UNIVERSITÀ CATTOLICA DEL SACRO CUORE

Sede di Piacenza

Dottorato di ricerca per il Sistema Agro-alimentare

Ph.D. in Agro-Food System

Cycle XXXVI

S.S.D. AGR 16-AGR 15

Set up Molecular Toolkit for Wheat Identification, Quality and Safety Assessment, and Design of Cereal-Based Foods with Enhanced Value

Coordinator:

Ch.mo Prof. Paolo Ajmone Marsan

Candidate:

Paola Carnevali

Matriculation n: 5014542

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Tutor:
Prof. Lorenzo Morelli
Dr. Valeria Terzi

Candidate:

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A Leonardo, Silvana, Alfredo

"...Leggendo non cerchiamo idee nuove, ma pensieri già da noi pensati, che acquistano sulla pagina un suggello di conferma..."

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Introduction

Plant-based diets have become popular means to reduce the environmental footprint of diets and promote human health and animal welfare (Alcorta et al., 2021).

Plant-based foods not only meet the nutritional requirement for a healthy diet and, are well accepted by consumers in terms of flavour and texture since they are produced in an environmentally friendly way and therefore undergo few industrial manipulations, hence meet consumer expectations.

Nowadays, consumers are aware of all aspects regarding food provenience and processing; of course, their food choices shape the evolution of this market.

Historically, cereals have been staple foods in cultures for millennia and the main dietary source of energy, carbohydrates, and plant proteins world-wide (Ritchie et al., 2022).

Currently, cereal grains are still the major constituent of human diets around the world: their grains are a major source of plant-based protein in human diet, the main protein reservoir is the storage proteins. Cereals are also key contributors to increase dietary energy, mainly through starch supply as well as being good sources of fibers, vitamins, and minerals (Wrigley, 2015). Based on the specific composition of the cereal, bioactive compounds, such as polyphenols, antioxidants, vitamins, and minerals, including calcium, magnesium, zinc, and iron (Zhou et al.,2012), may be present.

The most consumed cereals are wheat, rice and maize, other cereals and pseudo-cereals of interest including oat, millets, sorghum and spelt (Food and Agriculture Organization of the United Nations, 2016).

Besides having a high nutritional value, cereals contribute to the achievement of global food security: the annual cereal production amounts to approximately 2600 million tons (Mt) (Laskowski et al., 2019). Over the next ten years, cereal production expected to increase by 343 million tons (Mt) (+ 12%).

Yield improvement comes from ameliorated and more widely accessible seed varieties, greater efficiency in the use of inputs and better agricultural practices (Helia, 2022).

The interest in improving the health benefits of cereal foods is continuously increasing; this is due to the high frequency of their consumption worldwide, and to the chance of using them as carriers to provide health promoting components in the diet that may counteract the occurrence of non-communicable diseases (NCDs).

It should be an excellent contribution and opportunity to improve health maintenance and disease prevention.

The consumption of cereal-based foods, whole grain cereals, has been associated with ameliorated conditions in certain diseases, including type 2 diabetes, cardiovascular diseases, and certain types of cancer (Montonen et al.,2003).

The technological challenges present in this area of study and innovative strategies that are currently employed to produce cereal-based foods include foods quality and safety considerations (Revilla et al.,2009)

The understanding of cereals benefits must start from grain characterization, go through the production line, including the technological challenges and the innovative strategies underlying their use and the final product evaluation in respect of safety and quality, including the acceptability by the consumers (Estell et al.,2021). In fact, the increasing consumption of ready to eat meals, recognized to be rich in sugars and lipids, and poor in bioactive compounds and fibers, increase problems of overweight and obesity worldwide, and show an increasing trend in low-, middle-, and even high-income countries.

The improvement of the functional and technological properties of cereal wholemeal flours is a first and direct goal in favor of the production and spread of cereal-based foods with improved functional properties.

The challenge is to improve cereal foods not only for health benefits, but also for taste benefits that should satisfy and increase the consumer’s acceptance, at the same time assure the economic potential of new products (Laddomada et al.,2022). These studies intend therefore to contextualize cereal-based alternatives within the current frame of the plant-based food market (Beacom et al.,2021).

On the one hand, the research studies have been focused on genetic and technological aspects in order to improve the quality and safety of pasta chain and, on the other hand, studies have been focused on technological and nutritional aspects of rye and triticale, that are underutilized crops, contain health-promoting bioactive and are climate resilient. (Akib et al.,2023).

The research studies were focused on cereals' little-known aspects with a multi-disciplinary approach, i.e. innovative rye and triticale-based foods and wheat genomic.

DNA-based traceability support in pasta production chain

The Mediterranean Agrofood production is characterised by a great diversification, and a substantial part of it, is of great national and international prestige. Its competitiveness is based not only on quantitative aspects, but rather on the safety, quality, and authenticity of the products.

The culture of quality systems and related certification is therefore increasingly strengthening in the agricultural and food sectors, to offer the necessary guarantees to consumers, while at the same time safeguarding the market value of typical and quality products.

The research here developed is part of this logic and intends to introduce scientific and technological innovations based on DNA analysis to track aspects related to the authenticity and quality of raw materials and derived products in a key supply chain, such as the pasta production one. The study built on genomic data already developed and/or publicly available. Massive DNA sequence data for a wide range of organisms is presently available in online databases readily to be utilized. Genome sequencing data are available for most crops and animal, huge amount of SNP markers is derived from re-sequencing single genotypes. The basic idea is therefore to move from genomics and barcoding data acquisition to their practical exploitation in food supply chains.

The rationale behind the selection of DNA-based technologies is their flexibility, limited costs, and the possibility to be translated into fast and user-friendly systems.

Starting from such premises, the points reported below have been addressed with the overall aim to strengthen authenticity, quality and safety of durum wheat and pasta production chain**.**

The results obtained during this part of the study were the subject of the publications reported in chapter 1:

1. Digital PCR (d PCR) is a breakthrough molecular technology able to provide an absolute nucleic acid quantification. It is a third-generation PCR technology in the field of nucleic acid amplification. A unique feature of this molecular technique is that the sample is spitted into different many compartments, in each of which an independent amplification reaction takes place simultaneously. Instrumental platforms have been developed for this purpose, and different statistical approaches are now available to read the digital output data. Digital PCR assays developed so far in the plant science sector and, were identified in literature. The major applications, advantages, disadvantages, and applicative perspectives of the technique are considered in the review (Morcia et al.,2020).
2. Pasta, the Italian product of excellence, must derived from pure durum wheat. The use of *Triticum durum* semolina is mandatory for Italian pasta production, the presence of soft wheat flours less than 3% is permitted to be legally compliant (Legge n.580). Recently new generation methods, based on DNA (deoxyribonucleic acid) analysis, was developed with the aim of *T. aestivum* contamination as a tool recognition at specie level (Morcia et al., 2020).
3. A single nucleotide polymorphism (SNP) was identified as a reliable marker for wheat varietal discrimination, and a rapid test, which allows an easy and clear identification of specific wheat varieties, was also developed. The development of a colorimetric mono-varietal discriminating assay, aimed at improving traceability and quality control checks of durum wheat products, is described. Notably, an approach based on the loop-mediated isothermal amplification reaction (LAMP) as a SNP discrimination tool, in combination with naked-eye visualization of the results, was designed and optimized. Our assay was proven

to be effective in the detection of adulterated food products, including both substitution and mixing with different crop varieties (Cibecchini et al. 2020).

4. Digital polymerase chain reaction is characterized by unprecedented levels of precision as demonstrated when applied to quantify the presence of a specific plant genotype, in both raw materials and finished products, by exploiting a point polymorphism. As proof of concept, an Italian premium pasta production chain was considered, and digital PCR assay based on a durum wheat target variety private point mutation was designed and evaluated in supply-chain samples (Morcia et al., 2021).
5. Several food products, made from hulled wheats, are now offered by the market, ranging from grains and pasta with flour and bakery products. The possibility of verifying the authenticity of wheat species used at any point in the production chain is relevant, in defence of both producers and consumers. A chip digital PCR assay has been developed to detect and quantify percentages of hulless (i.e., common and durum wheat) and hulled (i.e., einkorn, emmer and spelt) wheats in flours and food products (Morcia et al., 2021).
6. *Fusarium* Head Blight (FHB) is one of the major diseases affecting small-grain cereals, worldwide spread and responsible for severe yield and quality losses annually. Diagnostic tool able to track *Fusarium* species even in the early stages of infection, can contribute to mycotoxins' risk control (Morcia et al., 2020).

Design of Cereal-Based Foods with Enhanced Value

Cereals provide nutrients such as lipids, carbohydrates, proteins, minerals, and vitamins and make-up human's healthy eating. Some cereals also contain a considerable number of dietary fibers (soluble and insoluble) (Food and Agriculture Organization of the United Nations, 2016). Cereals, when consumed as a whole or as colored varieties,

are sources of bioactive compounds with functional properties. Dietary fiber in whole grains has a unique blend of bioactive components such as resistant starch, vitamins, minerals, phytochemicals, and antioxidants with proven health benefits (M. Rawat et al., 2023).

Whole Grain products to provide health benefits due to the high content in dietary fibres (TDF) rich in bioactive phytochemicals (i.e., phenolic compounds, tocopherols, tocotrienols, carotenoids), plant sterols and lignans (Yang et al., 2021).

The bioactive compounds present in whole grain cereals can help in the prevention and/or control of certain diseases, such as reduce cardiovascular and cancer risk, type 2 diabetes, high blood pressure, improve gastrointestinal health and strengthen the immune system.

The consumer demands minimally processed foods that ensure both bioavailability of nutrients and improvement of metabolism, which are essential to maintain a health status. Therefore, development of products using whole cereals is of great interest to improve human health and safety (Rawat et al., 2023).

Therefore, cereals could be a natural source of healthy plant proteins and could play a role towards a more sustainable food system for healthy diets.

Moreover, shifting grain use from feed to traditional and new foods could improve protein security and alleviate climate change.

Rapid development of new grain-based food, such as dairy replacements and meat analogues, might accelerate the transition (Poutanen et al., 2022).The EAT-Lancet Commission, a multinational initiative launched for food system transformation, describes a universal healthy diet reference based on vegetables, fruits, whole grains, legumes, and nuts (EAT-Lancet Commission Summary Report, 2019).Whole grains and whole grain products, included at the base of the Food Guide Pyramid, contain unique phytochemicals, for instance ferulic acid, which are not present in significant quantities in fruits and vegetables (Liu, 2007).

Ferulic acid, like other bioactive components, is in the outer layers of the grain: the bran and the aleurone layer. This latter holds also soluble dietary fibres (SDF), composed of

arabinoxylans (65%), β-glucans (29%) and fructans; and insoluble dietary fibres (IDF), especially cellulose, hemicellulose and lignin (Lebert et al., 2022).

Soluble dietary fibres and fractions supply the most readily fermentable substrates for the human microbiota in large intestine, which may influence gut health. Arabinoxylans and β-glucans form viscous solutions and thereby slow intestinal transit, delay gastric emptying, and reduce glucose and sterol absorption by the intestine (Hübner, Arendt, 2013). Fructans are supposed to have prebiotic effects (Shewry et al., 2015) with the ability to promote absorption of calcium (Abrams et al., 2007).

The insoluble fraction of dietary fibres accelerates the intestinal transit time, which in turn may decrease the risk of constipation (Jonsson et al., 2018).

The aleurone cell-wall is also rich of phenolic compounds, the most common are phenolic acids and flavonoids. The major phenolic acids are ferulic acids and p-coumaric acid, the first is the main polyphenol present in cereals and the 92% of ferulic acids are in bounded form and are esterified to the hemicelluloses and arabinoxylans of the grain cell walls (Gani et al., 2012) (Lebert et al., 2022). The ferulic acids esterified to insoluble fibre are delivered into the colon, the fibres are fermented and the bounded ferulic acids, which have an antioxidant activity, are released, contributing to the reduction of colon-rectal cancer (Liu, 2007). Additionally, ferulic acids is known to decrease total cholesterol, increase vitamin-E bioavailability and to have anti-inflammatory activity (Rao et al., 2006). Coumaric acids are supposed have antioxidant effects and anti-cancer activity (Garrait et al., 2006). Other phenolic compounds of whole grains are alkyl resorcinols. These are phenolic lipids, with the ability to interfere with the solubilization of cholesterol micelles in human digestive tract and decrease cholesterol absorption. Rye has twice the amount of alkyl resorcinols of wheat, hence the cholesterol-lowering effect of rye (Jonsson et al., 2018).

The aleurone supply many minerals around 40% of total grain minerals, such as phosphate (80% in phytate form), magnesium, manganese, iron, potassium, and sodium. (Lebert et al., 2022)

Phytate is an undesirable compound, which forms complexes with minerals such as $Ca^{2+,}$ Fe^{3+}, Zn^{2+} and Mg^{2+} reducing their bioavailability (Šramková et al., 2009). However, Schlemmer et al. (2009) concluded that fermentation can transform the dietary fibres, in colon tract, by producing short chain fatty acids (SCFAs), that reduce the intestinal pH and foster the solubilization of complexes for colonic absorption of minerals. Nevertheless, a daily consumption of whole grains (3 servings per day) will contribute to a good mineral status (Brouns et al., 2012).

In the germ fraction, the mineral level is also high (4,5%) (Šramková et al., 2009).

The outer layers are also a source of vitamins, they hold larger quantities of the B-complex vitamins, in particular: niacin, thiamine and riboflavin.

The B-vitamins are also present in the endosperm and in the germ or embryo, which is also rich in essential vitamin E. Germ fraction shows the highest concentration of α-tocopherol, β-tocopherol and total tocols (Kumar, et al., 2011).

On the other hand, cereal grains have low content of the other lipid soluble vitamins (provitamin A, vitamin D and vitamin K) and are lacking in vitamin C (Brinch et al., 2007; Hübner et al., 2013).

Carotenoids are within the grain, in significant amounts are within the endosperm and germ. Carotenoids commonly found in whole-grain cereals are lutein, zeaxanthin, β-cryptoxanthin, β-carotene and α-carotene, components of the antioxidative defence systems (Liu, 2007; Gani et al., 2012).

For these reasons cereal grains have a lower environmental impact and excellent nutritional properties that prevent the incidence of diet-related diseases, small grains (wheat, oats, barley, rye, rice) are perfect candidates for the development of natural and healthy functional foods.

Summing up, cereal grains are essential for human diet and to lead a healthy life, reducing the risks of chronic lifestyle diseases (CVDs, cancer, overweight and obesity, diabetes) and helping gut health by boosting human immune system.

For these reasons, cereals and cereals products are the elective components of Mediterranean Diet (Giacosa et al., 2022) inscribed into the United Nations

Educational, Scientific and Cultural Organization (UNESCO) and as representative in List of Intangible Cultural Heritage of Humanity, since 2011 (Trichopoulou, 2021). This fact further underlines the importance of plant-based diet.

In this dissertation, studies were conducted on malted rye grains and relative flour was fermented (Mancino et al.,2022), and on malted triticale (Piazza et al., 2023) with the aim to design new foods. Rye is higher in fibers, vitamin E, riboflavin, folacin, pantothenic acid and has twice as much essential amino acid, namely lysine compared to wheat (Rakcejeva et al., 2008). Furthermore, rye contains a group of compounds, called benzoxazinoids, that have immunoregulatory, appetite- and weight-reducing, and anti-cancer effects (Adhikari et al., 2015).

Rye is also known for the "rye factor", the lower serum insulin release after intake of rye bread compared to wheat bread, despite similar serum glucose concentrations. This could be explained by slower intestinal glucose absorption from rye than wheat bread, due to the different types and amounts of dietary fibres, phytochemicals, amino acids, and fermentation products, particularly short-chain fatty acids (SCFAs), produced by gut microbiota (Jonsson et al., 2018).

Triticale (*Triticosecale* wittmack) is a wheat/rye hybrid grain with a world production that has consistently increased during the last two decades, reaching about 17 Mtons in 2014, is an anthropogenic cereal designed to incorporate the functionality and high yield of wheat (*Triticum* spp. Linnaeus 1753) and durability of rye (Secale cereale Linnaeus 1753). For instance, lunasin, a 43-amino acid-long peptide with cancer-preventive, cholesterol-reducing and anti-inflammatory properties, discovered in triticale with higher level (6.5 mg/g) than wheat (0.23 mg/g) and rye (1.5 mg/g) (Zhu, 2018).Triticale traditionally used as animal feed and for biofuel production, however, the growing demand for food resources and the current consumer trend of trying novel products has led to an increased interest in food production (McGoverin et al.,2011). In addition, triticale could be an important crop to ensure food security, due to its tolerance to drought, disease, more acid soils, low susceptibility to biotic stresses, and high grain yield even in marginal environments (Leon et al. 1996).

In the study presented triticale could satisfy the hopes originally placed upon it and may be useful to design foods with high nutritional values.

In fact, triticale offers advantages over other cereal grains, such as a better-quality protein in terms of amino acid composition and digestibility, higher content of soluble dietary fibres and minerals (Munoz-Insa et al., 2016).

During germination process, the starch hydrolysed by the action of amylolytic enzymes, and storage proteins hydrolysed into amino acids and low-molecular weight peptides by proteases, therefore starch becomes more digestible and there is an increase in amino acid bioavailability (Nako et al., 2022).

In addition, germination significantly enhance the levels of bioactive compounds such as folate, γ-aminobutyric acid (GABA), phenolic compounds and vitamins in cereal grains by activating the related enzyme activity (Singh et al., 2015). GABA is useful for disease prevention since it reduces symptoms of different disorders such as Type 2 diabetes, hypercholesterolemia, hypertension, some cancers (breast, colon, liver) and neurological disorders (Baranzelli et al., 2018). In Hübner et al. (2010) study, Insoluble Dietary Fibre concentration resulted increased in barley and oat malts, because of prolonged germination times: this fact could be partially explained by the loss of compounds such as starch; while the degradation and solubilisation of beta-glucans by β-glucanase and arabinoxylans due to a range of different enzymes (Hübner & Arendt, 2013) takes place. During germination, an increase in mineral content also occurs, because of the breakdown of phytate by phosphatases (endogenous phytase), that hydrolyse the phytates releasing their minerals (Brouns et al., 2012).

Moreover, cereals are one of the most suitable substrates for the development of foods holding probiotic microorganisms such as Lactic Acid Bacteria (LAB) and Bifidobacteria and may also have prebiotic properties due to the presence of nondigestible components of cereal matrix (Singh et al., 2015).

Natural Sourdough Fermentation (SD) offers advantages, it is recognized to play a key role to get improved flavour, texture, nutritional and shelf-life properties of many foods, we focused on bakery goods.

Nonetheless, the basic part of sourdough products is a sourdough starter, which is a culture of unique and complex microbial communities.

Industrial wheat bread production started in the middle of the 20th century, after the introduction of bakers' yeast as a fast and ready-to-use leavening agent and therefore ideal substitute for traditional sourdough or brewing yeast (Carnevali et al., 2012)

Even if liquid sourdough is still a world to be discovered, it is a promising flexible technology that can reserve an important level of exclusivity since its properties are not easily reproducible and can be a powerful tool for tailoring bread quality.

The technology of liquid sourdough can offer advantages with a real contribute to the creation of wide variety of bakery distinctive foods in flavour, texture and with health benefits (Carnevali et al., 2007).

An increasing number of studies are focused on innovative properties of foods are related to LAB addiction among these might be cited: improvement in control of pathogens and/or spoilage microorganisms (Settanni and Corsetti 2008); improvement of mineral bioavailability, reduction of glycaemic index, and improved quality of wholegrain or fiber-rich products (Poutanen et al., 2009); improved quality of gluten-free products (Moroni et al. 2009); increased content in exopolysaccharides (EPS) benefiting texture and shelf life (Di Cagno et al., 2006).

From a microbiological perspective, sourdough is to be considered as a specific and selective ecosystem, harboring yeasts and lactic acid bacteria (LAB), that is used for the production focus on baked goods. With respect to the metabolic impact of the sourdough microbiota, acidification (LAB), flavor formation (LAB and yeasts), and leavening (yeasts and heterofermentative LAB species) are most noticeable.

Three distinct types of sourdough fermentation processes can be discerned on the inocula applied, namely back slopped ones (type 1), those started with starter cultures (type 2), and those initiated with a starter culture followed by back slopping (type 3).

A sourdough-characteristic LAB species are *Fructilactobacillus sanfranciscensis* and *Lactiplantobacillus plantarum*, while yeast species are *Kazachstania humilis* and *Saccharomyces cerevisiae*.

It is however clear that sourdough-adapted microorganisms can withstand selective conditions encountered during their growth. Based on the technological setup, type 0 (pre doughs), type I (artisan bakery firm sourdoughs), type II (industrial liquid sourdoughs), and type III sourdoughs (industrial dried sourdoughs) can be distinguished. The production of all sourdoughs, independently of their classification, is based on intrinsic and extrinsic factors.

Both the flour (type, quality status, ….) and the process parameters (fermentation temperature, pH and pH evolution, dough yield, water activity, oxygen tension, back slopping procedure, and fermentation duration, and so on) can influence the dynamics and outcomes of the sourdough fermentation process. (De Vuyst L. et al. 2017).

Recently, however, naturally fermented sourdough bread has regained popularity at the industrial, artisan, and home-baking scales though all such scales of producing sourdough can differ drastically in their development, maintenance strategies, and quality. Compared to commercially leavened bread, breads and pastries made with sourdough starters have better shelf life as well as various positive nutritional and pleasant sensory attributes (Hutkins, 2019). The sourdough is a complex ecosystem that leverage the quality, the texture, the sensorial attributes in subsequent products.

The results obtained during this part of the studies were the subject of the publications reported in chapters 2 and 3, that highlighed the impact of technological strategy on design of new Rye and Triticale-Based Foods with Enhanced Value.

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Chapter 1

Development of a Molecular Toolkit for pasta chain authenticity and safety

biology

MDPI

Review

Digital PCR: What Relevance to Plant Studies?

Caterina Morcia [1], Roberta Ghizzoni [1], Chiara Delogu [2], Lorella Andreani [2], Paola Carnevali [3] and Valeria Terzi [1,*]

1 Consiglio per la Ricerca in Agricoltura e L'analisi Dell'economia Agraria-Centro di Ricerca Genomica e Bioinformatica (CREA-GB), via San Protaso 302, 29017 Fiorenzuola d'Arda, Italy; caterina.morcia@crea.gov.it (C.M.); roberta.ghizzoni@crea.gov.it (R.G.)

2 Consiglio per la Ricerca in Agricoltura e L'analisi Dell'economia Agraria-Centro di Ricerca Difesa e Certificazione (CREA-DC), via Emilia km 307, 26838 Tavazzano, Italy; chiara.delogu@crea.gov.it (C.D.); lorella.andreani@crea.gov.it (L.A.)

3 Barilla S.p.A., via Mantova 166, I-43122 Parma, Italy; paola.carnevali@barilla.com

* Correspondence: valeria.terzi@crea.gov.it; Tel.: +39-0523-983758

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Simple Summary: Digital PCR is a third-generation technology based on the subdivision of the analytical sample into numerous partitions that are amplified individually. This review presents the major applications of digital PCR (dPCR) technology developed so far in the field of plant science. In greater detail, dPCR assays have been developed to trace genetically modified plant components, pathogenic and non-pathogenic microorganisms, and plant species. Other applications have concerned the study of the aspects of structural and functional genetics.

Abstract: Digital PCR (dPCR) is a breakthrough technology that able to provide sensitive and absolute nucleic acid quantification. It is a third-generation technology in the field of nucleic acid amplification. A unique feature of the technique is that of dividing the sample into numerous separate compartments, in each of which an independent amplification reaction takes place. Several instrumental platforms have been developed for this purpose, and different statistical approaches are available for reading the digital output data. The dPCR assays developed so far in the plant science sector were identified in the literature, and the major applications, advantages, disadvantages, and applicative perspectives of the technique are presented and discussed in this review.

Keywords: digital PCR; genetic traceability; diagnostics; genetically modified organisms; species; copy number variation; gene expression

1. Introduction

Digital PCR (dPCR) is a breakthrough technology that is able to provide sensitive and absolute nucleic acid quantification [1]. It is a third-generation technology in the field of nucleic acid amplification. Starting from end-point PCR, which is able to provide a qualitative or semi-quantitative result, the second-generation of the technique—real time PCR or qPCR—gives a quantification of the target sequence (Figure 1). Digital PCR, the third-generation PCR technology, works by partitioning a sample of DNA or cDNA into a high number of single, parallel PCR reactions. The reactions are carried out in separated and numerous small volume compartments in which DNA or cDNA molecules of the sample are randomly distributed. Each compartment can host none, one, or many molecules. In ideal conditions (i.e., a low density of target DNA and a high number of compartments), each compartment holds one or none target molecule. In such conditions, after amplification and absorbance measurements, a compartment containing no target molecule is counted as 0, whereas a compartment with one target is counted as 1. From such data, considering the dilution factor, it is possible to calculate the target

copy number in an analytical sample. However, because a compartment can contain more than one target molecule in practice, correction factors based on Poisson statistical distribution—used to account for the probability of a partition initially containing more than one target—is used to achieve the final outcomes.

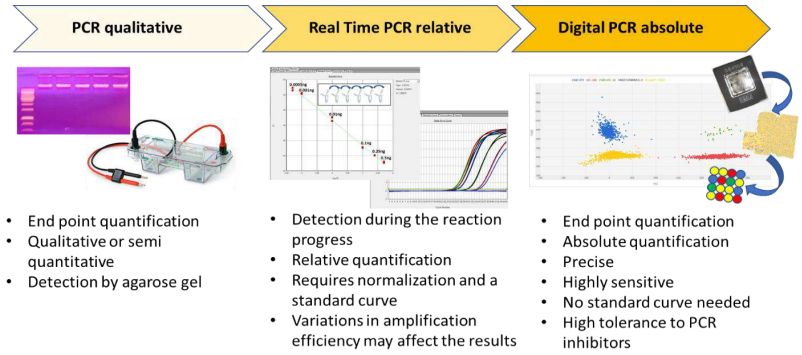

Figure 1. The three main PCR technologies are reported, and their main features are schematically presented.

The first pioneering achievement of this technique was realized in 1992 by Sykes et al. [2], who used limiting dilutions of their samples and end-point signal quantifications for gene mutation detection. A milestone for dPCR technology development was the study of Vogelstein and Kinzler [3], dated to 1999. These authors amplified individual molecules of a sample in parallel PCRs with fluorescent probes, therefore transforming the exponential, analog nature of the PCR into a linear, digital signal. With the introduction of microfluidics, proposed by Liu et al. [4] in 2003, a major improvement was completed, and the hitherto dormant technique became of considerable interest [5]. The partition of DNA samples was done with the aid of micropumps and microvalves, thus increasing the accuracy of the dilution step. In 2011, droplet digital PCR (ddPCR) was proposed by Quantalife Co, LT, as a cheaper approach in comparison to the microfluidic one.

Currently, several different platforms are used for PCR reaction compartmentalization, such as active partitioning platforms (based on mechanical aid for the compartment formation), passive partitioning platforms (based on fluidic effects to create sub-volumes), self-digitization platforms (which combine both passive filling and partitioning), and droplet-based platforms (in which aqueous droplets act as microreactors). The main differences among platforms are in the number of partitions and in the number of samples that can be processed in a run.

In dPCR, the detection of the target is achieved similarly to in qPCR, with two main families of chemistries: DNA intercalating dyes and hydrolysis-based probes. As in qPCR, there is the possibility to organize multiplex reactions to simultaneously follow more than one target.

