, 2009; Ferrari *et al.*, 2009; Hemery *et al.*, 2011). In fact, the particle size can influence also the health-related functionality of the raw material, as reported by the study of Hemery et al., 2011 regarding the lowering plasma cholesterol properties of fine particles, or the increased vitamin B group bioavailability reported by the same authors (Hemery *et al.*, 2007). Despite the application of particle size reduction and fractionation techniques on food matrices, very few are the data referring to the cereal by-products treatment. Therefore, the main objective of the present work was to study the effects of micronization and air classification on lipid component of industrial-scale rice bran, with particular attention to the mono-, di- and triacylglycerols and fatty acids.

Materials and Methods

Raw materials

Non-defatted rice bran (*Oryza sativa* L.), produced by industrial rice milling, was obtained from Grandi Riso (Codigoro, Italy). Sampling for bran fraction was carried out by five sub-samples of the same lot collected at different times and combined into one during the milling process. A 50 kg sample of freshly milled rice bran (with bulk density of about 0,40 Kg/dm^3) was subjected either to micronization (fine grinding) followed by air classification or to air classification only as reported by Laudadio *et al.*, 2013, with some modifications. Bran micronization was carried out with a self-cleaning micronizer, suitable for high-fat samples (model KMX-300, SeparMicroSystem sas, Flero, Italy). In this system, the reduction of particle size is obtained by mechanical impact against stator and rotor serrated surfaces operating at a peripheral speed of approximately 175 m/s, and by turbulent collision between bran particles. The processing time was set at 10 min. The large air volume could ensure a negligible temperature increase. Bran fractionation was performed on the micronized bran, or on rice bran as such, employing an air separator (model SXi-100, SeparMicroSystem s.a.s., Flero, Italy). The system is composed of a turbo separator – a highly modified cyclone – and a cyclone assembled in series, with an aspirating pump at the end of the system which drives the air flow, modifiable by an inlet valve. The operating settings were chosen to be suitable for low flow rate flours, targeting an inflow of 8 kg/h for micronized rice bran and 17 kg/h for rice bran. The apparatus was set at a 40 µm cut point to sort the flour into two portions: a CF and an FF, which were collected from the separator and the cyclone, respectively. Ultra-fine (Ff) fractions with a yield >1% were obtained and thus included in the analysis. The operating conditions were determined in function of the air flow inlet valve (see Table 1). Where the yield was very low (< 1% yield) samples were pooled. Samples were vacuum-packed in polyethylene bags and kept at -20°C until analysis.

Chemicals

1-monooleoyl-rac-glycerol (monoolein), 3-monopalmytoyl-sn-glycerol (monopalmitin), 3-monostearoyl-sn-glycerol (monostearin), 1-linoleoyl-rac-glycerol, 1-monolinolenoyl-rac-glycerol, glyceryl arachidate, 1-monopalmitoleoyl-rac-glycerol, mix of 1,2-dipalmitoyl-rac-glycerol (1,2-dipalmitin), 1,3-dipalmitoyl-rac-glycerol (1,3-dipalmitin) isomers, 1,3-distearoyl-rac-glycerol (1,3-distearin), 1,3-dilinoleoyl-rac-glycerol, glyceryl trioleate, glyceryl trilinoleate, glyceryl tristearate, were purchased from Nu-Check Prep, INC (Elysian, MN, 56028 USA). Commercial emulsifiers (E-471 additive) was purchased directly from the market (minimum 90 % of saturated fat, reported in label). Acetonitrile, methanol, dichloromethane, hexane and 2-propanol (IPA) were HPLC gradient-grade solvents and supplied by Merck (Darmstadt, Germany). Ultrapure water was used in all experiments (MilliQ System, Millipore, Bedford, MA, USA).

Particle size distribution (granulometry)

Granulometry analysis was performed manually using decreasing mesh sieves (from 500 to 125 μm, Endecotts Ltd, London, UK) loading between 100-200 grams of product and with a circular oscillation for 5 minutes.

Crude fat content (Soxhlet method)

The rice bran oil was extracted with diethyl-ether, utilizing an automatic extraction apparatus (SER148 Extractor unit, VELP Scientifica, Usmate (MB), Italy) with a total time of 120 min (90 min of immersion and 30 min of washing). Since fat extraction with the Soxhlet method could not be exhaustive in terms of recovery of MAG, DAG and TAG (Manirakiza, Covaci and Schepens, 2001), a further extraction was performed on the dried pellet resulted after the above mentioned process on 2 g of dried pellet residue, by stirring with a dichloromethane/methanol solution (20 mL, 50/50 v/v) during 30 minutes at room temperature.

The extract was taken to dryness under a gentle nitrogen flux and re-dissolved in an appropriate volume of IPA, methanol and water (65/30/5, v/v) before LC-MS/MS analysis.

Mono-, di- and triacylglycerols quantification and profiling with LC-MS/MS

The chromatographic separation was performed on a portion of the oil extracted and dissolved in an appropriate volume of IPA, methanol and water (65/30/5, v/v) using UHPLC Dionex Ultimate 3000 instrument (Thermo Fisher Scientific Inc., San Jose, CA, USA) equipped with a RP-C18 Kinetex EVO column 2.10×100 mm with a particle size of 2.6 µm (Phenomenex, Torrance, CA, USA).

The mobile phases used were: A, 5% of methanol and B, 60/35/5 (v/v) of IPA, methanol and MilliQ water, respectively, both acidified with formic acid (0.1 %) and added of 5mM ammonium formate. Flow rate was 0.45 mL/min. Mass spectrometer experiments were carried out using a triple quadrupole equipped with an electrospray source (ESI) (TSQ Vantage, Thermo Fisher Scientific Inc., San Jose, CA, USA). The elution gradient commenced from 10% (B) running an isocratic step of 1 min, increased at 50% in 6 min; then at 11 min was additionally increased at 80% and the flow was also increased to 0.5 mL/min. Then, the initial conditions were restored after 5 minutes. The total run time was 25 minutes, the column temperature was set at 60°C and the autosampler temperature at 24° C. The injection volume was settled at 2 µL for each sample. MAG, DAG and TAG were monitored in positive ionization mode (spray voltage = 4000 V) in the following conditions: capillary temperature, 250 °C; vaporizer temperature, 300 °C; sheath gas flow, 50 units; auxiliary gas flow, 5 units. An automatic function of the Xcalibur software (Thermo Fisher Scientific Inc., San Jose, CA, USA) was used to set the S-Lens RF amplitude and Collision Energy (CE) values, by tuning IPA/MeOH/H_2O (60/35/5, v/v) solutions of each considered molecule (1 mg/L). Detection was carried out using Multiple Reaction Monitoring (MRM) modality, using the specific transitions reported in **Table 14**.

For quantification, a stock solution of MAG, DAG and TAG was prepared using IPA/MeOH/H_2O (60/35/5, v/v) as solvent, then the calibration curve was made in the range 1-200 mg/L. MAG, DAG and TAG stereoisomer composition were analysed with the same equipment described before, using full scan MS as acquisition mode and comparing the characteristic retention times and neutral losses characteristics with those reported in the literature (Ham *et al.*, 2004; Moreau *et al.*, 2008; Geng, Harnly and Chen, 2015; Balgoma *et al.*, 2019).

Table 14. Mass spectrometry characteristics of monitored compounds.

Compound	Abbreviation (Carbon atom backbone:number of double bond)	Monitored Adduct	Ion *m/z*	Monitored transitions *m/z* (CE*)
Monoglycerides				
Palmitolein	Po (16:1)		329	219 (12) 135 (20) 121 (20)
Palmitin	P (16:0)		331	313 (6) - 95 (21)
Linolenin	Ln (18:3)		353	261 (14) 81 (14)
Linolein	L (18:2)	$[M+H]^+$	355	337 (10) 263 (5) 245 (11)
Olein	O (18:1)		357	339 (10) 247 (14) 135 (10)
Stearin	S (18:0)		359	341 (7) 95 (7)
Arachidin	A (20:0)		387	369 (9) 95 (9)
Diglycerides				
Dipalmitin	PP		547	237 (20) 95 (32) 81 (32)
Dilinolein	LL	$[M+H-H_2O]^+$	599	263 (22) 81 (33) 66 (36)
Distearin	SS		607	267 (22) 95 (32) 81 (32)
Triglycerides				
Tristearin	SSS		896	599 (27) 263 (35) 95 (45) 81 (45)
Triolein	OOO	$[M+NH_4]^+$	902	603 (25) 265 (35) 95 (44)
Trilinolein	LLL		897	607 (26) 267 (36)

* Collision Energy expressed as eV units

The results were expressed as mg of MAG, DAG or TAG/ 100 g of rice bran, considering the rice bran oil density (equation 1):

$$(((mg\ X)/(mL\ oil))/\delta) * 100$$

Where *X* is the compound belonging to the class of MAG, DAG or TAG and δ is the density (0.92 g/mL at 20° C, Kahlon, 2009) of rice bran oil.

Fatty acids (FAs) profile with GC-MS

The FA profile of the different samples was determined using gas chromatography coupled to mass spectrometry (GC–MS) after trans-esterification to FA methyl esters (EEC, 1991). A total of 100 mg of RB oil was dissolved in 4 mL of hexane, then 1 mL of tetracosane (50 mg/L in hexane) was added and mixed for few minutes with 0.2 mL of KOH 10% solution in methanol. The upper layer was separated and 1 µL was used for the GC−MS analysis (split mode, 1:20). The experiments were carried out on a Thermo Scientific Trace 1300 gas chromatograph coupled to a Thermo Scientific ISQ single quadrupole mass spectrometer (Thermo Fisher Scientific Inc., San Jose, CA, USA) equipped with an electronic impact (EI) source. A low-polarity capillary column (SLB-5 ms, Supelco, Bellafonte, PA) was used. Chromatograms were recorded in scan mode (40−500 m/z) with a programmed temperature gradient from 40 to 280 °C (40 °C for two minutes, increasing 15 °C/min until 280 °C and isothermal step at 280 °C for 2 min, total run time of 20 min). Results were reported as relative percentage calculated on the chromatographic area of each peak and expressed on dry matter content. Fatty acids were also reported according to their unsaturation degree, as saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids.

Statistical analysis

All analyses were performed at least in triplicate and the results were expressed on dry weight base as mean ± standard deviation. One-way ANOVA was used to compare the different results obtained for MAG, DAG, TAG and FAs content of the different rice bran samples. Data were analysed using Tukey-b's post hoc test with a significance level of $p<0.05$. Statistical analysis was carried out using SPSS Statistics 21.0 software (SPSS Inc., Chicago, IL).

Results and Discussion

Effect of air classification and particle size reduction on crude fat content of rice bran

After the micronization and air-classification treatments, the coarse and fine fractions of rice bran collected were characterized by a different particle size, a parameter recognised to affect texture and nutritional properties of food products (Protonotariou, Mandala and Rosell, 2015; Bressiani *et al.*, 2017; Tsatsaragkou *et al.*, 2017). In fact, the particle size distribution of fine fractions was characterized by finer particles than the coarse fractions (**Table 15**), which are probably composed by large fiber agglomerates particles (Laudadio *et al.*, 2013).

Table 15. Granulometry characteristics of coarse and fine rice bran fractions.

Granulometry characteristics	RBc	RBf	RBc1	RBf1	RBFf	MRBc	MRBf	MRBc1	MRBf1	MRBFf
Discard at 500 µm (%)	47.6±0.2^{h}	5.8±0.2^{b}	45.1±0.1^{g}	20.5±0.4^{d}	19.7±0.2^{d}	30.7±0.3^{e}	10.8±0.2^{c}	32.4±0.1^{f}	3.6±0.1^{a}	4.1±0.2^{a}
Discard at 250 µm (%)	29.2±0.3^{ab}	58.6±0.3^{d}	27.6±0.2^{a}	33.6±0.1^{b}	46.5±1.7^{c}	30.6±0.2^{ab}	40.2±1.0^{c}	30.1±0.2^{ab}	46.1±0.1^{a}	48.7±1.2^{a}
Discard at 125 µm (%)	22.4±0.2^{d}	19.4±0.3^{b}	16.4±0.2^{a}	30.4±0.2^{e}	21.3±0.6^{c}	21.2±0.2^{c}	30.4±0.4^{e}	22.9±0.2^{d}	31.9±0.1^{e}	33.1±0.8^{f}
Passage at 125 µm (%)	0.8±0.1^{a}	16.2±0.2^{f}	10.9±0.3^{b}	15.5±0.2^{e}	12.5±0.2^{c}	12.1±0.3^{c}	18.6±0.2^{g}	14.6±0.3^{d}	18.4±0.1^{g}	14.1±0.4^{d}

Values in the same row with different superscript letters differ significantly ($p<0.05$). RB, rice bran; RBc, coarse fraction of air-separated rice bran; RBf, fine fraction of air-separated rice bran; RBc1, coarse fraction of air-separated rice bran with different operating conditions; RBf1, fine fraction of air-separated rice bran with different operating conditions; RBFf, ultra-fine fraction of air-separated rice bran with different operating conditions; MRB, micronized rice bran; MRBc, coarse fraction of micronized and air-separated rice bran; MRBf, fine fraction of micronized and air-separated rice bran; MRBc1, coarse fraction of micronized and air-separated rice bran with different operating conditions; MRBf1, fine fraction of micronized and air-separated rice bran with different operating conditions; MRBFf, ultra-fine fraction of micronized and air-separated rice bran with different operating conditions.

On the latter fractions a fat extraction was performed: the total lipid content differed significantly, as reported in **Table 16**. The recovered oil was almost green to grey in colour, and with a matte glass texture. It is worth to mention that also the degree of the milling process can influence the amount and the quality of the extracted oil (Godber and Juliano, 2013). Fine rice bran fractions obtained higher crude fat content in respect to the coarse ones. Probably, a redistribution of the macro-components of rice bran occurred, increasing on one side the content of dietary fiber in coarse fractions and on the other side proteins and lipids in fine fractions, as reported by Rizzello, Coda, Mazzacane, Minervini, & Gobbetti, 2012, which used the same technology, hence similar results can be expected. The yield percentages reported always refer to the initial non-air-classified rice bran. Interestingly the increased fine fraction yields were obtained increasing the air valve regulation (**Table 16**). The applied treatments did not influence the moisture content of rice bran fractions.

Table 16. Processing parameters, yield, moisture content and crude fat content or rice bran samples.

Sample	Valve regulation	Yield (%)	Moisture content	Crude fat content
			mg/100g	
RB	-	100	11,0±1,1[a]	15,8±1,1[cd]
RBc	395	28	12,0±1,2[a]	8,8±0,1[a]
RBf		72	10,2±1,0[a]	17,7±1,4[def]
RBc1		82	10,4±1,0[a]	14,4±1,8[bcd]
RBf1	300	16	11,0±1,1[a]	18,7±0,3[ef]
RBFf		2	10,2±1,0[a]	18,7±1,5[ef]
MRB	-	100	10,7±1,1[a]	14,8±1,3[bca]
MRBc	365	24,1	11,0±1,1[a]	11,6±0,1[ab]
MRBf		75,9	10,3±1,0[a]	17,4±2,0[def]
MRBc1		78,2	10,3±1,0[a]	13,3±0,4[bc]
MRBf1	279	18,8	9,9±1,0[a]	19,6±2,0[f]
MRBFf		3	10,0±1,0[a]	18,7±1,2[ef]

Results represent a mean of three independent analyses. [a-f] Different superscript letters in the same row differ significantly ($p<0.05$) using *Tukey's b* post-hoc test.

Quantitative analysis of mono-, di- and triacylglycerols

Neutral lipids are the most abundant class of lipid compounds found in rice bran (~90%, Shin & Godber, 2002). These group comprises mono-, di- and triacylglycerols and free fatty acids. However, other minor lipid categories such as glycolipids and phospholipids have been studied in this matrix (Godber and Juliano, 2013). Concentrations of MAGs, DAG and TAGs determined by LC-MS/MS analysis in raw and treated rice bran are reported in **Table 17**. Overall, the concentration of each quantified compound reflected the trend of the crude fat content, showing a higher amount in finest rice bran fractions and a lower one in the coarse fraction.

MAGs are composed by a classic glycerol backbone in which one of the sn-1 or sn-2 positions is esterified with a fatty acid.

Their formation can occur both via lipid metabolism in plant and by some catabolic pathways of triacylglycerol compounds. Besides their fundamental role in lipid metabolism (Mu and Porsgaard, 2005), they have good emulsifying properties very important for sensory properties of food products. Among MAGs, oleic, linoleic, palmitic and stearic glycerides (in order of abundance) were quantified in rice brans. Among DAGs, only the dilinolein derivative was quantified in rice bran samples, meaning that no saturated DAGs were found. These compounds have been recently studied and they have been found to exert some beneficial effects on the lipid profile and cardiovascular risk factors in Wistar rats (Anikisetty *et al.*, 2018). Moreover, TAGs, the most abundant fraction of a lipid material, are important components of foods in terms of physical, sensory and nutritional impact. Amongst TAGs, trilinolein, triolein and tristearin were quantified in rice bran oils. Arguably, the mono-unsaturated triolein was found in higher content than the di-unsaturated dilinolein. The saturated tristearin was identified only in very low amount. The treatment applied to rice bran, deeply influenced the MAG, DAG and TAG content. In fact, in fine fractions (RbF1, RBFf, MRbf1 and MRBFf) the content of each compound quantified was higher and significantly different from the corresponding coarse fraction, the latter always lower than the starting raw material.

Table 17. Mono-, di- and triacylglycerols content of rice bran samples.

	MAG				DAG		TAG	
Sample	**P**	**L**	**O**	**S**	**LL**	**LLL**	**OOO**	**SSS**
					mg/100g RB			
RB	2.47±0.12^{c}	4.92±0.25^{a}	7.89±0.39^{b}	0.44±0.02^{b}	17.96±0.9^{b}	24.74±1.24^{c}	39.11±1.96^{c}	1.27±0.06^{bc}
RBc	0.34±0.02^{a}	<LOQ	0.95±0.05^{a}	0.11±0.01^{a}	2.70±0.14^{a}	7.45±0.37^{b}	22.83±1.14^{b}	0.92±0.05^{a}
RBf	2.93±0.15^{d}	5.51±0.28^{c}	9.54±0.48^{cd}	0.96±0.05^{c}	18.68±0.93^{b}	25.56±1.28^{cd}	41.70±2.09^{cd}	1.40±0.07^{bc}
RBc1	2.13±0.11^{b}	4.70±0.24^{a}	7.60±0.38^{b}	0.38±0.02^{b}	17.78±0.89^{bc}	24.73±1.24^{c}	38.45±1.92^{c}	1.23±0.06^{bc}
RBf1	3.50±0.18^{e}	6.64±0.33^{d}	10.42±0.52^{d}	1.14±0.06^{d}	19.73±0.99^{bc}	26.61±1.33^{cd}	41.94±2.1^{cd}	1.51±0.08^{c}
RBFf	3.32±0.17^{e}	5.74±0.29^{d}	10.34±0.51^{d}	1.13±0.06^{d}	19.45±0.97^{bc}	26.49±1.32^{cd}	41.73±2.09^{cd}	1.46±0.07^{c}
MRB	2.00±0.1^{b}	4.59±0.23^{a}	7.45±0.37^{b}	0.35±0.02^{b}	16.77±0.84^{b}	24.11±1.21^{c}	37.39±1.87^{c}	1.12±0.06^{b}
MRBc	<LOQ	<LOQ	0.54±0.03^{a}	<LOQ	0.85±0.04^{a}	1.44±0.07^{a}	16.01±0.8^{a}	0.87±0.04^{a}
MRBf	2.72±0.14^{d}	5.50±0.27^{c}	8.15±0.41^{c}	0.95±0.05^{c}	18.56±0.93^{bc}	25.02±1.25^{c}	40.37±2.02^{cd}	1.31±0.07^{bc}
MRBc1	1.92±0.10^{b}	4.26±0.21^{a}	6.85±0.34^{b}	0.32±0.02^{b}	15.32±0.77^{b}	23.36±1.17^{c}	36.40±1.82^{c}	0.96±0.05^{a}
MRBf1	5.26±0.26^{f}	8.69±0.43^{d}	13.94±0.70^{f}	1.23±0.06^{d}	26.17±1.31^{d}	28.69±1.43^{d}	43.62±2.18^{d}	1.95±0.1^{d}
MRBFf	3.57±0.18^{e}	6.69±0.33^{d}	10.50±0.52^{d}	1.15±0.06^{d}	21.59±1.08^{c}	28.31±1.42^{d}	42.76±2.14^{d}	1.55±0.08^{c}

Results reported as mean of three independent analyses. $^{a-f}$ Different superscript letters in the same column for significant differences ($p<0.05$) using *Tukey's b* post-hoc test. <LOQ: 0.1 mg/100 g

Considering the sum of quantified compounds for each glyceride category, the class of MAGs was the less abundant, while TAGs were found in higher concentration, as reported in **Table 18**. A further solid-liquid extraction was performed on the dried pellets with the aim to recover the lipid molecules. By this procedure, all the compounds were successfully extracted achieving an 87% recovery for total lipids (expressed as mean of all sample and referred to the sum of MAG, DAG and TAG identified) and a 90 %, 98% and 84% recovery for MAGs, DAGs and TAGs, respectively (**Figure 18**).