In 2004, Gachon et al. [6] published a review entitled "Real-time PCR: What Relevance to Plant Studies?" in which the "detection and quantification of foreign DNA" and the "quantification of specific transcripts" were reported as the major applications of the technology to the plant sector. Up to now, qPCR has had innumerable applications in the field of plant science, as expected. Now, dPCR has been proposed as a new technique applicable to the same targets of qPCR that is also able to provide answers to additional, biological questions. In the present review, we summarize the recent applications of dPCR in plant science. The literature of the last five years was screened, and 81 papers published in peer-reviewed international journals were found to present the development and application of new dPCR-based protocols. Figure 2 shows the main categories of dPCR applications and their percentages: more than 80% of the studies were focused on the detection and quantification of genetically modified plants (GMPs) and of plant pathogens, but other kinds of applications, such as plant species traceability, gene expression studies, and CNV (copy number variation) detection, have also been present.

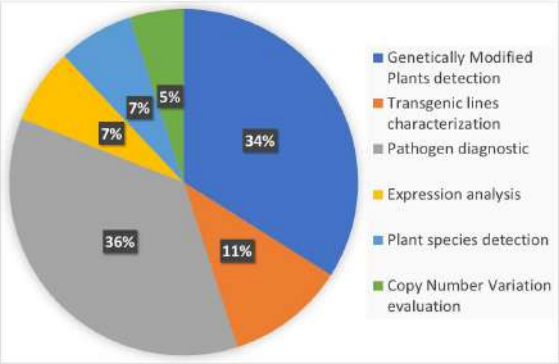

Figure 2. The main applications of digital PCR (dPCR) developed so far in the plant science sector are reported and classified into six main categories. The percentage of each category was calculated on the basis of number of recently published peer-reviewed studies. The literature screening was carried out using dPCR and its variants as main key words for an analysis of the recently published literature.

2. Genetically Modified Plants Detection

More than twenty years after the start of their commercialization, biotech crops currently cover around 200 million hectares worldwide [7]. Different labeling laws and voluntary labelling systems have been put into effect by various countries and groups with the aim of informing the consumer about the GMP content in products intended for consumption. Consequently, numerous analytical assays have been developed and validated over time with the aim of identifying and quantifying transgenic components in the various agri-food chains. A range of DNA-based methodologies have been developed using, among others, PCR, arrays, sequencing, and biosensor technologies. Currently, most of the validated assays are based on the qPCR method based on TaqMan probes. However, this analytical sector has shown strong interest in the adoption of dPCR assays, as reviewed by Demeke and Dobnik [8]. From the analysis of the literature of the last years, it is evident that a major sector in which dPCR has found application is the identification of GMP components in raw materials, as well as in derived food and feed. Thirthy-four percent of the published studies considered in this review were in fact focused on this goal.

The starting point of dPCR assays for GMP detection is the availability of primers/probes targeting a specific transgenic sequence and of primers/probes targeting an endogenous gene of the plant. This latter is not the modified gene. It is a single-copy, native gene that is strongly conserved in the species object of the analysis. The right choice of such an endogenous sequence is of great significance because it provides vital information on the stability and reliability of the detection system and it permits the quantification of genetically modified ingredients in mixtures. The analytical pipeline for the quantification through dPCR of GM components is not substantially different from that for qPCR and can be summarized in the following modules:

i. Sample preparation and DNA extraction.
ii. Digital PCR analysis for the amplification of the target transgenic sequence.
iii. Digital PCR analysis for amplification of a native, reference sequence.
iv. Data evaluation.

The ratio between transgene copy number and reference gene copy number provides the GM percentage present in a sample. However, EU legislation requires that the amount of GM content is estimated as a mass fraction, and a conversion factor was therefore established for each event to convert a copy number ratio into a mass fraction [9]

Table 1 summarizes some of the dPCR assays recently developed or evaluated for GMP traceability. In addition to the used instrumental platform and the plant species, the table reports the alfa-numeric identifier in the cases of authorized genetically modified lines (not available in case of not yet authorized experimental lines). Moreover, the plant endogenous gene(s) used as reference in the assay is reported.

Table 1. The table shows recently developed dPCR assays aimed at identifying and quantifying genetically modified plants and at characterizing transgenic lines.

Instrumental Platform	Plant Species	Genetically Modified Line	Endogenous Reference Gene(s)	Bibliography
Chamber-based digital PCR	*Zea mays*	NK603, MON810, MON863, Bt176, 3272, MIR162, MIR604	*Adh* and *hmg*	[10]
Droplet digital PCR	*Oryza sativa*	Kefeng-6	*Sps2*, *RBE4*, and *ppi-PPF*	[11]
Droplet digital PCR	*Zea mays* *Glycine max*	MON88017, MON87460, MON89034, MIR162 CV127, MON87701, and MON87705	-	[12]
Droplet digital PCR	*Oryza sativa*	TT51-1	*PLD*	[13]
Droplet digital PCR	*Glycine max*	A2704-12, 356043, 305423, and 40-3-2	*Lec-1*	[14]
Droplet digital PCR	*Brassica napus* *Glycine max*	OXY235 DP305423	*hmg* *Lec-1*	[15]
Droplet digital PCR	*Brassica napus*	HCN92	*Cruciferin*, *CruA*, *FatA*, and *hmg-I/Y*	[16]
Droplet digital PCR	*Glycine max*	MON87769, MON87708, MON87705, FG72	*Lec-1*	[17]
Droplet digital PCR	*Glycine max*, *Zea mays*	RR, MON89788, 2704, Bt176, Bt11, MON810, GA21, NK603, MON863, 59122, MIR604, TC1507, and T25	*Lec-1* *hmg*	[18]
Droplet digital PCR	*Zea mays*	IE034	*Adh*	[19]
Droplet digital PCR	*Saccharum officinarum*	Q208 and Q240	*ACT*	[20]
Digital PCR	*Glycine max*	40-3-2, MON89788	*Lec*	[21]
Droplet digital PCR	*Zea mays*	DAS1507, DAS59122, GA21, MIR162, MIR604, MON810, MON863, MON89034, NK603, T25, Bt11, and MON88017	*hmgA*	[22]

Table 1. *Cont.*

Instrumental Platform	Plant Species	Genetically Modified Line	Endogenous Reference Gene(s)	Bibliography
Droplet digital PCR	*Oryza sativa*, *Citrus*, *Solanum tuberosum*, *Zea mays*, *Lycopersicon esculentum*, *Triticum*	Non-commercial plants	*Rice-OsUBC*, *citrus-CsDHN*, *potato-StAAP2*, *maize-ZmADH1*, *tomato-SISYS*, and *wheat-PINb-D1b*	[23]
Droplet digital PCR	*Lolium*	Non-commercial plant	*LpCul4*	[24]
Droplet digital PCR	*Medicago sativa*	Non-commercial plants	-	[25]
Chamber-based digital PCR Droplet digital PCR	*Zea mays*	GA21	*Adh1*	[26]
Droplet digital PCR	*Zea mays*	MON810	*hmg*	[27]
Droplet digital PCR	*Nicotiana tabacum*	Non-commercial plants	*Ntactin* and *NtTubulin_1*	[28]
Droplet digital PCR	*Solanum tuberosum*	AV43-6-G7	*fru*	[29]
Droplet digital PCR	*Glycine max*	15 lines (authorized or with valid EFSA application)	*Lec-1*	[30]
Chamber-based digital PCR Droplet digital PCR	*Zea mays*	MON810, MON863, TC1507, MIR604, MIR162, GA21, T25, NK603, and BT176	-	[31]
Droplet digital PCR	*Zea mays*	MON863, MON810, DP98140, MIR604, GA21, MON89034, and MIR162	*hmgA*	[32]
Droplet digital PCR	*Arabidopsis thaliana*	Non-commercial plant	*AAP1*	[33]
Droplet digital PCR	*Triticum*	Non-commercial plant	*ssII-D* and *waxy-D1*	[34]
Droplet digital PCR	*Zea mays*	Certified reference materials	*hmg*	[35]
Droplet digital PCR	*Glycine max*	multitarget DNA molecule encoding for eight transgene soy traits	*Lec-1*	[36]
Droplet digital PCR	*Brassica napus*	Non-commercial transgenic lines	*CruA*	[37]
Droplet digital PCR	*Glycine max*	DAS-68416-4	-	[38]
Droplet digital PCR	*Zea mays*	DAS1507 and NK603	*hmg* and *Adh1*	[39]

Digital PCR approaches for transgene detection and quantification have been evaluated in cultivated GM crop and reference materials.

Various relevant points can be highlighted from the experiences made on dPCR-based GMP detection.

Iwobi et al. [12] compared qPCR and ddPCR performance on a panel of certified reference materials and GM samples arising from previous proficiency tests, and they demonstrated the applicability of ddPCR assays for the routine analysis of GM food and feed. Moreover, they demonstrated the better performance of ddPCR in comparison with qPCR in inhibitor-contaminated samples. The same results were obtained by Wang et al. [13] looking for transgenic rice in various processed samples. Other authors underlined the importance of selecting appropriate endogenous reference genes characterized by amplification efficiencies closely related to that of event-specific genes [11,16,20,34].

Several dPCR assays have been validated through proficiency studies in comparison with qPCR, and, in general, a similar performance has been found between the two techniques [12,18,19,21]. It has been found that sample pre-treatment prior to digital PCR can influence the results, and pretreatment-free detection is therefore preferential for achieving accurate results [26,31]. The convenience of digital PCR in real-life routine diagnostics has been verified on certified materials and analytical samples, and it has been found to useful for the routine quantification of GMP content in food and feed samples [12,23,27]. Multiplex assays for the quantification of several GM *Glycine max* lines were developed by Kosir et al. [30] and validated with an inter-laboratory trial. The assays performed well for key parameters such as the accuracy, robustness, and practicability and significant improvement in terms of cost efficiency has been demonstrated. The multiplexing of the assays therefore has the potentiality for further improvements of dPCR-based GMP traceability [32].

In conclusion, dPCR has several positive characteristics, such as its precision, accuracy even at very low target concentrations, suitability for routine analytics, and lower sensitivity to PCR inhibitors [8]. However, the main reason for the interest in this technique for GMP detection is its useful ability to perform an absolute quantification, independent of a reference standard/calibration curve. This means that differences in amplification efficiency due to matrix differences between a sample and its reference material do not affect the analytical results.

3. Transgenic Lines Characterization

A number of publications have reported the use of dPCR for the characterization of transgenic plants, here understood as experimentally transformed lines not proposed for cultivation and marketing. In this case, therefore, dPCR has a distinct use compared to what was reported in the previous paragraph. The purpose of dPCR analysis is, in fact, in this case not directed to the identification of transgenic components in food and feed but rather to the description and characterization of experimentally obtained transformed plants. In transformation projects, in fact, it is central to verify the success of transformation and to assess the inserted transgene copy number. Transformed lines carrying a single, full-length copy of the transgene are usually desirable to ensure a Mendelian inheritance of the transgene and to avoid silencing problems. Southern blot and qPCR analyses have been traditionally used to do such characterizations. However, the first technique is expensive and very laborious, and the second lacks the accuracy and precision needed to confidently characterize low copy number events. On the contrary, dPCR is emerging as a technology that is able to accurately identify transgene copy numbers, to discriminate between single and low transgene copies, and to do such characterization even in transgenic plants with large genomes. Moreover, the technique has been evaluated even for the determination of genome editing rates in CRISP/Cas9 (clustered regularly interspaced short palindromic repeats/associated protein 9) mutated plants [25]. Table 1 reports examples of dPCR applications to transformed plant characterization [19,20,23,28,33,37]. However, it is noteworthy that the published studies on this topic have apparently been much more numerous. The failure to enumerate other studies is probably due to the fact that in these works, the use of dPCR is not considered central and dPCR is therefore not included among the keywords, thus escaping the review.

Beyond this limit on our part, it can be concluded, from the identified experimental works, that the shared opinion is that dPCR is a particularly useful for the fast and accurate characterization of transgenic lines, able to identify both homozygous and hemizygous individuals among large number of transformants.

CNV refers to genome structural variations in which a specific DNA segment is repeated, and the number of repeats varies among different genotypes. In polyploids, CNV can also be defined as variations in allele dosage at a locus. The different allele dosages can be correlated with different phenotypes, e.g., barley genotypes with an increased copy number of HvCBF4 and HvCBF2 transcription factors showed greater frost resistance [40]. Recently, CNV has been recognized as a key mechanism for plant evolution and crop domestication, as reviewed by Lye et al. [41]. Cytogenetic techniques, especially fluorescence in situ hybridization (FISH), have been traditionally used to study CNV. However, the FISH technique can be limited by the subjective evaluation of images and the fact that the procedure is technically demanding and expensive. Moreover, it can efficiently identify only copy number variants of thousands of base pairs. Other hybridization-based techniques, such as array comparative genome hybridization (aCGH) and SNP microarrays, have been developed to study CNV in plants. Next generation sequencing (NGS)-based methods have been proposed for CNV discovery, and computational approaches have been developed to optimize output data. Even qPCR has been applied to estimate differences in CNV, showing two main limitations, i.e., many replicates are required to achieve sensitive discrimination of differences in copy number and a relative measure rather than an absolute quantification is obtained.

The results obtained in the leading field of human genetics have indicated that dPCR could serve as a sensitive and less technically challenging method in respect to those above-reported to detect CNV. It is a particularly promising approach, both in diploid and polyploid plants. Zmienko et al. [42] used dPCR to study the CNV variation naturally present in three *Arabidopsis thaliana* loci and found strong variations in their copy numbers among natural ecotypes, demonstrating the high plasticity of the *Arabidopsis* genome. These authors compared two different analytical approaches for CNV determination, namely MLPA (multiplex ligation-dependent probe amplification) and ddPCR and concluded that "dPCR should be the method of choice for discriminating loci with high copy numbers". Two studies applied dPCR to the high-level polyploid sugarcane, a challenging crop species because of its complex genetics. McCord [43] positively evaluated the ability of dPCR to identify genotypes with high copy numbers of the *Bru1* gene, characterized by a higher level of resistance to sugarcane brown rust. The information gained with such assay is now used in USDA breeding programs. Sun and Joyce [20] developed three dPCR assays to be used for the determination of ploidy level and CNV in different *Saccharum* species. Jouanin et al. [44] tested the accuracy of dPCR for the CNV analysis of the alfa-gliadin gene family in *Triticum aestivum*. The authors concluded that dPCR is suitable for the high-throughput screening of gene-edited and mutated wheat plants.

4. Expression Analysis and Regulation

Several technologies, such as real-time PCR, sequencing, and hybridization-based assays, are currently used for gene expression studies. The suitability of digital PCR for gene expression analysis has been demonstrated in several plant species, such as *Amaranthus cruentus* [45], *Beta vulgaris* [46], *Castanea* [47], and *Hordeum vulgare* [48]. The expression of genes involved in resistance to pathogens, in response to bioactive compounds, in starch synthesis, and in senescence developmental process have been considered. An increasing application of dPCR can be hypothesized in the field of cis-regulatory elements and epigenetics. In the sector of regulatory molecules, the more relevant role of dPCR can be in the quantification of long non-coding RNA regions and microRNAs. In this case, in fact, there is no need for standard or endogenous controls, and no competition between different targets can arise. Consequently, dPCR can ensure precise microRNA quantification and even the possibility to identify rare sequences. Interestingly, dPCR has been even applied to track dietary microRNAs. The levels of a

panel of *A. thaliana* microRNAs were evaluated in different *Plutella xylostella* tissues with the aim to study the cross-kingdom functions of microRNAs derived from host plants in insect herbivores [49].

5. Plant Species Traceability

Plant species traceability in food and feed chains can play a pivotal role in defending quality and safety. A dPCR assay is available to quantify *Triticum aestivum* and to differentiate this species from *Triticum durum*. This analytical tool has a great practical value in the Italian pasta production chain: the use of *Triticum durum* is, in fact, mandatory for pasta production, and *Triticum aestivum* is considered a contaminant whose percentage cannot exceed a maximum level of 3%. The assay developed by Morcia et al. [50] was demonstrated to suitable for *T. aestivum* traceability along the whole pasta production chain from grains to pasta. Digital PCR has been used as a tool to evaluate the quality and amplifiability of DNA extracted from a challenging matrix, such as olive oil [51]. Assays to quantify apricot kernels in marzipan and kidney beans in lotus seed paste were proposed, respectively, by Koppel et al. [52] and Dong et al. [53]. Starch used in the food industry can be obtained from different plant sources, such as potato, cassava, corn, and wheat. Additionally, depending on the raw materials used, starch can have different commercial values. Cassava starch is the main material in adulteration because of its lower price. To counteract potential fraud, Chen et al. [54] developed a dPCR assay for the specific detection of this plant species in starch products. Finally, to meet the need for quantification of allergens, a droplet digital PCR approach was shown to be reliable and sensitive enough to quantify the very common allergen soy in food [55].

6. Phytopathogens Diagnostics

dPCR is becoming an important new tool for use in the plant pathogen diagnostics and crop protection. Many examples of diagnostic assays ex novo developed or translated from similar qPCR assays have been recently published, as reported in Table 2. Several classes of pathogens have been targeted ranging from fungi and bacteria to viruses and phytoplasma. All the authors found several advantages over qPCR diagnostic assays, including absolute quantification without a standard curve, improved precision and accuracy, and more accurate quantitation. Moreover, the observed reduction of false negatives is critically important for the diagnosis of infections to be included in certification programs [56].

Table 2. The table shows recently developed dPCR assays aimed at identifying and quantifying plant pathogens.

Target Microorganism	Disease	Affected Crop	Reference
Candidatus Liberibacter asiaticus	Huanglongbing (HLB; yellow shoot disease)	*Citrus*	[56]
Candidatus Liberibacter asiaticus	Huanglongbing (HLB; yellow shoot disease)	*Citrus*	[57]
Acidovorax citrulli	Bacterial fruit blotch	*Cucurbitaceous*	[58]
Group 16SrIV phytoplasmas	Lethal yellowing (LY)	*Phoenix dactylifera*	[59]
Apscaviroid (apple chlorotic fruit spot viroid—ACFSVd)	Chlorotic fruit spots and bump-like symptoms on the skin of apples	*Malus*	[60]
Xanthomonas citri subsp. citri	Citrus bacterial canker	*Citrus*	[61]
Potato mop top virus	Potato mop top disease (tuber necrosis, internode reduction, foliar yellow spots, and plant chlorosis)	*Solanum tuberosum*	[62]
Ilyonectria	Black foot disease	*Vitis vinifera*	[63]

Table 2. *Cont.*

Target Microorganism	Disease	Affected Crop	Reference
Citrus yellow vein clearing virus (CYVCV)	Yellow vein disease	*Citrus*	[64]
Cadophora luteo-olivacea	Petri disease and esca of grapevine	*Vitis vinifera*	[65]
Fusarium graminearum, Fusarium culmorum, Fusarium sporotrichioides, Fusarium poae, Fusarium avenaceum	Fusarium head blight	Small grain cereals	[66]
Agrobacterium vitis	Crown gall	*Vitis vinifera*	[67]
Pepino mosaic virus (PepMV)	Fruit marbling, leaf, and stem necrosis	*Lycopersicon esculentum*	[68]
Aspergillus niger, Aspergillus welwitschiae, Aspergillus tubingensis, Aspergillus carbonarius	Bunch rots and mycotoxin production	*Vitis vinifera*	[69]
Phytophthora nicotianae	Root rot, crown rot, fruit rot, leaf infection, and stem infection	*Nicotiana tabacum*	[70]
Candidatus Phytoplasma asteris	Aster yellows (AY)	*Brassica*	[71]
Erwinia amylovora	Fire blight	*Malus*	[72]
Tilletia laevis	Common bunt	*Triticum*	[73]
Phytophtora infestans	Late blight	*Solanum*	[74]
Plasmodiophora brassicae	Clubroot	*Brassica*	[75]
Potato virus Y strains	Mosaic symptoms	*Solanum*	[76]
Spiroplasma citri	Citrus stubborn disease	*Citrus*	[77]
Erwinia amylovora and *Ralstonia solanacearum*	Fire blight of rosaceous plants, potato brown rot	*Solanaceae, Rosaceae*	[78]

Interestingly, Dreo et al. [78] demonstrated how ddPCR assays can be successfully performed on pure bacterial suspensions without any previous DNA extraction. This means that it is possible to quickly establish a correlation between the target concentrations and the starting CFUs (colony forming units). Moreover, this means that dPCR is even suitable for the preparation of in-house reference materials, which is particularly important in the field of plant pathogen diagnostics, where no reference materials are commercially available. Reverse-transcription (RT) digital PCR has been applied for the absolute quantification of viruses and viroids [60,64], with the aim of identifying the main transmission routes, main transmission vectors, and the plants carrying latent infections—a step of great relevance in the control and certification of nurseries' plants.

In addition to the identification of pathogens at the species level, dPCR can be applied to the identification of specific strains. An example is the study of Hua et al. [79], in which droplet digital PCR assays were developed to detect and characterize *Aspergillus* populations in soil with the aim to quantify the ratio between aflatoxigenic and atoxigenic strains. Zulak et al. [80] used dPCR to track two mutations in the *Blumeria graminis Cyp51* gene, which is able to confer high levels of resistance to triazoles in the field. The identification of such mutations in the pathogen field population has a key role in crop protection. In fact, the identification of resistant pathogenic strains carried out in an early stage of growth of the crop allows one to better modulate the consequent fungicidal treatments. Digital PCR has even been applied to the identification of non-pathogenic specific microorganism strains or populations present in the soil. A droplet digital PCR assay was developed by Xie et al. [81] for the quantitative detection of *Bacillus subtilis*, a typical plant growth-promoting bacterium, in rhizosphere samples. Its efficiency in the analysis of soil samples is one of the strengths of digital PCR that is considered less susceptible than qPCR to the PCR inhibitors present in DNA extracts. The success in DNA amplification is of paramount relevance for the soil samples [70] and dPCR can have a useful role in the study of environmental samples.

7. Other dPCR Applications

In addition to the macro categories discussed in the previous paragraphs, additional uses of the dPCR have been proposed both in applied and basic agricultural research. For example, dPCR has been applied to the single and/or multiple detection of SNPs linked to useful agronomic traits. An example in this sense is the study of Stevanato et al. [82], which aimed to quantitate an SNP able to distinguish between annual and biennial sugar beet flowering plants.

8. Minimum Information for Publication of Quantitative Digital PCR Experiments

In support of the scientific community that uses the dPCR, a dMIQE2020 (Minimum Information for Publication of Quantitative Digital PCR Experiments) was recently published by the dMIQE Group [83], and it is mainly intended to assist researchers in providing key experimental information and understanding of the associated experimental process. The first dMIQE guidelines were published in 2013 with the aim of improving the harmonization of dPCR results, data comparability, and reproducibility. The dMIQE guidelines provide a list of items to consider when publishing results from dPCR experiments. Such items can help in understanding, reproducing, and comparing dPCR results. The dMIQE guidelines therefore operate at the three levels: i) to ensure the replication of experiments, ii) to provide key information for researchers and reviewers to measure the technical quality of the analysis, and iii) to facilitate the harmonization of the reporting and comparison of the analytical data regardless of the dPCR instrumentation used. The adoption dMIQE2020, even by the scientific community involved in different plant science sectors, is a key point for the efficient use, exploitation, and further enhancement of dPCR technology.

9. Conclusions

In conclusion, digital PCR is, at least in the plant science sector, still a young but very promising technique.

The technology has many advantages and few disadvantages, which are summarized in Table 3. A disadvantage is that the assays can quantify within a narrower range of magnitude than qPCR. The major current limitation of the technique is its higher analytical costs and lower throughput compared to qPCR. Such disadvantages must be overcome with the refinement of technologies and the organization of multiplex reactions before dPCR competes with qPCR, which is now analytical labs' workhorse. Pecoraro et al. [84] underlined that the advantages of duplex and multiplex dPCR assays are "cost efficiency, due to the fact that multiple standard curves are not needed. In addition, for assays where the GM target(s) and the reference gene are analysed in the same partition (droplet or chamber), possible pipetting errors are reduced when relative concentrations are calculated".

Table 3. Main advantages and disadvantages of dPCR technology.

Advantages
Absolute quantification, no need to rely on reference or standard for several applications
Sensitivity and accuracy, useful to detect rare and low copy number targets
Suitability for allelic variant detection
Applicable to complex mixtures and complex background
Resistance to PCR inhibitors
Linear response to number of copies
Disadvantages
More expensive compared to qPCR, although questionable
Limited dynamic range of detection
Problems with very large amplicons
More complex work-flow compared to qPCR
More expensive instrumentation compared to qPCR

Speaking of the advantages, some are unique to the dPCR and particularly strong. One is the greater resilience of the reaction to the presence of inhibitors, and this characteristic therefore makes the technique particularly valuable for the study of environmental and soil samples [85]. Moreover, in general, authors agree in affirming that dPCR exhibits greater precision than qPCR with an equivalent or higher sensitivity. This is a valuable feature in the support of other technologies, e.g., next generation sequencing for which dPCR quantification is sufficiently accurate in counting amplifiable library molecules to justify elimination of titration techniques [86].

The main advantage of dPCR, however, remains the fact that the analysis operates an absolute quantification that is free from the need for standard reference curves for the quantification of the target sequence. This characteristic is fundamental in various fields of plant science—in particular for GMP detection, for which the transition from qPCR assays to dPCR ones is often considered advantageous. The EURL GM FF (European Union Reference Laboratory for GM Food and Feed, hosted by Joint Research Center, Ispra, Italy) is maintaining a database of species-, element-, and event-specific qPCR assays (see GMOMETHODS: EU Database of Reference Methods for GMO (genetically modified organism) Analysis [87] for more information), many of which have been validated in collaborative trials and are in current use in GMO laboratories around the world. It has been demonstrated that such qPCR assays can be converted into dPCR ones with only minor modifications. Such a transition is positively considered because, in contrast to qPCR, no standard curve is needed for dPCR in GMO quantification. This peculiarity also makes the technique fundamental to support qPCR, the accuracy and commutability of which may be improved with the implementation of standards and calibrants quantified by dPCR. In agricultural and environmental application fields, in fact, it is not trivial to have reference materials to build standard reference curves.

In human genetics, the technique has experienced great development in the last ten years, progressing from an expensive and niche approach to a plethora of applications. In plant science, as previously reported, the main applications are currently observed in the two sectors of GMO traceability [80] and pathogen diagnostics. However, other applications, such as the analysis of gene expression and the determination of structural gene variants and mutations, are present. It should be noted that some assays have been developed for the determination of plant species but not of variety. Moreover, dPCR is a very promising technology for epigenetic studies, targeted to characterize long and short non-coding RNAs and chromatin regulators. In food and environmental science, dPCR will likely find more and more applications in ingredient traceability, microorganism detection, microbial population description, the calibration of standards, and the analysis of inhibitory samples.

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A Chip Digital PCR Assay for Quantification of Common Wheat Contamination in Pasta Production Chain

Caterina Morcia [1,†], Raffaella Bergami [2,†], Sonia Scaramagli [2], Roberta Ghizzoni [1], Paola Carnevali [3] and Valeria Terzi [1,*]

1 Council for Agricultural Research and Economics, Research Centre for Genomics and Bioinformatics, Via San Protaso 302, I-29017 Fiorenzuola d'Arda PC, Italy; caterina.morcia@crea.gov.it (C.M.); roberta.ghizzoni@crea.gov.it (R.G.)
2 Coop Italia, Via del Lavoro, 6/8, I-40033 Casalecchio di Reno BO, Italy; raffaella.bergami@coopitalia.coop.it (R.B.); sonia.scaramagli@coopitalia.coop.it (S.S.)
3 Barilla S.p.A., Via Mantova 166, I-43122 Parma PR, Italy; paola.carnevali@barilla.com
* Correspondence: valeria.terzi@crea.gov.it; Tel.: +39-0523-983758
† These two authors contributed equally to the work.

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Abstract: Pasta, the Italian product par excellence, is made of pure durum wheat. The use of *Triticum durum* derived semolina is in fact mandatory for Italian pasta, in which *Triticum aestivum* species is considered a contamination that must not exceed the 3% maximum level. Over the last 50 years, various electrophoretic, chemical, and immuno-chemical methods have been proposed aimed to track the possible presence of common wheat in semolina and pasta. More recently, a new generation of methods, based on DNA (DeoxyriboNucleic Acid) analysis, has been developed to this aim. Species traceability can be now enforced by a new technology, namely digital Polymerase Chain Reaction (dPCR) which quantify the number of target sequence present in a sample, using limiting dilutions, PCR, and Poisson statistics. In our work we have developed a duplex chip digital PCR (cdPCR) assay able to quantify common wheat presence along pasta production chain, from raw materials to final products. The assay was verified on reference samples at known level of common wheat contamination and applied to commercial pastas sampled in the Italian market.

Keywords: pasta; *Triticum aestivum*; *Triticum durum*; genetic traceability; digital PCR; semolina; species

1. Introduction

Pasta production is a strategic chain in the Italian agri-food sector, covering around the 6% of total industrial output [1]. Italy is at the same time the world's leading pasta producer, with an annual production around 3.2 million tons and, in the same time, is the largest consumer of pasta (26 kg per capita). A pillar of Italian pasta production chain is the grain identity: The use of *Triticum durum* derived semolina is in fact mandatory for Italian pasta, in which *Triticum aestivum* species is considered a contamination that must not exceed the 3% maximum level, as indicated by Law n.580 of 1967 [2] and by subsequent Decreto del Presidente della Repubblica (D.P.R.) 187, 9 February 2001 [3] and D.P.R. 41, 5 March 2013 [4]. Traditional Italian pasta, according to such regulations, is therefore the result of the extrusion, rolling and drying of dough made exclusively from durum wheat and water. The choice of *Triticum durum* is based on its peculiarities, among others the hardiness of the caryopsis, the intense yellow color due to carotenoids, the gluten composition. Thanks to such specific properties, starch is not lost during cooking, avoiding sticking and ensuring a unique and authentic taste to pasta.

Beyond fraudulent behavior, dictated by the lower price of common wheat compared to durum, the purity of the semolina can also be compromised during the various processing stages of the supply chain, which range from harvesting in the field to storing the grains. Analytical methods have been proposed aiming at the detection and quantification of the possible presence of common wheat in semolina and pasta. In this perspective, over the last 50 years, various electrophoretic, chemical and immuno-chemical methods have been proposed aimed at detecting the purity of the semolina [5–9]. Such methods are based on the identification and quantification of specific protein, which, however, can be degraded by the high temperatures nowadays used to dry pasta. To overcome this gap and taking advantage of the remarkable thermic stability of DNA (DeoxyriboNucleic Acid), a new generation of methods, based on DNA analysis, has been developed during the last two decades. PCR (Polymerase Chain Reaction) based assays to identify common wheat by distinguishing it from durum one has been developed by Bryan et al. [10], by Arlorio et al. [11] and by Sonnante et al. [12], using respectively *Dgas44* gene sequence, puroindoline B and SSR (Simple Sequence Repeats) related sequences. Untargeted DNA fingerprinting through tubulin-based polymorphism (TBP) have been optimized by Casazza et al. [13] and by Silletti et al. [14] for the authentication of cereal species, including wheat and farro. qPCR assays for the quantification of *Triticum aestivum* species have been proposed by Alary et al. [15], Terzi et al. [16], Matsuoka et al. [17], and by Imai et al. [18]. These two last assays have been in-house verified and compared by Paterno' et al. [19], with the aim to select a taxon-specific assay useful for unauthorized GM (Genetically Modified) wheat detection in wheat samples. An inter-laboratory validation in collaboration with public and private laboratories has been even reported by Morcia et al. [20] to determine the performance parameters of a qPCR assay based on the primers designed on puroindoline-b gene by Alary et al. [15] and on low molecular weight glutenin encoding sequence by Terzi et al. [16].

Species traceability can be now enforced by a new technology, namely digital PCR (dPCR) which quantify the number of target sequence present in a sample, using limiting dilutions, PCR and Poisson statistics [21]. The PCR mix is compartmentalized across a large number of partitions or droplets containing zero, one or more copies of the target sequence. After endpoint PCR amplification, a partition can be positive ("1'', the presence of PCR product) or negative ("0'', the absence of PCR product). The absolute number of target nucleic acid molecules contained in the original sample before partitioning can be calculated directly from the ratio of the number of positive to total partitions, obtained using Poisson statistics. It is an absolute quantification strategy because there is not the need to have a standard curve as reference for quantification. In the past several years, dPCR has achieved progress in in agri-food sector, especially for GMO (Genetically Modified Organism) testing [21,22] and for pathogen diagnostics and, at more limited extent, to the detection of animal- and plant-derived ingredients in food adulteration control [23].

The aim of this work has been to develop a chip digital PCR (cdPCR) assay able to quantify common wheat presence along pasta production chain, from raw materials to final products. The assay was verified on reference samples at known level of common wheat contamination and applied to commercial pastas sampled in the Italian market.

2. Materials and Methods

2.1. Mono-Species Flour Samples Preparation and DNA Extraction

Certified *Triticum durum* (Claudio variety) and *Triticum aestivum* (Eureka variety) seeds were obtained from CREA DC (Tavazzano, Italy). Such first-reproduction seeds are controlled and certified both at species and variety levels. In major details, at species purity level, the maximum admitted contamination is of 7 seeds belonging to different cereal species/500 g of certified seeds, according to the Italian D.P.R. n. 1065, 8 October 1973. The seeds were milled using a Cyclotec (FOSS Italia S.r.l., Padova, Italy) at 0.2 mm grid diameter, avoiding any contamination between samples. Samples of

100% durum wheat semolina and 100% common wheat flour were separately stored at controlled temperature and humidity conditions until further use.

DNA were extracted from three biological replicates of milled *Triticum aestivum* and *Triticum durum* seeds using the DNeasy mericon Food Kit (Qiagen, Milan, Italy), that is based on an improved cetyltrimethylammonium bromide (CTAB) extraction of total cellular nucleic acids. The flour samples (2 g) were extracted according to manifacturer's instructions. The evaluation of quality and quantity of extracted DNA was done using Qubit™ fluorometer in combination with the Qubit™ dsDNA BR Assay kit (Invitrogen by Thermo Fisher Scientific, Monza, Italy).

2.2. Mixed Species DNA Samples Preparation

Triticum aestivum and *Triticum durum* DNA, extracted from the mono-species flours described in point 2.1, were mixed to obtain the following samples:

- *T.durum* DNA 99.7% + *T.aestivum* DNA 0.3%;
- *T.durum* DNA 98.5% + *T.aestivum* DNA 1.5%;
- *T.durum* DNA 97% + *T.aestivum* DNA 3%;
- *T.durum* DNA 95.5% + *T.aestivum* DNA 4.5%;
- *T.durum* DNA 70% + *T.aestivum* DNA 30%

2.3. Mixed Species Flour Samples Preparation and DNA Extraction

Common wheat flour was used to contaminate durum wheat semolina with the aim to produce durum wheat samples containing 0.3, 1.5, 3, 4.5, and 30% of common wheat. After weighing the common and durum wheat flour, samples containing different percentages of the two species were homogenized for 10 min. DNA were extracted from flours (2 g) with the DNeasy mericon Food Kit (Qiagen, Milan, Italy), as previously described. The evaluation of quality and quantity of extracted DNA was done using Qubit™ fluorometer in combination with the Qubit™ dsDNA BR Assay kit (Invitrogen by Thermo Fisher Scientific, Monza, Italy).

2.4. Reference and Commercial Pasta Samples and DNA Extraction

Four reference pasta samples were prepared by mixing tap water and wheat flours containing the following common wheat percentages: 1.5%, 3%, 4.5%, 10%. The samples were dried in oven at 80 °C for 1 hour, followed by 3 hours at decreasing temperature. Such desiccation thermal profile is those commonly used for commercial pasta preparation. DNA were extracted from two biological replicates of reference pasta using the DNeasy mericon Food Kit (Qiagen, Milan, Italy), Twenty commercial pasta samples of different brands were purchased from the market. The pasta samples were milled with M20 Universal Mill (IKA). Samples (2 g) were extracted in single replicate with the DNeasy mericon Food Kit (Qiagen, Milan, Italy), as previously described. The DNA obtained was measured using Qubit™ fluorometer in combination with the Qubit™ dsDNA BR Assay kit (Invitrogen by Thermo Fisher Scientific, Monza, Italy).

2.5. Primers and Probes

Primers and probes (Table 1) were designed using Primer Express 3.0.1 Software (Life Technologies Corporation). Each primer was checked for absence of self-complementarity and primer dimer formation with other primer pairs using the online tool Multiple Primer Analyzer (Thermo Fisher Scientific, Monza, Italy). Primer specificity was checked by blasting in EnsemblPlants (https://plants.ensembl.org/index.html) against the *Triticum aestivum* database.

Table 1. Primers and probes.

Name	Primer Sequence (5′-3′)	Gene	Target
GranoCO2- Forward	TGCTAACCGTGTGGCATCAC	*Triticum TaHd1*	*Triticum genus*
GranoCO2 Reverse	GGTACATAGTGCTGCTGCATCTG		
GranoCO2 probe	VIC- CATGAGCGTGTGCGTG -MGB		
TritA_APX Forward	AGGAGCGGCCGAAGCT	*Pinb-D1*	*Triticum aestivum*
TritA_APX Reverse	TGTGAAACATCGCTCCATCAC		
TritA_APX probe	FAM-AGCTCTTGCAAGGAT -MGB		

2.6. Real-Time PCR

The reaction mixture was prepared in a final volume of 25 µL consisting of 12.5 µL of SYBR Green PCR, 2× GoTaq qPCR Master Mix (Promega Italia, Milan, Italy), 0.25 µl of 100× Reference Dye (Promega Italia, Milan, Italy), 0.5 µL of each primer at 10 µM (final concentration 200 nmol), 4 µL of DNA template serial dilution (10, 5, 2.5, 0.5, 0.25 and 0.025 ng/µL) and water to 25 µL. Three technical real-time PCR replicates were done for each sample and control. The PCR mixture was activated at 95 °C for 10 min. Forty amplification cycles were carried out at 95 °C for 15 s followed by 60 °C for 1 min. A melting curve analysis was included in each run.

2.7. Chip Digital PCR

Chip digital PCR was performed using QuantStudioTM 3D Digital PCR System (Applied Biosystems by Life Technologies, Monza. Italy). The reaction mixture was prepared in a final volume of 16 µLconsisting of 8 µL QuantStudioTM 3D Digital PCR 2X Master Mix, 0.72 µL of each primer at 20 µM (final concentration 900 nmol), 0.32 µL of FAM and VIC-MGB probes at 10 µM (final concentration 200 nmol), 2 µl of DNA (40 ng/µL) and nuclease free-water. Also, a negative control with nuclease free-water as template was added. A total volume of 15 µL reaction mixture was loaded onto the QuantStudioTM 3D Digital PCR chips using QuantStudioTM 3D Digital chip loader, according to manufacturer protocol. Amplifications were performed in ProFlexTM 2Xflat PCR System Thermocycler (Applied Biosystems by Life Technologies, Monza, Italy) under the following conditions: 96 °C for 10 min, 45 cycle of 55 °C annealing for 2 min and 98 °C denaturation for 30 s, followed by 60 °C for 2 min and 10 °C. End-point fluorescence data were collected in QuantStudioTM 3D Digital PCR Instrument and files generated were analyzed using cloud-based platform QuantStudioTM 3D AnalysisSuite dPCR software, version 3.1.6. Each sample was analyzed in triplicate.

2.8. Triticum aestivum Percentage Calculation

For the common wheat percentage calculation, we start from the absolute copies/µL yielded by the QuantStudioTM 3D Analysis Suite dPCR software. In our assay the *T. aestivum* target sequence is marked with FAM, whereas the taxon target sequence is marked in VIC. Equation 1 was used to calculate the percentage of common wheat copies in the sample, in which FAM stands for the number of FAM copies/µL and VIC for the number of VIC copies/µL:

$$\frac{FAM}{\frac{VIC-3*FAM}{2}+FAM}*100 \quad (1)$$

3. Results

3.1. Reference Samples

Several factors are important for accurate quantification of multiplexed assays, including target linkage, probe specificity and differential PCR efficiencies.

The absence of linking between the two targets has been evaluated through literature and bioinformatic analysis. Nemoto et al. [24] demonstrated, through Southern blot analysis, that the *Triticum TaHd1* gene is present in single copy on each A, B and D genomes of wheat and maps on long arm of chromosome 6. *Pinb-D1*gene maps in D sub-genome and is located on chromosome 5 at the Hardness (Ha) locus. The two targets are therefore not linked.

Primers/probes specificity have been preliminarily evaluated in qPCR, finding that TritA_APX assay gives a signal only in hexaploid wheat, whereas GranoCO2 assay gives a signal both in hexaploid and tetraploid wheats (including farro dicoccum and Kamut).

Amplification efficiency and reproducibility for each primer set were examined through a standard curve qPCR assay, using bread and durum wheat DNA dilutions (Figure 1). Efficiency of reactions were calculated from the slope using the formula E = 10−1/slope. The slope values obtained were of −3.44 for GranoCO2 primers, and of −3.17 was obtained for TritAPX primers. Amplification efficiencies were of 99.6 and 104%, respectively.

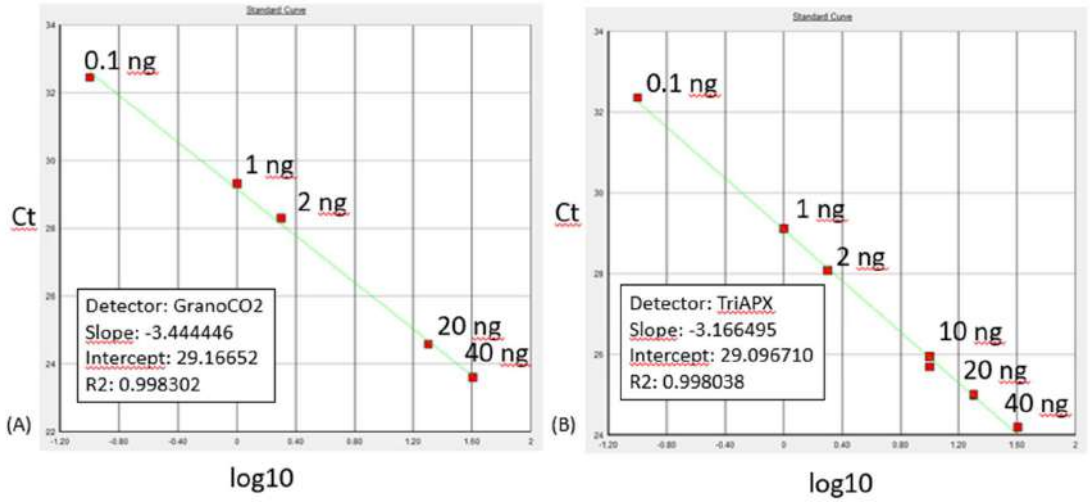

Figure 1. qPCR standard curves obtained after amplification of the DNA dilutions reported in the graph with GranoCO2 primers (**A**) and with TriAPX primers (**B**).

The duplex method was then optimized in cdPCR system for specificity on the reference samples described in Materials and Methods. The concentrations of primers and probes were optimized at 900 nmol and 200 nmol respectively and the annealing temperature was fixed at 55 °C. The resolution of the clusters (Figure 2) was obtained in absence of restriction digestion of the samples, therefore this time-consuming procedure was omitted from the protocol.

The mean common wheat percentages experimentally determined in "mixed flour" and "mixed DNA" samples in comparison with actual percentages are reported in Table 2. The SD values reported in the same table express the precision of the method, i.e., the closeness of agreement between replicate measurements. At 3% level, the SD values are <35% for all the samples and therefore the precision is acceptable, according to Codex Alimentarius Commission/Guidelines 74–2010 [25]. In Table 2 are even reported some values informative about the precision and the accuracy of the method, such as the coefficient of variation (CV), the absolute error and the relative error.

The trueness of the method is usually defined as the degree of agreement of the expected value with the true value or accepted reference value. In GMO testing the trueness must be within 25% of the accepted reference value [25]. The trueness of our method fits the purpose: The estimated concentrations over the dynamic range tested were within the ± 25% acceptable bias as recommended by GMO analytical guidelines [26]. In particular, at 3% level the experimentally determined percentages are very close to the true one. In the evaluated dynamic range, the LOD (Limit of Detection) of

the method has been found at 0.3% common wheat contamination, whereas the LOQ (Limit of Quantification) at 1.5% level.

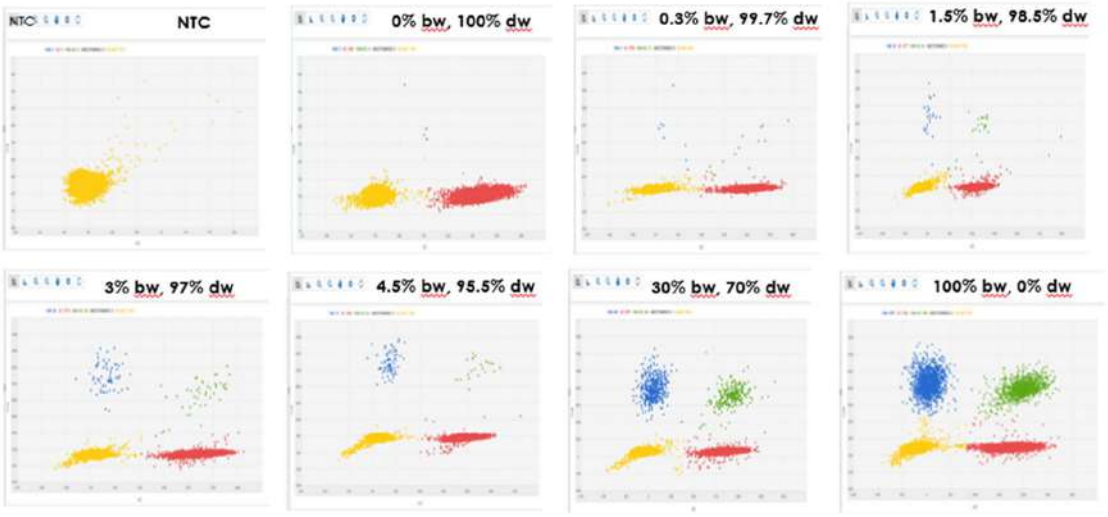

Figure 2. Two-dimensional scatter graphs generated by chip digital PCR (cdPCR) analysis of eight different samples. NTC (No Template Control) is a blank sample without DNA; The other samples are made of durum wheat (dw) DNA or common wheat (bw) DNA or a mix of the two, as indicated in the figure; In this graph a partition can fall into one of four possible clusters: negative partition that contain no amplified targets (yellow), single positive partition for *Triticum* genus (red), single positive partition for common wheat (blue) and positive partitions that contain a positive signal for both targets (green, double-positive partitions).

Table 2. Actual common wheat percentages in comparison with those experimentally determined in two different classes of samples. "Mixed DNA" samples were obtained by mixing DNA extracted from pure common and durum wheat species. "Mixed flour" samples were obtained by extracting DNA from of common and durum wheat flours mixed at different percentages). CV: Coefficient of variation.

Actual Common Wheat %	Mixed DNA					Mixed Flour				
	Mean Common Wheat %	Std Dev	CV	Absolute Error	Relative Error	Mean Common Wheat %	Std Dev	CV	Absolute Error	Relative Error
0	0.12	0.05	0.39	0.12		0.09	0.06	0.65	0.09	
100	105.00	7.00	0.07	5.00	0.05	94.40	6.85	0.07	5.60	0.06
0.3	0.43	0.05	0.12	0.13	0.43	0.37	0.12	0.34	0.07	0.23
1.5	1.37	0.07	0.05	0.13	0.09	1.43	0.28	0.19	0.07	0.05
3	3.06	0.05	0.01	0.06	0.02	2.86	0.32	0.11	0.14	0.05
4.5	4.50	0.04	0.01	0.00	0.00	3.93	0.51	0.13	0.57	0.13
30	25.90	0.46	0.02	4.10	0.14	24.90	1.68	0.07	5.10	0.17

The Pearson's r between the expected and calculated common wheat percentages were determined in mixed DNA samples and in mixed flour samples. The correlation values found are respectively of 0.9985 and of 0.9993. Extracting DNA from mixed flours and their subsequent amplification is much more realistic model of real foods, rather than mixing DNA from different species/samples. However, the preparation of mixtures of flours can be potentially affected by weighting errors and by heterogeneity problems, due, for example, to variation in granulometry, in mixing and blending. On the other hand, DNA mixtures can be affected by errors in DNA quantification and mixing. Therefore, with the intent to minimize the inaccuracy of the reference materials we decided to prepare two series

of blends using the two different options. After analyses, the two classes of reference materials gave the same results. No statistically significant differences were found among mean common wheat % values determined from mixed DNA samples and from mixed flours. It is therefore possible to conclude that the two classes of reference materials prepared worked in agreement.

Since 3% common wheat threshold is in percentage of mass ratio (% *m/m*) and since the analytical output is in number of common wheat and taxon target copies, a conversion factor is needed. This conversion factor, CF, mainly depends on the zygosity, but even on differences linked to DNA extraction and varieties. CF for GMO detection is available for each CRM (Certified Reference Material) [21].

For our homozygous samples, for which certified reference materials are not available, a conversion from % (copy/copy) to % (m/m) can be hypothesized. This same approach has been used in the study of Dong et al. [23] aimed to quantify kidney bean in lotus seed paste.

In 3% common wheat reference samples, a mean percent recovery of 100.44 has been obtained, that fully fits with the acceptable range for major components in low complexity matrices (95–105%).

3.2. Reference and Commercial Pasta Samples

The applicability of duplex dPCR assay to pasta was evaluated in two different groups of samples: 4 reference pasta samples prepared in our laboratory and contaminated with 1.5%, 3%, 4.5%, and 10% common wheat and on 20 pasta samples of different brands commercialized in Italy.

The results are reported in Figure 3, from which it can be observed that the duplex dPCR assay performs well on reference pasta, with a correlation value of 0.99 among actual and measured percentages and a mean relative error of 0.07.

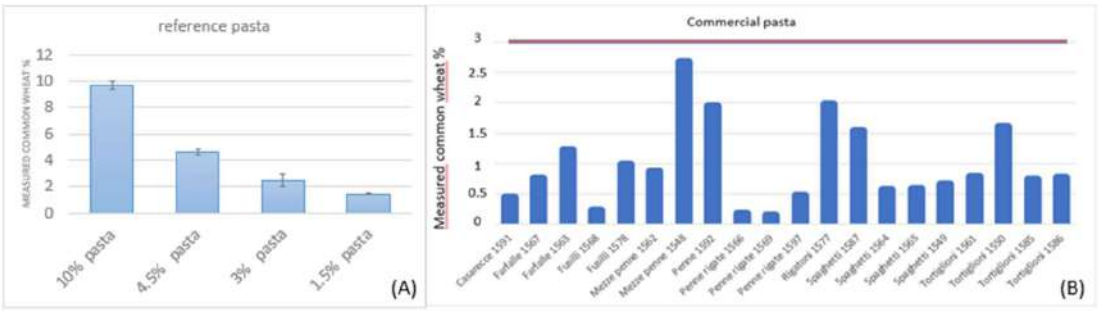

Figure 3. Common wheat percentages determined in 4 reference pasta (**A**) and in 20 commercial pasta samples (**B**) with duplex digital PCR (dPCR) assay. In (A) the percentages values before the word "pasta" indicate the common wheat contaminations. In (B) the red horizontal line indicates the maximum level of common wheat contamination allowed by law.)

As previously introduced, a body of Italian laws and regulations rule the product named "pasta" [2–4]. The denomination "pasta" strictly defines a product obtained after drawing, rolling and subsequent drying of a dough exclusively made from durum wheat (flour or semolina or whole semolina) and water. In the final product the humidity must not exceed 12.50%. The production of pasta with common wheat flour is forbidden, but a maximum level of 3% common wheat flour is tolerated as result of accidental contamination during the production chain. The inclusion of ingredients different from durum wheat and water is reserved to "special pasta". The special pastas must be offered for sale in Italy with the name durum wheat semolina pasta supplemented by the mention of the ingredient used and, in the case of several ingredients, of that or the characterizing ones. Anyway, even in special pasta, common wheat is a contaminants. The special pasta represents a minor sector of Italian pasta production and consumption. Therefore, as representative of the market, pasta of different brands has been considered in this study. The analyzed samples were all labelled as "pasta" and all reported, as ingredients, durum wheat and water. According to Italian laws, a maximum 3% common wheat

presence is expected. All the commercial samples have been found below the 3% common wheat contamination threshold. The analytical data confirm that all the samples comply with the Italian laws.

4. Discussion

We have developed a duplex chip digital PCR analytical protocol to identify and quantify common wheat contamination in pasta production chain. The reason for developing such new assay is related to dPCR particularities. In comparison with conventional end-point PCR and qPCR, this technique has been reported to have many advantages (reviewed by Demeke et al. [27]), the major the absolute quantification of a target without reference to a standard/calibration curve. This fact reduces the errors deriving from the comparison of different matrices, i.e., the calibrant and the test sample. Moreover, because of the high-level sample partitioning, dPCR is less sensitive to PCR inhibitors and the results obtained are potentially very precise and accurate [27,28]. Thanks to the high resilience to inhibitors, the efficiency and the reproducibility on different platforms, dPCR is candidate as higher-order reference measurement methods and as the method for value assignment of reference materials [28]. On the other hand, a limitation of such approach is that it is more expensive than qPCR, but the use of multiplex approaches moves the scales in favor of dPCR [27]. From a technology transfer point of view, both the pasta industry and the large consumer cooperative, between the other involved in this work, expressed interest in developing and applying a dPCR strategy for control pasta chain. The key control points are in the passage of the grains from stackers to the mills, of semolina batches from the mills to the pasta factory and in the final product, the pasta. The pasta chain stakeholders interested in such analytical tool are therefore the farmer associations, the stackers, the mills, the pasta industry, the consumer associations and the public and private control bodies. All the stakeholders have the interest to share a method for common wheat contamination control in grains, semolina, and pasta. Several assays has been developed and validated for such purpose, but are all dependent on a calibration curve and suffer from the loss of certified reference materials for the construction of such curves. DigitalPCR, that works without the need of calibrants, can fill this gap. It can in fact be proposed as method for the validation of reference materials to be used for qPCR standard curves and as higher order reference measurement method. This hypothesis to apply dPCR technology to prepare reference materials has been advanced by other authors, e.g., Mehle et al. [29] in plant pathogen detection, by Dong et al. [30] in environmental microbiology and by Pavšič et al. [31] in microbial diagnostics. The potential for synergy of qPCR and dPCR has been underlined by Debski et al. [32]. in the field of medical diagnostics. In conclusion, the opportunity to complement and strengthen the cheaper qPCR analyses justify the higher cost of dPCR assays.