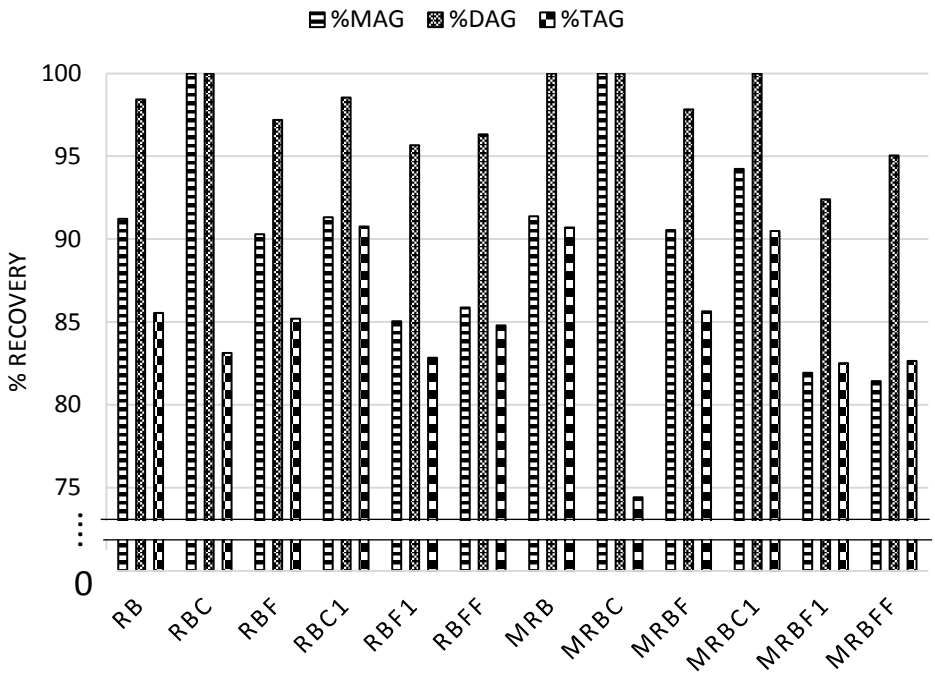

Figure 18. Recovery of MAG, DAG and TAG, calculated as percentage (%) (see supplementary material **Table A1** for more information).

For comparison, we analysed the lipid composition of a commercial emulsifier (E-471 additive, data not shown): only short chain saturated MAGs (Palmitin and Stearin) and DAGs (Stearin) were found, while no TAGs were present.

The E-471 additive had a ~10-fold higher content of these compounds compared to the obtained rice bran fractions.

Table 18. Characteristic lipid attributes of RB samples.

Sample	% MAG*	% DAG*	% TAG*	% Saturated MAG**	Unsaturated TAG/Saturated TAG
RB	15.9±0.8$^{bc\ C}$	18.2±0.9$^{cd\ B}$	65.9±3.3$^{ab\ A}$	18.5±0.9^{ab}	50.1±2.5^{cd}
RBc	4.0±0.2$^{b\ C}$	7.7±0.4$^{b\ B}$	88.4±4.4$^{c\ A}$	32.2±1.6^{d}	32.8±2.6^{b}
RBf	17.8±0.9$^{cde\ B}$	17.6±0.9$^{c\ B}$	64.6±3.2$^{ab\ A}$	20.6±1.0^{bc}	48.2±1.4^{c}
RBc1	15.3±0.8$^{b\ C}$	18.3±0.7$^{cd\ B}$	66.4±3.3$^{ab\ A}$	17.0±0.8^{a}	51.2±3.3^{cd}
RBf1	19.5±1.0$^{e\ B}$	17.7±0.4$^{c\ C}$	62.8±3.2$^{ab\ A}$	21.4±1.1^{c}	45.5±2.6^{c}
RBFf	18.7±0.9$^{de\ B}$	17.7±0.9$^{c\ B}$	63.5±3.1$^{ab\ A}$	21.7±1.3^{c}	46.7±2.3^{c}
MRB	15.3±0.8$^{b\ C}$	17.9±0.7$^{c\ B}$	66.8±3.3$^{ab\ A}$	16.3±0.8^{a}	54.9±2.7^{d}
MRBc	2.7±0.1$^{a\ C}$	4.3±0.2$^{a\ B}$	93.0±4.6$^{c\ A}$	N.F.	20.1±2.1^{a}
MRBf	16.9±0.8$^{bcd\ B}$	18.1±1.0$^{cd\ B}$	65.0±4.3$^{ab\ A}$	21.2±1.2^{c}	50.0±2.5^{cd}
MRBc1	14.9±0.7$^{b\ C}$	17.1±0.9$^{c\ B}$	67.9±3.4^{bA}	16.7±0.6^{a}	62.1±3.1^{e}
MRBf1	22.5±1.1$^{f\ B}$	20.2±0.8$^{e\ C}$	57.3±2.9$^{a\ A}$	22.3±1.3^{c}	37.1±2.9^{b}
MRBFf	18.9±0.9$^{de\ B}$	18.6±0.9$^{cd\ B}$	62.5±3.1$^{ab\ A}$	21.5±1.0^{c}	45.7±2.3^{c}

* calculated as a function of the total MAG, DAG and TAG content; ** in respect to the total amount of MAG. N.F., not found. [a-f] Different superscript letters in the same column, or [A-C] rows for significant differences ($p<0.05$) using *Tukey's b* post-hoc test. RB, rice bran; RBc, coarse fraction of air-separated rice bran; RBf, fine fraction of air-separated rice bran; RBc1, coarse fraction of air-separated rice bran with different operating conditions; RBf1, fine fraction of air-separated rice bran with different operating conditions; RBFf, ultra-fine fraction of air-separated rice bran with different operating conditions; MRB, micronized rice bran; MRBc, coarse fraction of micronized and air-separated rice bran; MRBf, fine fraction of micronized and air-separated rice bran; MRBc1, coarse fraction of micronized and air-separated rice bran with different operating conditions; MRBf1, fine fraction of micronized and air-separated rice bran with different operating conditions; MRBFf, ultra-fine fraction of micronized and air-separated rice bran with different operating conditions.

Qualitative analysis of mono- and diacylglycerols

Since the MS/MS analysis only permits the quantification of compounds for which an analytical standard is available, results for the other DAGs (A) and TAGs (B) species found in rice bran are reported in **Fig. 2**. TAGs and DAGs have already been studied using LC-MS methods (Ham *et al.*, 2004; Moreau *et al.*, 2008; Geng, Harnly and Chen, 2015; Balgoma *et al.*, 2019): they tend to fragment into their main components during ionization, showing *m/z* characteristic of glycerol and of fatty acids in the mass spectrum (neutral losses). In DAGs fraction, the OL (as sn-1,2, sn-2,3 and sn-1,3 mixture) was the most abundant. In the TAG fraction, PLO, OLO and LLO isomers were the highest found in rice bran oil, as also reported by other study (Jin *et al.*, 2016). Among them, the class formed by unsaturated fatty acids (i.e.: oleic or linoleic acid) was the most abundant (in terms of area under the peak compared to the area of the most similar standard reference, see Fig. 2), and the regiospecific distribution showed oleic and linoleic acid (18:1 and 18:2, respectively) in sn-1 position, and palmitic acid (16:0) in sn-1,3 position, as reported by Berger *et al.*, 2005.

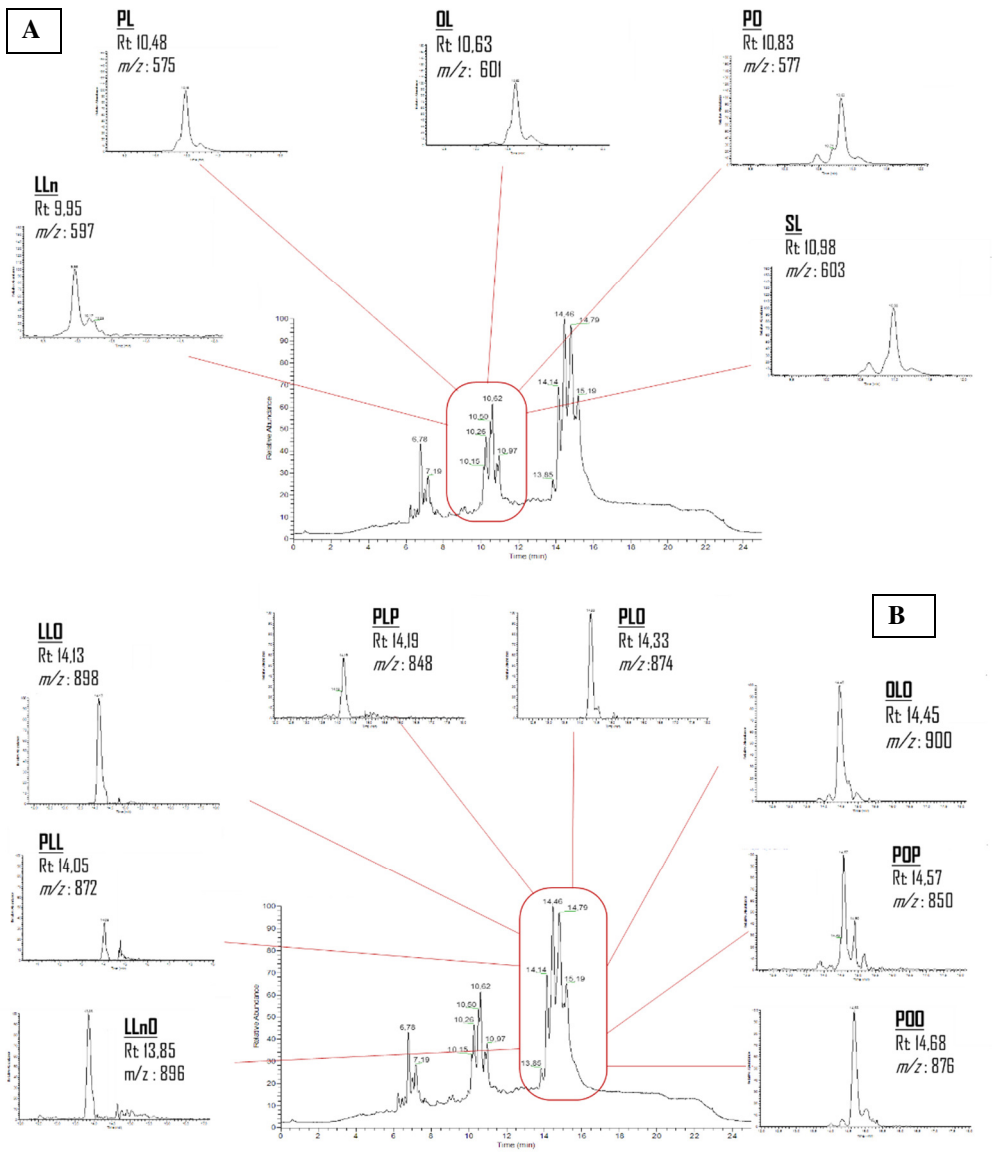

Figure 19. Total ion chromatogram (TIC, in the centre) and extracted ion chromatograms (EICs) of diglycerides (DAG, A) and triglycerides (TAG, B) stereoisomers found in rice bran oil. For each compound the retention time (Rt) and molecular ion (m/z) are reported. The abbreviation corresponds to the fatty acid composing the di- or triacylglycerol (see **Table A3** in supplementary material for more information).

Fatty acids profile of rice bran

The fatty acid (FA) profile of rice bran oil is in agreement with the results obtained by LC-MS/MS analysis, as oleic, linoleic and palmitic acid were the most abundant FAs found in rice bran (**Fig. 3**). Rice bran oil is prone to be highly acidic (Capellini *et al.*, 2017), on account of the fast and easy release of free FAs upon lipid degradation by enzymatic processes (both microbial and endogenous) (Kim, Chung and Lim, 2014). In general, the free FAs content of rice bran oil ranges 9-20 % (w/w of crude fat), depending on the milling degree and the storage practices (Godber and Juliano, 2004).

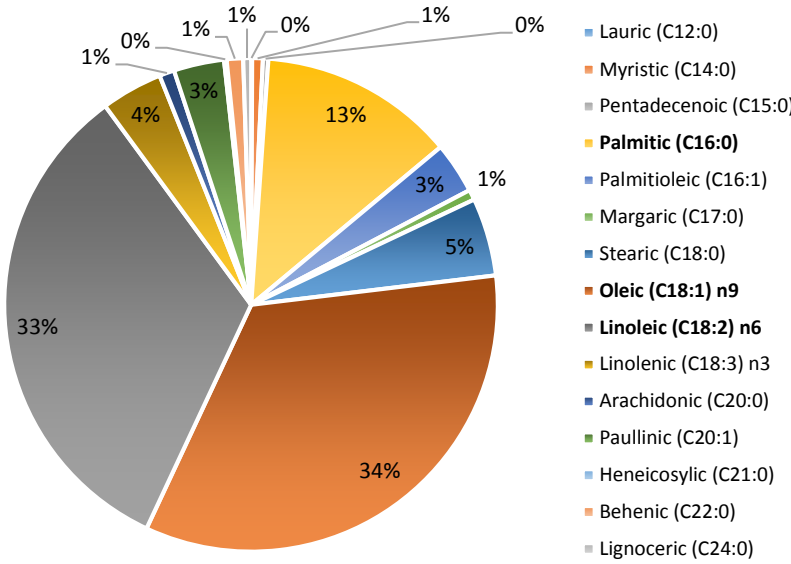

Figure 20. Fatty acid profile as %, of raw rice bran. The most abundant FAs are depicted in bold.

However, these compounds are also important (Park, Lee and Choi, 2017) for human nutrition, mostly for their rich unsaturated fraction. The composition of rice brain oil examined in this study resulted in 22.5% of SFA, 40.5% of MUFA, 37% of

PUFA and with a PUFA/SFA ratio of 1.45, which is considered a healthy parameter (<3, EFSA, 2010).

Many other studies reported a similar composition for RBO (Latha and Nasirullah, 2014). In the tested conditions, the applied processing treatments did not significantly influence the FAs content, confirming that no lipid degradation occurred.

Conclusion

Rice bran is an interesting by-product rich of several valuable components, in particular the lipid fraction. To the best of our knowledge, the application of air classification and micronization to rice bran lipid component was never investigated. Using these technologies different fractions with coarse and fine granulometry were obtained. Moreover, compared to the coarse fraction functional lipid compounds, such as monoacylglycerols, were concentrated in fine rice bran fractions. These findings could be of relevance to the food industry because these molecules could provide a textural improvement in food products (i.e.: gluten-free foodstuffs). Finally, from the nutritional point of view, RB is characterised by a good FA profile, with a higher content of MUFA and PUFA than SFA. Nevertheless, further studies are needed in order to confirm the enhanced textural properties in food product formulation using air classified and micronized rice bran.

Authors contribution

MS, CD, GG, SF and RR designed the study. SF and RR collected the sample set. MS and LR performed the analysis. MS, LR, CD, SF, RR and GG interpreted the results. MS wrote the manuscript. All the author contributed to the critical review of the work.

Supplementary information

Table A1. Mono-, di- and triacylglycerols content in rice bran pellet after the Soxhlet extraction.

	MAG				DAG	TAG		
Sample	**P**	**L**	**O**	**S**	**LL**	**LLL**	**OOO**	**SSS**
				mg/100g				
RB	0.90±0.05	<LOQ	0.61±0.03	<LOQ	3.28±0.16	7.73±0.39	0.90±0.1	N.F.
RBc	<LOQ	<LOQ	<LOQ	<LOQ	0.64±0.03	5.69±0.38	<LOQ	
RBf	1.20±0.06	<LOQ	0.83±0.04	<LOQ	3.76±0.19	8.17±0.41	1.20±0.4	
RBc1	0.86±0.04	<LOQ	0.55±0.3	<LOQ	0.79±0.04	5.77±0.29	0.86±0.03	
RBf1	1.35±0.07	0.92±0.05	1.55±0.08	0.90±0.01	5.11±0.26	9.40±0.47	1.35±0.01	
RBFf	1.22±0.06	0.72±0.04	1.44±0.07	0.74±0.04	4.11±0.21	8.40±0.42	1.22±0.4	
MRB	0.81±0.04	<LOQ	0.55±0.03	<LOQ	0.67±0.3	5.74±0.29	0.81±0.7	
MRBc	<LOQ	<LOQ	<LOQ	<LOQ	0.61±0.03	5.69±0.67	<LOQ	
MRBf	1.19±0.06	<LOQ	0.61±0.3	<LOQ	3.42±0.17	7.77±0.48	1.19±0.3	
MRBc1	0.60±0.03	<LOQ	0.21±0.1	<LOQ	0.64±0.03	5.74±0.14	0.60±0.18	
MRBf1	2.03±0.1	2.09±0.01	2.30±0.12	2.15±0.11	5.54±0.38	10.20±0.51	2.03±0.12	
MRBFf	1.48±0.7	1.92±0.1	1.60±0.08	1.13±0.06	5.52±0.28	9.74±0.49	1.48±0.08	

Results represent a mean of three independent analyses. N.F., not found. <LOQ: 0.1mg/100g

Table A2. Mass spectrometry characteristic of putative di- and triacylglycerols stereoisomers found in rice bran oil samples.

Putative Compound	Ion *m/z*	Rt	Monitored adduct	Neutral loss	Relative abundance*
			DAG		
LLn	597	9.95		541, 337	-
PL	575	10.48		313, 263	+
OL	601	10.63	$[M+H-H_2O]^+$	545, 339	++
PO	577	10.83		313, 265	+
SL	603	10.98		341, 263	
			TAG		
LLnO	896	13.85		879, 601, 599, 597	+
PLL	872	14.05		855, 575	--
LLO	898	14.13		811, 601, 599	++
PLP	848	14.19	$[M+NH_4]^+$	831, 575	-
PLO	874	14.33		601, 577, 575	+
OLO	900	14.45		883, 603, 601	++
POP	850	14.57		577, 511	-
POO	876	14.68		603, 577	+

* Calculated in function of the most similar analytical standard reference (i.e.: for OLO was used the OOO peak area).

References

Balgoma, D. *et al.* (2019) 'Modeling the fragmentation patterns of triacylglycerides in mass spectrometry allows the quantification of the regioisomers with a minimal number of standards', *Analytica Chimica Acta*, 1057, pp. 60–69. doi: 10.1016/j.aca.2019.01.017.

Berger, A. *et al.* (2005) 'Similar cholesterol-lowering properties of rice bran oil, with varied γ-oryzanol, in mildly hypercholesterolemic men', *European Journal of Nutrition*, 44(3), pp. 163–173. doi: 10.1007/s00394-004-0508-9.

Bottega, G. *et al.* (2009) 'The debranning of common wheat (Triticum aestivum L.) with innovative abrasive rolls', *Journal of Food Engineering*, 94(1), pp. 75–82. doi: 10.1016/j.jfoodeng.2009.03.002.

Bressiani, J. *et al.* (2017) 'Properties of whole grain wheat flour and performance in bakery products as a function of particle size', *Journal of Cereal Science*, 75, pp. 269–277. doi: 10.1016/j.jcs.2017.05.001.