Our cdPCR assay is based on duplex non-competing reactions: two amplicons are generated from two primer sets and the signal generated from a probe specific for each amplicon enable to distinguish the two targets within a single reaction. Such concurrent amplifications reduce technical errors, reagent and time needed. One of the target is a D-genome specific genic sequence and the other a *Triticum* specific genic sequence present in A, B and D genomes. This taxon-specific assay was designed on *TaHd1* gene sequence. Such gene, involved in the photoperiodic flowering pathway, has been demonstrated to be present in single copy in each of the A, B and D *Triticum* genomes [20]. The bread wheat specific assay was designed on *Pinb-D1*, a single-copy gene encoding for puroindoline b protein [15,33]. This gene belongs to the Ha locus, occurring only on chromosome 5D in common wheat [26]. Accordingly, we have developed the formula reported in Materials and Methods for the common wheat % calculation. In the formula we have considered:

- The different level of ploidy between common wheat (hexaploid, with the three A, B and D genomes) and durum wheat (tetraploid, with A and B genomes);
- the fact that *TaHd1* gene is present in single copy/A, B and D haploid genomes;
- the fact that *Pinb-D1* gene is present in single copy/D haploid genome; and
- the comparable amplification efficiency of the two targets

The *Pinb-D1* gene sequence has been used to target common wheat in cqPCR assays previously developed, whereas the *TaHd1* gene sequence has never been used in pasta authenticity assessment.

As verified on reference samples, the proposed protocol highly performs to track 3% common wheat contamination, that is the critical value fixed by law as limit between accidental contamination and fraud. Its applicability has been evaluated on reference and commercial pasta samples. In conclusion, a cdPCR duplex assay has been developed to control pasta production chain from an economically motivated adulteration, that is the use of cheaper ingredient (i.e., common wheat) instead of durum wheat for pasta manufacturing. It is possible to quantify the mass of common wheat directly in flours and in highly processed food, such as pasta. The inter-laboratory validation of the method can be proposed as further step.

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Article

Digital PCR for Genotype Quantification: A Case Study in a Pasta Production Chain

Caterina Morcia [1], Valeria Terzi [1,*], Roberta Ghizzoni [1], Chiara Vaiuso [1], Chiara Delogu [2], Lorella Andreani [2], Andrea Venturini [2], Paola Carnevali [3], Pier Paolo Pompa [4] and Giorgio Tumino [5]

1 Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria-Centro di Ricerca Genomica e Bioinformatica (CREA-GB), Via San Protaso 302, 29017 Fiorenzuola d'Arda, Italy; caterina.morcia@crea.gov.it (C.M.); roberta.ghizzoni@crea.gov.it (R.G.); chiara.vaiuso97@gmail.com (C.V.)
2 Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria-Centro di Ricerca Difesa e Certificazione (CREA-DC), Via Emilia km 307, 26838 Tavazzano, Italy; chiara.delogu@crea.gov.it (C.D.); lorella.andreani@crea.gov.it (L.A.); andrea.venturini@crea.gov.it (A.V.)
3 Barilla S.p.A., Via Mantova 166, I-43122 Parma, Italy; paola.carnevali@barilla.com
4 Nanobiointeractions & Nanodiagnostics, Istituto Italiano di Tecnologia, Via Morego 30, 16163 Genova, Italy; pierpaolo.pompa@iit.it
5 Plant Breeding, Wageningen University Research, Droevendaalsesteeg 1, 6708PB Wageningen, The Netherlands; giorgio.tumino@wur.nl
* Correspondence: valeria.terzi@crea.gov.it; Tel.: +39-0523-983758

Simple Summary: Digital polymerase chain reaction (dPCR) is a breakthrough technology able to provide an absolute quantification of the target sequence through the compartmentalization of the sample and independent amplifications of the numerous separate compartments. Such technology has recently found several applications in plant science; however, to the best of our knowledge, it has never been applied until now for the detection and quantification of a specific plant variety along a production chain. As proof of concept, a dPCR assay targeted to the quantification of a durum wheat variety routinely used in an Italian premium pasta production chain has been developed.

Abstract: Digital polymerase chain reaction (dPCR) is a breakthrough technology based on the partitioning of the analytical sample and detection of individual end-point amplifications into the separate compartments. Among the numerous applications of this technology, its suitability in mutation detection is relevant and characterized by unprecedented levels of precision. The actual applicability of this analytical technique to quantify the presence of a specific plant genotype, in both raw materials and transformed products, by exploiting a point polymorphism has been evaluated. As proof of concept, an Italian premium pasta production chain was considered and a dPCR assay based on a durum wheat target variety private point mutation was designed and evaluated in supply-chain samples. From the results obtained, the assay can be applied to confirm the presence of a target variety and to quantify it in raw materials and transformed products, such as commercial grain lots and pasta. The performance, costs, and applicability of the assay has been compared to analytical alternatives, namely simple sequence repeats (SSRs) and genotype-by-sequencing based on Diversity Arrays Technology sequencing (DArTseq™).

Keywords: digital PCR (dPCR); simple sequence repeats (SSRs); genotype-by-sequencing (GBS); varietal confirmation; molecular traceability; durum wheat; pasta; quantification of variety; private allele; allelic discrimination

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1. Introduction

Traceability is currently considered an important issue for food supply chains in defense of different characteristics of a product, including quality and safety, healthiness, origin of production, and sustainability, among others. Several protocols, devices, and sensors have been developed supporting food traceability to answer to legal requirements

and/or voluntary regulations and certifications, as reviewed by Espineira and Santaclara [1]. The traceability is therefore a complex issue, with peculiarities for both food and feed production chains.

This study focuses on a particular traceability request for high-quality pasta production. Italian legislation requires pasta to be produced exclusively with durum wheat semolina. Soft wheat is considered a contaminant for this product and its accidental presence cannot exceed 3%, as indicated by Law *n*.580 of 1967 [2], and by subsequent Decreto del Presidente della Repubblica (D.P.R.) 187, 9 February 2001 [3] and D.P.R. 41, 5 March 2013 [4]. The pasta supply chain requires that the ingredients have to be checked at the level of the plant species used. The control of pasta production at the plant-species level is therefore a legal requirement.

At the variety level, no legal requirement is requested to check the presence of one or more specific cultivars, unless the label states that specification. However, wide variability exists among durum wheat cultivars from the technological and qualitative points of view [5,6]. Moreover, some cultivars are legacies of the past, linked to traditional uses, including bread and pasta [7]. Currently, the commercial interest in bread and pasta made from a single variety or with a prevalence of one or few specific varieties is growing [8]. Consequently, there is increasing interest in the tracking of specific genotypes along the supply chain, from seeds to grains and transformed products.

To ensure both the various players in the supply chain and consumers of the effective presence of a particular cultivar in the finished products, new approaches to track specific varieties are needed, to better defend and valorize specialty products. Many DNA-based technologies have so far been evaluated for plant-variety protection and registration [9] and for wheat genetic-diversity estimation [10]. Simple sequence repeats (SSRs), array-based genotyping, and genotyping-by-sequencing (GBS) are the most popular techniques to return certain identity of a cultivar. All three families of techniques, in order to limit the costs, are based on a reduction of the genome complexity, and are obtained with very different strategies. The genome complexity reduction in GBS is linked to the restriction enzymes used [11], while in SSR markers and SNPs (single nucleotide polymorphisms), arrays are linked to the starting set of genotypes [12]. SSRs have been evaluated as complementary traits in DUS testing of wheat [13]. Seven SNP arrays that are now available in wheat (Wheat 9K, 15K, 35K, 55K, 90K, 660K, and 820K SNP array) have been widely used mainly to detect trait-related genetic loci by QTL mapping and GWAS [14]. Among GBS technologies, Diversity Arrays Technology sequencing (DArTseq™), which starts from a smart reduction of the complexity of the genome to produce both sequence data and SNP markers, was recently applied to a diversity analysis of 80,000 wheat accessions by Sansaloni et al. [15].

The three technologies (SSRs, SNP array, DArTseq) are undoubtedly useful for genetic diversity-based studies and for varietal fingerprinting; however, the last two in particular seem too complex and time-consuming to be used in a production chain to track one specific genotype.

Digital PCR is a breakthrough technology able to provide an absolute quantification of the target sequence through the compartmentalization of the sample and independent amplifications of the numerous separate compartments. Such technology has recently found several applications in plant science, as reviewed by Morcia et al. [16]. However, to the best of our knowledge, it has never been applied until now for the detection and quantification of a variety along a production chain.

As proof of concept, a dPCR assay targeted to the quantification of a wheat variety routinely used in Italian premium pasta production chain has been developed. The performance, costs, and applicability of the assay have been evaluated and compared with other analytical technologies.

2. Materials and Methods

2.1. Study Workflow

The workflow of the study is summarized below:

1. Establishment of a working collection of 28 durum wheat varieties, selected from among the most extensively cultivated in Italian environments [17];
2. Selection of a durum wheat target variety (TV), whose name cannot be reported in accordance with the protection requirements of sensitive industrial data;
3. Genotype-by-sequencing through DArTseq analysis of the DNAs extracted from certified seeds of TV and of all the cultivars, included in the working collection, and establishment of a SNP database;
4. SNP screening directed to the identification of a private allele of the target variety;
5. Development of a chip digital PCR assay designed for such private allele to identify and quantify the target variety;
6. Evaluation of the applicability of the dPCR assay on reference grain, flour, and pasta samples;
7. Digital PCR analysis of five commercial grain samples;
8. DArTseq analysis of five commercial grain samples;
9. SSR analysis of five commercial grain samples;
10. Comparison among the fingerprinting methods.

2.2. Materials

Table 1 summarizes the seed, grain, flour, and pasta samples used in this work.

Table 1. The samples used in this work and the techniques used for their analysis.

Sample	dPCR	DArTseq	SSR
Working collection of certified seeds	+	+	+
100% TV flour	+	–	–
90% TV flour	+	–	–
80% TV flour	+	–	–
70% TV flour	+	–	–
60% TV flour	+	–	–
50% TV flour	+	–	–
40% TV flour	+	–	–
30% TV flour	+	–	–
20% TV flour	+	–	–
0% TV flour	+	–	–
Pasta 100% TV	+	+	+
Pasta 90% TV	+	+	+
Pasta 70% TV	+	+	+
Pasta 50% TV	+	+	+
Pasta 20% TV	+	+	+
Grain commercial lots	+	+	+

2.2.1. Seed Samples

Certified seeds of the durum wheat working collection (varieties: Achille, Antalis, Anvergur, Aureo, Babylone, Bronte, Claudio, Core, Iride, Fabulis, Kyle, Kronos, Levante, Maestrale, Marco Aurelio, Miradoux, Monastir, Navigator, Normanno, Odisseo, Orizzonte, Pigreco, Relief, Rusticano, Saragolla, Simeto, Svevo, Tirex) were obtained from the Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria-Centro di ricerca Difesa e Certificazione (Tavazzano, Italy) seed repository or from the breeders responsible for their maintenance in purity. The seeds were milled using a Cyclotec (FOSS Italia S.r.l., Padova, Italy) at 0.2 mm grid diameter, avoiding any contamination between samples. DNA was extracted from three biological replicates of milled seeds using the DNeasy mericon Food Kit (Qiagen, Milan, Italy), according to manufacturer's instructions. The evaluation of quality and quantity of the extracted DNA was performed using a Qubit™ fluorometer in

combination with the Qubit™ dsDNA BR Assay kit (Invitrogen by Thermo Fisher Scientific, Monza, Italy).

2.2.2. Flour Samples

Flours were obtained from the target variety and nontarget variety (Odisseo cultivar) grains using a Cyclotec (FOSS Italia S.r.l., Padova, Italy) mill at 0.2 mm grid diameter, avoiding any contamination between samples. The flour samples reported in Table 1 containing TV percentages ranging from 90 to 20% were obtained by mixing TV and Odisseo flours. After weighing the two wheat flours, the samples containing different percentages of the two cultivars were homogenized for 10 min. DNAs were extracted from flours (2 g) with the DNeasy mericon Food Kit (Qiagen, Milan, Italy), as previously described. The evaluation of quality and quantity of extracted DNA was performed as described above.

2.2.3. Pasta Samples

Pasta samples were obtained by mixing tap water and wheat flours containing the following TV percentages: 100%, 90%, 70%, 50%, and 20%. The samples were dried in oven at 80 °C for 1 h, followed by 3 h at decreasing temperatures. Such a desiccation thermal profile is among those currently used for industrial pasta preparation. DNAs were extracted from two biological replicates of reference pasta using the DNeasy mericon Food Kit (Qiagen, Milan, Italy). The pasta samples were milled with an M20 Universal Mill (IKA®-Werke GmbH & Co., Staufen, Germany). Samples (2 g) were extracted with the DNeasy mericon Food Kit (Qiagen, Milan, Italy). The DNA obtained was measured as described in Section 2.2.1.

2.2.4. Commercial Grain Lots

Five commercial grain lots expected to consist of the target variety only were found, and 50 g of bulked grains were sampled from each lot. DNA was extracted from each sublot using the DNeasy mericon Food Kit (Qiagen, Milan, Italy) as already reported. These DNA samples were used for the SSR and dPCR analyses. For the DArTseq analysis, the DNAs were extracted from single seeds using the same method. Each commercial sample was represented for DArTseq analysis by 15 single seeds.

2.3. Chip Digital PCR Assay

Genotype-by-sequencing based on DArTseq technology and SNP screening for a TV private allele were performed according to Cibecchini et al. [18]. After screening, the SNP 12876 was selected. It is localized on chromosome 7A of durum wheat, where the reference allele is G and the alternative is T. The T allele is present only in the TV, while the other varieties tested were homozygous G/G. The BLAST of the SNP 12876 flanking sequence on the Svevo durum wheat reference genome highlighted even the presence of a homeologous region on chromosome 7B with high identity. The homeologous chromosome 7B held a nonpolymorphic G/G locus in all the tested varieties.

Primers and MGB probes were designed on the SNP 12876 sequence using the Custom TaqMan® SNP Genotyping Assay procedure (Thermo Fisher Scientific, Monza, Italy), and are available as Assay ID ANXGZMY, Catalog *n*. 4332077 (Thermo Fisher Scientific, Monza, Italy). In the dPCR assay developed, the TV target allele was marked with FAM, whereas the alternative, non-TV allele was marked with VIC.

Chip digital PCR was performed using the QuantStudio™ 3D Digital PCR System (Applied Biosystems by Life Technologies, Monza, Italy). The reaction mixture was prepared in a final volume of 16 µL consisting of 8 µL QuantStudio™ 3D Digital PCR 2X Master Mix, 0.4 µL of Custom TaqMan® SNP Genotyping Assay 40X (Catalogue number 4332077, Applied Biosystems by Life Technologies, Monza, Italy) containing primer and VIC/FAM-MGB probes, 1 µL of DNA (10 ng/µL), and nuclease-free water. In addition, a negative control with nuclease-free water as a template was added. A total volume

of 15 µL of reaction mixture was loaded onto the QuantStudio™ 3D Digital PCR chips using the QuantStudio™ 3D Digital chip loader, according to the manufacturer's protocol. Amplifications were performed in a ProFlex™ 2Xflat PCR System Thermocycler (Applied Biosystems by Life Technologies, Monza, Italy) under the following conditions: 96 °C for 10 min, 47 cycle of 60 °C annealing for 2 min, and 98 °C denaturation for 30 s, followed by 60 °C for 2 min and 10 °C. End-point fluorescence data were collected in a QuantStudio™ 3D Digital PCR Instrument, and the files generated were analyzed using cloud-based platform QuantStudio™ 3D AnalysisSuite dPCR software, version 3.1.6. Each sample was analyzed in triplicate.

2.4. SSR Analysis

For the analysis of TV, Odisseo certified seeds, commercial grain lots, and pasta samples, 14 SSR markers were used as described in the *International Rules for Seed Testing 2021* [19] for wheat varieties. For each DNA sample, the amplification was performed in duplicate.

Each SSR forward primer was labeled with a fluorescent dye on the 5′ end (6-FAM, VIC, HEX, NED, PET) and the 14 SSRs were amplified in two multiplex PCR reactions. PCR amplifications were performed in 10 µL reaction volumes containing 1 µL of 10 ng/µL genomic DNA as a template, 5 µL of 2X Type-it Multiplex PCR Master Mix (Qiagen, Milan, Italy), 1 µL 10x primer mix (2 µM each primer, Thermo Fisher Scientific, Monza, Italy), and 3 µL RNase-free water. The PCR program consisted of initial denaturation for 5 min at 95 °C, followed by 26 cycles of 30 s at 94 °C, 90 s at 57 °C, 30 s at 72 °C, and 1 cycle of 30 min at 72 °C. The PCR products were separated by capillary electrophoresis on the 3500 Genetic Analyzer (Applied Biosystems by Life Technologies, Monza, Italy). For each amplified fragment base pair size, height and peak area were measured by v5 GeneMapper software (Applied Biosystems by Life Technologies, Monza, Italy). The TV was quantified based on the relative quantities of its specific allelic fragment vs. all amplified alleles for the concerned locus, in terms of peak area [20,21]. The estimated percentage was calculated as the average of the peak area value obtained from the polymorphic loci. Figure 1 shows an example of polymorphisms between the TV and Odisseo cultivar and the different peak sizes according to different TV and Odisseo percentages.

2.5. DArTseq Analysis

According to point 8 of the study workflow cited in Section 2.1, DNAs extracted from 15 single seeds of five commercial grain samples, together with the DNAs extracted from 15 single seeds sampled from the TV certified seed lot, were sent to Diversity Arrays Technology Pty Ltd. (http://www.diversityarrays.com, accessed on 8 May 2021, Canberra, Australia) for sequencing, and SNP marker identification was done by DArTseq genotyping. The data were curated to include only SNP markers with NA <5% and MAF >5%. The final data set included 6249 SNPs. Euclidean genetic distances were calculated between each pair of samples and further used in clustering analysis (R stats: hclust, method = "average"). The intravarietal genetic distance present in the TV cultivar was calculated on the data obtained from the 15 certified single seeds. Such value was considered as the maximum intravariety distance present in the TV cultivar. Single seeds present in the five commercial samples with higher values of genetic distance were considered different from the TV. The TV percentages in the five commercial samples were then calculated as TV seeds/15 seeds.

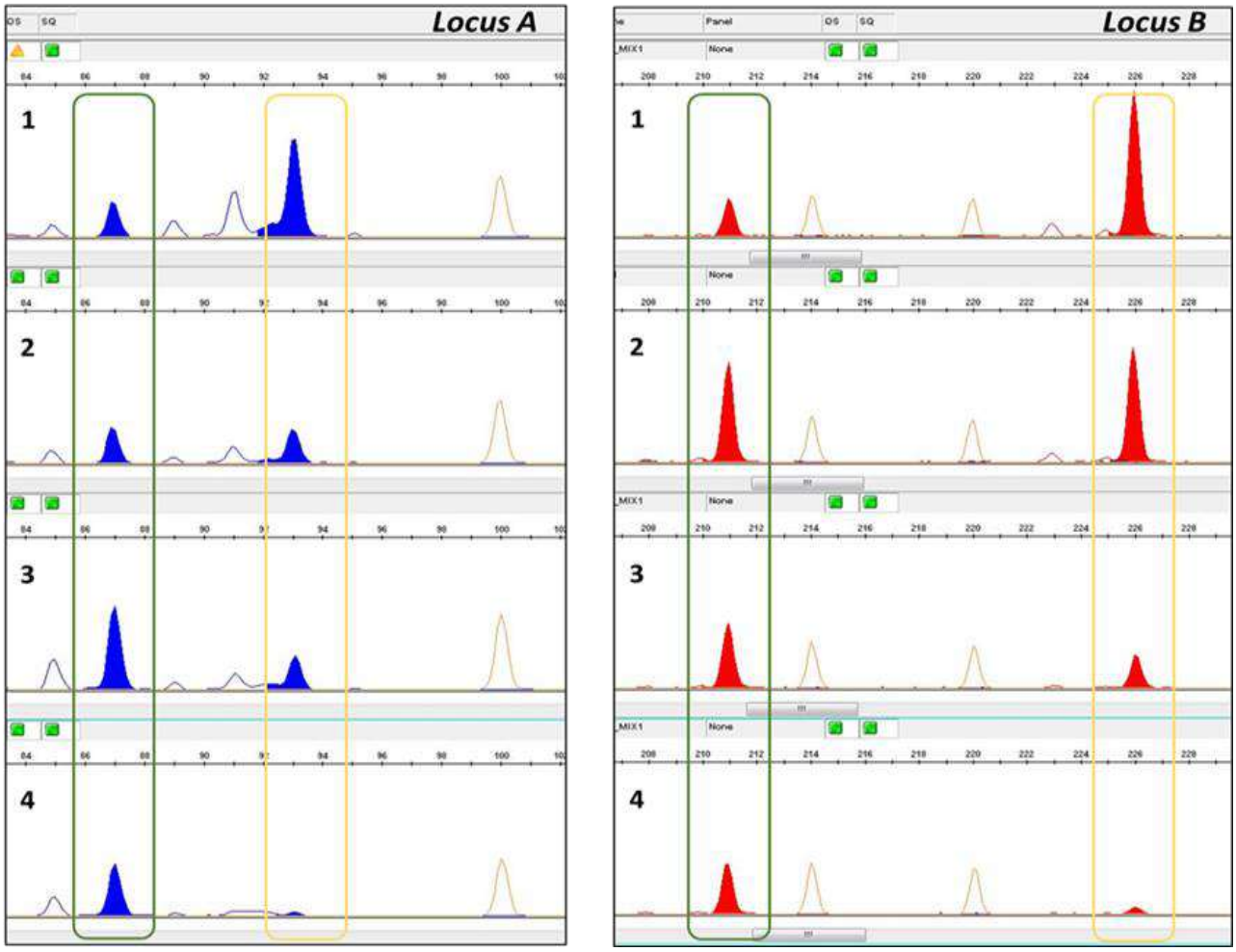

Figure 1. Electropherograms showing amplicons at two polymorphic loci between the TV and Odisseo. The green line highlights the TV alleles, while the yellow line highlights the Odisseo alleles. Plots of Locus A and Locus B from 1 to 4 show the electropherogram obtained from the pasta samples (1: TV 20%–Odisseo 80%, 2: TV 50%–Odisseo 50%; 3: TV 70%–Odisseo 30%; 4: TV 90%–Odisseo 10%).

3. Results

3.1. Digital PCR Assay

3.1.1. Specificity

The assay was developed with the aim to be a confirmation assay; that is, with the aim of verifying whether the target variety was actually present in the supply chain sample and in what quantities. According to this objective, the assay was built on target variety private alleles; that is, present in only one of the analyzed varieties. As reported in the Materials and Method section, in the dPCR assay, the mutated T allele (only present in target variety) was marked with FAM, whereas the wild type G allele, present in all the varieties, was marked with VIC. The dPCR assay developed was specific for target variety identification and quantification, as verified in DNA samples extracted from certified seeds of the TV cultivar and of the durum wheat varieties of the working collection. Examples of amplification patterns obtained are reported in Figure 2.

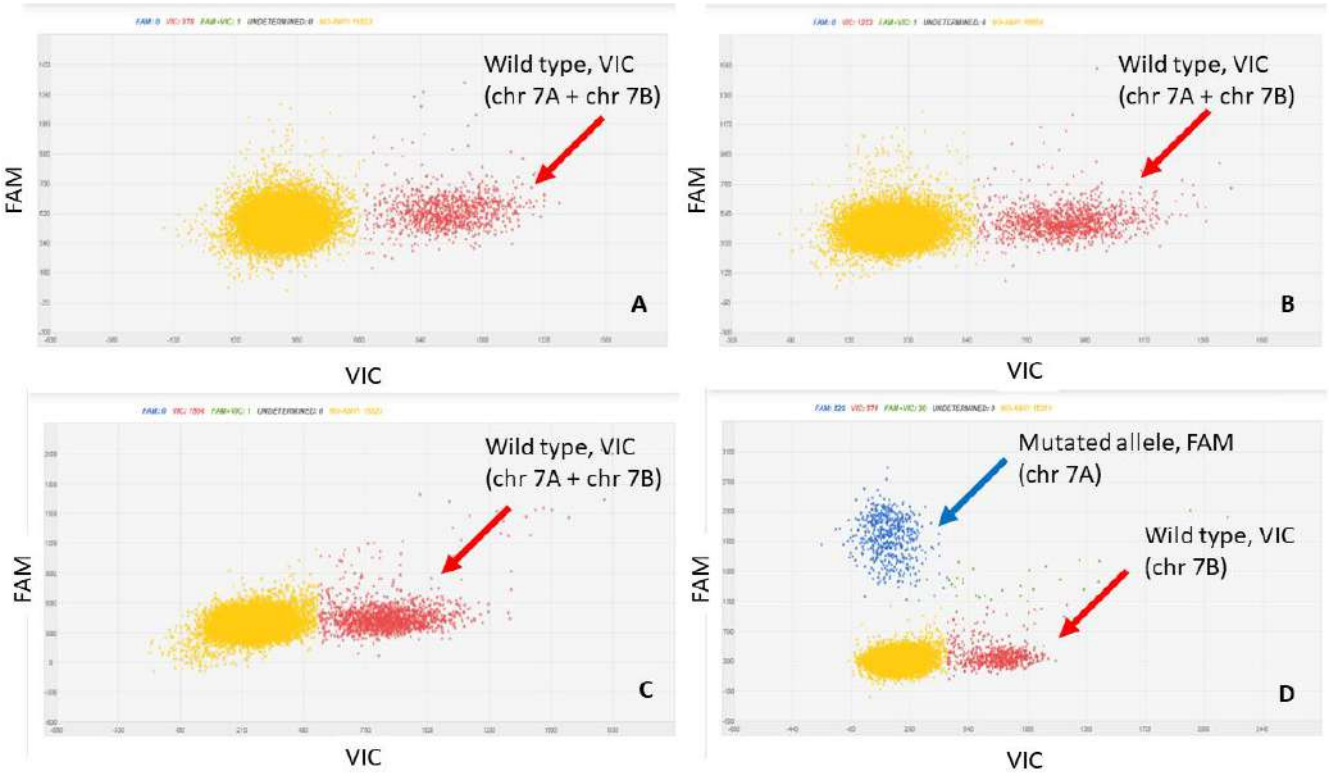

Figure 2. Two-dimensional scatter graphs generated by chip digital PCR (cdPCR) analysis of DNAs from the durum wheat varieties: Miradoux (**A**), Maestrale (**B**), Odisseo (**C**) and target variety (**D**). The G allele is marked by VIC (red cloud), and the T allele by FAM (blue cloud). All the patterns have a yellow cloud due to DNA-empty wells.

The dPCR assay on the 100% target variety sample detected both alleles with equal concentration in terms of copies/µL. The test in fact amplified the SNP target region on chromosome 7A (TT in target variety and GG in the other varieties) and a second locus on the homeologous chromosome 7B (nonpolymorphic, GG in all varieties). Supplementary Figure S1, shows the alignment of the chromosome 7A SNP 12876 sequence to the durum wheat Svevo reference genome. A high-identity region was mapped on homeologous chromosome 7B as well.

3.1.2. Precision, Accuracy, Trueness, and Sensitivity

The dPCR assay was applied to TV quantification in the reference flours obtained by mixing TV and non-TV flours in percentages ranging from 100% TV to 0% TV (Table 1). As reported in the Materials and Methods section, the TV target allele was marked with FAM, whereas the alternative allele was marked with VIC. Both FAM and VIC signals were present in equal quantity in the TV pure samples, because TV chromosome 7A carries the T allele, whereas TV chromosome 7B carries the G allele, as already reported. On the contrary, all the other varieties had VIC signals only, because G alleles were present in both chromosomes 7A and 7B. Moreover, in 100% TV samples, the G and T alleles were amplified with very similar efficiency, as demonstrated by the FAM/VIC ratio of 0.98 ± 0.4 experimentally found. Consequently, the curve reported in Figure 3 and the related polynomial function were calculated to correlate the TV percentage present in a theoretical sample and the expected FAM/VIC ratio. Such polynomial function (Figure 3) was experimentally validated in the flour and pasta reference samples reported in Table 1. Table 2 shows the obtained results.

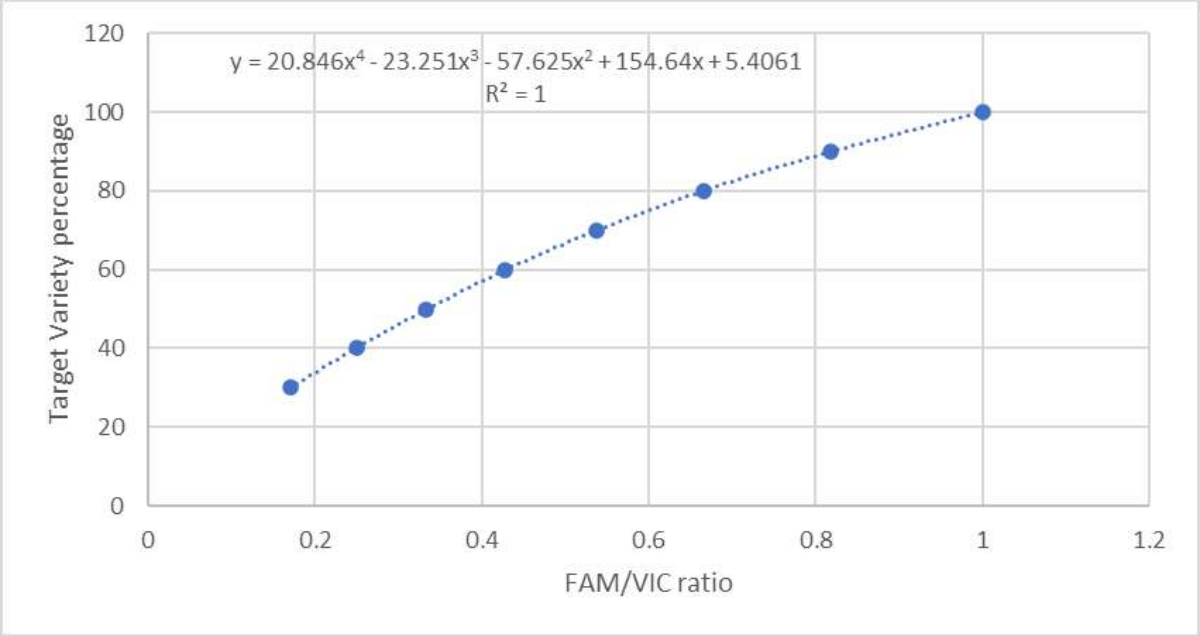

Figure 3. Theoretical correlation between TV percentage and FAM/VIC ratio.

Table 2. Actual TV percentages in comparison with those dPCR experimentally determined ("Mean TV% in flour") in mixed flour samples, prepared as described in the Materials and Methods section.

Actual TV% in Flour	Mean TV% in Flour	Std Dev	Absolute Error	Relative Error
100%	96.6	0	3.4	0.03
90%	90.9	0.07	0.95	0.01
80%	84.2	0.47	4.2	0.05
70%	70.3	0.56	0.3	0.004
60%	55.7	2.48	4.25	0.07
50%	48.7	1.63	1.25	0.025
40%	39.7	2.62	0.25	0.006
30%	31.4	1.84	1.4	0.04
20%	26.1	0.92	6.15	0.3
0%	0	-	-	-

The SD values (Table 2) were <35% for all samples and therefore the precision of the method was acceptable, according to the Codex Alimentarius Commission/Guidelines 74–2010 [22]. The accuracy of the method was evaluated by calculating the absolute and relative errors (Table 2). The trueness of the dPCR assay fit with the GMO analytical guidelines [23] because the estimated percentages over the dynamic range tested were within the ±25% acceptable bias, as recommended. A mean bias of 0.05 ± 0.09 was in fact found between the theoretical and experimentally determined estimated percentages in the flour samples. The sensitivity of the assay was found to be a 0.124% level of contamination. The Pearson's r between the actual and the experimentally determined percentages was 0.995 for the flour samples.

3.2. Digital PCR Assay Validation on Reference Pasta and Comparison with SSR Analysis

Four reference pasta samples, prepared as described in the Material and Methods section, starting from mixed TV + Odisseo flours, were analyzed with the dPCR technique. The results obtained are reported in Table 3. The Pearson's r between the actual and the experimentally determined percentages in pasta samples was 0.991.

Table 3. Actual TV percentages in comparison with those dPCR and SSR experimentally determined in reference pasta samples prepared with mixed TV and non-TV flours.

Actual TV% in Pasta	Mean TV% in Flour (dPCR)	Std Dev	Absolute Error	Relative Error
90%	88.7	1.34	1.25	0.01
70%	63.4	2.69	6.6	0.09
50%	48.4	2.05	1.55	0.03
20%	26.1	0.92	6.15	0.31
Actual TV% in Pasta	**Mean TV% in Flour (SSR)**	**Std Dev**	**Absolute Error**	**Relative Error**
90%	89	0.02	1	0.01
70%	66	0.01	4	0.06
50%	49	0.03	1	0.02
20%	20.5	0.01	0.5	0.025

The same four reference pasta samples also were analyzed with the SSR technique. As a preliminary step, the certified seed samples of the TV and Odisseo were genotyped using the 14 SSR markers to obtain the molecular profile. The Odisseo variety and the TV showed two different polymorphic alleles at two SSR loci, considered as "specific marker alleles". The pasta samples were genotyped and then screened at the two selected SSR marker loci to detect TV and non-TV marker alleles.

In all the pasta samples, it was possible to recognize the alleles of the TV and Odisseo varieties, and then proceed to the detection of the peaks parameters useful for the quantification. The values obtained were repeatable in the different loci and the two replicate samples of each mixture. The mean values were consistent with the actual values of the TV percentage in pasta as reported in Table 3. The Pearson's r between the actual and the experimentally determined percentages in the pasta samples was 0.998 with SSR analysis.

3.3. Digital PCR Assay Application to Commercial Grain Samples and Comparison with SSR and DArTseq Analyses

The dPCR assay was used to evaluate the TV percentages present in five TV-declared grain commercial lots, but suspected to be contaminated by non-TV varieties. The same samples also were evaluated with SSR and DArTseq approaches, with the same objective; i.e., to evaluate the TV percentages. Figure 4 shows the results obtained with the three different analytical methods.

The Pearson's r between the TV percentage determined in the commercial lots with dPCR and SSR was 0.991, whereas the Pearson's r between the TV percentages calculated according to the dPCR and DArTseq analyses were 0.852 and 0.834 for the SSR and DArTseq approaches. In particular, the DArTseq analysis seemed to overestimate the TV percentage in commercial lot B.

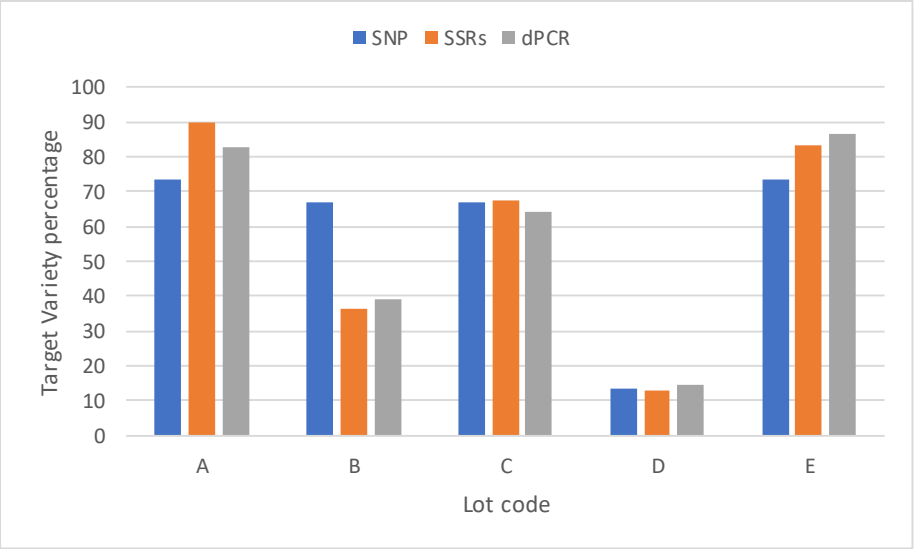

Figure 4. TV percentages found in five grain commercial lots measured with three different approaches (dPCR, SSR, and DArTseq).

4. Discussion

A new dPCR-based assay was developed to track a specific genotype. The assay can be applied to confirm the presence of such genotype and to quantify it in raw materials and transformed products. The working hypothesis, i.e., the possibility of exploiting a point polymorphism to confirm or not and quantify the presence of a genotype mixed with others, was fully confirmed. To the best of our knowledge, this is the first example of dPCR application to the quantification of a cultivar obtained after the conventional breeding procedure. Several dPCR assays have in fact been developed to track genetically modified events, but not conventional varieties [16,24]. This last goal raised the level of complexity related to the development of the assay. In fact, in the case of GM events, the target sequence is a priori known, requiring, for the purposes of authorization for cultivation and use, detailed information on the transgenic sequences inserted and on the surrounding genomic areas. On the contrary, in the case of a conventional variety, it is necessary to identify, as the first step of the workflow, one or few private polymorphisms that uniquely identify the target variety. To this purpose, a database of SNP profiles derived from DArT-based genotyping by sequencing characterization was exploited. A panel of durum wheat varieties was selected from among those extensively cultivated in Italy, and therefore at greater risk of being confused or harvested and stored together with the target variety. This therefore confirms the existence of other cultivars with the same sequence polymorphism used for this discrimination assay. However, the chosen polymorphism is effective for an application in the actual supply chain under consideration. Moreover, it can always be accompanied by further markers if such a need arises in the production chain.

From the obtained results, it can be concluded that the dPCR technique demonstrated to be usefully applied for varietal quantification not only in grains and flours, but also in processed products; e.g., in pasta, which can be subjected to high temperatures during drying. The reliability of dPCR for analysis of heat-treated samples has been previously demonstrated, and dPCR proved to be superior to real-time quantitative PCR in testing for genetically engineered events in such heat-treated samples [25].

To compare the dPCR approach with other analytical alternatives, a common set of commercial grain samples was analyzed not only with dPCR, but also with two other techniques, based on SSR and SNP markers.

SSR are consolidated markers in the varietal fingerprinting of many agricultural species and, for some of these, sets of internationally shared SSR markers have been identified. In the case of the *Triticum* species, including durum wheat, the International Seed Testing Association (ISTA) developed and published a standard protocol based on 14 SSRs

internationally agreed upon for variety testing and evaluation of seed lots [19]. The 14 SSR markers used showed a high level of polymorphism. Three to six different polymorphic loci were recognized in the commercial grain samples. In lot D, with an estimated TV percentage of 13.2%, 18 different alleles were scored, while the TV and Odisseo were differentiated by two loci. The ISTA protocol is based on a semiperformance approach: in case it is necessary to distinguish very similar varieties, the number of SSR markers can be increased to improve the discrimination power of the protocol. However, the multiplex PCR assay reduces the time and costs of the analysis. The quantification is the result of the qualitative–quantitative evaluation of the total polymorphic alleles obtained. The evaluation can be time-consuming when the nontarget varieties are numerous. In brief, due to the robustness of SSR in varietal fingerprinting, the CE (capillary electrophoresis) quantification method could give results close to those obtained with more innovative technologies.

SNPs are the markers of choice for mapping traits of interest, to assess the level of genetic diversity of a population, to study its structure, or to reconstruct genetic relationships among accessions. SNP markers were used to evaluate the varietal identity in commercial lots using a single-seed analytical procedure. Each commercial batch was represented by 15 seeds, which were genotyped individually. Lower correlations among SNP-based results and dPCR- or SSR-based ones in commercial lots were observed, in comparison with the very high correlations found between dPCR and SSR data for the same samples. This evidence leads to the hypothesis that this method, although able to give a careful description of the genotype, was more sensitive to sampling than the others. For SNP analysis, in fact, 15 seeds were genotyped, whereas for the dPCR and SSR analyses, the DNAs were extracted starting from 50 g of bulked and milled grains, which meant more than 1000 seeds. On the other hand, the SNP-based analysis was the key step for the identification of private alleles needed for dPCR assay development.

5. Conclusions

This is the first example of development and application of a dPCR assay aimed at confirming the authenticity of a supply-chain product. The approach was fully satisfactory in terms of precision, accuracy, trueness, sensitivity, and applicability. It can therefore open the way to subsequent applications in various production chains. Moreover, compared to the other reference techniques, it is the only one to have the characteristics close to a DNA barcoding, intended as taxonomic method that uses one short genetic sequence for identification at the species level. The same target genetic sequence can be exploited in fast methods, as reported for instance in [18]. This latter point-of-care (POC) method is user-friendly and fast, yet the dPCR assay is able to provide an accurate quantification. SSR analysis also proved to be effective in quantifying a target variety in raw materials and processed products, with sensitivity close to dPCR. On the contrary, the DArTseq approach, which is positioned at the opposite extreme of the concept of barcoding, cannot be proposed for efficient quantification of varietal components.

By focusing on analytical costs, digital PCR analysis is in the lower price class when compared to other analytical approaches. Table 4 shows the approximate analytical costs related to the characteristics of each assay for the quantification of a specific variety in one single sample. In addition to the methods reported in this work, other approaches that proved to be useful for genotype quantification (reviewed by Madesis et al. [26]) have been considered, such as high-resolution melting (HRM) single-assay platforms, qPCR and SSR, or high-throughput genotyping. The costs were partly deduced from rate tables present on the Italian market and were grouped into classes that included ranges of costs.

Table 4. Cost evaluation for the quantitative detection of a specific genotype in one wheat grain sample. Class A includes costs up to EUR 100, class B in the range of EUR 100–200, and class C above EUR 200. The need for a reference curve or for single-seed based analyses is reported as + (needed) or − (not needed). qPCR = quantitative PCR; Bar = barcoding; KASP = kompetitive allele specific.

Analytical Technique	Cost Class	Need of Reference Curve	Need of Single-Seed Analysis
Digital PCR	A	−	−
qPCR	B	+	−
Bar-HRM, SSR-HRM, SNP-HRM	B	+	−
SSR-peak area	A	−	−
SSR genotyping	C	−	+
KASPar SNP genotyping	C	−	+
SNP genotyping	C	−	+
GBS genotyping	C	−	+

Considering a single-sample analysis, the dPCR and SSR peak area evaluations are the cheaper methods, as they do not need to develop a reference curve for quantitation, or a single-seed based analysis. Increasing the number of samples to be analyzed at the same time, the qPCR and HRM-based methods also fall into class A of costs, since the development cost of the reference curve is amortized by the number of analytical samples. On the contrary, all the genotyping methods that need to analyze several sampled seeds to obtain a quantitative result maintain a high cost.

In conclusion, starting from the pilot work developed here, it can be said that the dPCR has a useful role in verifying and confirming the authenticity of agro-food products. This applicability is strengthened by the lower analytical costs and by the reduced analytical times compared to other methods, as well as by its precision in quantitative analyses in comparison with POC approaches.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/biology10050419/s1, Figure S1: Alignment of a 630 bp fragment (from 323 to 955 of the SNP12876 sequence) showing 95% identity with a region of chromosome 7B. SNP 12876 is green highlighted.

Author Contributions: Conceptualization, V.T., P.C., and P.P.P.; methodology, C.M., L.A., and G.T.; software, G.T.; validation, R.G., A.V., and C.V.; data curation, V.T.; writing—original draft preparation, C.M. and L.A.; writing—review and editing, V.T. and C.D.; funding acquisition, V.T. and C.D. All authors have read and agreed to the published version of the manuscript.

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A Fast, Naked-Eye Assay for Varietal Traceability in the Durum Wheat Production Chain

Giulia Cibecchini [1,2,†], Paola Cecere [1,†], Giorgio Tumino [3,†], Caterina Morcia [3], Roberta Ghizzoni [3], Paola Carnevali [4], Valeria Terzi [3,*] and Pier Paolo Pompa [1,*]

1 Istituto Italiano di Tecnologia, Nanobiointeractions & Nanodiagnostics, Via Morego 30, 16163 Genova, Italy; giulia.cibecchini@iit.it (G.C.); paola.cecere@iit.it (P.C.)
2 Department of Chemistry and Industrial Chemistry, University of Genova, Via Dodecaneso 31, 16146 Genova, Italy
3 Council for Agricultural Research and Economics, Research Centre for Genomics and Bioinformatics, Via San Protaso 302, 29017 Fiorenzuola d'Arda PC, Italy; giorgiotumino@hotmail.it (G.T.); caterina.morcia@crea.gov.it (C.M.); roberta.ghizzoni@crea.gov.it (R.G.)
4 Barilla S.p.A., Via Mantova 166, 43122 Parma PR, Italy; paola.carnevali@barilla.com
* Correspondence: valeria.terzi@crea.gov.it (V.T.); pierpaolo.pompa@iit.it (P.P.P.)
† These authors contributed equally to this work.

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Abstract: The development of a colorimetric mono-varietal discriminating assay, aimed at improving traceability and quality control checks of durum wheat products, is described. A single nucleotide polymorphism (SNP) was identified as a reliable marker for wheat varietal discrimination, and a rapid test for easy and clear identification of specific wheat varieties was developed. Notably, an approach based on the loop-mediated isothermal amplification reaction (LAMP) as an SNP discrimination tool, in combination with naked-eye visualization of the results, was designed and optimized. Our assay was proven to be effective in the detection of adulterated food products, including both substitution and mixing with different crop varieties.

Keywords: durum wheat variety; genetic traceability; single nucleotide polymorphisms; colorimetric tests; authenticity; point of care test

1. Introduction

Food traceability currently has a key role in the agrifood sector, bringing many benefits to both industries and consumers, such as supply chain optimization, improvement in food safety and quality, and an increase in controls and in the consumers' confidence. Mandatory traceability regulations have been established by several countries with different level of stringency for both domestic and imported commodities and agrifood products (for a review, see Charlebois et al., 2014 [1]). Mandatory traceability actions are flanked by voluntary ones, characterized by the identification of standards related, for example, to the valorization of traditional food products and local economy development [2]. In this framework, the world leader in Italian pasta manufacturing has started a pilot study equipping its products with a digital passport, i.e., a QR code capturing the entire food journey from "field to fork" [3]. Such an approach can enhance transparency and safety, and creates a connection between the consumers and the regions where their food was produced. A pillar of Italian durum wheat pasta production chain is, however, the grain identity, which is mandatory at the species level. The use of durum wheat semolina is, in fact, compulsory for Italian pasta, in which the *Triticum aestivum* species is considered a contamination that must not exceed the 3% maximum level [4]. However, the quality of the end products can be related not only to species, but also to the cultivar [5]. At the industrial

level, pasta is commonly made by mixtures of different durum wheat cultivars. At the same time, there is an increasing presence in the market of high-value mono-varietal pasta from both local producers and industrial ones. In such productions, there is great interest in developing a voluntary traceability system to identify specific varieties along the production chain.

Varietal fingerprinting approaches have been developed from mainly to protect intellectual property (IP) of wheat variety to guarantee plant breeders' rights (PBR). Distinctness, uniformity, and stability are among the basic requirements for a variety. The traditional morpho-physiological descriptors, collected during the whole plant life cycle, are classically used for such evaluation purposes. In a further step, morphological descriptors have been flanked by biochemical markers, e.g., the isoforms of seed storage proteins. Such markers can be informative when applied to the grains, but are not effective on transformed products, in which technological processes can impair the proteins' integrity. The subsequent advancement in varietal fingerprinting was based on the introduction of DNA markers that are independent of the environmental influence and available in theoretically unlimited numbers. Many classes of DNA molecular markers have been developed, ranging from inter simple sequence repeats (ISSRs) to amplified fragment length polymorphism markers (AFLP) and random amplified polymorphic DNA (RAPD), as reviewed by Pasqualone, 2011 [6]. SSRs (simple sequence repeats), considered the second generation of molecular markers and characterized by a simple technique, high polymorphism rate, and robustness, have been widely employed in wheat [7,8]. SNPs (single-nucleotide polymorphisms) are the third generation of molecular markers, with several advantages, including high frequency across the whole genome, ease of detection, and cost efficiency [9]. Classical techniques for SNP genotyping are currently sequencing, SNP array, or PCR-based methods, such as the restriction fragment length polymorphism (RFLF) and amplification refractory mutation system (ARMS). In particular, for the wheat genome, SNP arrays are available and permit the identification of 9000 to 90,000 polymorphisms [10–12]. Moreover, with the development of next-generation sequencing (NGS) technology, increasing numbers of SNPs have been discovered, building the basis for the development of diagnostic SNP barcodes [13]. Now, the "universalization and minimization of SNP number without compromising identification accuracy is the major challenge in development of varietal profile by rapid genotype assay", as reported by Singh et al., 2019 [14]. This step of identifying a small panel of informative SNPs is accompanied by the need to have efficient and low-cost techniques for their identification in large numbers of samples. However, classical methods are time consuming, expensive, and require costly instrumentations and specialized personnel, limiting their practical applicability for quality control screenings in the food supply chain. A rapid, on-site technique for SNP discrimination would thus be of great industrial interest. In this work, starting from a detailed SNP analysis on a panel of durum wheat, we set up a pilot study aimed at developing a point of care (POC) testing method to track, in a fast and inexpensive way, the presence of a specific wheat variety (Aureo) in grains and flours. In particular, we first identified an SNP allele specific for the focus variety, then we developed a portable colorimetric method based on the loop isothermal amplification reaction (LAMP) technique [15,16]. Our assay enables on-site, rapid quality control analyses by untrained personnel through simple visual inspection.

2. Materials and Methods

2.1. Plant Material and DNA Extraction

The following 28 durum wheat varieties were used: Iride, Rusticano, Saragolla, Odisseo, Maestrale, Bronte, Antalis, Fabulis, Core, Svevo, Orizzonte, Aureo, Achille, Monastir, Claudio, Tirex, Pigreco, Normanno, Marco Aurelio, Relief, Miradoux, Babylone, Simeto, Anvergur, Navigator, Levante, Kyle, and Kronos. Moreover, the tetraploid *Triticum turanicum*, variety QK-77 (traded as Kamut®), was included in the analysis. All of the varieties were certified foundation seeds produced directly from the breeder responsible for their maintenance in purity. Ten certified seeds of each variety were sown in duplicates in pots, and the foliar tissues were harvested at the three-leaves stage. DNA samples

were isolated and purified using the cetyl trimethyl ammonium bromide method [17]. The quality check, quantification, and concentration adjustment were accomplished with a NanoDrop2000C Spectrophotometer (Thermo Fisher Scientific, Monza, Italy). The concentration of each sample was adjusted to 50 ng/µL. Two biological replicates of DNA extracts were used for each variety.

2.2. Genotyping by Sequencing and SNP Data Analysis

Purified DNA samples (1 µg for each sample) were sent to Diversity Arrays Technology Pty Ltd. (http://www.diversityarrays.com/, Canberra, Australia) for sequencing, and SNP marker identification was done by DArTseq genotyping. Sequences of the genomic representations were obtained on a HiSeq2500 instrument.

The set of SNP markers generated by DArTseq was curated, removing markers with more than 25% of missing values (null alleles were considered as missing). SNPs with low frequent alleles were kept in the dataset to allow the research of private alleles of Aureo. This resulted in a dataset containing 20,198 SNPs and 58 samples (two genotyping replicates for each variety). After marker curation, all samples had a percentage of missing values below 10% and a percentage of heterozygous calls below 12%. Genetic distances were calculated based on simple matching.

A custom R script was used to select SNPs with no missing values and a private allele of both replicates of Aureo. Candidate SNPs were mapped on the durum wheat genome sequence [18] by BLAST (Basic Local Alignment Search Tool). Starting from a panel of 11 candidates, an SNP mapped on chromosome 7A was selected for further analyses.

2.3. Primer Design

LAMP primers were designed from the 7A chromosome of durum wheat. Primer sequences are shown in Table 1:

Table 1. Names and Primer sequences used in the study.

Name of Primers	Sequence (5′–3′)
FIP	TGCAGTGACTGATTGTACTGTCCACCACTTCCTCAGGTA
BIP	GTACTGCACTACTGCACCATTCTCGCCTGCAAACACAC
F3	CTGCCGTTGCCAACA
B3	TATCCGCACGCACC
LoopT	TGCAGGCGATG
LoopG	GCAGGCGAGG

2.4. LAMP Reactions for RealTime

LAMP reactions were performed in 25 µL of a mixture containing 1.6 µM each of inner primers, 0.4 µM each of outer primers, 0.8 µM of loop primers (Integrated DNA Technologies, Coralville, IA, USA), 1 M of betaine (VWR International SRL, Milano, Italy), 2.5 µL of 10× LAMP buffer (200 mM of Tris-HCl, 100 mM of $(NH_4)2SO_4$, 20 mM of $MgSO_4$, 500 mM of KCl, and 1% *v*/*v* Tween 20), 2 mM of $MgSO_4$, 0.8 mM each of dNTPs (Promega, Madison, WI, USA), 0.112 U/µL of Bst 2.0 WarmStart DNA Polymerase (New England BioLabs, Ipswich, MA, USA), and 5 µL of DNA template at the concentration of 5 ng. DNA-free LAMP reactions were included as negative controls. Amplification reactions were performed using forward and backward inner primers (FIP and BIP), forward and backward outer primers (F3 and B3), and one loop primer (LoopT or LoopG). For real-time fluorescent LAMP, 1/50,000 diluted SYBR Green (Thermo Fisher Scientific, Waltham, MA, USA) was pre-added to the reaction mix. Real-time amplifications were performed on an Applied Biosystem real-time instrument (Thermo Fisher Scientific, Waltham, MA, USA) with StepOne Software v2.3 at 63 °C.

2.5. LAMP Reactions for Colorimetric Assay

LAMP colorimetric reactions were performed as reported above, using 1 M of betaine (VWR International SRL, Milano, Italy), 2.5 µL of 10× LAMP buffer (100 mM of $(NH_4)2SO_4$, 20 mM of $MgSO_4$, 500 mM of KCl, and 1% *v/v* Tween 20), 2 mM of $MgSO_4$, 0.8 mM each of dNTPs (Promega, Madison, WI, USA), 0.112 U/µL of Bst 2.0 WarmStart DNA Polymerase (New England BioLabs, Ipswich, MA, USA), and 5 µL of DNA template at the concentration of 5 ng. DNA-free LAMP reactions were included as negative controls. For visualized detection, 0.06 mM of Cresol Red (Sigma-Aldrich, St. Louis, MO, USA) was pre-added to the reaction mix. The amplification efficiency was verified by color changing of the reaction mix. Colorimetric LAMP reactions were performed on a T100 Thermal Cycler (BIO-RAD, Hercules, CA, USA) at 63 °C for 1 h, followed by heat inactivation at 90 °C for 2 min.

3. Results and Discussion

In this work, we aimed at developing a rapid assay for Aureo varietal discrimination based on a single SNP suitable for POC settings. A preliminary genotyping study had the objective of identifying a set of SNPs capable of discriminating Aureo within a panel of 29 wheat varieties. The DArTseq methodology applied on this panel of varieties produced a set of 20,198 good-quality SNPs (with a percentage of missing values below 25%). In order to develop a single-SNP assay, we selected SNPs tagging a private allele of Aureo. The genotyping of two replicates per variety reduced the risk of selecting a private SNP allele caused by a genotyping error. The average genetic distance between two replicates was 98.5% (simple matching), suggesting a genotyping error rate of 1.5% on average. A set of 11 candidate SNPs carrying a private allele of Aureo was identified, with six of them presenting no missing values. To ensure the selection of a private SNP allele, the SNP marker to be used for development of the LAMP assay was selected among those six SNPs with complete genotypes. The assay developed in this study was based on an SNP located on Chr 7A.