Capellini, M. C. *et al.* (2017) 'Rice bran oil extraction using alcoholic solvents: Physicochemical characterization of oil and protein fraction functionality', *Industrial Crops and Products*, 104, pp. 133–143. doi: 10.1016/j.indcrop.2017.04.017.

Coda, R. *et al.* (2014) 'Effect of bioprocessing and particle size on the nutritional properties of wheat bran fractions', *Innovative Food Science and Emerging Technologies*, 25(C), pp. 19–27. doi: 10.1016/j.ifset.2013.11.012.

Coda, R., Katina, K. and Rizzello, C. G. (2015) 'Bran bioprocessing for enhanced functional properties', *Current Opinion in Food Science*, 1(1), pp. 50–55. doi: 10.1016/j.cofs.2014.11.007.

European Commission (2008) 'COMMISSION REGULATION No 2568/91 of 11 July 1991 on the characteristics of olive oil and olive-residue oil and on the relevant methods of analysis', *Official Journal of the European Communities*, L 248(2568), p. 112. doi: 2004R0726 - v.7 of 05.06.2013.

Ferrari, B. *et al.* (2009) 'Optimization of air classification for the production of β-glucan-enriched barley flours', *Journal of Cereal Science*, 50(2), pp. 152–158. doi: 10.1016/j.jcs.2009.04.007.

García-Lara, S. (2010) *Cereal Grains: Properties, Processing and Nutritional Attributes*, *Crop Science*. doi: 10.2135/cropsci2010.12.0005br.

Geng, P., Harnly, J. M. and Chen, P. (2015) 'Differentiation of Whole Grain from Refined Wheat (T. aestivum) Flour Using Lipid Profile of Wheat Bran, Germ, and Endosperm with UHPLC-HRAM Mass Spectrometry', *Journal of Agricultural and Food Chemistry*, 63(27), pp. 6189–6211. doi: 10.1021/acs.jafc.5b01599.

Godber, J. S. and Juliano, B. O. (2004) 'Chapter 7: Rice Lipids', in *RICE: Chemistry and Technology*, pp. 163–190. doi: 10.1094/1891127349.007.

Ham, B. M. *et al.* (2004) 'Identification, quantification and comparison of major non-polar lipids in normal and dry eye tear lipidomes by electrospray tandem mass spectrometry', *Journal of Mass Spectrometry*, 39(11), pp. 1321–1336. doi: 10.1002/jms.725.

Hemery, Y. *et al.* (2007) 'Dry processes to develop wheat fractions and products with enhanced nutritional quality', *Journal of Cereal Science*, 46(3), pp. 327–347. doi: 10.1016/j.jcs.2007.09.008.

Hemery, Y. *et al.* (2011) 'Potential of dry fractionation of wheat bran for the development of food ingredients, part I: Influence of ultra-fine grinding', *Journal of Cereal Science*, 53(1), pp. 1–8. doi: 10.1016/j.jcs.2010.09.005.

Jin, J. *et al.* (2016) 'Production of rice bran oil with light color and high oryzanol content by multi-stage molecular distillation', *JAOCS, Journal of the American Oil Chemists' Society*, 93(1), pp. 145–153. doi: 10.1007/s11746-015-2747-8.

Kahlon, T. S. (2009) 'Rice bran: Production, composition, functionality and food applications, physiological benefits', in *Fiber Ingredients: Food Applications and Health Benefits*, pp. 305–321. doi: doi:10.1201/9781420043853-c14.

Kim, S. M., Chung, H. J. and Lim, S. T. (2014) 'Effect of various heat treatments on rancidity and some bioactive compounds of rice bran', *Journal of Cereal Science*, 60(1), pp. 243–248. doi: 10.1016/j.jcs.2014.04.001.

Latha, R. B. and Nasirullah, D. R. (2014) 'Physico-chemical changes in rice bran oil during heating at frying temperature', *Journal of Food Science and Technology*, 51(2), pp. 335–340. doi: 10.1007/s13197-011-0495-9.

Laudadio, V. *et al.* (2013) 'Production of low-fiber sunflower (Helianthus annuus L.) meal by micronization and air classification processes', *CYTA - Journal of Food*, 11(4), pp. 398–403. doi: 10.1080/19476337.2013.781681.

Metzger, J. O. and Bornscheuer, U. (2006) 'Lipids as renewable resources: Current state of chemical and biotechnological conversion and diversification', *Applied Microbiology and Biotechnology*, 71(1), pp. 13–22. doi: 10.1007/s00253-006-0335-4.

Moreau, R. A. *et al.* (2008) 'The identification of mono-, di-, tri-, and tetragalactosyl-diacylglycerols and their natural estolides in oat kernels', *Lipids*, 43(6), pp. 533–548. doi: 10.1007/s11745-008-3181-6.

Mu, H. and Porsgaard, T. (2005) 'The metabolism of structured triacylglycerols', *Progress in Lipid Research*, pp. 430–448. doi: 10.1016/j.plipres.2005.09.002.

Orthoefer, F. (2008) 'Applications of emulsifiers in baked foods', in *Food Emulsifiers and Their Applications: Second Edition*. doi: 10.1007/978-0-387-75284-6_9.

Park, H. Y., Lee, K. W. and Choi, H. D. (2017) 'Rice bran constituents: immunomodulatory and therapeutic activities', *Food and Function*, 8(3), pp. 935–943. doi: 10.1039/c6fo01763k.

Pelgrom, P. J. M. *et al.* (2013) 'Dry fractionation for production of functional pea protein concentrates', *Food Research International*. doi: 10.1016/j.foodres.2013.05.004.

Pfaltzgraff, L. A. *et al.* (2013) 'Food waste biomass: A resource for high-value chemicals', *Green Chemistry*, pp. 307–314. doi: 10.1039/c2gc36978h.

Protonotariou, S., Mandala, I. and Rosell, C. M. (2015) 'Jet Milling Effect on Functionality, Quality and In Vitro Digestibility of Whole Wheat Flour and Bread', *Food and Bioprocess Technology*, 8(6), pp. 1319–1329. doi: 10.1007/s11947-015-1494-z.

Puupponen-Pimiä, R. *et al.* (2016) 'Fermentation and dry fractionation increase bioactivity of cloudberry (Rubus chamaemorus)', *Food Chemistry*. doi: 10.1016/j.foodchem.2015.11.061.

Rizzello, C. G. *et al.* (2012) 'Micronized by-products from debranned durum wheat and sourdough fermentation enhanced the nutritional, textural and sensory features of bread', *Food Research International*, 46(1), pp. 304–313. doi: 10.1016/j.foodres.2011.12.024.

Sairam, S., Gopala Krishna, A. G. and Urooj, A. (2011) 'Physico-chemical characteristics of defatted rice bran and its utilization in a bakery product', *Journal of Food Science and Technology*, 48(4), pp. 478–483. doi: 10.1007/s13197-011-0262-y.

Schutyser, M. A. I. *et al.* (2015) 'Dry fractionation for sustainable production of functional legume protein concentrates', *Trends in Food Science and Technology*. doi: 10.1016/j.tifs.2015.04.013.

'Scientific Opinion on Dietary Reference Values for fats, including saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, trans fatty acids, and cholesterol' (2016) *EFSA Journal*, 8(3). doi: 10.2903/j.efsa.2010.1461.

Shin, T. S. and Godber, J. S. (1996) 'Changes of endogenous antioxidants and fatty acid composition in irradiated rice bran during storage', *Journal of Agricultural and Food Chemistry*, 44(2), pp. 567–573. doi: 10.1021/jf950386a.

Tsatsaragkou, K. *et al.* (2017) 'Improving Carob Flour Performance for Making Gluten-Free Breads by Particle Size Fractionation and Jet Milling', *Food and Bioprocess Technology*, 10(5), pp. 831–841. doi: 10.1007/s11947-017-1863-x.

Wang, J. *et al.* (2016) 'Dietary fibre enrichment from defatted rice bran by dry fractionation', *Journal of Food Engineering*, 186, pp. 50–57. doi: 10.1016/j.jfoodeng.2016.04.012.

Zhang, L. *et al.* (2019) 'Arabinoxylans-enriched fractions: From dry fractionation of wheat bran to the investigation on bread baking performance', *Journal of Cereal Science*. doi: 10.1016/j.jcs.2019.02.005.

CHAPTER III

Recovery of wheat bran by-product: nutritional features improvement

Evaluation of bioactive properties, methyl donor compounds absorption and intestinal glucose uptake inhibition of pre-fermented bran enriched bread in *in vitro* cell line models.

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Preface

Supported by the results derived from the study presented in **Chapter 2.1** and looking towards a potential application of wheat bran milling-by-products, this last part of the work started with the formulation of a lab-scale wheat bread enriched with fermented wheat bran. In order to highlight potential health related properties differences, additional bread without wheat bran and adding raw wheat bran were produced and compared. The experiments were performed using *in vitro* cell culture models, which are important methods in order to gain further knowledge on a specific topic and crucial for the implementation of future *in vivo* studies.

The assumptions taken as the basis were the increased content of bioactive compounds found in fermented wheat bran, which they could possibly lead to beneficial effects on health, in particular the protective actions against oxidative stress. These molecular defending mechanisms have been assigned to the phenolic components, like flavonoid or phenolic acids, which may be have inter-related or synergic effects [1]. However, in the context of a complex matrix, such as bread, the whole food system plays a role on the given beneficial function.

[1] Anson, N. M. (2010). Bioactive compounds in whole grain wheat. Maastricht University.

Abstract

The re-introduction of cereal milling by-products, such as bran, in baked food is a good strategy for the improvement of both nutritional quality and sustainability. Bread enriched with previously fermented wheat bran (BFB), white bread (BB) and a bread enriched with raw bran (BWB) were formulated and compared in terms of bioactive properties and biological effects. Breads were *in vitro* digested, the soluble fractions recovered were analysed in their antioxidant potential and used for the treatment of IEC-6 and CaCo-2 epithelial cells. In addition, the potential intestinal glucose uptake inhibitory activity and methyl donor compounds uptake were determined. The digestas did not affect the cellular viability, in fact any cytotoxicity effect was found (concentration not exceeding 1 mg mL^{-1}). A diminishing tendency of ROS generation was found for both BWB and BFB, but no differences were found between these samples. The BFB extract significantly reduced the NO production, in respect to the other breads. Moreover, BFB could delay the glucose absorption, although significantly differed from BWB only in CaCo-2 cells. Overall, breads enriched with bran had a higher content and absorption rate of betaine and choline. This study represents a progress for the evaluation of improved characteristics of high nutritional value wheat-based baked products.

Abbreviations

AAPH, disodium salt and 2,2'-azobis (2-methylpropionamidine), dihydrochloride ABTS, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, AsA, Ascorbic acid, BB, white bread, BFB, bread with pre-fermented wheat bran, BWB, bread with raw wheat bran, CaCo-2, colorectal adenocarcinoma cells from human, CE, Collision Energy, DCFH-DA, 20,70-dichlorofluorescin diacetate, DMSO, Dimethyl sulfoxide DPPH, 2,2-diphenyl-1-picrylhydrazyl, d.w., dry weight, FBS, Fetal Bovine Serum, FL, fluorescein, FRAP, ferric reducing antioxidant power, GAE, gallic acid equivalent, GLU, glucose, GLUT-2, glucose transporter, HPLC, high performance liquid chromatography, IEC-6, Epithelial small intestine cells from rat, LPS, lipopolysaccharide from *E. coli* O55:B5 (L2880), MTT, [3-(4,5-dimethylthyazol-2-yl)-2,5-diphenyltetrazolium bromide], NO, nitric oxide, OAC, overall antioxidant capacity, ORAC-FL, oxygen radical absorbance capacity, PBS, phosphate buffer solution, RAW 264.7, Abelson murine leukaemia virus transformed macrophages, ROS, reactive oxygen species, SGLT-1, sodium glucose transport protein, *t*-BOOH, tert-Butyl hydroperoxide, TEER, transepithelial electrical resistance TPC, total phenolic content, Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, UHPLC-MS, ultra high-performance liquid chromatography coupled to mass spectrometry.

Introduction

Cereal grains and cereal-derived products constitute the most of the developed and non-developed countries daily diet [1]. During their journey, cereals are subjected to several treatments, such as milling, which lead to the separation of the bran and germ fractions from the seed endosperm, generating a relevant amount of agri-processing derived by-products. From the chemical point of view, bran and external seed layers have different composition compared to the endosperm, in fact they are characterised by a higher content of nutrients, dietary fibres and bioactive compounds [2]. Therefore, the feed formulation remains the main fate of cereal milling by-products, leading to huge economical losses and an increased environmental load. However, sustainability and food nutritional value are nowadays important driving factors in modern food systems [3], especially for staple foods like cereal. Although the whole grains consumption has been associated to numerous health benefits, the inclusion of this nutrient-rich outermost layers in baked and non-baked products is still scarce and challenging, probably because of their poor sensorial and technological quality [4]. Nevertheless, the study and application of innovative techniques for agri-food by-products valorisation is a promising research path and innovation field which look at the healthy diet promotion in a sustainable context. Lactic acid fermentation of food processing by-products, for example, has been recently recognized as a valuable tool for the overall functionalization of such residues [5–8]. For instance, has been observed that fermentation of wheat bran using lactic acid bacteria led to a higher content of soluble phenolic compounds [9], a solubilization of dietary fibers and arabinoxylans [10], a decreased content of phytic acids [8], the production of aromatic compounds and also novel metabolites with potential bioactivity Spaggiari *et al*., 2019 (**Chapter 2.1**).

Moreover, the todays developed countries diet is characterized by a high carbohydrate consumption, including simple sugars like glucose.

The glucose homeostasis in human organisms is primarily regulated by insulin hormone excreted by pancreas. The excess of glucose ingestion and the consequently insensitivity of organs to the latter hormone could lead to type 2 diabetes, a continuously increasing chronic pathology [11]. In this context, the modulation of glucose uptake and absorption by the enterocytes through a limited carbohydrates digestion and/or assimilation is crucial. Several compounds belonging to different classes and present in cereals, such as polyphenols [12,13] and soluble dietary fibers [14], have shown glucose uptake inhibition at intestinal level via different mechanisms, today still not clear. In addition, wheat and cereal related products have been recognized as good source of methyl donor compounds [15]. Betaine and choline are important chemicals which can positively influence the homocysteine metabolism and methionine cycle at liver level [16], showing their important role in chronic diseases prevention [17].

Therefore, in this study the pre-fermented wheat bran was used for the formulation of a common fibre-rich bread. Moreover, after a simulated *in vitro* gastrointestinal digestion step, the (1) overall antioxidant capacity (OAC) and total phenolic content (TPC) of the soluble fraction of the digested bread were evaluated. This is supported by the fact that the assessment of beneficial health-related properties should not be performed on simple extract since it is far from the physiological situation. Then, a general health benefit is scarcely related to a specific compound, instead the whole food matrix must be taken into account [18]. Afterword, (2) the cytotoxicity and the counteraction of intracellular reactive oxygen species (ROS) production of treatments at different scalar concentrations of digested bread (0.1-2 mg mL^{-1}) were evaluated using two different cellular model (IEC-6 and CaCo-2), in basal and inflammation-induced conditions. Furthermore (3) the modulation of pro-inflammatory mediator secretion (nitric oxide, NO) using an established RAW 264.7 murine macrophages assay, (4) the influence on the intestinal glucose uptake using a well-accepted model of intestinal absorption cell monolayer model [19]

and finally (5) the absorption of important methyl donor nutrients, such as betaine and choline, were also studied.

Experimental

Chemicals

Human saliva α-amylase type IX-A (A0521-5KU, 160 U mg^{-1} solid), porcine gastric pepsin (P6887, 3260 U mg−1 solid), pancreatin from porcine pancreas (P1625, 8×USP), porcine bile salts extract (B8631), MTT [3-(4,5-dimethylthyazol-2-yl)-2,5-diphenyltetrazolium bromide], buffer salts Na_2HPO_4 and KH_2PO_4, KCl, NaCl, HCl 37%, $CH_3COONa*3(H_2O)$, Folin-Ciocalteu's reagent, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), fluorescein (FL) disodium salt and 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH), 2,2-diphenyl-1-picrylhydrazyl (DPPH), sulfanilamide (S9251), N-(1-napthyl)ethylenediamine dihydrochloride (33461), Phosphoric acid (P5811), $NaNO_2$, lipopolysaccharide (LPS) from *E. coli* O55:B5 (L2880), potassium persulfate (99,9%), iron (III) chloride, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (97 %), gallic acid (>98%), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ, >98%), HPLC-grade acetonitrile (>99.9%), betaine solution (in H_2O 0.1 M) and choline chloride (>99%), NH_4HCO_2, phlorizin dihydrate (274313), phloretin (P7912), tert-Butyl hydroperoxide (tBOOH) solution, ascorbic acid, Lucifer yellow and formic acid were purchased from Sigma-Aldrich (St. Louis, Missouri, US). Dulbecco's modified Eagle's medium (DMEM) and Trypsin (BE17-161E) were from Lonza (Basel, Switzerland). D-glucose content was determined using Megazyme K-SUFRG test kit (Chicago, IL, USA). Dimethyl sulfoxide (DMSO) from MERK Millipore (Darmstadt, Germany). Ultrapure MilliQ water was used for the elaboration of all solutions.

Wheat bread formulation

Three different types of bread, named white bread (BB), bread with raw wheat bran (BWB) and bread with pre-fermented wheat bran (BFB), were manufactured using a domestic bread maker (Backmeister mod. 8650/68511/series

Linus Pro.). The recipe and ingredients are reported in **Table 19**. The baking program included a pre-warming step (17 min.), dough mixing (18 min), three sequential leavening phases (45, 18 and 45 min.) and oven cooking (55 min.). Wheat bran (*Triticum durum* subsp. *turanicum*) was kindly donated by local milling plant, while the other ingredients were purchased at local market. All the bread tested were produced using the same ingredients batch. Consequently, wheat bran was fermented using a *Lactobacillus rhamnosus* 1473 following the procedure described by Spaggiari *et al*, 2019. Once baked, breads were cooled to room temperature, cut into homogeneous pieces (containing both crumb and crust), lyophilized, accurately minced and frozen at -20 °C until analysis or *in vitro* digestion. The bread making was performed twice for each bread. The proportion of wheat bran added did not exceeded the 20 % (as weight of flour) of the total flour amount, as reported to maintain acceptable sensory quality features of the final product [20].

Table 19. Recipe for the bread preparation.

Ingredients	**BB**	**BWB or BFB**
	g	
All-purpose wheat flour	250	210
Raw wheat bran/pre-fermented wheat bran	-	40
Sugar	10	10
Salt	7	7
Baker's yeast powder	7.5	7.5
Water	160	160
Olive oil	7.5	7.5

Simulated *in vitro* gastrointestinal digestion

Bread samples were digested according to Hollebeeck *et al*., 2013 [21] method with some modifications. The digestion process was performed in parallel on 1.2 g of bread, 1.2 g salt and digestion solutions (blank A) or 1.2 g of bread using previously irreversibly denatured enzymes by pH (i.e. pepsin) and temperature

(i.e. α-amylase, pancreatin), allowing the comparison of digested and non-digest bread at the same extractive conditions (i.e. time, temperature and volume). The process consisted in three stages starting from 2 min of oral phase, 120 min of gastric digestion and 150 min of intestinal digestion at 37 °C in mixing water bath placed in the dark. For each phase, enzyme treatments took place by the addition of human α-amylase IX-A (210 units/mg solid, 2400 units/mg protein) solution made in alkaline phosphate buffer solution (PBS) to obtain 90 units mL-1, simulated gastric juice (containing 1:10,000, 460 units/mg solid, 1020 units/mg protein of pepsin) at acid pH in HCl 0.1 M; this solution was prepared freshly for each experiment day. Moreover, a simulated pancreatic juice (containing porcine bile extract mixture and pancreatin with an activity equivalent to 4 X United States Pharmacopeia [USP] specification, at a constant ratio of 6:1) at neutral pH in $NaHCO_3$ 1 M was also used. Finally, samples were rapidly immersed in liquid nitrogen for a snap freezing and stocked at -20 °C. The day after samples were thawed and centrifuged at 10000 rpm for 15 min at 4 °C, and the supernatants were treated with a previously activated cholestyramine resin [22] in a 1:10 ratio for 1 hour under constant agitation aiming to the bile salts removal. Consequently, the solutions were centrifuged at 10000 rpm for 15 min at 4 °C and gravimetrically filtered using filter paper. This soluble fraction derived from two independent digestions of the same bread were mixed, lyophilized and kept at -20 °C until experiments.