The low missing rate and the number of SNPs generated in this study enabled the identification of six SNPs as potential candidates for the assay. This was sufficient for developing our single-SNP approach, which aimed at the discrimination of a variety within a relatively small panel. Such an approach would be very suitable for specific industry requirements.

Such a need is relevant in several high-value agrifood products. In Italy, for example, some PGI (protected geographical indication) products, such as Amarene brusche di Modena, Ciliegia di Vignola, and Pera dell'Emilia-Romagna, are regulated by product specifications that require the use of specific varieties. Moreover, worldwide wine production is linked to the use of specific grape varieties. Various wine laws include appellation-based regulations that cover not only the area of production and the wine-making practices, but even the permitted grape varieties. European PDO (protected designation of origin) and PGI are examples of such regulations directed to guarantee that a certain wine is made from a percentage of grapes belonging to a specific variety or to a specific panel of varieties. However, the genotyping effort required to identify a private SNP allele might be higher in situations with a larger panel of varieties or lower genetic diversity. To overcome this issue, a multi-SNP assay could be developed, since a private allele can be tagged more easily using more than one SNP.

In this framework, LAMP has been recently proven to be an interesting tool to achieve SNP genotyping [19]. In general, LAMP strategies for SNP detection are based on suitable modifications of the primer design, in which two versions of the same primer are typically used to distinguish both the wild-type and the polymorphic allele. The most common approaches exploit the design of the inner primers, hybridizing to the polymorphic or the wild-type base with the 5′ end of both FIP and BIP [20,21], or with the second base at the 5′ end of FIP and BIP [22]. Other methods use allele-discriminating outer primers, placing the mutation at the 3′ end of the F3 primer [23] or one inner primer with the mutated base at the 5′ end [24], or exploit peptide nucleic acid-locked nucleic acid (PNA-LNA) mediated LAMP reactions [25]. In several cases, however, these approaches lead to high background and low discrimination efficiency due to the poor inhibition of the non-specific amplification. Herein, we focused on the optimization of a recently published methodology proposed

for the detection of salivary SNPs [26] based on the use of loop primers as allele-discriminating primers. In this strategy, two reactions are performed in parallel, one with the loop primer hybridizing with the wild-type base and one with the loop primer perfectly matched to the polymorphic one. Since loop primers are designed short, one mismatch significantly destabilizes the annealing, and the obtained effect is comparable to that of a reaction performed without loop primers. For instance, in the case of a wild-type target gene, the reaction performed with the polymorphic loop primer resulted in a strongly decreased amplification rate, creating a significant time gap between the two reactions [26].

In this work, we optimized this approach for Aureo varietal discrimination, exploiting the high tolerance of LAMP to contaminants and the possibility of instrument-free visualization of the results through pH-sensitive dyes [27,28], which together may provide an excellent portable tool for colorimetric SNP discrimination in food products/ingredients in industrial and supply chain settings.

The design of loop primers has a pivotal role in this strategy. In particular, the approach relies on the stabilization of the annealing between short loop primers and the perfectly complementary sequence, while the non-specific one is disfavored. Loop primers act as accelerating primers, speeding up the onset of the amplification. Hence, the reaction in which the loop primer is perfectly hybridized will be properly enhanced, unlike the one with the unstable annealing of the primer, which will be delayed (Figure 1). A single mismatch located on the short primer will significantly impair the annealing, reducing the efficiency of the amplification. Loop primers were constructed as the following: loop primer T, complementary to the SNP that identifies Aureo variant, and loop primer G, perfectly matching the SNP distinctive of all of the other durum wheat variants (see also Experimental). For each sample, two reactions were carried out in parallel to ensure that the same target was tested using both loop primer sets T and G. The results of the SNP-sensitive LAMP reactions on real wheat samples, followed in real-time, are reported in Figure 2. The reaction with the Aureo variant had a perfect annealing when performed with loop primer T, so in this case, the amplification was properly enhanced (ca. 40 min, see Figure 2a). On the other hand, with the same template, the reaction performed with loop primer G was destabilized and significantly delayed. The resulting time gap between the two reactions was about 40 min, allowing for an excellent discrimination. At the same time, the reaction performed on a non-Aureo genetic variant (Marco Aurelio, Figure 2b) revealed the inverted behavior, with similar high temporal discrimination between the fast, matched (G) and non-matched (T) reactions. This preliminary result demonstrated that, in this configuration, a single mismatch can properly modulate the amplification efficiency of LAMP; consequently, it is possible to identify one specific variant of durum wheat with high discrimination effectiveness.

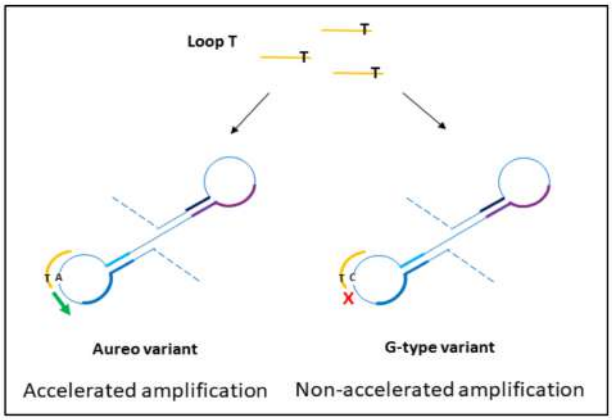

Figure 1. Schematic representation of the hybridization of loop primers to amplicon loops from durum wheat variants. During the exponential phase of loop-mediated isothermal amplification (LAMP) reaction, the perfect match between loop T and the complementary region on the Aureo intermediate loops accelerates the amplification, while the presence of the single mismatch between loop T and G-variant amplicon loops causes the short primer destabilization and a significant reaction delay. The results are reversed for loop G mediated reactions.

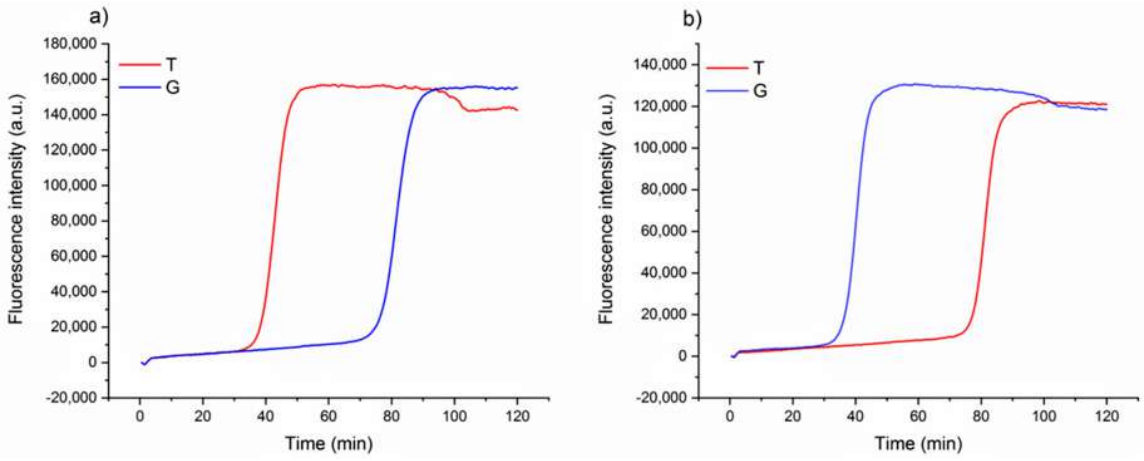

Figure 2. Real-time fluorescence detection of genetic variants of wheat. LAMP reaction with loop primer T (red line) and G (blue line) of Aureo variant genome (**a**) and one G-type variant genome (Marco Aurelio) (**b**). The time gap between fast and slow reactions is ca. 40 min. All of the reactions were performed at 63 °C.

This strategy was applied to the assessment of a wide range of different wheat varieties to analyze the reliability of the technique in a real scenario of quality control. Interestingly, the method was able to efficiently discriminate all of the 13 tested varieties of wheat versus Aureo (Figure 3), with an average time gap of ca. 35–40 min between the fast and the slow reactions. Moreover, it is notable that the time gap was nearly constant among all the tested cases.

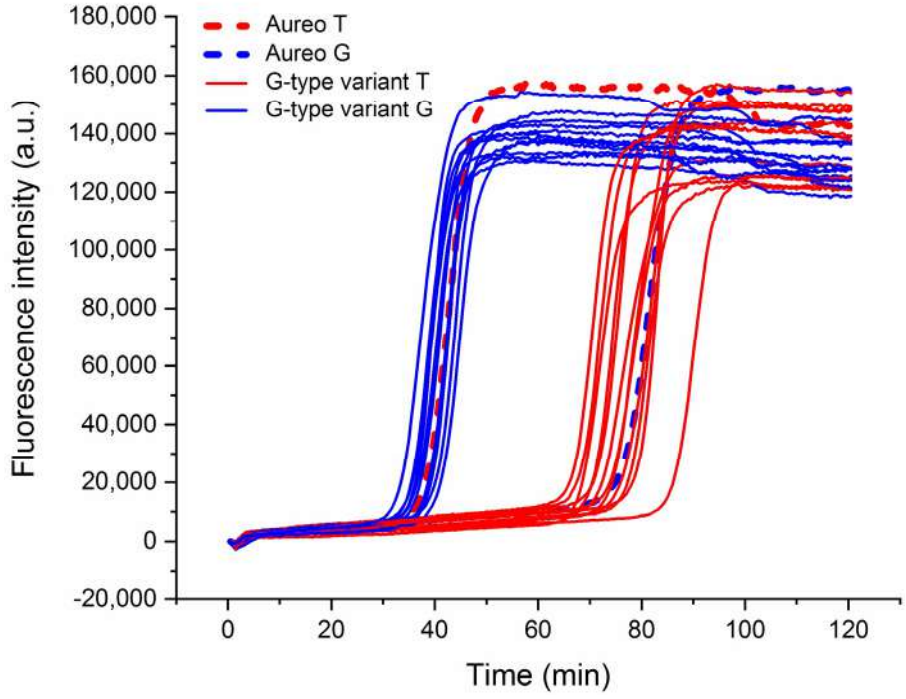

Figure 3. Real-time fluorescence detection of 13 genetic variants of wheat. LAMP reactions with loop primer T (red lines) and loop primer G (blue) of Aureo and G-type variants (Aureo: dashed lines). The time gap between all of the fast and all of the slow reactions was 35–40 min. All of the reactions were performed at 63 °C.

Once we assessed that the method could consistently detect the possible substitution of the Aureo with another variety, we investigated a more complex scenario of food fraud, in which the Aureo species is adulterated (mixed) with another variety, with different levels of contaminations. This is

important in industrial settings to guarantee the mono-varietal nature of some productions. To this purpose, different mixtures of Aureo with decreasing contaminations of a G-type variant (from 50 to 10%) were prepared to probe the sensitivity of the strategy to also detect minor adulterations. As reported in Figure 4, the method can effectively discriminate the 50/50 (Aureo/G-type) mixture, with the two reactions displaying similar efficiency. In such a case, the two reactions both behave like fast reactions, because the half amount of template of each variant is sufficient for the optimized functioning of the amplification (Figure 4c). Overall, the mixture appeared as a heterozygous sample. Comparable results were obtained with a 25% contamination with the G-type variant (Figure 4b). Although the amount of G-type genome was lower, the reaction with loop primer G worked efficiently, exhibiting a negligible delay. Interestingly, our LAMP test was also able to discriminate an adulteration with the G-type variant as low as 10% (Figure 4a). In this latter case, we observed a small delay of the loop primer G reaction due to the significantly lower (10%) amount of G-type variant present in the mixture. However, the test behaved very differently with respect to the result obtained with 100% pure Aureo mixture, overall indicating that the proposed strategy enables accurate discrimination of relevant adulterations, including both substitution and mixing of wheat varieties (see also in the following).

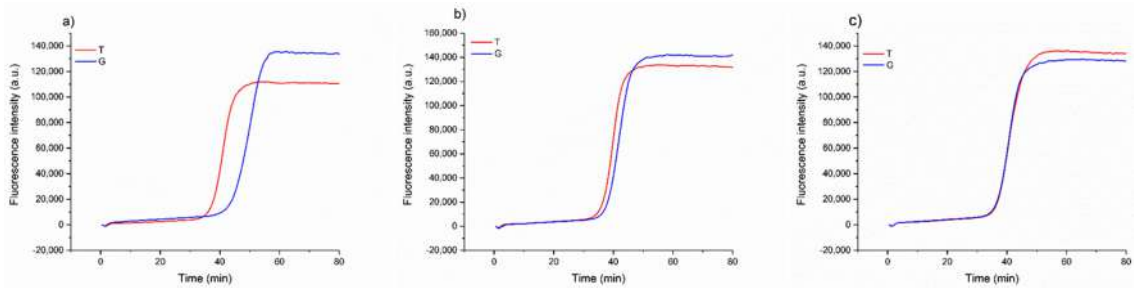

Figure 4. Real-time fluorescence detection of a simulated adulteration, in which a sample of Aureo was mixed with different proportions of a G-type variant (Babylone). (**a**) 90% of Aureo with 10% of G-type variant; (**b**) 75% of Aureo with 25% of G-type variant; (**c**) 50% of Aureo with 50% of G-type variant. The LAMP reactions were performed with loop primer T (red line) and G (blue line) at 63 °C.

After characterizing and optimizing the performance of our SNP test, we attempted to develop a simplified, instrument-free colorimetric assay with a naked-eye readout. Visual interpretation of the varietal discrimination was made possible by the reproducible and large time gap achieved between the fast and the slow reactions, in combination with pH-sensitive dyes that could provide a clear color change of the reaction tubes upon target amplification [20,21]. In particular, we used Cresol Red dye, which shifts from purple to yellow during amplification, and a reaction time of 1 h to fully discriminate between the two reactions. Based on the results showed above (Figures 2–4), the fast reaction should display clear color change after 1 h, unlike the unmatched slow reaction. This was expected to enable visual discrimination of all of the adulteration cases analyzed so far. In line with previous experiments, reactions were carried out in tube A with loop primer T and in tube B with loop primer G. In more detail, as reported in the scheme in Figure 5a, the combination of the yellow/purple colors of the two reactions performed in parallel allowed detecting the possible scenarios, i.e., pure Aureo, the presence of contaminations (variety mixing), or full substitution. We tested all of the contamination cases, mixing Aureo with different fractions of a G-type variety (from 0 to 100%). What's noteworthy is that the colorimetric visual test was proven to be effective and highly specific (Figure 5b). In particular, in the case of the pure Aureo variant, the reaction performed with loop primer T turned yellow (positive result), while the reaction performed with loop primer G remained purple (negative result) (Figure 5b, sample 1). On the contrary, the full substitution of Aureo with a G-type variant exhibited the inverted result, with tube A appearing purple and tube B yellow (Figure 5b, sample 5). Interestingly, all of the adulterated samples with different levels of contaminations (down to 10% adulteration) were clearly detected, with both of the tubes exhibiting yellow color (samples 2–4) due to the amplification of both genomic variants. Hence, this direct and instrument-free strategy was proven to be specific and had

enough sensitivity for quality control purposes, with the visual inspection of the results being an additional advantage for on-field applications.

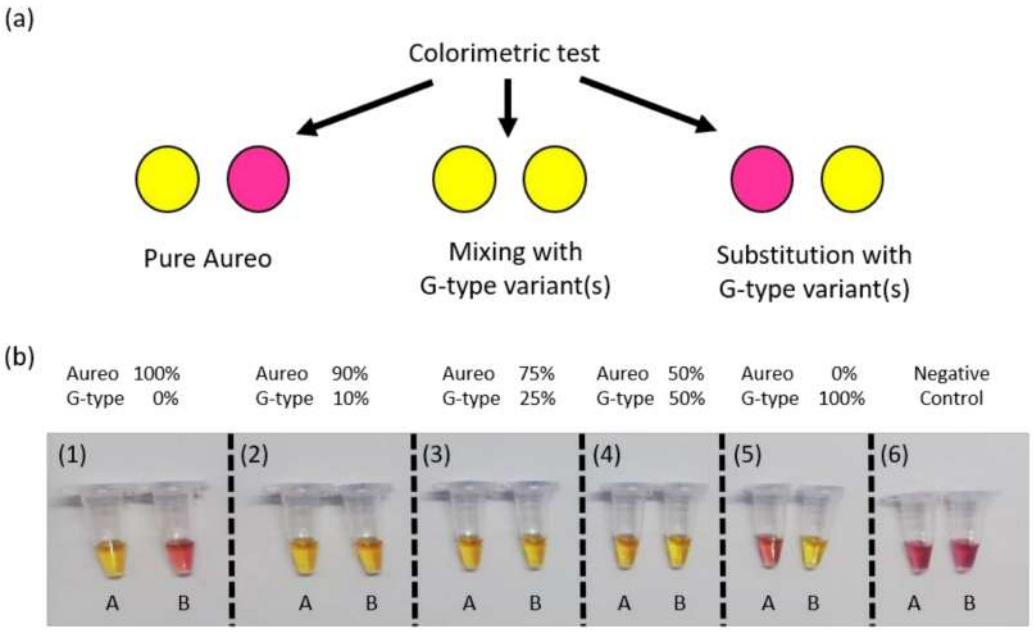

Figure 5. (**a**) Schematic of the colorimetric test displaying the possible yellow/purple color combinations: pure Aureo variety (yellow/purple), adulterated mixture with G-type varieties (yellow/yellow), or fully substituted product (purple/yellow). (**b**) Colorimetric detection of possible adulteration cases of Aureo with a G-type wheat variant (here, Babylon is used as an example). LAMP reactions with loop primer T (tube A) and loop primer G (tube B) were all performed at 63 °C for 1 h. From left to right, targets were composed of 100% of the Aureo variant of wheat (sample 1), then mixtures of Aureo and G-type variant (from 10 to 100%, samples 2–5), and a negative control (sample 6).

To completely validate our system, a more complex case of adulteration was tested, in which the Aureo was mixed with two different G-type varieties of wheat (from 50 to 10% total contaminations). Remarkably, also in this case, the strategy revealed its high specificity, being able to correctly identify the different genomes in a complex mixture and even in the situation in which the total amount of the two contaminations was as low as 10% (Figure 6).

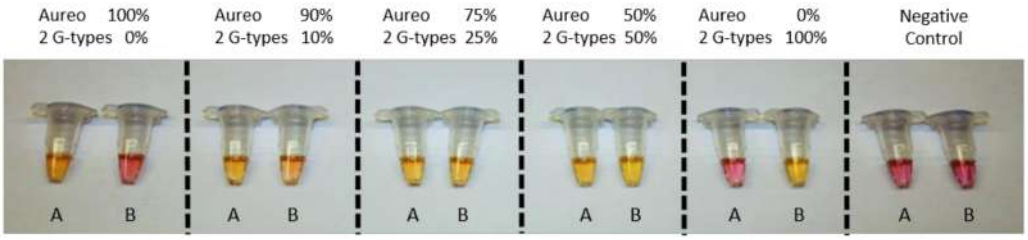

Figure 6. Colorimetric detection of a complex adulteration of Aureo with two G-type wheat variants. LAMP reactions with loop primer T (tube A) and loop primer G (tube B) were all performed at 63 °C for 1 h. From left to right, targets were composed of 100% Aureo variant, then mixtures of Aureo and two G-type variants (Babylon + Marco Aurelio) with a total contamination from 10 to 100%, and a negative control.

4. Conclusions

In conclusion, we designed a single-SNP discriminating strategy to correctly identify mono-varietal productions based on Aureo, and we set up a direct colorimetric test able to discriminate, by simple

visual inspection, the purity or the adulteration of Aureo with a wide range of G-type variants. The test exhibited high efficiency and specificity in complex cases of adulteration, in which different varieties were mixed, even at a 10% level of contamination. The isothermal nature of the LAMP amplification together with the naked-eye readout of the results make this approach potentially very useful in real quality control applications in the industry and food supply chain.

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Article

A Digital PCR Assay to Quantify the Percentages of Hulled vs. Hulless Wheat in Flours and Flour-Based Products

Caterina Morcia [1], Raffaella Bergami [2], Sonia Scaramagli [2], Chiara Delogu [3], Lorella Andreani [3], Paola Carnevali [4], Giorgio Tumino [5], Roberta Ghizzoni [1] and Valeria Terzi [1,*]

1 Consiglio Per la Ricerca in Agricoltura e L'analisi Dell'economia Agraria-Centro di Ricerca Genomica e Bioinformatica (CREA-GB), Via San Protaso 302, 29017 Fiorenzuola d'Arda, PC, Italy; caterina.morcia@crea.gov.it (C.M.); roberta.ghizzoni@crea.gov.it (R.G.)
2 Coop Italia, Via del Lavoro, 6/8, I-40033 Casalecchio di Reno, BO, Italy; raffaella.bergami@coopitalia.coop.it (R.B.); sonia.scaramagli@coopitalia.coop.it (S.S.)
3 Consiglio Per la Ricerca in Agricoltura e L'analisi Dell'economia Agraria-Centro di Ricerca Difesa e Certificazione (CREA-DC), Via Emilia km 307, 26838 Tavazzano, LO, Italy; chiara.delogu@crea.gov.it (C.D.); lorella.andreani@crea.gov.it (L.A.)
4 Barilla S.p.A., Via Mantova 166, I-43122 Parma, PR, Italy; paola.carnevali@barilla.com
5 Plant Breeding, Wageningen Plant Research, Wageningen University & Research, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands; giorgio.tumino@wur.nl
* Correspondence: valeria.terzi@crea.gov.it; Tel.: +39-0523-983758

Simple Summary: The agri-food market is currently showing interest in hulled wheat-based products, in particular emmer and spelt. These wheats were rediscovered as ingredients for both traditional and innovative food products. Since hulled wheats' commodity value is higher than common and durum wheat, it is useful to have an analytical system that allows to control and quantify the actual presence of einkorn, emmer and spelt and, therefore, check the authenticity of derived products. With this aim, we developed an analytical assay based on digitalPCR, which has been able to discriminate between hulled (i.e., einkorn, emmer and spelt) and common or durum wheats and to give a quantification. The assay can be used along production chains, from raw materials to final food products.

Abstract: Several food products, made from hulled wheats, are now offered by the market, ranging from grains and pasta to flour and bakery products. The possibility of verifying the authenticity of wheat species used at any point in the production chain is relevant, in defense of both producers and consumers. A chip digital PCR assay has been developed to detect and quantify percentages of hulless (i.e., common and durum wheat) and hulled (i.e., einkorn, emmer and spelt) wheats in grains, flours and food products. The assay has been designed on a polymorphism in the miRNA172 target site of the AP2-5 transcription factor localized on chromosome 5A and involved in wheat spike morphogenesis and grain threshability. The assay has been evaluated even in a real-time PCR system to assess its applicability and to compare the analytical costs between dPCR and real-time PCR approaches.

Keywords: hulled wheats; einkorn; emmer; spelt; *Triticum monococcum*; *Triticum dicoccum*; *Triticum spelta*; authenticity; quantification; dPCR

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1. Introduction

The ethnobotanical Italian term "farro" indicates the complex of einkorn, emmer and spelt, the earliest wheats to be cultivated, and therefore, identified as "ancient grains". Ancient wheats are characterized by three different levels of ploidy: einkorn (*T. monococcum* L.) is diploid (AA; $2n = 2x = 14$), emmer (*T. turgidum* L. spp. *dicoccum* Schrank ex Schübler) is tetraploid (AABB; $2n = 4x = 28$), whereas spelt (*T. aestivum* subsp. *spelta*) is hexaploid (AABBDD; $2n = 6x = 42$). These plants, primarily domesticated in the Fertile Crescent area

where their wild ancestors are still present [1], are among the founder crops of agriculture [2].

Einkorn is today present in isolated mountain areas of a few Mediterranean and European countries only [3] and can be considered a relic crop.

Emmer, domesticated 10,000 years ago from *Triticum dicoccoides* [4] represents today about 1% of the total world wheat area. It is cultivated as a minor crop in Iran, Eastern Turkey, Transcaucasia, the Volga Basin, ex-Yugoslavia, Central Europe, Italy and Spain [5], even though it remains an important plant in India, Ethiopia and Yemen [6]. In Europe, spelt became cultivated as far back as 7000–8000 BCE in the Neolithic period [7] and became the most important cereal in Northern and Central Europe starting from the Bronze Age. Today, spelt cultivation is mainly restricted to marginal areas in eastern Europe, Germany, Belgium, Austria, Switzerland, Slovenia, the Asturias region of Spain and Italy [8].

Ancient hulled wheats, widely cultivated in the past, were, starting from 19th century, replaced by naked wheats; however, in the last years, a trend reversal supported by consumers' interest in traditional crops and derived food, nutritional peculiarities of hulled wheats and their aptitude to organic farming has been observed [9–11]. As a result of this market trend, an increase in cultivation area has been observed in several countries, including Italy.

In Italy, the hulled wheat that is typically used is emmer and the most important area of its cultivation, estimated around 4000 ha, is Central/Southern Italy. Within this area, specific ecotypes have been fixed by long time in situ reproduction. Such landraces, therefore, are typical of their own cultivation area. This cultivated area hosts emmer varieties both selected among landraces and modern cultivars, obtained by crossing cultivated emmer and durum wheat [12].

Several food products, made from hulled wheats, are now offered by the market, ranging from grains and pasta to flour and bakery products. Farro price on the Italian cereal market is significantly higher in comparison with naked wheats, ranging from 30% higher than common wheat to 15% higher than durum wheat [13]. This significant difference in commodity value, due to the easier handling and processing of naked wheats, can be the motives for alimentary frauds based on farro replacement with common or durum wheats. This implies that the possibility of verifying the authenticity of the wheat species used at any point in the production chain is relevant, in defense of both producers and consumers.

Some analytical procedures, reported in Table 1, have been proposed to track hulled wheats.

Table 1. Analytical assays proposed for the identification and quantification of hulled wheats.

Analytical Target	Method	Reference
Spelt	Fatty acids profile	[14]
Triticum species	PCR-RFLP (*Q-locus*)	[15]
Spelt	RLP-LOC-CE, Real-time PCR (*γ-gliadin*)	[16]
Farro della Garfagnana in cereal mixtures	padlock probe ligation and multiplex microarray	[17]
Spelt	LC-MS peptide markers identification	[18]
Einkorn, emmer and spelt	tubulin-based polymorphism (TBP)	[19]
Spelt	PCR (*γ-gliadin*, *Q-locus*); NIR	[20]
Italian emmer landraces	Spectroscopy and chemometrics	[21]
Spelt	Duplex droplet digital PCR (*Q-locus*)	[22]

Most of the proposed assays are DNA-based methods used for the identification and quantification of spelt. Moreover, assays developed by Voorhuijzen et al. [17] and by Foschi et al. [21] are focused on the traceability of accessions specifically cultivated in

Italian environments. Tubulin-based DNA barcode, multiple gene targets, *γ-gliadin* polymorphisms and *Q-locus* polymorphisms have been exploited using different technologies, ranging from PCR to microarray, up to the very recent digitalPCR [22].

The tubulin-based polymorphism (TBP) profiling developed by Silletti et al. [19] has the peculiarity to be the only DNA-based untargeted method, not requiring any prior genome sequence information and able to profile any plant species with universal primer pairs. The authors suggest that this method is a useful first screening step, which can be complemented by target quantitative analysis, performed by qPCR or other methods.

γ-gliadin polymorphisms were exploited in the analytical protocols developed by Mayer et al. [16] and by Curzon et al. [20].

Mayer et al. [16] proposed two alternative methods for the detection and quantification of spelt flour "adulteration" with soft wheat: a restriction fragment length (RFLP) analysis, combined with lab-on-a-chip capillary gel electrophoresis (LOC-CE) for the simple detection and a real-time PCR for the quantification of soft wheat "adulterations" in spelt.

The study of Curzon et al. [20] has the same objective, i.e., the identification of common wheat adulteration in spelt. In this study, markers for *γ-gliadin-D*, *γ-gliadin-B* and the *Q*-gene were used, alongside a phenotypic assessment based on near-infrared spectroscopy (NIRS). The *γ-gliadin* markers demonstrated low diagnostic power in comparison to the *Q*-gene marker and to the NIR predictions.

Asakura et al. [15] developed a method based on polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) to distinguish between the Q and q alleles. PCR-RFLP analysis was extended to six conserved single nucleotide polymorphisms in common wheat and wild and cultivated einkorn, emmer and timopheevi wheat.

Q locus polymorphism was exploited even by Voorhuijzen et al. [17] to develop a DNA-based multiplex detection tool based on padlock probe ligation and microarray detection (PPLMD) for the detection of (un-)intentional adulteration of Farro della Garfagnana with different species. This approach, developed on grain samples, is sensitive enough to track the presence of 5% contaminant plant species, and therefore, it can be applied to check the purity of a premium food such as Farro della Garfagnana grains.

The recent study of Köppel et al. [22] describes a duplex droplet digital PCR (ddPCR) for the detection and quantification of contaminations by common wheat in food products made from spelt. The authors take into account both a single nucleotide polymorphism (SNP) in the Q locus, as well as a short sequence of the γ-gliadin gene. The SNP in the Q locus was able to discriminate all the tested spelt cultivars from common wheat cultivars.

The choice, made by several authors, of the Q locus as a useful analytical target to discriminate between free- and not-free threshing wheats is the logical consequence of the historical use of key morphological descriptors. Common and durum wheat kernels are naked, whereas einkorn, emmer and spelt are hulled. The naked/hulled seed trait is, therefore, one of the major morphological characters to discriminate the common and durum wheat grains from einkorn, emmer and spelt grains. This trait is genetically determined, not influenced by the cultivation environment, and with a key role in the wheat domestication process. Several studies have contributed to highlight the genetics of the trait. Pioneering in this regard was the work of Nilsson-Ehle [23], in which for the first time the Q-locus was identified as involved in wheat spike morphology and pleiotropically, affecting many other agronomic traits, such as free-threshing. Q-locus was assigned to the long arm of chromosome 5A and its molecular cloning showed that it belongs to the APETALA2 transcription factors [24]. Q-locus, similar to other AP2-like genes, has a miR172 target site within the coding region that can modulate the mRNA stability and can have an impact on several developmental processes in several species, including maize [25]. In wheat, Debernardi et al. [26] demonstrated the miR172 key role in spike morphogenesis and a sequence variation at the miR172 target site between Q and q alleles involved in the grain threshability trait.

Such polymorphism has been exploited in our study and a new digitalPCR assay has been developed that can identify and quantify all kind of hulled wheats, i.e., einkorn,

emmer and spelt, and can distinguish them from naked wheats in both raw materials and processed products.

2. Materials and Methods

2.1. Samples

The wheat genotypes reported in Table 2 have been used across the study. The hulled wheat accessions selected are all present in the Italian National Register of Variety. Moreover, two durum wheat varieties (Aureo and Iride) and two common wheat varieties (Bologna and Palesio) have been selected because they are widely cultivated in Italian environments. Durum wheat Cappelli and common wheat Apulia have been selected as representative of Italian traditional varieties.

Table 2. Hulled and hulless wheat varieties used and their maintainers, defined as the natural or the legal person identified by the national seed law as responsible for maintaining the variety in purity, very often coincident with the breeder [27]. A = Fondazione M. Bolognini, Sant'Angelo Lodigiano (Lodi), Italy; B = CREA—Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria, Italy; C = Prometeo s.r.l., Urbino (PU), Italy; D = Istituto Di Genetica Vegetale CNR, Bari, Italy; E = Agribosco s.r.l., Sigillo (PG), Italy; F = Società Produttori Sementi, Bologna, Italy; G = Società Italiana Sementi, San Lazzaro di Savena (BO), Italy.

Botanical Species	Variety	Maintainers
Triticum monococcum L.	Antenato	A,B
Triticum monococcum L.	Hammurabi	A,B
Triticum monococcum L.	Monili	A,B
Triticum monococcum L.	Monlis	B,C
Triticum monococcum L.	Norberto	A,B
Triticum dicoccum Schubler	Augeo	E
Triticum dicoccum Schubler	Farvento	D
Triticum dicoccum Schubler	Giovanni Paolo	B
Triticum dicoccum Schubler	Hervillum	E
Triticum dicoccum Schubler	Padre Pio	B
Triticum dicoccum Schubler	Rosso Rubino	C
Triticum dicoccum Schubler	Yakub	C
Triticum dicoccum Schubler	Zefiro	C
Triticum dicoccum Schubler	Sephora	-
Triticum spelta L.	Benedetto	A,B
Triticum spelta L.	Forenza	D
Triticum spelta L.	Giuseppe	A,B
Triticum spelta L.	Maddalena	B
Triticum spelta L.	Pietro	A,B
Triticum spelta L.	Rita	B
Triticum spelta L.	Rossella	B
Triticum durum	Aureo	F
Triticum durum	Iride	F
Triticum durum	Cappelli	B,G
Triticum aestivum	Apulia	B
Triticum aestivum	Bologna	G
Triticum aestivum	Palesio	G

Moreover, seeds of barley (cv Fibar), oat (cv Buffalo) and rice (cv Vialone nano), included in the CREA-GB germplasm collection, have been used to evaluate the species-specificity of the assay.

A panel of different foods, labeled as containing hulled wheats and commercially available in Italy, were bought on the market. Flour samples containing different percentages of hulled and naked wheats were produced by weighing the wheat flours and homogenizing them for 10 min. Moreover, mixed flour samples containing hulled and hulless wheats and barley have been prepared using the same approach.

2.2. Methods

2.2.1. DNA Extraction

The seeds were milled using a Cyclotec (FOSS Italia S.r.l., Padova, Italy) at a grid diameter of 0.2 mm, avoiding any contamination between samples. DNA was extracted from three biological replicates of milled seeds using the DNeasy mericon Food Kit (Qiagen, Milan, Italy), according to manufacturer's instructions. The evaluation of quality and quantity of the extracted DNA was performed using a Qubit™ fluorometer in combination with the Qubit™ dsDNA BR assay kit (Invitrogen by Thermo Fisher Scientific, Monza, Italy).

The same procedure was applied for the DNA extraction from flour and food samples, starting from 2 g. The evaluation of quality and quantity of extracted DNA was performed as described above.

2.2.2. Chip Digital PCR

Primers and MGB probes were designed on the C/T mutation that lies within the miR172 target site in exon 10 of the AP2-5 transcription factor [23]. The Custom TaqMan® SNP Genotyping assay procedure (Thermo Fisher Scientific, Monza, Italy) was used to design the allelic discrimination assay, and primers and probes are available as assay ID ANH6NUZ, Catalog n. 4332077 (Thermo Fisher Scientific, Monza, Italy). In the dPCR assay developed, the recessive allele carrying cytosine was marked with VIC, whereas the dominant allele, carrying timyne, was marked with FAM. Chip digital PCR was performed using the QuantStudioTM 3D Digital PCR System (Applied Biosystems by Life Technologies, Monza, Italy). The reaction mixture was prepared in a final volume of 16 µL consisting of 8 µL QuantStudioTM 3D Digital PCR 2X Master Mix, 0.4 µL of Custom TaqMan® SNP Genotyping assay 40X (Catalogue number 4332077, Applied Biosystems by Life Technologies, Monza, Italy) containing primer and VIC/FAM-MGB probes, 1 µL of DNA (10 ng/µL) and nuclease-free water. In addition, a negative control with nuclease-free water as a template was added. A total volume of 15 µL of reaction mixture was loaded onto the QuantStudioTM 3D Digital PCR chips using the QuantStudioTM 3D Digital chip loader, according to the manufacturer's protocol. Amplifications were performed in a ProFlexTM 2Xflat PCR System Thermocycler (Applied Biosystems by Life Technologies, Monza, Italy) under the following conditions: 96 °C for 10 min, 47 cycles of 60 °C annealing for 2 min, and 98 °C denaturation for 30 s, followed by 60 °C for 2 min and 10 °C. The fluorescent signals were detected at the end of the amplification, in an end-point mode [28]. The fluorescence data were collected in a QuantStudioTM 3D Digital PCR Instrument, and the files generated were analyzed using cloud-based platform QuantStudioTM 3D AnalysisSuite dPCR software, version 3.1.6. Each sample was analyzed in triplicate.

All the commercial samples were analyzed by the two laboratories of CREA and of Coop Italia.

2.2.3. Hulless Wheat Percentage Calculation

The polynomial curves reported in Figure 1 were developed and used for hulless wheat percentage calculations. The theoretical curves were built starting from the above-listed premises and from the genetic information reported in Table 3:

1. The C allele, marked with VIC, is present in miRNA172 target site of the AP2-5 transcription factor localized on chromosome 5A in hulled wheats;
2. The T allele, marked with FAM, is present in miRNA172 target site of the AP2-5 transcription factor localized on chromosome 5A in hulless wheats;
3. The C allele is present in miRNA172 target site homoeologous regions of chromosome 5B and 5D in all wheats.

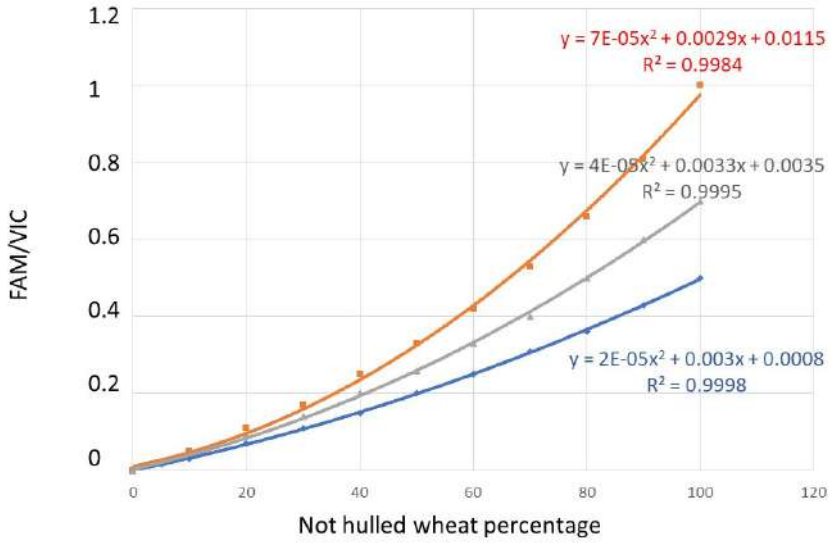

Figure 1. Polynomial curves reporting the theoretical relationships among FAM/VIC ratios and hulless/hulled wheats. The red curve refers to the case of durum wheat mixed with hulled wheats of any ploidy level; the blue curve refers to the case of common wheat mixed with hulled wheats of any ploidy level; the grey curve refers to a "mean situation" in which a 50:50 mixture of durum and common wheat is mixed with hulled wheats of any ploidy level.

Table 3. Sequence variation at the miR172 target site in different homoeologs and in wheat species different in ploidy level and grain threshability.

Wheat Species and Threshing Habit	Chromosome 5A miR172 Target Site	Chromosome 5B miR172 Target Site	Chromosome 5D miR172 Target Site
Einkorn Non-free-threshing (GenBank MK101270.1)	gct gca gca tca tca gga tt**c** tct	-	-
Emmer Non-free-threshing (GenBank MK493450.1)	gct gca gca tca tca gga tt**c** tct	gct gca gca tca tca gga tt**c** tct	-
Spelt Non-free-threshing (GenBank MK450625.1)	gct gca gca tca tca gga tt**c** tct	gct gca gca tca tca gga tt**c** tct	gct gca gca tca tca gga tt**c** tct
Durum wheat Free-threshing (GenBank KY924305.1)	gct gca gca tca tca gga tt**t** tct	gct gca gca tca tca gga tt**c** tct	-
Common wheat Free-threshing (GenBank JF701619.1)	gct gca gca tca tca gga tt**t** tct	gct gca gca tca tca gga tt**c** tct	gct gca gca tca tca gga tt**c** tct

The curve was, therefore, developed considering the fact that all hulled wheats, regardless of their ploidy level, will only give a VIC signal, since the three genomes A, B and D all carry the allele with C base. On the contrary, both the durum and common wheat gave a double signal, both VIC and FAM, as both carry the T allele in genome A and the C allele in genomes B and D. In the case of monospecies hulled samples, it was, therefore, easy to highlight the exclusive presence of hulled wheats because only the VIC signal is present. In the case of monospecies samples of durum wheat, a double VIC and FAM signal of equal intensity was observed, and the FAM/VIC ratio will be equal to 1. In the case of common wheat, the VIC signal was double the FAM signal, as genome A carries the T allele, while the other two genomes B and D carry the C allele. Starting from these premises, the polynomial curve was constructed by calculating the theoretical ratios FAM/VIC in

the case in which hulled and hulless are mixed. Therefore, the polynomial curve has been constructed taking into account 100% hulled wheat samples, 100% common wheat sample, 100% common wheat sample and mixed hulled/hulless samples. In Figure 1, the red curve was drawn using 100% einkorn or emmer or spelt samples, 100% durum wheat sample and samples of hulled/durum wheat in mixed percentages (between 100% hulled and 100% hulless) in 10% increments. The same approach was used to draw the blue curve, using 100% einkorn or emmer or spelt samples, 100% common wheat sample and mixed samples of hulled/common wheat between 100% hulled and 100% hulless in 10% increments. The grey curve was drawn using the 100% hulled sample, 100% hulless sample (made of 50% durum and 50% common wheat) and mixed samples of hulled/hulless wheats between 100% hulled and 100% hulless in 10% increments.

2.2.4. Real-Time PCR

The same primers/probes of the digitalPCR assay were used in real-time PCR analysis. The reaction mixture was prepared in a final volume of 20 µL consisting of 10 µL of Master Mix, 0.5 µL of Custom TaqMan® SNP Genotyping assay 40X (Catalogue number 4332077, Applied Biosystems by Life Technologies, Monza, Italy), 5 µL DNA template diluted at 20 ng/µL) and 4.5 µL of water. Three technical real-time PCR replicates were done for each sample and control. The PCR mixture was heated at 50 °C for 2 min and activated at 95 °C for 10 min. Forty amplification cycles were carried out at 95 °C for 15 s followed by 60 °C for 1 min. The signal detection was performed at each cycle, in real-time mode [28]. The percentage of hulled/hulless wheat was calculated as the ratio of the copy number of the hulled target gene sequence to the copy number of the target hulless gene sequence.

Different dilutions of standard samples of known hulled/hulless concentration were amplified to obtain two regression curves (one for the VIC—hulled—and one for the FAM—hulless) with the number of copies on the abscissa and the corresponding CTS (Cycle Threshold) in ordinate. In parallel to standard samples, analytical samples were amplified. The number of copies of the analytical samples was obtained by interpolation on the standard curves using the corresponding CTS. The software used is Sequence Detection Software 1.4.2-Applied Biosystems (Monza, Italy).

3. Results

3.1. Mono-Species Samples

The dPCR assay was evaluated for the specificity in wheat discrimination on the DNA extracted from the genotypes listed in Table 2. The DNA quantity extracted from such samples ranged from 19.5 to 42 ng/mg of sample, with a 1.80 mean ratio of absorbance at 260 nm and 280 nm, indicating an acceptable purity level.

All the einkorn, emmer and spelt varieties showed VIC signal only, with an absent or negligible FAM signal (Figure 2). All the varieties showed FAM/VIC = 0, with a Pearson's r correlation of 1 between experimentally and expected values. An FAM/VIC = 0, according to the calculation formulas of Figure 1, predicts 0% of durum or common wheats, as expected.

The durum and bread wheat varieties showed both VIC and FAM signals (Figure 2). The mean FAM/VIC ratio of common wheat samples was 0.52 ± 0.02 and those of durum wheat varieties was 0.95 ± 0.05, which are very close to the expected values of, respectively, 0.5 and 1. A Pearson's r of 0.9989 was found between theoretical and experimentally obtained FAM/VIC ratios in hulless wheats. The FAM/VIC ratios experimentally obtained after analysis of nominal 100% common wheat and nominal 100% durum wheat samples were used to calculate the experimentally determined percentages using the polynomial curve of Figure 1. According to the calculation formulas of Figure 1, the mean, experimentally measured value for nominal 100% common wheat sample was 99.8 ± 0.44% and those for nominal 100% durum wheat sample was of 100%.

A subset of 18 DNA samples extracted from hulless and hulled wheats were analyzed independently by the CREA and CoopItalia laboratories and a Pearson's r of 0.999 was found among the results obtained.

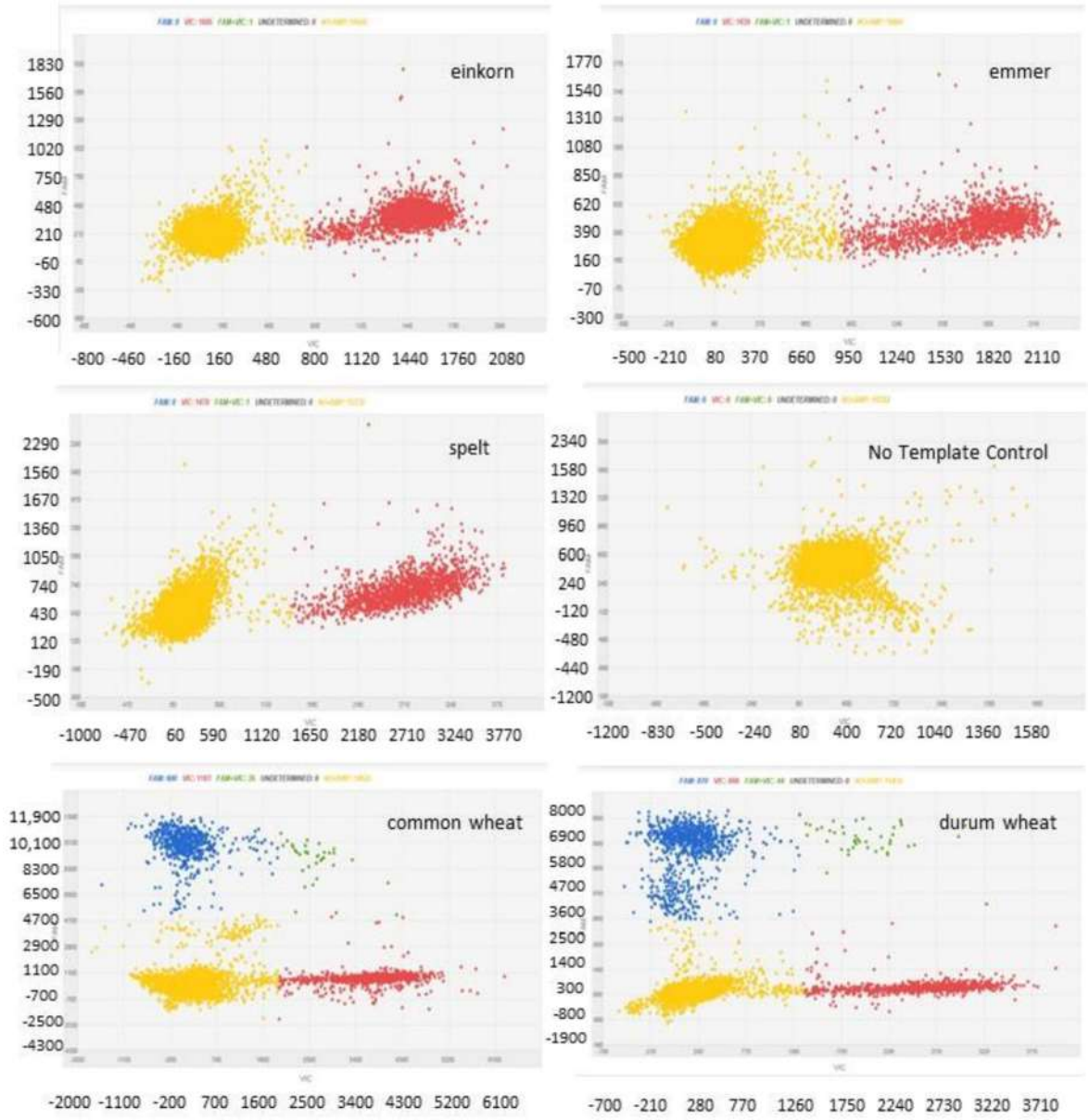

Figure 2. Two-dimensional scatter graphs generated by chip digital PCR (cdPCR) analysis of 100% hulled and hulless wheats. No template control is a blank sample without DNA, with negative partitions that contain no amplified targets (yellow signals). X axis stands for the VIC signal, whereas Y axis stands for the FAM signal. Einkorn, emmer and spelt carrying the recessive alleles show red (VIC) signal only, whereas hulless wheats, carrying both recessive and dominant alleles, show both red (VIC) and blue (FAM) signals. Green signals stand for partitions in which a co-amplification of both VIC and FAM targets occurs.

3.2. Mixed-Species Samples

The dPCR assay has been applied to the hulless wheat quantification in mixed-species samples prepared by mixing DNAs extracted from einkorn, emmer, spelt, durum and common wheat in the percentages reported in Table 4. The Pearson's r between the expected and calculated hulless wheat percentages was 0.97. In the same Table 4 are reported the absolute and relative errors, as informative values about the precision and the accuracy of the method.

Table 4. Actual hulless wheat percentages in comparison with those experimentally determined in samples obtained by mixing DNA extracted from pure einkorn, emmer, spelt, common and durum wheats.

Nominal Hulled to Hulless Ratio in Mixed Samples	Measured Hulless Wheat Percentage	Absolute Error	Relative Error
80% einkorn, 20% durum wheat	20%	0	-
80% emmer, 20% durum wheat	25%	5	0.20
80% spelt, 20% durum wheat	24%	4	0.16
50% einkorn, 50% durum wheat	50%	0	-
50% emmer, 50% durum wheat	55%	5	0.09
50% spelt, 50% durum wheat	50%	0	-
40% einkorn, 60% durum wheat	55%	5	0.09
40% emmer, 60% durum wheat	69%	9	0.13
40% spelt, 60% durum wheat	64%	4	0.06
50% einkorn, 50% common wheat	48%	2	0.04
50% emmer, 50% common wheat	52%	2	0.04
50% spelt, 50% common wheat	54%	4	0.07

3.3. Commercial Samples

The dPCR assay has been applied to the panel of foods reported in Table 5, labeled as made from farro or containing farro among the ingredients. The products are commercially available and have been sampled on an Italian market. The products were chosen as representative of different food categories. A DNA quantity ranging from 1.5 to 17.5 ng/mg of sample has been obtained from commercial samples, with a 1.87 mean ratio of absorbance at 260 nm and 280 nm, indicating an acceptable purity level.

Table 5. Commercially available food sampled, their cereal content as reported in the label and hulled wheat percentages determined by the two CREA and CoopItalia laboratories using dPCR assay.

Commercial Sample	Cereal Formulation in the Label	Farro % (dPCR Determined by CREA Lab)	Farro % (dPCR Determined by CoopItalia Lab)
Cookie 1	Farro 54%, common wheat	54%	56%
Breakfast cereals	Whole farro flakes 100%	100%	97%
Pearled farro	Farro 100%	100%	100%
Bread substitute 1	Farro 99.8%	99%	99%
Mix for bread making	Whole farro flour 7%, common wheat flour 93%	25%	25%
Flour	Spelt flour	90%	91%
Bread substitute 2	Common wheat flour, common wheat flakes 5.1%, toasted wheat bran, whole farro flour 2.1%, malted common wheat flour	7%	0%
Pasta	Farro flour	78%	76%
Egg pasta	Farro flour 80.64%	81%	83%
Bread substitute 3	Common wheat flour, farro flour 30.4%, malt, oat flakes	25%	25%
Cookie 2	Farro flour	100%	100%
Baby food	Farro 100%	100%	100%

Table 5 shows the products and the cereal ingredients reported in the label. The percentages of farro experimentally determined with dPCR analysis by the two independent laboratories of CREA and CoopItalia are shown in the table. The data obtained by the CREA and CoopItalia laboratories are very close, with a Pearson's r of 0.99. For several products (i.e., cookie 1, breakfast cereals, pearled farro, bread substitute 1, egg pasta, cookie 2 and baby food), the experimentally determined percentages fully confirm those reported in the labels, with a Pearson's r of 0.99. The "Mix for bread making" sample showed a higher percentage of farro in comparison with those reported in the label, whereas the pasta sample contains farro as major ingredients, but even a percentage of hulless wheat.

3.4. Specificity

The specificity of the assay has been evaluated considering cereals that can be present in the mixture of ingredients in a food sample. Barley, oat and rice fail to give amplification signals, or give very low signals, as reported in Figure 3. Moreover, mixtures of hulled and hulless wheats with or without barley have been evaluated, as reported in the same Figure 3. Very close FAM and VIC amplification signals were obtained for the mixtures with or without barley, suggesting that the presence of barley has no significant impact on the results of the analysis, supporting the assay specificity.

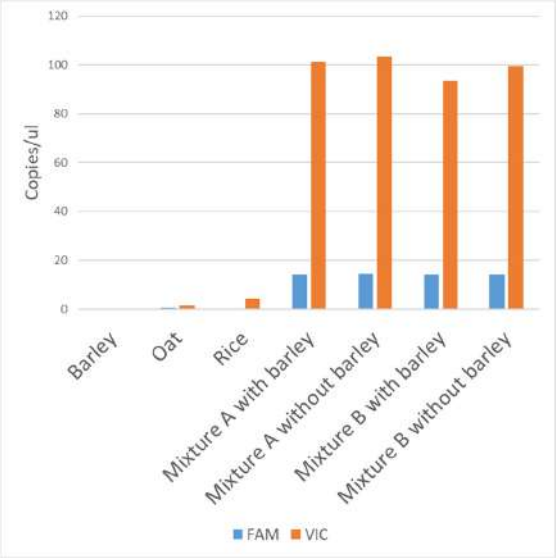

Figure 3. Copies/µL of FAM and of VIC targets. A total of 20 ng of barley, oat and rice DNA was amplified in dPCR. Mixture A was obtained by mixing 15 ng of hulled wheat DNA plus 4 ng of hulless wheat, with or without 1 ng of barley DNA. Mixture B was obtained by mixing 14 ng of hulled wheat DNA plus 4 ng of hulless wheat, with or without 2 ng of barley DNA.

3.5. Real-Time PCR Assay

The same commercial samples reported in Table 5 have been even analyzed with the real-time qPCR assay (Table 6), based on the use of the same primers/probes of dPCR and of the same calculation approach reported in Section 2.2.3. In the qPCR analysis, a double standard curve has been generated, considering dilutions of hulled and hulless wheats.

Table 6. Commercially available food sampled, their cereal content as reported in the label and hulled wheat percentages determined using a qPCR assay.