Overall antioxidant capacity (AOC) and total phenolic content (TPC)

The overall antioxidant capacity and total phenolic content (following Folin-Ciocalteu's method [23]) of soluble fraction of undigested and digested breads were determined by microplate reader adaptation of the DPPH radical scavenging activity assay [24], $ABTS^{\bullet+}$ radical cation scavenging [25], ferric reducing antioxidant power (FRAP) [26] and ORAC-FL [27] assays. Results were expressed as mg gallic acid equivalents (GAE) g^{-1} and µmol Trolox equivalent (TEAC) g^{-1} soluble fraction of digested bread dry weight (dw), for TPC and OAC, respectively.

UHPLC-MS analysis for the betaine and choline quantification

The soluble fraction of digested breads, the apical and basal supernatant recovered from the absorption assay were transferred to LC-MS vials and opportunely diluted in a CH_3CN/H_2O (8/2 v/v) for the analysis. A HILIC XBridge BEH column (2.5µm, 150x3 mm; Waters, Massachusetts, USA) was used for the analyte separation. The eluents used were CH_3CN (phase A), MilliQ H_20 (phase B) both acidified with formic acid (0.2%) and 20 mM NH_4HCO_2 1% formic acid (phase C). The elution gradient started from 1% of B, 10% of C and 89% of A and, after an initial isocratic step of 1 min, B increased at 10% in 3 min; then at 5 min the percentage of B was further increased at 63% and, after a flashing step of 2 minutes, the initial conditions were restored, with a total run time of 13 minutes. The temperature of the column hoven was set at 35 °C and the flow maintained at 0.4 mL min^{-1}. 3 µL was the sample injection volume. Betaine and choline were monitored in positive ionization mode using a spray voltage of 4000V. The capillary and vaporizer temperature were set at 325 °C, the sheath gas flow was set at 50 units and the auxiliary gas flow at 5 units. The S-Lens RF amplitude and Collision Energy (CE) values were obtained and set by tuning methanolic solutions of each considered molecule at concentration of 1 µg mL^{-1} using an automatic function of the XCalibur Thermo-Fischer software. The SRM modality was used for the compounds detection by using the following transitions: betaine *m/z* 118→42 (CE = 32 eV), *m/z* 118→58 (CE = 24 eV), *m/z* 118→59 (CE = 19 eV); choline *m/z* 104→58 (CE = 33 eV), *m/z* 104→60 (CE = 25 eV). After, a calibration set was prepared for betaine and choline (0.01-10 µM) using commercial standards and the regression curve obtained was used for quantification.

Cell lines, culture conditions and treatments

Normal epithelial small intestine cells from rat (IEC-6), colorectal adenocarcinoma cells from human (CaCo-2) and Abelson murine leukaemia virus transformed macrophages (RAW 264.7) were obtained from the *American Type*

Culture Collection (ATCC; Manassas, Virginia, United States). The cells were routinely seeded and grown in DMEM (Lonza Bioscience, Switzerland) containing 10% v/v heat inactivated fetal bovine serum (FBS) (Sigma, Saint Louis, USA), 1% v/v L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate and sodium bicarbonate (1.5 g L^{-1}) in T75 flasks. The medium was also supplemented with 1% v/v penicillin and streptomycin. Cell cultures were incubated at 37 °C and 100% humidity in a 5% CO_2 atmosphere. The cell culture lines were sub-cultured using trypsin after reaching 80% confluence. Passages ranging from 10–17 (CaCo-2), 13-19 (IEC-6) and 6-10 (RAW 264.7) were used for all the experiments. All the cell lines were analysed for negative presence of mycoplasma contamination following the Hoechst DNA staining procedure. Before the cell treatments, the soluble fractions resulted from the *in vitro* digested breads and controls were filtered using syringe filters with porous size of 0.22 µm. In basal condition, the treatments were dissolved directly in DMEM or PBS (pH 7) at scalar concentration ranging 0.1-2 mg mL^{-1}.In inflamed conditions, after 24 h the treatments were removed and incubated for further time (specified for each assay, see methods) with new supplementation containing LPS (1 µg mL^{-1}) or tBOOH (1µM). All experiments were repeated at least 3 independent times (n = 3) and each experiment was performed in triplicate (3 wells per treatment). To avoid any interference cells were supplemented with the same amount of digestion blank A. Prior experiments were carried out to check potential detrimental effects in cell viability. No significant differences were observed (data not shown).

Cell viability assay (MTT)

Cell viability was measured using the 3-(4,5-dimethyldiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. First, IEC-6, CaCo-2 and RAW 264.7 cells were cultured at a density of 2×10^4, 5×10^4 and 8×10^4 cells per well, respectively, in a 96-well plate for 24 h. Then, cells were treated with BB, BWB, BFB and controls for 24 h. In order to induce an inflammation status in cell cultures, the

LPS (1 μg mL^{-1}) was added to treatments and cell were cultivated for further 30 min. Subsequently, cells were incubated with MTT labelling reagent for 1-4 h (depending on cell type) at 37 °C and 100 μL of solubilization solution (DMSO) was added. Then, the optical density of each well was read at 570 nm using a microplate reader (Synergy HT, Biotek, Winooski, VT, US). Results were expressed as the percentage of viability (%) with respect to the control.

Intracellular ROS scavenging activity

Intracellular ROS concentration was observed fluorometrically by measuring the oxidation of the probe 20,70-dichlorofluorescin diacetate (DCFH-DA). Briefly, DCFH-DA, 2 mM in dimethyl sulfoxide (DMSO) was prepared and kept in the dark at -20 °C until use. Cells were treated for 24 h and, in basal condition, 10 μL DCFH-DA mL^{-1} medium were added to IEC-6 and CaCo-2 cells 30 min before supplementation with BB, BWB, BFB and controls, while DCFH-DA was added 30 min before the inflammation status induced by tBOOH (1mM, final concentration) within treatments. After, DCF fluorescence intensity was detected in supernatants using a λ of excitation of 485 nm and λ of emission of 528 nm. Next, the cell viability was determined as described above. Results were normalized for the cell viability value and expressed as percentage of ROS production (%) in respect to the medium-only treated cells, cells treated with an ascorbic acid solution (10 μg mL^{-1}, antioxidant control) and cells treated with tBOOH solution (1 mM, oxidative control).

Nitric oxide (NO) production in RAW 264.7 macrophages

The anti-inflammatory activity was studied determining the production of the nitric oxide according to Benayad *et al*., 2014 [28]. The cell viability was previously assessed (see supplementary material, **Figure A1**). After the cell seeding in 96-well plate and 24 h of incubation, the cultures were treated with 1 μg mL^{-1} LPS with or without different concentrations of BB, BWB, BFB and controls for 24 h. Then the supernatant was collected, and NO production was measured adding an equal

amount of the Griess reagent constituted by 1% (w/v) sulphanilamide and 0.1% (w/v) N-1-(naphthyl) ethylenediamine dihydrochloride in 2.5% (v/v) H_3PO_4. The plate was incubated in the dark for 5 min and the absorbance measured at 550 nm in a microplate reader. The amount of NO was calculated using a sodium nitrite standard curve (1-10 µg mL^{-1}). Results were expressed as µg of NO mL^{-1} of medium.

Study for the inhibitory potential of glucose intestinal uptake

For the glucose uptake inhibition study the guide lines reported by Hubatsch et al., 2007 [29] and Abbasi et al., 2016 [30] were followed for CaCo-2 and IEC-6 cell lines, respectively. The CaCo-2 and IEC-6 cells were seeded at a density of $30x10^4$ and $7.6x10^4$ cells per well, respectively on 12 wells polycarbonate inserts plate (VWR, PA, USA) with a mean pore size of 0.4 µm. For CaCo-2, the culture was cultivated up to 21 days to allow differentiation, while for IEC-6 around 13 days, refreshing the both medium at least every two days. The intestinal barrier function was evaluated every medium refreshment step, before and after the treatments by measuring the transepithelial electrical resistance (TEER) using a epithelial Volt/Ohm Meter (World Precision Instruments Sarasota, FL, US) [31]. To ensure monolayer integrity, only wells containing cells with not less than 350 [32] or around 120 Ohm (Ω) $cm^{2\ -1}$, for CaCo-2 and IEC-6, respectively, were used for the experiments.

Regarding the inhibitory potential of glucose intestinal uptake study, cells were washed with tempered PBS in order to remove the medium and incubated for 30 min in PBS to induce the cell starvation.

After, PBS was removed and cells were treated with 500 µL digested bread solutions (1 mg mL^{-1}, apical chamber) supplemented with glucose (25 mM) and 1.5 mL of PBS was added to the basolateral chamber. Phloretin (1mM) and phlorizin (3mM) were used as inhibitory control. Sampling from basolateral compartment was made at 10, 30, 45, 60, 90, 120 and 240 minutes, replacing with the same volume of PBS. Samples were kept at -80 °C until glucose content analysis. Hexose content was determined using Glucose-TR reagent kit assay (Spinreact, Girona,

Spain) following the instruction reported by the producers and using a glucose calibration curve for quantification (1-25 mM).

The effect of the treatments on epithelial barrier function was assessed with the Lucifer yellow [33], a molecular probe (molecular weight 444.24 $g \cdot mol^{-1}$). Briefly, after the last sampling (240 min), apical and basolateral compartments were washed three times with PBS. Then 500 µL of Lucifer yellow solution (50 µM in PBS) was added to the apical compartment, and 1.5 mL of PBS was added to the basolateral chamber. After an incubation step of 3 h at 37°C in a humidified incubator with 5% CO_2, 250 µL of supernatant was taken from the basolateral compartment. Fluorescence of both apical and basolateral compartments was determined using an excitation and emission wavelengths of 485 and 535 nm, respectively. Values were expressed as a percentage of Lucifer yellow passage across the cell monolayer and permeability coefficient. Values lower than 5 % were considered acceptable.

Statistical analysis

Results are reported as mean ± standard deviation (SD) for the OAC, TPC and UHPLC-MS analysis, and ± standard error (SE) for the *in vitro* cell culture assays. Statistical analyses were carried out using SPSS Statistics 21.0 software (SPSS Inc., Chicago, IL, USA). ANOVA (1-way) followed by *Tukey-b*'s post hoc analyses were performed to evaluate the equality of variances and statistical differences between the treatments. The values <alpha>=0.05 or 0.01 (*, **) were indicated as statistically significant. Moreover, *Pearson correlation* analysis was performed to measure the relationship between TPC and OAC tests.

Results and Discussion

Overall antioxidant capacity (OAC) and total phenolic content (TPC) of the *in vitro* digested breads

The OAC and TPC of samples was determined in both soluble fractions of non-digested and digested samples; results are reported in **Table 20**. In this study four rapid methods based on different mechanisms of action for the evaluation of the OAC were used, as largely recommended [34]. Evidently, the replacement of refined flour for wheat bran led to a significantly (<alpha>=0.05) increased OAC and TPC content, as reported by other studies [35,36]. The addition in bread formulation of wheat bran significantly increased both OAC and TPC, resulting in 1.5-fold increment (as average) confirming that this cereal fraction has a high amount of bioactive compounds compared to the endosperm flour. These results were already reported in literature [37]. Interestingly, the digested bread containing fermented wheat bran presented a significantly higher OAC and TPC in respect to other breads, emphasizing the effect of lactic acid bacteria metabolism. During digestion several changes could occur, mostly regarding the breakdown of macro-nutrients such as starch and proteins. However, other compounds may be susceptible to the quick pH changes and enzymes activity. In fact, phenolics have been showed a mid-high sensitiveness to low pH and enzymes [37]. Besides, thanks to these reactions, the releasing of phenolic acids presents in wheat bran as conjugated to vegetable cell insoluble components might occur [38], leading to a higher concentration in soluble form.

Generally, a good correlation between OAC and TPC can be found, meaning a significant contribution of phenolics, but not total, to the OAC [39]. However, since the Folin-Ciocalteus's assay is not phenolic-specific, also other compounds like peptides could interfere during the analysis. Therefore, it is possible that a portion of this bioactivity could be generated by the latter compounds [40].

Moreover, the soluble portions of digested bread are complex mixtures which contain different classes of molecules interacting between each other, meaning a complicate prediction of OAC [41]. In this study an acceptable positive correlation between the OAC and TPC tests (**Table 20**).

Among the ingredient used for the breads formulation, the olive oil can be a source of phenolics and more in general bioactive compounds. Nevertheless, all the breads contained the same amount of ingredient, hence they would be affected by an equal amount of these substances.

Table 20. Overall antioxidant capacity (OAC) and total phenolic content (TPC) of the soluble fraction of *in vitro* digested wheat bread (BB), bread enriched with raw wheat bran (BWB) and bread enriched with pre-fermented wheat bran (BFB) before and after *in vitro* gastrointestinal digestion.

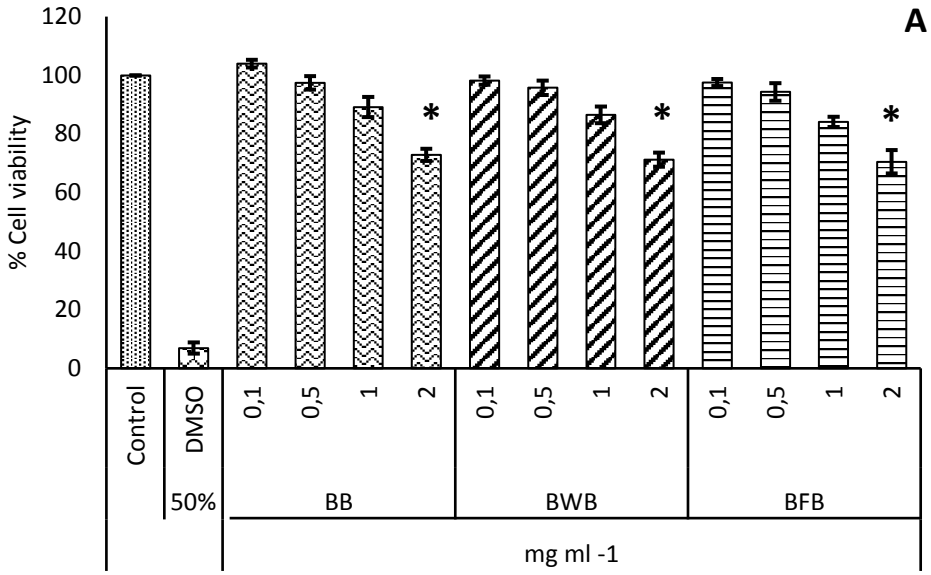
Samples		FRAP	DPPH	ABTS	ORAC-FL	TPC
		μM TEAC g^{-1} d.w.				mg GAE g^{-1}
BB	Non-digested*	23.5±1.0 ^{a}	5.3±0.2 ^{a}	71.7±2.8 ^{a}	1.7±0.2 ^{a}	0.72±0.06 ^{a}
	Digested	31.2±3.3 ^{b}	6.9±0.1 ^{b}	80.7±0.3 ^{ab}	2.3±0.2 ^{b}	0.96±0.05 ^{b}
BWB	Non-digested	57.9±2.7 ^{c}	7.2±0.4 ^{b}	81.3±1.3 ^{ab}	2.3±0.1 ^{b}	0.96±0.08 ^{b}
	Digested	70.6±1.1 ^{d}	8.2±0.7 ^{c}	87.4±4.3 ^{bc}	2.6±0.1 ^{bc}	1.07±0.02 ^{c}
BFB	Non-digested	70.3±2.5 ^{d}	8.4±0.1 ^{cd}	95.6±1.4 ^{cd}	2.5±0.1 ^{bc}	1.19±0.02 ^{d}
	Digested	81.1±6.4 ^{e}	9.3±1.2 ^{d}	104.1±3.3 ^{d}	2.7±0.2 ^{c}	1.25±0.02 ^{d}
r^{2} **	Non-digested	0.87	0.88	0.84	0.73	
	Digested	0.79	0.77	0.72	0.41	

Results were reported as mean ± standard deviation (*n=6*). Different superscript letters $^{a-e}$ indicate a significant difference (<alpha>=0.05). TEAC, Trolox equivalent antioxidant capacity; GAE, Gallic acid equivalent. *For non-digested samples the enzymatic treatments were excluded in order to maintain the same extractive conditions (i.e. volumes, temperature and time). ** *Pearson*'s correlation coefficient between OAC and TPC tests.

A targeted UHPLC-MS/MS method was used to quantify the most abundant phenolic acids present in samples (see **Table A1** and **Figures A2** in supplementary material for additional information). In digested samples the phenolic acids were not identifiable. This could be mainly explained by the likely modifications in terms of chemical structure as results of degradation processes, such as fermentation and baking. These results were also reported by Antognoni *et al.* [42]. In non-digested sample hydroxybenzoic (2-HB), p-coumaric, caffeic, ferulic (t-Fer) and sinapic acids were found only in BWB and BFB, probably for the addition of wheat bran in recipe. 2-HB and t-Fer were the most abundant, however no information regarding their potential GLU transporters inhibitory activity was found in literature.

Cell viability of digested bread soluble fractions supplementation

The cell viability after treatments with digested bread soluble fractions was monitored using the MTT assay. This preliminary screening should be mandatory before every *in vitro* study carried out using cell culture models [44]. The BB, BWB and BFB soluble fractions generated after digestion at concentration of 0.1 and 0.5 mg mL^{-1} had a similar behaviour as the negative control (cell supplemented with DMEM only). Among the concentration range studied (0.1-2 mg mL^{-1}), the highest amount supplemented triggered a cell damage mechanism in all cell cultures since their viability value was lower than 80 %, as reported in literature [45] (**Figure 21 A, B, C, D)**. The MTT test measures the mitochondrial respiratory activity in living cells [46], considered as an indirect result of cell vitality, thus no information regarding the cell number was obtainable. Slight differences were found between cell type.


A
% Cell viability
120
100
80
60
40
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Control
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50%
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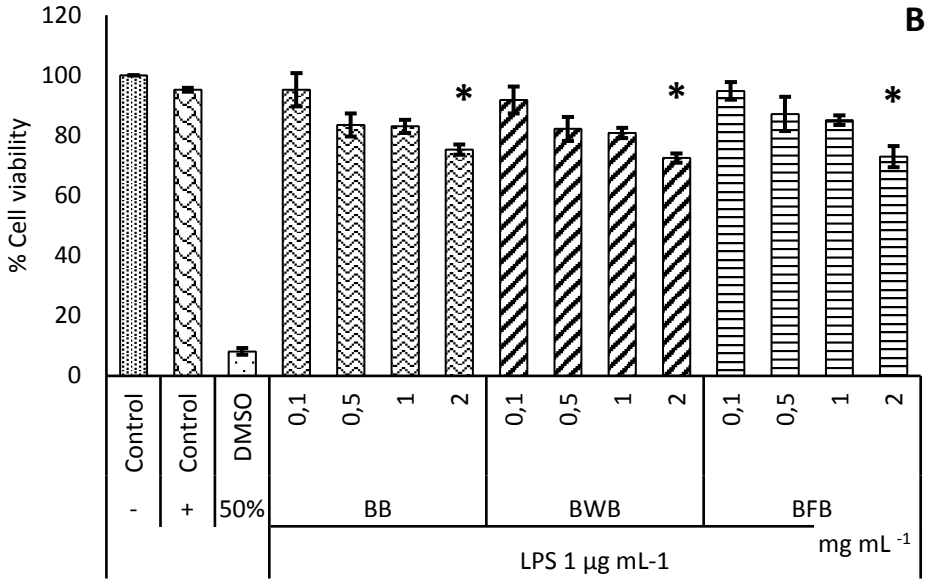
0,5
1
2
BB
BWB
BFB
mg ml -1


B
% Cell viability
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80
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- Control
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DMSO
50%
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LPS 1 µg mL-1
mg mL -1

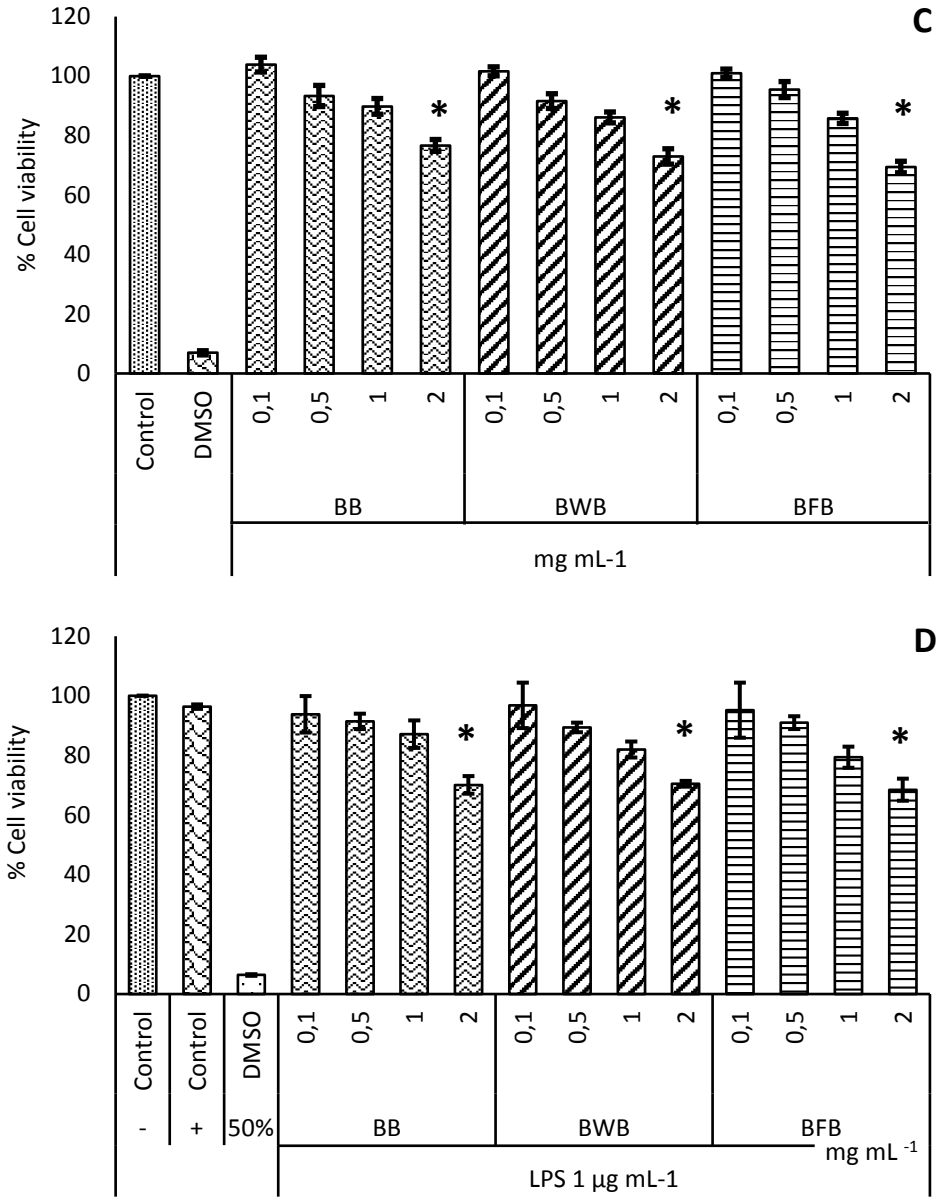


Figure 21. Cell viability of IEC-6 (A), CaCo-2 (C) in basal and inflammation induced conditions (B and D, respectively) supplemented with 0.1, 0.5, 1 and 2 mg mL^{-1} of digested bread soluble fractions. Results are reported as mean ± standard error of triplicates, each repeated three times (*n=9*) and are expressed as percentage (%) value in respect to the control cells ("- control", medium-only treated, 100%). 50% DMSO solution in DMEM. "+ control", cells treated with LPS 1 µg mL^{-1} in DMEM. BB, white bread, BFB, bread with pre-fermented wheat bran, BWB, bread with raw wheat bran. CTRL, medium-only treated cells.

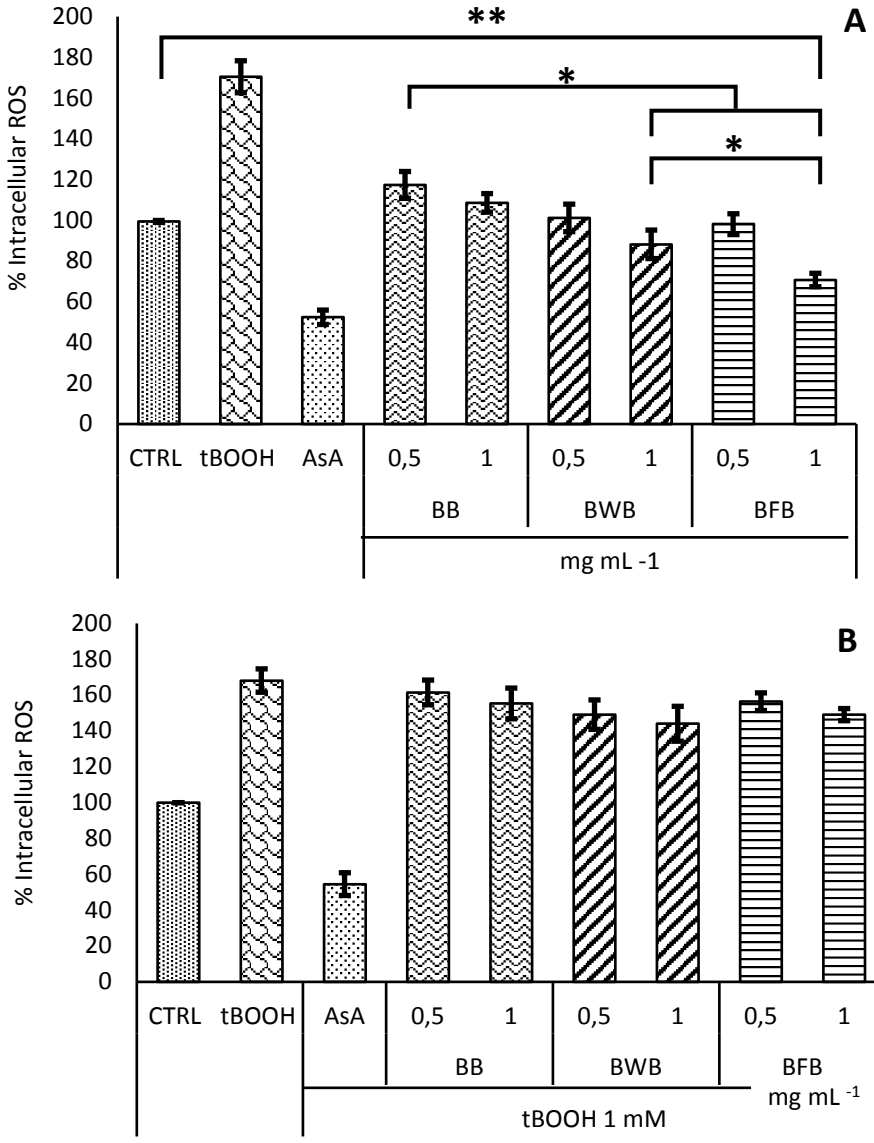
Besides, when cells were induced to an inflammation status by the addition of LPS, the toxicity mechanism was enhanced at the highest concentration of treatments used and in both cell culture models (**Figure 21 B** and **D**). Similar results were also obtained for the RAW 264.7 macrophages (see supplementary material, **Figure A1).**

Based on these findings, for all the subsequent analyses the cells were supplemented with the highest amounts of digested bread (0.5 or 1 mg mL $^{-1}$). In addition, any injurious effects can be ascribed to the lack of nutrients, since the digested bread suspension were suspended in medium.

Intracellular reactive oxygen species (ROS) generation

ROS generation is a mechanism which influences the cell redox homeostasis implicated in apoptosis and signalling processes [47] which finally might lead to the induction and progression of disease status [48]. The mere chemical analysis of food products should not be considered a direct measure of the corresponding nutritional value, since the physiological effects must be assessed.

The cells supplemented with BWB and BFB decreased significantly the ROS production in basal condition and in both cell lines, as reported in (**Figure 22 A** and **C**), suggesting a protective effect of treatments against the oxidative damage. However, no differences were found among these two digested breads (in CaCo-2). Furthermore, when the cells were induced to an inflammation status, the supplementation with soluble digested bread did not counteract the ROS generation, possibly because of the high oxidative environment. However, no increasing ROS level was found (**Figure 22 B** and **D**). These results are in agreement with those reported by Valli *et al*., 2018 [49], where they evaluated the ROS generation counteraction of soluble fractions of digested bread formulated using ancient and modern wheat heritages.


A
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200
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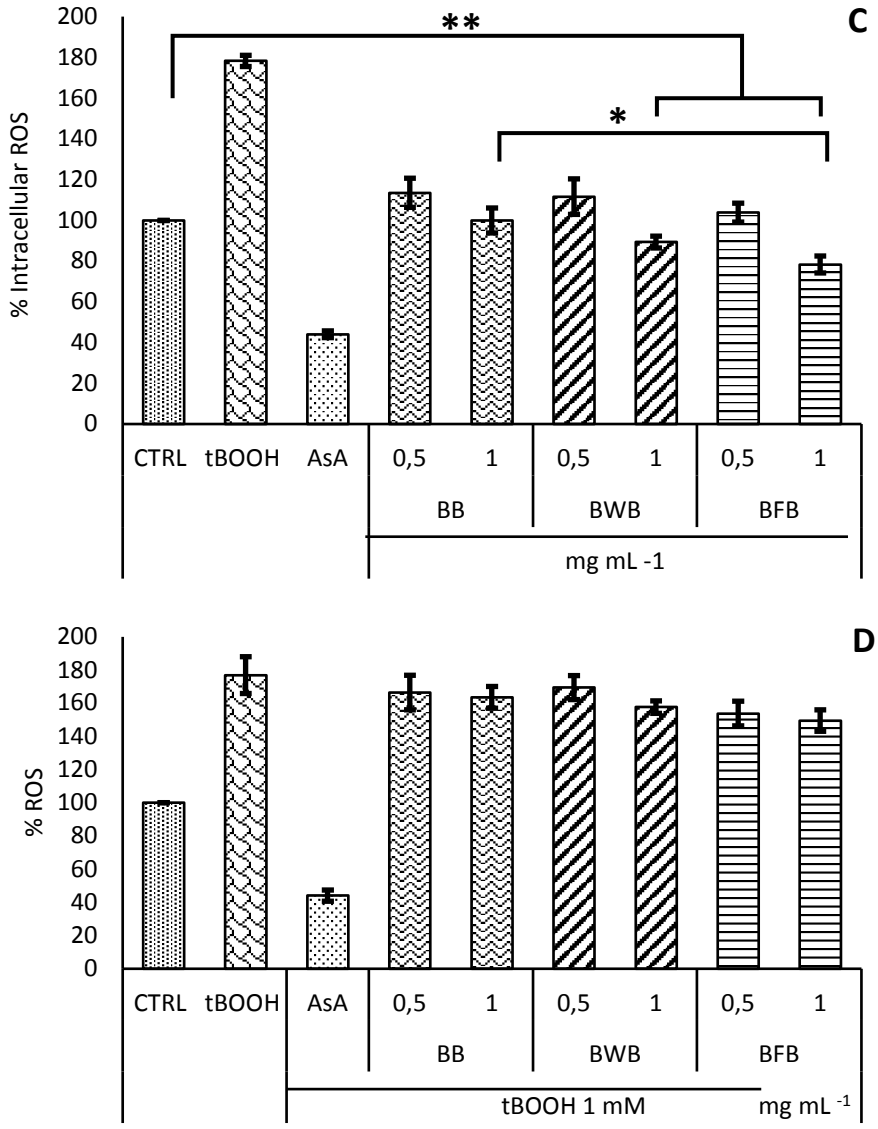

Figure 22. Intracellular ROS production IEC-6 (A) and in CaCo-2 (C) under basal and inflammation-induced conditions (B and D, respectively), supplemented with 0.5, and 1 mg mL^{-1} of digested bread soluble fractions. Results are reported as mean ± standard error of triplicates, each repeated three times (*n=9*) and are expressed as percent value in the control cells (medium-only treated, 100%), normalized for the MTT value. Differences were statistically significant at * <alpha>=0.05 and ** <alpha>=0.01, as results of the ANOVA followed by *Tukey's b* post-hoc test. AsA, ascorbic acid. BB, white bread, BFB, bread with pre-fermented wheat bran, BWB, bread with raw wheat bran. CTRL, medium-only treated cells, t-BOOH, tert butyl superoxide 1 mM in DMEM.

The latter protective effect could be ascribed to the higher soluble phenolic content and antioxidants present in BWB and BFB (**Table 20**). Besides, also the bread making process could produce compounds derived from the Maillard reaction which contribute to the antioxidant activity of the matrix, such as melanoidins, highly present in bread crust [50]. Related to this, the fiber and polysaccharides degradation activity of lactic acid bacteria could release an amount of simple reducing sugars capable to react with amino acids to form the above citated substances. In support to the oxidative response study, both oxidative (*t*-BOOH, 1mM in DMEM) and antioxidant (Ascorbic acid, AsA, 10 µg mL^{-1} DMEM) were tested concomitantly with samples. As reported in **Figure 22,** the AsA effectively contrasted the ROS generation in both cells and conditions, whereas the oxidative control induced a massive production of oxidant species.

Anti-inflammatory activity measured as nitric oxide (NO) production

NO cells secretion is considered as a signalling inflammation biomarker produced by cells in response to external stimulus, and it is generally formed by nitric oxide synthase (NOS) enzymes [51] In this study, the latter activity was stimulated by the LPS produced from *E. coli*, as reported in **Figure 23**.

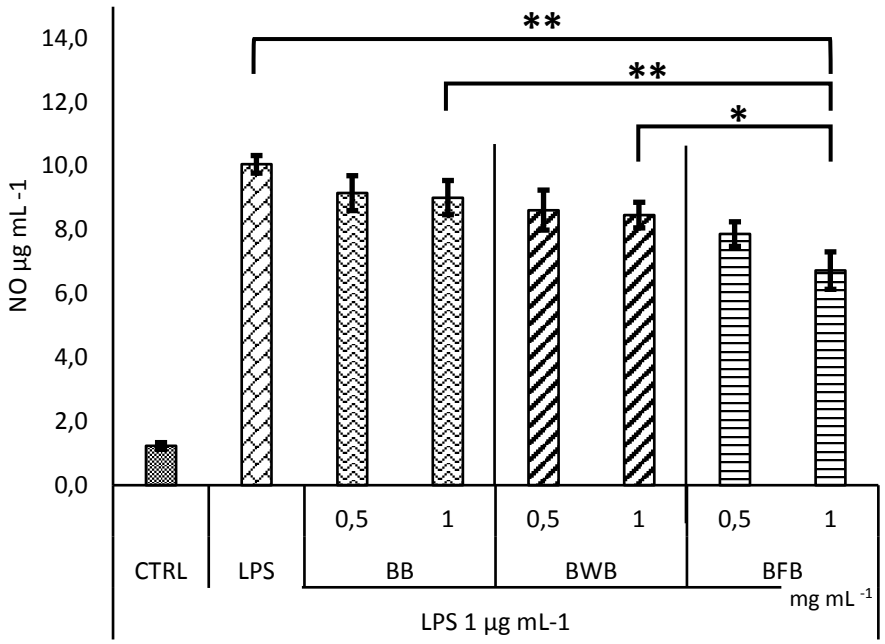

Figure 23. Nitric oxide secretion in RAW 264.7 cells supplemented with 0.5 and 1 mg mL^{-1} of digested bread soluble fractions. Results are reported as mean ± standard error of triplicates, each repeated three times (*n=9*) and are expressed as µg NO mL^{-1} medium, normalized for the MTT value. Differences were statistically significant at * <alpha>=0.05 and ** <alpha>=0.01 as results of the ANOVA followed by *Tukey's b* post-hoc test. BB, white bread, BFB, bread with pre-fermented wheat bran, BWB, bread with raw wheat bran. CTRL, medium-only treated cells, LPS, lipopolysaccharide 1 µg mL^{-1} in DMEM.

The concentration of extracellular NO was significantly lower in BFB treated macrophages at concentration of 1 mg ml^{-1}, in respect to the corresponding BB and BWB supplementations. The lowering inflammation molecule production provided by BFB could be possibly due to the higher content of soluble antioxidants and phenolics which can modulate the NOS expression [52].

Inhibition potential of intestinal glucose uptake

Simple carbohydrates uptake in small intestine is regulated by glucose transporters localized on the enterocyte cell membrane. These transporters are known as SGLT-1 (sodium-glucose transport protein) and GLUT-2 (glucose

transport). The first, is a sodium dependent channel expressed mainly on the apical part of intestine cells, while GLUT-2 participate in uptake and export, hence is found in basolateral compartment under specific conditions [53,54]. In blood, glucose concentration can quickly rise from 4 to 12 mM after meal [55]. However, in small intestine could reach even higher levels [56], hence 25 mM is considered a medium to high glucose concentration value. IEC-6 rat small intestine cells have been already used for the study of the blunting effect of beta-glucan on intestinal glucose transporter, confirming the suitability of this model for the latter analysis [30]. The mainly advantage is that, firstly these are normal cells (not cancerous), and secondly, they are easily cultivable than the CaCo-2. However, it has been showed that at lower glucose concentration these cells could not express the apical GLUT-2 function, leading to potential incorrect results [57], although these topic is highly controversial [30,58]. Moreover, the glucose transport system appear to saturate more rapidly than other *in vitro* models [57].

The glucose concentration in samples after digestion process did not show a significant difference (see supplementary material, **Table A1**), taking in consideration the presence of sucrose in recipe, such amount was not a source of variation. In fact, sucrose is mainly added as nutrient supplementation for baker's yeast growth. Moreover, breads herein produced included also NaCl, likewise in PBS, hence it could be a favourable factor since SGLT-1 is a sodium dependent transporter.

The cumulative % of glucose uptake over time (10-240 minutes) was calculated in IEC-6 and CaCo-2 using cell supplemented with GLU 25 mM as control reference. In both cell models, BWB and BFB slightly, but significantly, inhibited the absorption of GLU at 10 minutes, in respect to BB (data not shown). However, only in IEC-6 differed significantly. Moreover, the cumulative % of GLU uptake started an asymptotic trend at 60 minutes, meaning the effectiveness of transepithelial hexose transport. Therefore, the inhibition of glucose uptake at the latter time is depicted in **Figure 24** for IEC-6 (**A**) and CaCo-2 (**B**) cells.

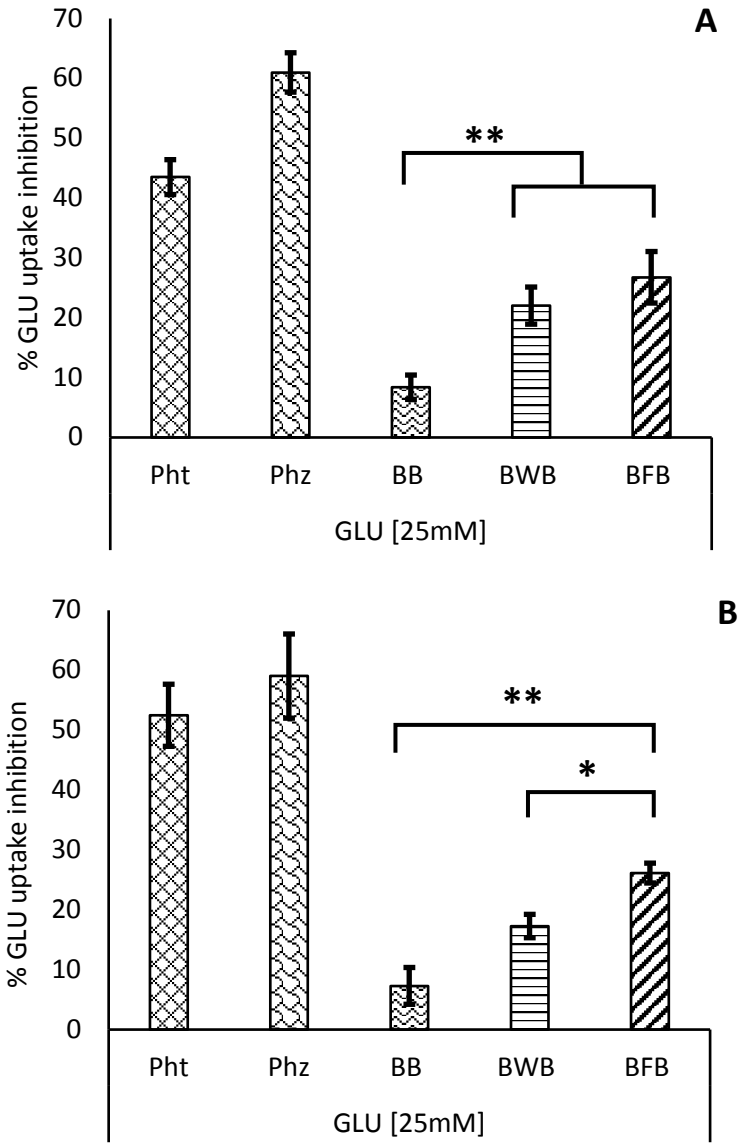

Figure 24. The IEC-6 (A) and CaCo-2 (B) cells were treated for 240 minutes with 25 mM glucose in presence of the digested bread at concentration of 1 mg mL^{-1}. Histograms represent the % of GLU uptake inhibition at the stage of 60 min. Values are reported as mean ± standard error of triplicates, each repeated two times (*n=6*). Results are expressed as % of GLU uptake inhibition calculated in respect to the cumulative uptake of only GLU 25 mM treated cells. Differences were statistically significant at * <alpha>=0.05 and ** <alpha>=0.01 as results of the ANOVA followed by *Tukey's b* post-hoc test. Pht, phloretin, Phz, phlorizin, BB, white bread, BFB, bread with pre-fermented wheat bran, BWB, bread with raw wheat bran.

The GLU uptake inhibition in CaCo-2 model was different among cells supplemented with the bread digestas, and BFB totalised the higher uptake inhibition rate. This difference was no longer significant in IEC-6 model, although the uptake inhibition of BWB and BFB treated cells significantly differed from the BB treated cells.

A possible explanation of the observed blunting effect of BFB and BWB could be ascribed to the higher presence of soluble dietary fibers in respect to the with bread, leading to an increased viscosity of the medium [30]. Furthermore also phenolic derived compounds found in cereals, such as feruloylated arabinoxylans, have been assigned with this inhibitory function [13,14]. In addition, the whole food matrix plays an important role in the functionality of single compound, of which depend their releasing degree. The glucose concentration was also determined in the apical chamber at the final stage of the assay to calculate the total GLU concentration, avoiding errors due to the portions of glucose which could be possibly metabolised or internalised by cells, which is a potential limitation of this study.

Additionally, the compounds phloretin (Pht) and phlorizin (Phz) have been recognised as effective intestinal glucose transporter inhibitor, since they interact with GLUT-2 and SGLT-1, respectively, compromising the uptake enzymes activity [59]. In this study, these substances were also tested for their ability inhibit the transporters. In fact, when cells were supplemented with both glucose and Pht or Phz, the glucose uptake was drastically inhibited (**Figure 24 A** and **B**) achieving an almost 70 % of GLU absorption inhibition.

Intestinal uptake of betaine and choline

Free betaine and choline concentration in non-digested and digested samples were significantly different. The addition of wheat bran significantly increased the content of both methyl donor compounds (**Figure 25**), since these cereal by-products are particularly rich in these substances [60]. However, in BFB the betaine and choline content were lower than BWB.

This could be explained by the fact that, betaine, at least, is used by microorganisms (baker's yeast and lactic acid bacteria) as source of nitrogen [60]. Moreover, there is no information in literature that betaine can form crosslinks to matrix components. Also in the study of Koistinen *et al*., [61] the authors found a decreased content of glycine betaine, nevertheless some betainised metabolites were found in bread produced using rye a raw material (i.e. amino acid derived betaines), mainly related to the lactic acid bacteria used for the sourdough formation. Furthermore, betaine was present in higher concentration in respect to choline, as mentioned in literature [62].

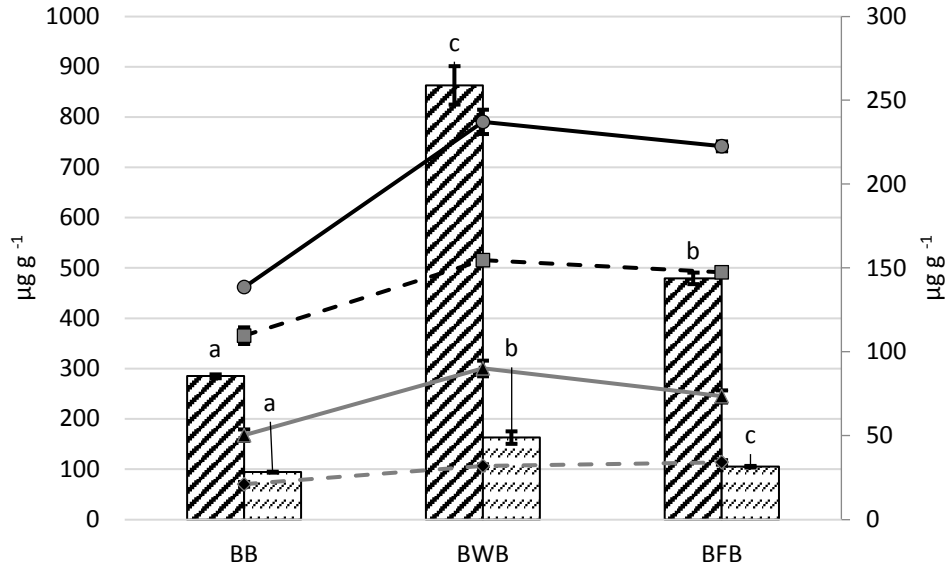

Figure 25. Betaine (▨) and choline (▨) content in digested samples, left y axis. The IEC-6 (grey lines) and CaCo-2 (black lines), right y axis, cells were treated with digested bread at concentration of 1 mg mL^{-1}, the uptake is reported at 10 minutes after supplementation. Values are reported as mean ± standard error of triplicates, each repeated two times (*n=6*). Results are expressed as µg g^{-1} of digested bread. Different letters (a-c) indicate statistical difference at <alpha>=0.05, as results of the ANOVA followed by *Tukey's b* post-hoc test.

Cells were treated with soluble fractions derived from the bread digestion, and the methyl donor compounds uptake was measured in basal and apical compartments using a UHPLC-MS/MS method. In both models, the absorption rate was very rapid (10 minutes), as suggested by other researchers [63,64] (**Figure 25 A**). The uptake in epithelial cells showed no differences in absorption behaviour of cells supplemented with BWB and BFB, indicating that the fermentation process only influenced the final amount of these substances. This information suggest that the methyl donors compounds absorption might only be directly related to their initial quantity in food products. Nevertheless, whole grain and more in general bran enriched baked products persist as a good sources of methyl donor compounds, since the uptake at intestinal level was 1.7, 4.8-fold (as average of bread containing bran) for betaine and choline respectively, in respect to the BB supplemented cells.

Betaine uptake is ascribed to sodium or chloride active transport or else to passive independent systems [65]. It is also worth to mention that betaine do not interfere in cellular metabolism nor enzyme functionality [16]. Moreover, human trial studies have shown a quick absorption and redistribution in organism [63,66,67]. Since choline is similar to betaine, from the chemical structure point of view, a comparable and parallel absorption behaviour can be supposed. Additionally, choline can be synthesized from betaine and other precursors in human organism. Betaine and choline can occur in different forms in foods and cell metabolism can lead to the production of multiple metabolites. Since the quantification method used in this study is specific for free betaine and choline only, this could be considered as a limitation. For this reason, a non-targeted method for the identification of important metabolites would help to fill this gap.

Conclusion

In conclusion, the inflammation, the oxidative protection and nutrient absorption properties of digested bread enriched with wheat bran, and supplemented in intestinal cell culture models, were highlighted in this study. Moreover, since the preliminary fermentation step applied to this cereal by-product caused deep changes in metabolites profile, inducing a significantly improved bioactivity, this procedure may be confirmed as valuable tool for wheat bran whole valorisation through a higher nutritional value.

To the best of our knowledge, this is the first research study analysing the glucose intestinal uptake inhibition potential and methyl donor compounds absorption of *in vitro* digested bread enriched with pre-fermented wheat bran using intestinal cell culture models.

Although no specific functionally active compounds were studied in this work, a broader framework which considers the food matrix – bioactivity relationship under physiological conditions was deemed. Therefore, further *in vitro* and *in vivo* studies might be focussed to deepen the present topic, showing the interactions between highly consumed foods and effects on human health.

Author contributions

MS and MDdC: designed the study. MS: performed the analysis, interpreted the results and wrote the manuscript. MS, GG, CD and MDdC: interpreted the results and edited the manuscript. All authors contributed to the critical review of the manuscript.

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Supplementary material

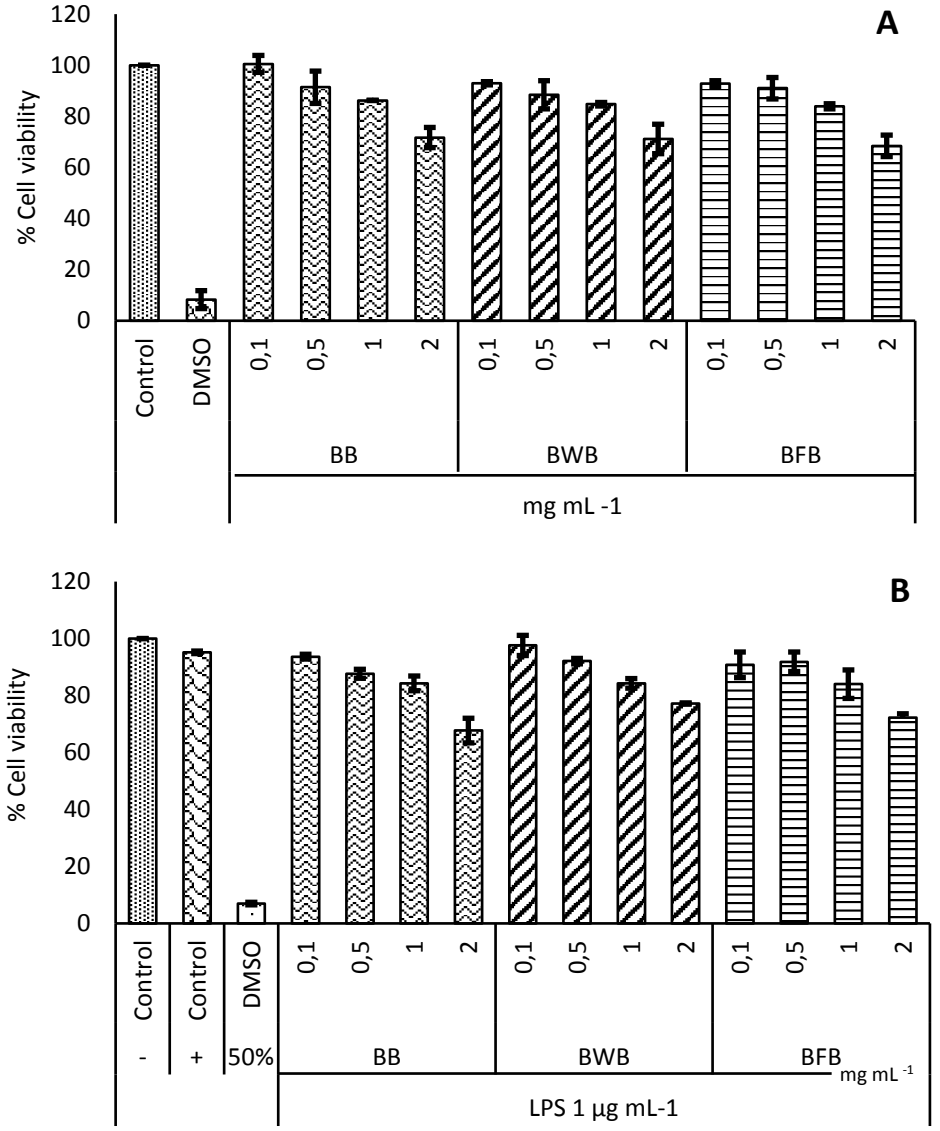

Figure A1. Cell viability of RAW 264.7 macrophages in basal and inflammation induced conditions (A and B, respectively) supplemented with 0.1, 0.5, 1 and 2 mg mL^{-1} of digested bread soluble fractions. Results are reported as mean ± standard error of triplicates, each repeated three times (*n=9*) and are expressed as percentage (%) value in respect to the control cells ("- control", medium-only treated, 100%). 50% DMSO solution in DMEM. "+ control", cells treated with LPS 1 µg mL^{-1} in DMEM. BB, white bread, BFB, bread with pre-fermented wheat bran, BWB, bread with raw wheat bran. CTRL, medium-only treated cells.

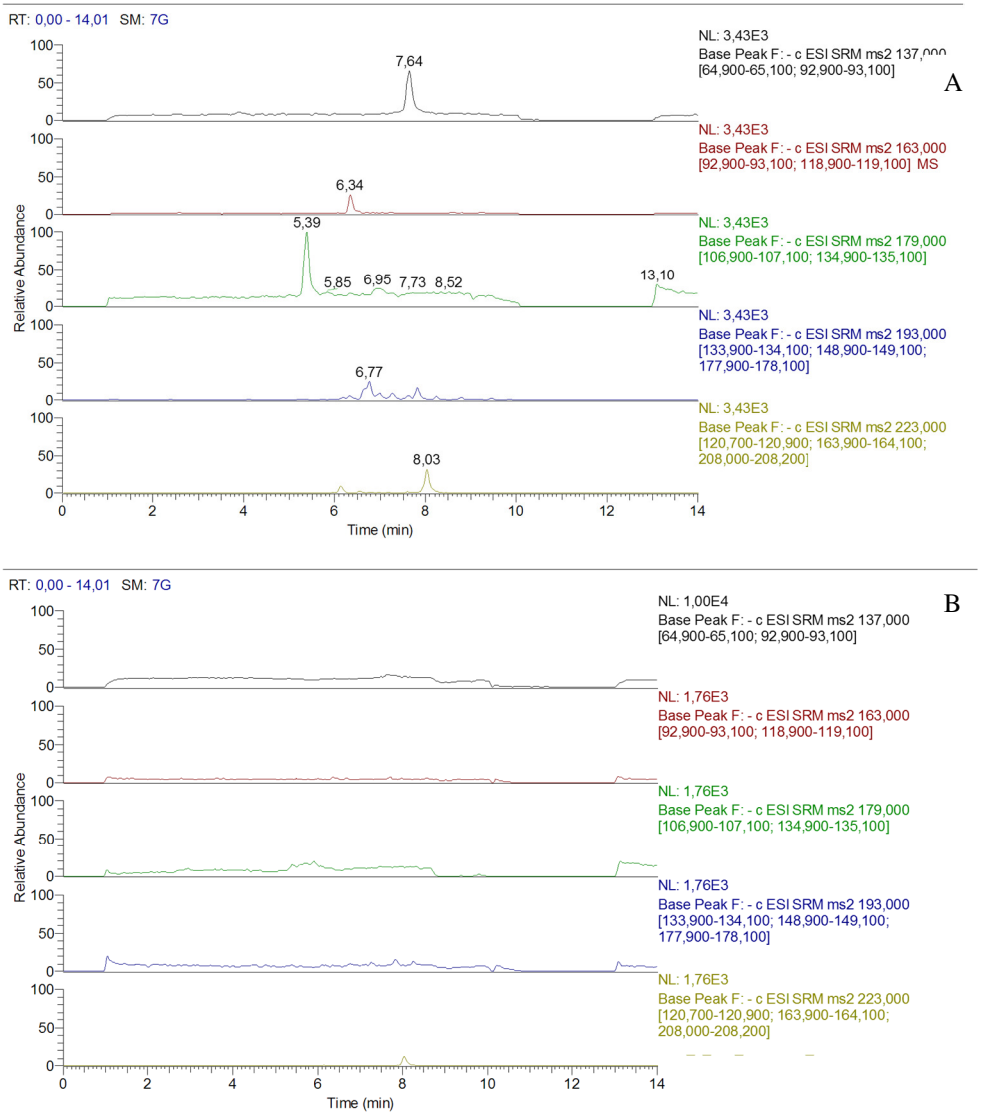

Figure A2. Extracted Ion Chromatograms (EIC) of the phenolic acids found in non-digested (A) and digested (B) BWB. Signals were normalized for the highest peak.

Table A1. Soluble phenolic acid content in non-digested samples and glucose concentration in digested breads.

Sample	*2-HB* **	*p-C*	*Caff*	*t-Fer*	*Sin*	*GLU* *
			µg g^{-1} d.w.			mM
BB	<LOQ [a]	<LOQ [a]	<LOQ [a]	<LOQ [a]	<LOQ [a]	3.63±0.23 [a]
BWB	5.01±0.39 [b]	0.36±0.04 [b]	<LOQ [a]	2.14±0.13 [c]	0.74±0.1 [c]	2.91±0.25 [a]
BFB	4.98±0.01 [b]	0.44±0.12 [b]	0.21±0.01 [b]	1.58±0.09 [b]	0.37±0.06 [b]	2.69±0.38 [a]

Results are expressed as mean ± standard deviation (*n=6*) Different letters [a-c] in the same column mean a significant difference (<alpha>=0.05) between samples, following the *Tukey* b's post-hoc test. <LOQ 0.05 µg g^{-1}. BB, white bread. BWB, bread enriched with raw bran. BFB, bread enriched with pre-fermented wheat bran. 2-HB, 2-hydroxybenzoic acid. p-C, para-Coumaric. Caff, caffeic acid. t-Fer, trans-Ferulic acid. * measured in soluble fraction of digested bread samples and expressed as means ± standard deviation (*n=6*). ** the 4-HB isomer was not identified.

References

(1) McKevith, B. Nutritional Aspects of Cereals. *Nutr. Bull.* **2004**, *29* (2), 111–142. https://doi.org/10.1108/nfs.2004.01734fab.017.

(2) Prückler, M.; Siebenhandl-Ehn, S.; Apprich, S.; Höltinger, S.; Haas, C.; Schmid, E.; Kneifel, W. Wheat Bran-Based Biorefinery 1: Composition of Wheat Bran and Strategies of Functionalization. *LWT - Food Sci. Technol.* **2014**, *56* (2), 211–221. https://doi.org/10.1016/j.lwt.2013.12.004.

(3) Béné, C.; Oosterveer, P.; Lamotte, L.; Brouwer, I. D.; de Haan, S.; Prager, S. D.; Talsma, E. F.; Khoury, C. K. When Food Systems Meet Sustainability – Current Narratives and Implications for Actions. *World Dev.* **2019**, *113*, 116–130. https://doi.org/10.1016/j.worlddev.2018.08.011.

(4) Heiniö, R. L.; Noort, M. W. J.; Katina, K.; Alam, S. A.; Sozer, N.; de Kock, H. L.; Hersleth, M.; Poutanen, K. Sensory Characteristics of Wholegrain and Bran-Rich Cereal Foods - A Review. *Trends Food Sci. Technol.* **2016**, *47*, 25–38. https://doi.org/10.1016/j.tifs.2015.11.002.

(5) Filannino, P.; Di Cagno, R.; Gobbetti, M. Metabolic and Functional Paths of Lactic Acid Bacteria in Plant Foods: Get out of the Labyrinth. *Curr. Opin. Biotechnol.* **2018**, *49*, 64–72. https://doi.org/10.1016/j.copbio.2017.07.016.

(6) Arte, E.; Rizzello, C. G.; Verni, M.; Nordlund, E.; Katina, K.; Coda, R. Impact of Enzymatic and Microbial Bioprocessing on Protein Modification and Nutritional Properties of Wheat Bran. *J. Agric. Food Chem.* **2015**, *63* (39), 8685–8693. https://doi.org/10.1021/acs.jafc.5b03495.

(7) Messia, M. C.; Reale, A.; Maiuro, L.; Candigliota, T.; Sorrentino, E.; Marconi, E. Effects of Pre-Fermented Wheat Bran on Dough and Bread Characteristics. *J. Cereal Sci.* **2016**, *69*, 138–144. https://doi.org/10.1016/j.jcs.2016.03.004.

(8) Zhao, H. M.; Guo, X. N.; Zhu, K. X. Impact of Solid State Fermentation on Nutritional, Physical and Flavor Properties of Wheat Bran. *Food Chem.* **2017**, *217*, 28–36. https://doi.org/10.1016/j.foodchem.2016.08.062.

(9) Filannino, P.; Bai, Y.; Di Cagno, R.; Gobbetti, M.; Gänzle, M. G. Metabolism of Phenolic Compounds by Lactobacillus Spp. during Fermentation of Cherry Juice and Broccoli Puree. *Food Microbiol.* **2015**, *46*, 272–279. https://doi.org/10.1016/j.fm.2014.08.018.

(10) Katina, K.; Juvonen, R.; Laitila, A.; Flander, L.; Nordlund, E.; Kariluoto, S.; Piironen, V.; Poutanen, K. Fermented Wheat Bran as a Functional Ingredient in Baking. *Cereal Chem.* **2012**, *89* (2), 126–134. https://doi.org/10.1094/CCHEM-08-11-0106.

(11) Chatterjee, S.; Khunti, K.; Davies, M. J. Type 2 Diabetes. *Lancet* **2017**, *389* (10085), 2239–2251. https://doi.org/10.1016/S0140-6736(17)30058-2.

(12) Shamloo, M.; Jones, P. J. H.; Eck, P. K. Inhibition of Intestinal Cellular Glucose Uptake by Phenolics Extracted from Whole Wheat Grown at Different Locations. *J. Nutr. Metab.* **2018**, *2018*, 1–10. https://doi.org/10.1155/2018/5421714.

(13) Malunga, L. N.; Eck, P.; Beta, T. Inhibition of Intestinal α -Glucosidase and Glucose Absorption by Feruloylated Arabinoxylan Mono- and Oligosaccharides from Corn Bran and Wheat Aleurone. *J. Nutr. Metab.* **2016**, *2016*, 1–9. https://doi.org/10.1155/2016/1932532.

(14) Malunga, L. N.; Izydorczyk, M.; Beta, T. Antiglycemic Effect of Water Extractable Arabinoxylan from Wheat Aleurone and Bran. *J. Nutr. Metab.* **2017**, *2017*, 1–6. https://doi.org/10.1155/2017/5784759.

(15) Kojić, J.; Krulj, J.; Ilić, N.; Lončar, E.; Pezo, L.; Mandić, A.; Bodroža Solarov, M. Analysis of Betaine Levels in Cereals, Pseudocereals and Their Products. *J. Funct. Foods* **2017**, *37*, 157–163. https://doi.org/10.1016/j.jff.2017.07.052.

(16) Craig, S. A. S. Betaine in Human Nutrition. *American Journal of Clinical Nutrition*. 2004, pp 539–549. https://doi.org/10.1093/ajcn/80.3.539.

(17) Wang, H.; Li, S.; Fang, S.; Yang, X.; Feng, J. Betaine Improves Intestinal Functions by Enhancing Digestive Enzymes, Ameliorating Intestinal Morphology, and Enriching Intestinal Microbiota in High-Salt Stressed Rats. *Nutrients* **2018**, *10* (7), 907. https://doi.org/10.3390/nu10070907.

(18) Slavin, J. L.; Jacobs, D.; Marquart, L. Grain Processing and Nutrition. *Crit. Rev. Biotechnol.* **2001**, *21* (1), 49–66. https://doi.org/10.1080/20013891081683.

(19) Van Breemen, R. B.; Li, Y. Caco-2 Cell Permeability Assays to Measure Drug Absorption. *Expert Opinion on Drug Metabolism and Toxicology*. 2005, pp 175–185. https://doi.org/10.1517/17425255.1.2.175.

(20) Wang, J.; Rosell, C. M.; Benedito de Barber, C. Effect of the Addition of Different Fibres on Wheat Dough Performance and Bread Quality. *Food Chem.* **2002**, *79* (2), 221–226. https://doi.org/10.1016/S0308-8146(02)00135-8.

(21) Hollebeeck, S.; Borlon, F.; Schneider, Y. J.; Larondelle, Y.; Rogez, H. Development of a Standardised Human in Vitro Digestion Protocol Based on Macronutrient Digestion Using Response Surface Methodology. *Food Chem.* **2013**, *138* (2–3), 1936–1944. https://doi.org/10.1016/j.foodchem.2012.11.041.

(22) Edwards, A. D.; Slater, N. K. H. Protection of Live Bacteria from Bile Acid Toxicity Using Bile Acid Adsorbing Resins. *Vaccine* **2009**, *27* (29), 3897–3903. https://doi.org/10.1016/j.vaccine.2009.04.006.

(23) Singleton, V. L.; Orthofer, R.; Lamuela-Raventós, R. M. Analysis of Total Phenols and Other Oxidation Substrates and Antioxidants by Means of Folin-Ciocalteu Reagent. In *Methods in Enzymology*; 1999; Vol. 299, pp 152–178. https://doi.org/10.1016/S0076-6879(99)99017-1.

(24) Brand-Williams, W.; Cuvelier, M. E.; Berset, C. Use of a Free Radical Method to Evaluate Antioxidant Activity. *LWT - Food Sci. Technol.* **1995**, *28* (1), 25–30. https://doi.org/10.1016/S0023-6438(95)80008-5.

(25) Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant Activity Applying an Improved ABTS Radical Cation Decolorization Assay. *Free Radic. Biol. Med.* **1999**, *26* (9–10), 1231–1237. https://doi.org/10.1016/S0891-5849(98)00315-3.

(26) Pulido, R.; Bravo, L.; Saura-Calixto, F. Antioxidant Activity of Dietary Polyphenols as Determined by a Modified Ferric Reducing/Antioxidant Power Assay. *J. Agric. Food Chem.* **2000**, *48* (8), 3396–3402. https://doi.org/10.1021/jf9913458.

(27) Ou, B.; Hampsch-Woodill, M.; Prior, R. L. Development and Validation of an Improved Oxygen Radical Absorbance Capacity Assay Using Fluorescein as the Fluorescent Probe. *J. Agric. Food Chem.* **2001**, *49* (10), 4619–4626. https://doi.org/10.1021/jf010586o.

(28) Benayad, Z.; Martinez-Villaluenga, C.; Frias, J.; Gomez-Cordoves, C.; Es-Safi, N. E. Phenolic Composition, Antioxidant and Anti-Inflammatory Activities of Extracts from Moroccan Opuntia Ficus-Indica Flowers Obtained by Different Extraction Methods. *Ind. Crops Prod.* **2014**, *62*, 412–420. https://doi.org/10.1016/j.indcrop.2014.08.046.

(29) Hubatsch, I.; Ragnarsson, E. G. E.; Artursson, P. Determination of Drug Permeability and Prediction of Drug Absorption in Caco-2 Monolayers. *Nat. Protoc.* **2007**, *2* (9), 2111–2119. https://doi.org/10.1038/nprot.2007.303.

(30) Abbasi, N. N.; Purslow, P. P.; Tosh, S. M.; Bakovic, M. Oat β-Glucan Depresses SGLT1- and GLUT2-Mediated Glucose Transport in Intestinal Epithelial Cells (IEC-6). *Nutr. Res.* **2016**, *36* (6), 541–552. https://doi.org/10.1016/j.nutres.2016.02.004.

(31) Srinivasan, B.; Kolli, A. R.; Esch, M. B.; Abaci, H. E.; Shuler, M. L.; Hickman, J. J. TEER Measurement Techniques for In Vitro Barrier Model Systems. *Journal of Laboratory Automation*. 2015, pp 107–126. https://doi.org/10.1177/2211068214561025.

(32) Behrens, I.; Kissel, T. Do Cell Culture Conditions Influence the Carrier-Mediated Transport of Peptides in Caco-2 Cell Monolayers? *Eur. J. Pharm. Sci.* **2003**, *19* (5), 433–442. https://doi.org/10.1016/S0928-0987(03)00146-5.

(33) Puthia, M. K.; Sio, S. W. S.; Lu, J.; Tan, K. S. W. Blastocystis Ratti Induces Contact-Independent Apoptosis, F-Actin Rearrangement, and Barrier Function Disruption in IEC-6 Cells. *Infect. Immun.* **2006**, *74* (7), 4114–4123. https://doi.org/10.1128/IAI.00328-06.

(34) Abramovič, H.; Grobin, B.; Poklar Ulrih, N.; Cigić, B. Relevance and Standardization of In Vitro Antioxidant Assays: ABTS, DPPH, and Folin–Ciocalteu. *J. Chem.* **2018**, *2018*, 1–9. https://doi.org/10.1155/2018/4608405.

(35) Jensen, S.; Ostdal, H.; Skibsted, L. H.; Thybo, A. K. Antioxidants and Shelf Life of Whole Wheat Bread. *J. Cereal Sci.* **2011**, *53* (3), 291–297. https://doi.org/10.1016/j.jcs.2011.01.010.

(36) Dziki, D.; Rózyło, R.; Gawlik-Dziki, U.; Świeca, M. Current Trends in the Enhancement of Antioxidant Activity of Wheat Bread by the Addition of Plant Materials Rich in Phenolic Compounds. *Trends in Food Science and Technology*. 2014, pp 48–61. https://doi.org/10.1016/j.tifs.2014.07.010.

(37) Laddomada, B.; Caretto, S.; Mita, G. Wheat Bran Phenolic Acids: Bioavailability and Stability in Whole Wheat-Based Foods. *Molecules*. 2015, pp 15666–15685. https://doi.org/10.3390/molecules200915666.

(38) Szawara-Nowak, D.; Bączek, N.; Zieliński, H. Antioxidant Capacity and Bioaccessibility of Buckwheat-Enhanced Wheat Bread Phenolics. *J. Food Sci. Technol.* **2016**, *53* (1), 621–630. https://doi.org/10.1007/s13197-015-2074-y.

(39) Verardo, V.; Glicerina, V.; Cocci, E.; Frenich, A. G.; Romani, S.; Caboni, M. F. Determination of Free and Bound Phenolic Compounds and Their Antioxidant Activity in Buckwheat Bread Loaf, Crust and Crumb. *LWT - Food Sci. Technol.* **2018**, *87*, 217–224. https://doi.org/10.1016/j.lwt.2017.08.063.

(40) Žilić, S.; Janković, M.; Barać, M.; Pešić, M.; Konić-Ristić, A.; Hadži-Tašković Šukalović, V. Effects of Enzyme Activities during Steeping and Sprouting on the Solubility and Composition of Proteins, Their Bioactivity and Relationship with the Bread Making Quality of Wheat Flour. *Food Funct.* **2016**, *7* (10), 4323–4331. https://doi.org/10.1039/c6fo01095d.

(41) Katina, K.; Laitila, A.; Juvonen, R.; Liukkonen, K. H.; Kariluoto, S.; Piironen, V.; Landberg, R.; Åman, P.; Poutanen, K. Bran Fermentation as a Means to Enhance Technological Properties and Bioactivity of Rye. *Food Microbiol.* **2007**, *24* (2), 175–186. https://doi.org/10.1016/j.fm.2006.07.012.

(42) Di Nunzio, M.; Valli, V.; Tomás-Cobos, L.; Tomás-Chisbert, T.; Murgui-Bosch, L.; Danesi, F.; Bordoni, A. Is Cytotoxicity a Determinant of the Different in Vitro and in Vivo Effects of Bioactives? *BMC Complement. Altern. Med.* **2017**, *17* (1), 453. https://doi.org/10.1186/s12906-017-1962-2.

(43) Irakli, M.; Mygdalia, A.; Chatzopoulou, P.; Katsantonis, D. Impact of the Combination of Sourdough Fermentation and Hop Extract Addition on Baking Properties, Antioxidant Capacity and Phenolics Bioaccessibility of Rice Bran-Enhanced Bread. *Food Chem.* **2019**, *285*, 231–239. https://doi.org/10.1016/j.foodchem.2019.01.145.

(44) Di Nunzio, M.; Valli, V.; Tomás-Cobos, L.; Tomás-Chisbert, T.; Murgui-Bosch, L.; Danesi, F.; Bordoni, A. Is Cytotoxicity a Determinant of the Different in Vitro and in Vivo Effects of Bioactives? *BMC Complement. Altern. Med.* **2017**, *17* (1), 453. https://doi.org/10.1186/s12906-017-1962-2.

(45) Kumar, P.; Nagarajan, A.; Uchil, P. D. Analysis of Cell Viability by the MTT Assay. *Cold Spring Harb. Protoc.* **2018**, *2018* (6), 469–471. https://doi.org/10.1101/pdb.prot095505.

(46) Raza, H.; John, A.; Benedict, S. Acetylsalicylic Acid-Induced Oxidative Stress, Cell Cycle Arrest, Apoptosis and Mitochondrial Dysfunction in Human Hepatoma HepG2 Cells. *Eur. J. Pharmacol.* **2011**, *668* (1–2), 15–24. https://doi.org/10.1016/j.ejphar.2011.06.016.

(47) Zhang, J.; Wang, X.; Vikash, V.; Ye, Q.; Wu, D.; Liu, Y.; Dong, W. ROS and ROS-Mediated Cellular Signaling. *Oxid. Med. Cell. Longev.* **2016**, *2016*, 1–18. https://doi.org/10.1155/2016/4350965.

(48) Li, S.; Tan, H.-Y.; Wang, N.; Zhang, Z.-J.; Lao, L.; Wong, C.-W.; Feng, Y. The Role of Oxidative Stress and Antioxidants in Liver Diseases. *Int. J. Mol. Sci.* **2015**, *16* (11), 26087–26124. https://doi.org/10.3390/ijms161125942.

(49) Valli, V.; Taccari, A.; Di Nunzio, M.; Danesi, F.; Bordoni, A. Health Benefits of Ancient Grains. Comparison among Bread Made with Ancient, Heritage and Modern

Grain Flours in Human Cultured Cells. *Food Res. Int.* **2018**, *107*, 206–215. https://doi.org/10.1016/j.foodres.2018.02.032.

(50) Michalska, A.; Amigo-Benavent, M.; Zielinski, H.; del Castillo, M. D. Effect of Bread Making on Formation of Maillard Reaction Products Contributing to the Overall Antioxidant Activity of Rye Bread. *J. Cereal Sci.* **2008**, *48* (1), 123–132. https://doi.org/10.1016/j.jcs.2007.08.012.

(51) ALDERTON, W. K.; COOPER, C. E.; KNOWLES, R. G. Nitric Oxide Synthases: Structure, Function and Inhibition. *Biochem. J.* **2001**, *357* (3), 593. https://doi.org/10.1042/0264-6021:3570593.

(52) Costa, G.; Francisco, V.; C. Lopes, M.; T. Cruz, M.; T. Batista, M. Intracellular Signaling Pathways Modulated by Phenolic Compounds: Application for New Anti-Inflammatory Drugs Discovery. *Curr. Med. Chem.* **2012**, *19* (18), 2876–2900. https://doi.org/10.2174/092986712800672049.

(53) Merigo, F.; Brandolese, A.; Facchin, S.; Missaggia, S.; Bernardi, P.; Boschi, F.; D'Incà, R.; Savarino, E. V.; Sbarbati, A.; Sturniolo, G. C. Glucose Transporter Expression in the Human Colon. *World J. Gastroenterol.* **2018**, *24* (7), 775–793. https://doi.org/10.3748/wjg.v24.i7.775.

(54) Wright, E. M.; Hirayama, B. A.; Loo, D. F. Active Sugar Transport in Health and Disease. In *Journal of Internal Medicine*; 2007; Vol. 261, pp 32–43. https://doi.org/10.1111/j.1365-2796.2006.01746.x.

(55) Wright, E. M.; Martín, M. G.; Turk, E. Intestinal Absorption in Health and Disease - Sugars. *Bailliere's Best Pract. Res. Clin. Gastroenterol.* **2003**, *17* (6), 943–956. https://doi.org/10.1016/S1521-6918(03)00107-0.

(56) Ferraris, R. P.; Yasharpour, S.; Lloyd, K. C. K.; Mirzayan, R.; Diamond, J. M. Luminal Glucose Concentrations in the Gut under Normal Conditions. *Am. J. Physiol. Liver Physiol.* **1990**, *259* (5), G822–G837. https://doi.org/10.1152/ajpgi.1990.259.5.G822.

(57) Zheng, Y.; Scow, J. S.; Duenes, J. A.; Sarr, M. G. Mechanisms of Glucose Uptake in Intestinal Cell Lines: Role of GLUT2. *Surgery* **2012**, *151* (1), 13–25. https://doi.org/10.1016/j.surg.2011.07.010.

(58) Zheng, Y.; Sarr, M. G. Translocation of Transfected GLUT2 to the Apical Membrane in Rat Intestinal IEC-6 Cells. *Dig. Dis. Sci.* **2012**, *57* (5), 1203–1212. https://doi.org/10.1007/s10620-011-1984-4.

(59) Idris, I.; Donnelly, R. Sodium-Glucose Co-Transporter-2 Inhibitors: An Emerging New Class of Oral Antidiabetic Drug. *Diabetes, Obes. Metab.* **2009**, *11* (2), 79–88. https://doi.org/10.1111/j.1463-1326.2008.00982.x.

(60) Filipčev, B.; Kojić, J.; Krulj, J.; Bodroža-Solarov, M.; Ilić, N. Betaine in Cereal Grains and Grain-Based Products. *Foods* **2018**, *7* (4), 49. https://doi.org/10.3390/foods7040049.

(61) Koistinen, V. M.; Mattila, O.; Katina, K.; Poutanen, K.; Aura, A. M.; Hanhineva, K. Metabolic Profiling of Sourdough Fermented Wheat and Rye Bread. *Sci. Rep.* **2018**, *8* (1), 5684. https://doi.org/10.1038/s41598-018-24149-w.

(62) Ross, A. B.; Zangger, A.; Guiraud, S. P. Cereal Foods Are the Major Source of Betaine in the Western Diet - Analysis of Betaine and Free Choline in Cereal Foods and Updated Assessments of Betaine Intake. *Food Chem.* **2014**, *145*, 859–865. https://doi.org/10.1016/j.foodchem.2013.08.122.

(63) Pekkinen, J.; Rosa-Sibakov, N.; Micard, V.; Keski-Rahkonen, P.; Lehtonen, M.; Poutanen, K.; Mykkänen, H.; Hanhineva, K. Amino Acid-Derived Betaines Dominate as Urinary Markers for Rye Bran Intake in Mice Fed High-Fat Diet-A Nontargeted Metabolomics Study. *Mol. Nutr. Food Res.* **2015**, *59* (8), 1550–1562. https://doi.org/10.1002/mnfr.201500066.

(64) Kärkkäinen, O.; Lankinen, M. A.; Vitale, M.; Jokkala, J.; Leppänen, J.; Koistinen, V.; Lehtonen, M.; Giacco, R.; Rosa-Sibakov, N.; Micard, V.; et al. Diets Rich in Whole Grains Increase Betainized Compounds Associated with Glucose Metabolism. *Am. J. Clin. Nutr.* **2018**, *108* (5), 971–979. https://doi.org/10.1093/ajcn/nqy169.

(65) Niculescu, M. D.; Zeisel, S. H. Diet, Methyl Donors and DNA Methylation: Interactions between Dietary Folate, Methionine and Choline. *J. Nutr.* **2002**, *132* (8), 2333S-2335S. https://doi.org/10.1093/jn/132.8.2333s.

(66) Bostom, A. Short Term Betaine Therapy Fails to Lower Elevated Fasting Total Plasma Homocysteine Concentrations in Hemodialysis Patients Maintained on Chronic Folic Acid Supplementation. *Atherosclerosis* **1995**, *113* (1), 129–132. https://doi.org/10.1016/0021-9150(94)05466-V.

(67) Steenge, G. R.; Verhoef, P.; Katan, M. B. Betaine Supplementation Lowers Plasma Homocysteine in Healthy Men and Women. *J. Nutr.* **2003**, *133* (5), 1291–1295. https://doi.org/10.1093/jn/133.5.1291.

CONCLUSIONS

New insights for a stronger bioeconomy chain

The recovery of industrial cereal processing side streams, their innovative improving treatment and the consequent introduction on a novel food sector are the core goals of the bioeconomy system. The near future of the circular economy will need solid basis for an effective food processing by-products recovery to reduce the environmental load.

In the present Ph.D. thesis, the potential of cereal milling by-products has been pointed out. The information presented herein are novel and of interesting from the socio-economic point of view. These three years project contributed to increase the knowledge regarding the food by-products recovery and valorization through a multidisciplinary approach, gathering together nutrition, health and sustainability. This field is nowadays a research priority, continuously exploring innovative strategies for the efficient resources exploitation with respect to the consumer acceptance criteria and safety safeguarding.

The first section was dedicated to the assessment of the impact of milling process on safety and quality aspects of cereal grains. The distribution of mycotoxins, important harmful plant secondary metabolites, was studied in different cereal species and corresponding pearled fractions. The occurrence of modified mycotoxin forms exploiting a high-resolution mass spectrometry (HRMS) technique was the main goal of the latter study. These compounds were highly concentrated in the outermost cereal fractions, in fact DON and its modified forms were the most abundant. Nevertheless, pearling effectively mitigated the mycotoxins content in the endosperm, achieving an almost total mycotoxin removal. Moreover, oligoglycoside and acetylated glucoside forms of the latter toxin were firstly identified in naturally contaminated wheat samples, stressing the increased importance of HRMS application in routine quantification processes.

Beyond toxic compounds, bioactives in cereal are specialized metabolites deriving from plants metabolism, used mainly as defense mechanism. However, they are getting more attention because of their human health-related properties. The distribution of phenolic acids, betaine and choline in six, old and modern, wheat (*Triticum* spp.) species and corresponding industrial milling by-products was studied, providing a more realistic occurrence scenario. A deep analysis of soluble and bound phenolic acids components was performed using modern analytical techniques. Likewise mycotoxins, these compounds were highly present in bran and middlings, the mainly produced side-stream at industrial level. The milling process, in this sense, had a detrimental pauperization of bioactive compounds effect in flour and semolina, the end products. The same effect was found for betaine and choline.

"Cereal milling by-products, such as bran and seed germ, are a good source of bioactive compounds. However, more attention should be paid on the parallel study of mycotoxins, since they can be concentrated as an effect of milling process."

In the second section, two different innovative techniques were applied to wheat and rice bran for their potential valorization, lactic acid fermentation and dry fractionation. The results of the first study demonstrated that fermentation with lactic acid bacteria led to a significant improvement of nutritional properties of wheat bran producing a different material with increased antioxidant activity, concentration of soluble phenolic acids, rich in *ex-novo* produced bioactive compounds and with lower antinutritive phytic acid content, generally characterized by different organoleptic characteristics.

Beside the use of microorganism's metabolism activity, the air classification and micronization applied to rice bran resulted in a higher content of lipid molecules in the finest fractions produced, mainly composed by unsaturated mono-, di and triacylglycerols. The combined action of the particle size reduction and the accumulation of important lipid molecules could lead to a high vale raw material.

These results are encouraging since rice bran could be re-utilized in food manufacturing as ingredient, improving the texture and nutritional characteristics of novel food products.

"The quality characteristics improvement of cereal bran achieved through the application of both bioprocessing and/or physical treatment are promising paths to follow. Nevertheless, further study must be performed in combination with industries regarding the potential scalability."

In the last section the fermented wheat bran was used for the formulation of a high-fiber content bread, a type of product widely present in nowadays food markets. After an *in vitro* simulated gastrointestinal digestion, the potentially improved bioactive properties were assessed in two different intestinal cell culture models (IEC-6 and CaCo-2). These results were compared with the classic white bread and wheat bran enriched bread. Since, the bioaccessible phenolic and antioxidant fractions of bran enriched breads were higher in respect to the white bread ones, the corresponding protection toward reactive oxygen species generation was improved. Furthermore, the nitric oxide formation after an inflammatory stimulus was efficiently lowered by the bread containing the fermented wheat bread. Likewise, the intestinal glucose transport was highly delayed by the supplementation to cells with enriched bran bread, although no clear improvements were found when the fermented bran was used.

"The formulation of a highly consumed baked products using previously fermented wheat bran is a good form for the substantial recovery of cereal milling by-products. Moreover, significant beneficial effects, like a blunted intestinal glucose absorption and a protection against oxidative damage, might results from the ingestion of such products. However, more in vivo studies must be performed for the definitive confirmation of such properties."

Future perspectives

The future research scenario in an innovative and sustainable food system is starting to connect different disciplines for a bigger common objective: **the creation of a strong and efficient circular system.**

In light of these results, cereal milling by-products represent a huge opportunity for the food industry, since they can be used as sustainable ingredients for several technological and nutritional purposes in food formulation. It could mean a double advantage: a **lower environmental impact on agri-food** chain with a concomitant **diversification of food products**. However, the future priority must be the collaboration and joint actions of food nutritionists, technologists and scientist with industries, to efficiently face the next global challenges:

- higher consumer consciousness, perception and concerns about all the food chain aspects;
- to fight the increasing occurrence of chronic diseases (i.e. epidemic Type II diabetes).

APPENDIX I

SENSORY PROPERTIES EVALUATION OF FORMULATED WHEAT BREAD

Since the sensory aspect of foods is an essential parameter in nowadays food market, the preliminary assessment of organoleptic features of the newly produced bread containing pre-fermented wheat bran was studied.

This part of the work was performed with the support of Prof. María Jesús Callejo at the Universidad Politécnica de Madrid (Spain).

Method

The bread samples (white bread, bran bread and fermented bran bread) were analysed by conventional sensory profiling using ten trained panellists.

The trained panellists used a list of 24 sensory attributes, and recorded the values using an anchored continuous line scale (see **Figure Ax1**)**.** Breads were cut into slices of 1.5 cm thick served the same day of preparation, at room temperature, in a randomised order. Judges cleaned their mouth with not refrigerated water between each sample tasting.

Wheat bread sensory analysis questionnaire

Visual phase

Colour (crust)	white	dark
Colour (crumb)	white	dark
Alveolus size	small	big

Olfactory phase

Yeasty	absent	intense
Floury/cereal	absent	intense
Buttery/lactic	absent	intense
Nuts	absent	intense
Acid	absent	intense
Grassy/herbaceous	absent	intense
Cooked cereal	absent	intense
Toasted	absent	intense
Other (specify)		

Tactile phase

Firmness (by hand)	soft	hard
Elasticity (by hand)	soft	hard
Humidity (in mouth)	soft	hard
Cohesiveness (in mouth)	soft	hard
Adhesiveness (in mouth)	soft	hard

Oral phase

Sweet taste	absent	intense
Salty taste	absent	intense
Bitter taste	absent	intense
Acid taste	absent	intense
Herbaceous	absent	intense
Cooked cereal	absent	intense
Overall flavour	absent	intense
Others (specify)		

Panellist name:_____ **Sample code:**_____

Date:_____

Figure Ax1. Sensory analysis sheet.

Preliminary results

In **Figure Ax2** are reported the mean value of the attributes evaluated by the trained sensory panellists for each bread tasted.

Although all breads were appreciated, those containing fermented and non-fermented wheat bran by-product showed a more complex overall flavour. Moreover, breads fortified with wheat bran were characterized by a darker colour of both crumb and crust and a higher firmness evaluated by hand. Interestingly, in terms of odour and taste, bread enriched with fermented bran was similar to white bread (i.e. cooked cereal taste and buttery/milky smell). However, the dried fruits and toasted odour perception was noticed by the judges in both breads enriched with fermented and raw wheat bran.

The perception of rancidity, typical of baked products containing a high amount of wheat bran tissue [1], was found in raw bran enriched bread. In fact, few panellists have specified the presence of plastic-like odour in comment section with a relatively high intensity. This characteristic is mainly related to the presence of highly oxidised unsaturated fatty acid due to enzymatic activities.

These results suggest that, besides the previous accurate selection of raw material, lactic acid fermentation could be a useful strategy for the sensory improvement of high bran content baked foods.

Future studies will correlate results of trained sensory panellists and consumers (i.e. CATA analysis or preference studies) with biochemical parameters through a sensomics approach [2].

[1] Heiniö, R. L., Noort, M. W. J., Katina, K., Alam, S. A., Sozer, N., De Kock, H. L., ... & Poutanen, K. (2016). Sensory characteristics of wholegrain and bran-rich cereal foods–A review. *Trends in Food Science & Technology*, *47*, 25-38.

[2] Sahin, B., & Schieberle, P. (2019). Characterization of the Key Aroma Compounds in Yeast Dumplings by Means of the Sensomics Concept. *Journal of agricultural and food chemistry*, *67*(10), 2973-2979.

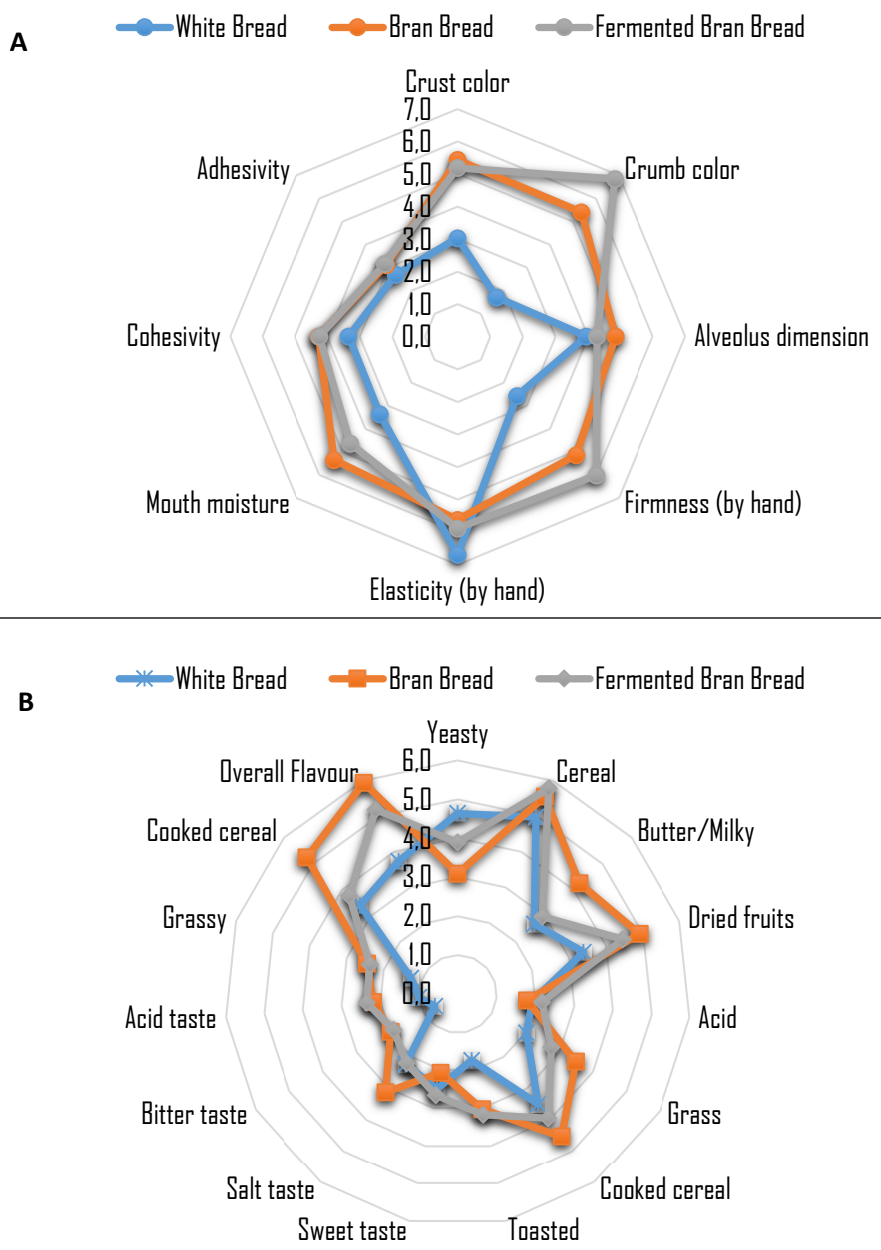

Figure Ax2. Spider web graph showing the values attributed by panel judges after the visual-tactile phases (A) and oral-olfactory phases (B). BB, white bread, BWB, bread with raw wheat bread, BFB, bread with pre-fermented wheat bran.

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Academic career

Marco Spaggiari got the master's degree in Food Science and Technology (University of Parma, Italy) in July 2016 with a project titled "*Effect of Encapsulation by Freeze-drying Technique on the Antioxidant Properties of Orange Fruit* (*Citrus sinensis* var. Navel) *Polyphenolic Compounds: Preliminary study*". His master's degree thesis focused on the development of functional foods and took place at the Polytechnic University of Valencia (Valencia, Spain) for a period of 8 months (2016). During his studies, he volunteered in Food Chemistry lab. of Food and Drug Department of University of Parma. Afterward, in November 2016, Marco Spaggiari started his Ph.D. in Food Science (University of Parma, Italy), under the supervision of Prof. Gianni Galaverna and Prof. Chiara Dall'Asta. The PhD research work has been associated with a strong collaboration with the Department of Food Science (University of Parma, Italy) and Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM, Madrid, Spain) where he carried out a short-term visit, 8 months exploiting the potential of *in vitro* cell culture models for the assessment of bioactive properties of food products.

His doctoral research aimed to the overall valorisation of cereal milling by-products for the sustainability and healthy diet promotion using a multidisciplinary approach. He has been also involved in a national project dealing with competitiveness increase of high hill and mountain farms through cereal biodiversity valorisation under organic farming (Bio^2, Rural Development Programme 2014-2020 of the Emilia-Romagna Region).

PH.D. TRAINING ACTIVITIES

WORKSHOPS and Academic SCHOOLS

24th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Firenze, Italy (September 2019). Oral presentation: *Cereal milling by-products valorisation: from unexploited materials to valuable resources.*

23rd Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Oristano, Italy (September 2018). Poster contribution: *Innovative methodologies for the recovery of cereal milling by-products*.

The conduct of Clinical trials in nutrition: a 4-days course to understanding key Aspects. February 2nd, at 10:00, class I, (Q02), Professors Cintia Bladè and Joseph del Bas.

The Italian Food Valley: The heritage of the Mediterranean diet between safeguarding tradition, sustainability and economic value. Dept. of Food and Drug, University of Parma, September 27th, Room A Q02, University of Parma.

ILSI Europe Seminar on Process-Related Compounds & Natural Toxins. Dept. of Food and Drug, University of Parma, September 18th, 2018, Aule delle Scienze, University of Parma.

Strategies and methods in toxicity testing in vitro: food contaminants of plant origin and process-related toxicants. Dept. of Food and Drug, University of Parma, June 18th, 2018, University of Parma.

"Genetic taste & Food Preferences", Dept. of Food and Drug, University of Parma, May 3rd, 2018, Room A, Q02 Building, University of Parma.

QUADRAM Institute visit in Parma, Dept. of Food and Drug, University of Parma, September 3rd, 2018, Room M (Q02), University of Parma.

"*Industry meets Academia – Advances in food processing, food security, and bioeconomy through research*" held at the University of Hohenheim, Stuttgart, Germany on 03 July 2018.

"*Metabolomics workshop*" organised by Waters, held at the University of Padova on 13 November 2017

22nd Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Bolzano, Italy (September 2017). Poster contribution*: Co-Products recovery from cereal grain milling process to increase sustainability and competitiveness of the agri-food chain*.

"*MS Technology Day 2017*" organised by Waters, Milan, Italy on 19 September 2017.

Course on "*Come scrivere un progetto MSCA di successo nel programma Marie Sklodowska-Curie Action in H2020*", held at the University of Parma on 03 July 2017.

"*Metabolomics Seminar*" organised by Agilent Technologies, held at the University of Parma, Parma, Italy on 16 March 2017.

2nd MetaboMI meeting organized by IFOM and IRCCS held at the Ospedale San Raffaele, Milan, Italy on 09 February 2017.

"*NMR & MS in metrabolomica*" organized by GIDRM and IMaSS, held at University of Padova, Department of Chemistry, Italy on 03 February 2017.

AWARDS and PRIZES

Phenolic acids distribution in "Carnaroli" rice (Oryza sativa L.) and corresponding milling fractions.
Authors: M. Spaggiari, S. Folloni, R. Ranieri, C. Dall'Asta, G. Galaverna.
Conference: 5th MS Food Day, Bologna, Italy
"**Best Poster Award winner**" (October 2017).

Determination of betaine, choline and γ-aminobutyric acid in Triticum durum and Triticum turanicum whole grain and milling co-products.
Authors: M. Spaggiari, L. Righetti, S. Folloni, R. Ranieri, C. Dall'Asta, G. Galaverna.
Conference: XIX EUROFOODCHEM Conference, Budapest, Hungary.
"**Best Poster Award winner**" (October 2017).

TEACHING AND DIDACTIC ACTIVITIES

Master's degree course in "*Xenobiotics in Foods*" for the academic years 2016/2017 and 2017/2018

- 27 hours of laboratory experience
  Lecturer: Prof. Chiara Dall'Asta

Bachelor's degree course in "*Food Chemistry"* for the academic years 2017/2018

- 16 hours of laboratory experience
  Lecturer: Prof. Martina Cirlini

Lectures dedicated to MSc in *Food Science and Technology* students held at the University of Parma, Department of Food and Drug on 02 and 03 October 2017:

- Chemical composition of foods;
- Principles of Food Chemistry and Food Analysis.

Tutoring of bachelor's and master's degree in *Food Science and Technology* students during their traineeship and thesis project period. Some examples of thesis are reported below:

- *Comparative study of in vitro digestion methods: wheat bread nutrients bio-accessibility;*
- *Old and modern cereal grain evolutionary populations: what's beyond safety and quality aspects? ;*
- *Biotechnologies and food safety: new frontiers of decontamination processes;*
- *The aromatic profile of "Lemons and Liquors of Sorrento (Italy)": an authentic study.*

OTHER ACTIVITIES

Attendance to seminar cycle "*Fundamentals in toxicology"* held at the University of Parma, Italy by Professor Doris Marko (University of Wien) on 16-19 September 2019.

Active collaboration with "*Food Hub*" magazine, writing article for divulgation purposes (https://www.foodhubmagazine.com/):

- "I cereali senza le vesti: sottoprodotti della macinazione", 25 April 2019 (https://www.foodhubmagazine.com/2019/04/25/i-cereali-senza-le-vesti-sottoprodotti-della-macinazione/)
- "Tecnologie innovative per il recupero dei sottoprodotti della lavorazione dei cereali", Jul-Sept. 2019 (https://www.foodhubmagazine.com/innovazione-tecnologica/)

Participation as **invited speaker** at seminar cycle for students held at the University of Parma, Department of Food and Drug, Italy on 12 and 19 June 2018:

- Analysis and interpretation of EFSA's Scientific Opinions;
- Effective communication of EFSA's Scientific Opinions.

Participation at seminar cycle (**invited speaker**) for students, undergraduates and secondary school children *"Workshops for the "International Day of Women and Kids in Science*" held at Gastronomy Innovation Centre and International Institute Rosa Chacel, Madrid, Spain on 8 and 15 February 2019:

- Innovative foods and drinks produced from coffee processing by-products: a bioeconomy focus.

Participation at the joint seminar (**invited speaker**) "*Ph.D. students from Parma and Wageningen*" held at the University of Parma, Centro Santa Elisabetta, Italy on 18 October 2018.

Oral presentation: "*Innovative methodologies for the recovery of cereal milling by-products*"

Cereal milling by-products:

from unexploited residues to valuable resources

The research presented in this Ph.D. thesis is part of the project "*Agro-food industry by-products recovery for the competitiveness improvement of local companies*" (POR/FSE 2014-2020), financed by Regione Emilia-Romagna through the European Social Fund. Following an integrated and multidisciplinary approach the research goals were:

- To assess the **impact of milling process** on whole grain quality and safety traits;
- To study and apply **innovative technologies** for the total recovery and valorisation of cereal milling by-products;
- To evaluate **the nutritional properties** of a novel wheat bread enriched with pre-fermented wheat bran.

The main findings suggest that these unexploited materials, largely produced nowadays, can offer a great potential for their valorisation as innovative food ingredients for the formulation of high-value products.

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The End

(...to be continued)