Commercial Sample	Cereal Formulation in the Label	Farro % qPCR Determined
Cookie 1	Farro 54%, common wheat	58%
Breakfast cereals	Whole farro flakes 100%	100%
Pearled farro	Farro 100%	100%
Bread substitute 1	Farro 99.8%	100%
Mix for bread making	Whole farro flour 7%, common wheat flour 93%	0%
Flour	Spelt flour	95%
Bread substitute 2	Common wheat flour, common wheat flakes 5.1%, toasted wheat bran, whole farro flour 2.1%, malted common wheat flour	0%
Pasta	Farro flour	90%
Egg pasta	Farro flour 80.64%	94%
Bread substitute 3	Common wheat flour, farro flour 30.4%, malt, oat flakes	27%
Cookie 2	Farro flour	100%
Baby food	Farro 100%	100%

Table 6 shows the percentages of farro experimentally determined with qPCR analysis. A Pearson's r of 0.99 has been found between the hulled wheat percentages reported in the label and the values experimentally determined with qPCR analysis. The mean values obtained with dPCR and qPCR analyses on this set of commercial samples are very close, with a Pearson's r of 0.98.

4. Discussion

A chip digital PCR assay has been developed for the discrimination between hulled and hulless wheats and their quantification along food chains. The assay is based on an allelic variation linked to the hulless/hulled seed morphology in wheats. Because einkorn, emmer and spelt seeds have the common characteristic to be hulled, whereas common and durum wheats are hulless, the dPCR assay can be used for the discrimination of the two wheat classes (i.e., hulless vs. hulled). The polymorphism targeted by the assay is localized in miRNA172 target site of the AP2-5 transcription factor on chromosome 5A that is involved in wheat threshing [26]. Polynomial curves have been developed starting from the nominal FAM/VIC ratios to calculate the hulled/hulless wheats percentages in experimental samples.

The assay has been evaluated on a panel of samples, including pure common and durum wheats, einkorn, emmer and spelt, hulless/hulled mixtures and commercial samples. From the results obtained, it can be concluded that the assay can be efficiently applied to the precise quantification of einkorn, emmer and spelt in mixture with common wheat or in mixture with durum wheat.

To evaluate the trueness of the method, defined as the degree of agreement of the expected value with the true value, the guidelines for GMO testing has been adopted. The main reason to adopt such guidelines lies in the fact that dPCR has so far been massively applied especially in the traceability of GMOs [29,30], which implies even the species traceability. The guidelines suggested for GMO can, therefore, likely be exploited for the traceability of *Triticum* species and subspecies. The same approach to evaluate trueness has been used by Köppel et al. [22] for the quantification of common wheat in spelt. According to such guidelines, the difference between the analytical value found compared to the certified, reference value must remain within the limits of 25% [31,32]. The trueness of our method fits the purpose: the estimated percentages were within the recommended ±25% acceptable bias.

The accuracy of the method decreases in samples in which, in addition to hulled wheats, blends of common and durum wheat are present in not-declared relative percentages. This situation, i.e., blends in unknown proportion of common and durum wheats together with hulled wheats, is probably not frequent. Durum wheat is in fact classically used in the pasta supply chain or the preparation of special types of bread. However, in such infrequent samples, the mean polynomial curve can be used for the percentage calculation of farro, as shown in Figure 1, with the awareness of obtaining an average quantification. The acceptability of this average quantification depends on the labeling requirements. Alternatively, it can be proposed to use two digitalPCR methods in series: the first, published by Morcia et al. [33], allows to quantify the percentage of hexaploid wheat compared to diploid wheat, while the second assay, proposed in this paper, provides the precise quantification of einkorn, emmer or spelt in samples containing common wheat, durum wheat or their mixtures at known percentages. Very recently, Köppel et al. [22] proposed an efficient dPCR assay designed on the Q-locus for the quantification of contaminations by common wheat in spelt-based products. However, in Italy and in other Mediterranean countries, emmer and durum wheat are the species of greater diffusion among, respectively, hulled and hulless wheats. Consequently, the dPCR assay developed in this study adds the ability to quantify hulless wheat contaminations in einkorn, emmer and spelt flours and derived food products. In addition to contamination by common wheat, our assay also takes into consideration that of durum wheat, a species of greater diffusion in Italy [34] and in other Mediterranean environments than common wheat.

Moreover, the analytical problems due to the co-presence of common and durum wheat as contaminants are also introduced.

The primers/probes developed firstly for dPCR application have been additionally evaluated in a real-time PCR system. Two main reasons suggested to check the applicability of the assay with a real-time PCR instrument. The first reason is that the dPCR instruments are not, at the present time, as widespread as real-time ones, and therefore, there may be an interest, for laboratories that do not have the dPCR, to exploit the assay with a real-time PCR machine. The second reason is linked to the comparative evaluation of the analytical costs of the assay using the dPCR in comparison with a real-time one. Taking into account the reagents' costs and the analytical time required, the cost of the dPCR approach in our hands is reduced by 30% compared to real-time PCR. The increased cost in real-time PCR is due to the higher amount of reagents required for each reaction and the need of standard curves' development.

5. Conclusions

A new dPCR assay to quantify hulless wheat contamination in raw materials and premium food made of einkorn, emmer or spelt has been developed. The innovation in comparison with already available DNA-based methods is in the technique adopted, simpler and faster and able to do a precise quantification. This same technique has been exploited in the study of Köppel et al. [22] using a different polymorphism of the Q-locus and focusing mainly on the discrimination between common wheat and spelt. In our work, all hulled wheat species (i.e., einkorn, emmer and spelt) and all hulless (i.e., durum and common wheats) were considered. In conclusion, dPCR is confirmed as a particularly promising analytical method for the identification and quantification of plant species, in defense of the authenticity of the product. The potential of this technique has been understood by the food industry and retailers, which collaborate in the development and validation of these methods. Contrary to what was thought at the dawn of its diffusion, this technique allows considerable savings, both in terms of analytical times and reagents, as verified in our work. This aspect is also even more true by observing the recent advances in digital PCR instrumentation, which allow for flexibility and scalability of the analyses such as to further reduce analytical costs.

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Communication

Moving from qPCR to Chip Digital PCR Assays for Tracking of some *Fusarium* Species Causing *Fusarium* Head Blight in Cereals

Caterina Morcia [1], Giorgio Tumino [1], Giulia Gasparo [1], Caterina Ceresoli [1], Chiara Fattorini [1], Roberta Ghizzoni [1], Paola Carnevali [2] and Valeria Terzi [1,*]

[1] Council for Agricultural Research and Economics, Research Centre for Genomics and Bioinformatics, I-29017 Fiorenzuola d'Arda PC, Italy; caterina.morcia@crea.gov.it (C.M.); giorgiotumino@hotmail.it (G.T.); giulia.gasparo@gmail.com (G.G.); caterinaceresoli@gmail.com (C.C.); chiarafattorini@alice.it (C.F.); roberta.ghizzoni@crea.gov.it (R.G.)

[2] Barilla S.p.A., I-43122 Parma PR, Italy; paola.carnevali@barilla.com

* Correspondence: valeria.terzi@crea.gov.it; Tel.: +39-0523-983758

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Abstract: *Fusarium* Head Blight (FHB) is one of the major diseases affecting small-grain cereals, worldwide spread and responsible for severe yield and quality losses annually. Diagnostic tools, able to track *Fusarium* species even in the early stages of infection, can contribute to mycotoxins' risk control. Among DNA-based technologies for *Fusarium* detection, qPCR (single and multiplex assays) is currently the most applied method. However, pathogen diagnostics is now enforced by digital PCR (dPCR), a breakthrough technology that provides ultrasensitive and absolute nucleic acid quantification. In our work, a panel of chip digital PCR assays was developed to quantify *Fusarium graminearum*, *F.culmorum*, *F. sporotrichioides*, *F. poae* and *F. avenaceum*. The primers/probes combinations were evaluated on pure fungal samples with cdPCR technique, in comparison with the qPCR approach. Moreover, the cdPCR assays were applied to quantify *Fusarium* in durum wheat and oat samples, naturally contaminated or spiked with fungal DNA. For a better evaluation of infection level in plants, duplex assays were developed, able to co-amplify both plant and fungal DNA. To the best of our knowledge, this is the first study directed to the application of digital PCR to *Fusarium* diagnosis in plants.

Keywords: molecular diagnostics; *Fusarium*; chip digital PCR; qPCR

1. Introduction

Fusarium Head Blight (FHB) is one of the major diseases affecting small-grain cereals, it is worldwide spread and responsible for severe yield and quality losses annually.

Several fungal species, mainly of the *Fusarium* genus, have been identified as the etiological agents of such a disease. In European cultivation environments, FHB occurs, mainly, because of *Fusarium graminearum* and *Fusarium culmorum* presence, but also *Fusarium poae*, *Fusarium pseudograminearum*, *Fusarium avenaceum*, *Fusarium sporotrichioides* and *Fusarium langsethiae*. Most of the species associated with FHB, in advantageous environmental conditions, invade the ear of the cereals and produce toxic secondary metabolites—mycotoxins—that contaminate the grain. FHB, therefore, compromises not only the yield but also the grain safety and quality due to the accumulation of mycotoxins in infected kernels. Depending on species and chemotypes, *Fusarium* can produce A and B trichothecenes: type A trichothecenes include highly toxic mycotoxins, such as T-2 and HT-2, meanwhile type B trichothecenes include, among others, deoxynivalenol (DON), nivalenol (NIV) and acetyl-NIV. Moreover, fumonisins, zearalenone, beauvericin and enniatin B can

accumulate in cereal grains after *Fusarium* infection. All these fungal secondary metabolites can cause a wide range of diseases as well as death in humans and animals [1].

Although contamination from mycotoxins occurs in the field, it has repercussions throughout the whole supply chain, including storage periods and transformation steps in the agri-food industries. Physical, chemical, and biological treatments might be defective for the purpose of total decontamination and/or detoxification, with permanence of active mycotoxins along the production cycle as consequence [2].

The fungal load, an environment conducive to the fungus development and a suitable host are indispensable prerequisites for the success of *Fusarium* infection. Consequently, several strategies can be activated to counter FHB. From an agronomic point of view, the fungal load can be reduced adopting Good Agricultural Practices (GAPs) [3]: e.g., optimizing the seeding density and fertilization levels, introducing crop rotation and soil practices that reduce crop residues [4]. The effectiveness of fungicidal treatments is related to their administration coinciding with the restricted period in which the initial infection of the plant can occur [5]. Starting from the information collected in monitoring actions, prediction models were developed. Such models, by weighing the various environmental and agronomic factors, give predictive indications on the risk of infection in cultivation areas [6]. To these strategies, the genetic one is implemented as well, that aims at obtaining, through different breeding approaches, naturally resistant varieties and, at the same time, maintaining the environmental impact of agriculture as low as possible [7]. The epidemiological characterization of present and emerging fungal species is also a useful tool in mycotoxins' risk control, together with the possibility of having facile, affordable, and early applicable diagnostic methods.

A molecular diagnostic approach has been proposed as an alternative strategy to traditional microbiological techniques for the identification and quantification of *Fusarium* species [8]. A bibliographic search, focusing on DNA-based methods and aiming to conduct *Fusarium* diagnostics in small-grain cereals, was published in 2009–2019 [9]. By applying the appropriate filters, 50 publications have been selected and analyzed to derive information, among others, on the molecular technology used. The obtained results are schematically shown in Figure 1, from which it can be inferred that the qPCR (single and multiplex assays) is the most widespread method, followed by multiplex PCR, LAMP-based protocols and metabarcoding.

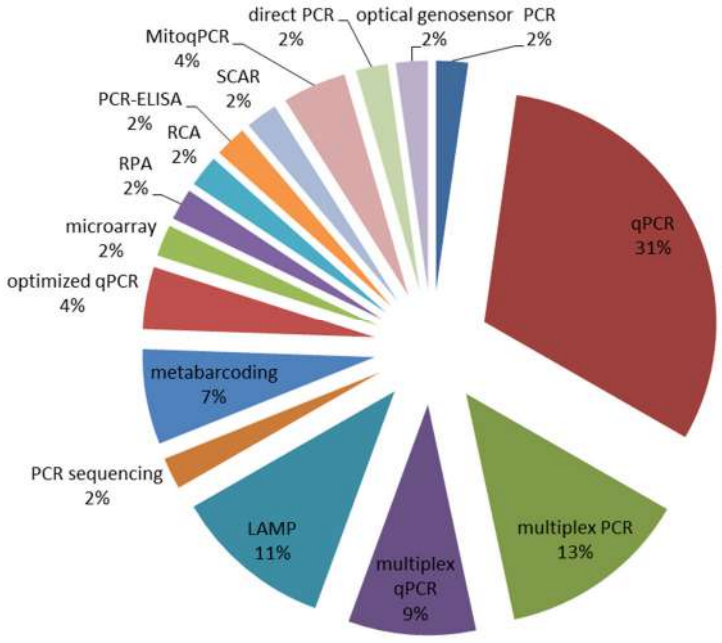

Figure 1. Relative percentages of molecular technologies currently applied to *Fusarium* detection in small-grain cereals. The data have been extracted from a panel of 50 peer-reviewed publications spanning 2009–2019 and specifically focused on the use of DNA tracking for *Fusarium* diagnosis in small-grain cereals [9].

Phytopathogen diagnostics can be now enforced by digital PCR (dPCR), a new PCR application in which the reaction volume is split over a high number of small-volume partitions or droplets [10]. After end-point amplification, each partition can be positive or negative, depending on the presence of a target sequence, therefore giving a binary or digital read-out. Poisson statistics are then used to determine the absolute quantity of target DNA in a sample. It is an absolute quantification strategy, therefore there is no need to have a standard curve reference. Moreover, the end-point measurement enables quantification independently of the reaction efficiency, thereby digital PCR can be used for low-target quantification even in variable contaminated samples [11,12]. Digital PCR is now considered an important tool in plant pathology laboratories [13] both for diagnostics [14] and pathogen biology studies [15]. The aim of our work was to develop a panel of dPCR assays to identify and quantify *Fusarium* species widely spread in cereals crop, starting from qPCR-based assays. The chip digital PCR (cdPCR)-developed assays were evaluated in durum wheat and oat samples naturally contaminated or spiked with *Fusarium* DNA. To the best of our knowledge, dPCR assays have not been evaluated until now for *Fusarium* diagnosis.

2. Materials and Methods

2.1. Fungal Samples

F.culmorum (MPVP/70) and *F. avenaceum* (MPVP/66) strains were obtained from Università Cattolica del Sacro Cuore, Piacenza, Italy. *F. graminearum* (ITEM 6477), *F. sporotrichioides* (ITEM 194) and *F. poae* (ITEM 10402) were provided by ISPA, Institute of Sciences of Food Production, CNR-National Research Council, Bari, Italy and belong to the ISPA collection of toxigenic fungi of agro-food interest (www.ispa.cnr.it/Collection).

Strains were stored on potato dextrose agar (PDA, Liofilchem, Teramo, Italy) at 4 °C until use. Fungal DNA was extracted from lyophilized mycelium previously grown on PDA medium, according to the procedure described by Al-Samarrai and Schmid [16]. DNA concentrations were determined using Qubit® fluorimeter (Life Technologies™, Invitrogen, Monza, Italy)

2.2. Plant Samples

In the study 19 *Triticum durum* (cultivars Claudio, Simeto, Aureo, Svevo and Creso) and 4 *Avena sativa* (cultivars Buffalo and Tardis) grain samples were used.

The plants were grown in the experimental fields of Research Centre for Genomics and Bioinformatics, in the 2015 and 2016 seasons, without any fungicide treatment. The 23 wheats and oats were grown in 3-m^2 plots, in triplicate. At maturity, the plots were harvested, and 20 gr of grains were sampled from each of the three plots and bulked. The 60-gr bulked sample was then milled into a fine powder using an analytical mill (IKA Universal mill M20, IKA-Werke GmbH, Staufen, Germany) and stored at 4 °C until analysis.

Plant genomic DNA was extracted in triplicate from 100 mg samples from the 60-gr bulked milled grains using DNeasy Plant Mini Kit (Qiagen Italia, Milano, Italy) according to the manufacturer's instructions. DNA concentrations were determined using Qubit® fluorimeter (Life Technologies™, Invitrogen, Monza, Italy). The DNA extracted were analyzed with qPCR to evaluate the presence of *Fusarium*. A subset of these samples was analyzed with cdPCR for *Fusarium* quantification. Moreover, a second subset of plant DNA samples was spiked with fungal DNA. For the preparation of such samples, batches of 20 ng plant DNA were added with 250, 100, 10, and 1 pg of fungal DNA.

2.3. Design of Primers and Probes

Table 1 reports the primers and probes sequences. Primer Express 3.0.1 Software (Life Technologies™, Invitrogen, Monza, Italy) was used to design *F. spo*, *F. gram*/*culm* and Avena dig assays. Multiple Primer Analyzer (Thermo Fisher Scientific, Monza, Italy) was used to verify the absence of self-complementarity and primer dimer formation.

Table 1. Primer and probe sequences used in the study to target different *Fusarium* and plant species.

Assay Code	Probe and Primers	Biological Target	Target Gene	Reference
F. spo	Pr: FAM-CTGCATCACAACCC-MGB F: GCAAGTCGACCACTGTGAGTACA R: TGAAACTACCCCGCCAAGTC	*F. sporotrichioides*	*tef1* GenBank: MN120771.1	This work
F. gram/culm	Pr: FAM-ATCAGTGCTTAAATGCA-MGB F: CAGTAGAGTCGACAAGATCTGCAATC R: TGAAAGTCGCGTAGCTGGAA	*F. graminearum* *F. culmorum*	*Tri* GenBank: MH514957.1	This work
F. poae	Pr: FAM-AAAGCGGTCGAGTCTG-MGB F: GCGGCCGCTTTTGTCA R: GCCTTTCCAGCAAGAGATGGT	*F. poae*	*esyn1*	[17]
F. avetric	Pr: FAM- CCGTCGAGTCCTCT -MGB F: AGCAGTCGAGTTCGTCAACAGA R: GGCYTTTCCTGCGAACTTG	*F. avenaceum*, *F.tricinctum*	*esyn1*	[17]
Grano CO2	Pr: VIC- CATGAGCGTGTGCGTG -MGB F: TGCTAACCGTGTGGCATCAC R: GGTACATAGTGCTGCTGCATCTG	*Triticum* genus	*Triticum TaHd1*	[18]
Avena dig	Pr: VIC- ACAATCTTTGCTTGTTCTT-MGB F: TCGTTGATTTTTGGTTGCTTTG R: AGCCTTTGCAATCCACATCTG	*Avena*	*actin 1*, GenBank: AF234528.1	This work

2.4. qPCR

Real-time reactions were prepared in duplicate with 8 µL of QuantStudioTM 3D Digital PCR 2X Master Mix (Applied Biosystems by Life Technologies, Monza, Italy), 900 nM forward and reverse primers, 200nmol of FAM and VIC-MGB probes, 20 ng of DNA template and water to 16 µL. The amplifications were done in a 7300 RealTime PCR System (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). The best amplification conditions obtained after optimization step for all assays are reported in Table 2.

Table 2. Optimized amplification conditions applied in qPCR and dPCR assays.

Assay Code	Initial Activation Step	Denaturation/Annealing/Amplification Step	N. of Cycles
F. sporo	95 °C, 10 min	95 °C, 30 s; 58 °C, 2 min	47
F. gram/culm	95 °C, 10 min	95 °C, 30 s; 60 °C, 2 min	45
F. poae	95 °C, 10 min	95 °C, 30 s; 60 °C, 2 min	45
F. avetric	95 °C, 10 min	95 °C, 30 s; 59 °C, 2 min	47
Grano CO2	96 °C, 10 min	98 °C, 30 s; 58–60 °C, 2 min	45–47
Avenadig	96 °C, 10 min	95 °C, 30 s; 58–60 °C, 2 min	45–47

For the determination of reaction efficiencies, standard curves were generated by plotting the Ct (Cycle Threshold) values versus the log10 amount of pure DNA of the different *Fusarium* (10-fold dilution series).

2.5. Chip Digital PCR

QuantStudioTM 3D Digital PCR System (Applied Biosystems by Life Technologies, Monza, Italy) was used for Chip digital PCR assays. The reaction was done in a final volume of 16 µL obtained by mixing 8 µL of QuantStudioTM 3D Digital PCR 2X Master Mix, 0.72 µL of each primer at 20 µM (final concentration 900 nmol), 0.32 µL of FAM and VIC-MGB probes at 10 µM (final concentration 200 nmol), 2 µL of DNA and nuclease free-water. Nuclease-free water as template was used in the negative control. The reaction mixture of 15 µL was loaded onto the QuantStudioTM 3D Digital PCR chips using QuantStudioTM 3D Digital chip loader, according to manufacturer instructions. Amplifications were performed in ProFlexTM 2Xflat PCR System Thermocycler (Applied Biosystems

by Life Technologies, Monza, Italy) under the same conditions used for qPCR amplifications and reported in Table 2. End-point fluorescence data were collected in QuantStudioTM 3D Digital PCR Instrument and files generated were analyzed using cloud-based platform QuantStudioTM 3D AnalysisSuite dPCR software, version 3.1.6. Each sample was analyzed in duplicate.

3. Results

3.1. Fungal Samples

The fungal DNA stocks were initially quantified with Qubit and the same dilutions of *F. sporotrichioides*, *F. graminearum*, *F. culmorum*, *F. poae* and *F. avenaceum* DNA were amplified with both qPCR and cdPCR techniques. A dynamic range of 0.5–0.0005 ng of fungal DNA was considered in all the assays. The same primers/probe combinations and the same amplification conditions were applied in both techniques. Table 3 reports the qPCR assays' efficiencies, as determined with qPCR.

Table 3. The R2 coefficients and amplification efficiencies of the four assays targeting *Fusarium* species were calculated in qPCR with the standard curve approach, using six calibration points with three PCR replicates each and the formula E = 10-^{1}/slope.

qPCR assay name	R2 coefficient	Amplification efficiency
F. sporo	0.984	104%
F. gram/culm	0.969	124%
F. poae	0.997	100%
F. avetric	0.991	106%

Figure 2 reports examples of the amplification patterns obtained after cdPCR analysis of some fungal DNA samples.

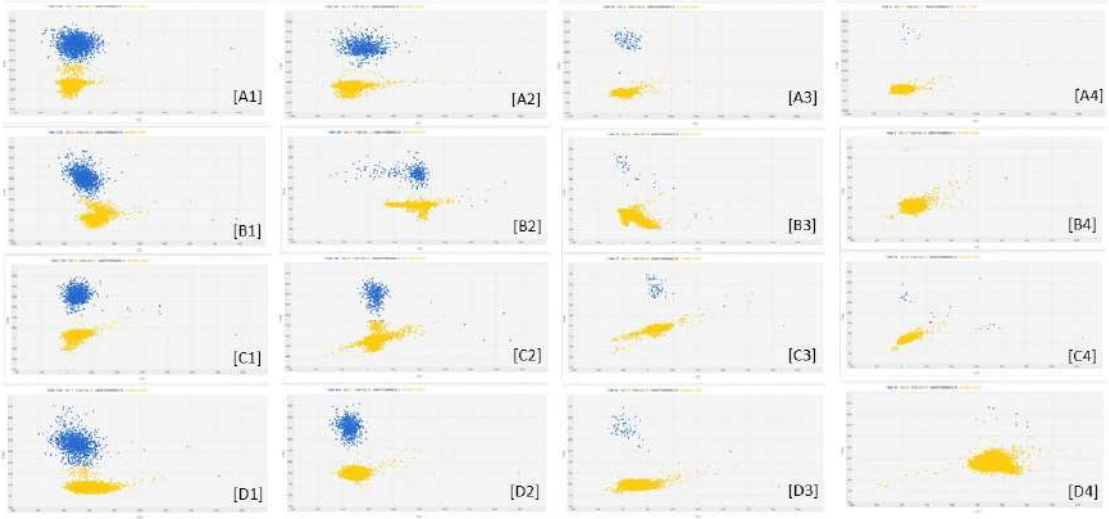

Figure 2. cdPCR amplification plots of *Fusarium* DNA dilutions. The letters indicate the different fungal species: [**A**] = *F. poae*; [**B**] = *F. sporotrichioides*; [**C**] = *F. graminearum*; [**D**] = *F. avenaceum*. The numbers indicate the dilutions factors: 1 means 0.25 ng of fungal DNA as Qubit quantified; 2 means sample 1 diluted 2.5 times; 3 means sample 2 diluted 10 times; 4 means sample 3 diluted 10 times. The blue dots are the PCR partitions resulted positive to amplification of the target; the yellow dots are the PCR partitions negative to amplification of the target.

Linearity between DNA dilution factors and copies/uL (cdPCR determined) has been found for all the assays (Figure 3) as well as high correlation levels between theoretical and cdPCR measured copies/µL for *F. sporotrichioides* (R^2 = 0.987), *F. graminearum* (R^2 = 0,999), *F. poae* (R^2 = 0.999) and *F. avenaceum* (R^2 = 0.999).

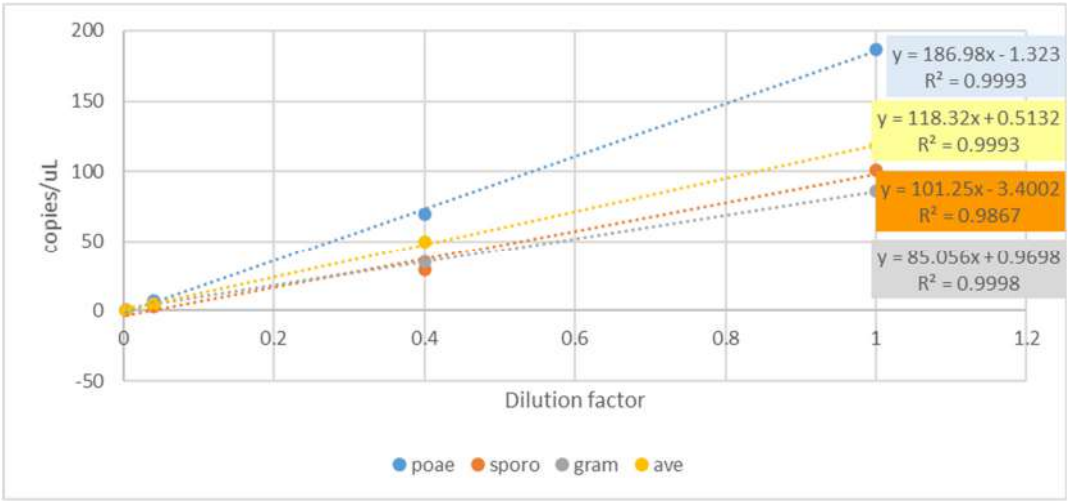

Figure 3. Linear regression among DNA dilution factors vs fungal copies numbers/µL as determined by cdPCR amplification of *F. poae* (blue dots), *F. sporotrichioides* (red dots), *F. graminearum* (grey dots) and *F. avenaceum* (yellow dots).

The Limit of Detection (LOD) and the sensitivity in cdPCR, for the four assays tracking *Fusarium*, were calculated with QuantStudioTM 3D AnalysisSuite dPCR software, as reported in Table 4.

Table 4. Limit of detection expressed as copies/µL and sensitivity of the four cdPCR assays.

Target	Limit of Detection	Sensitivity
F. sporotrichioides	10	0.466%
F. graminearum	13	0.6%
F. poae	2	0.134%
F. avenaceum	8	0.636%

Precision refers to the ability of distinguish between two measurements with a certain confidence. The AnalysisSuite TM Software calculates precision as the ratio of the maximum deviation of the confidence interval to the mean value, therefore it expresses the tightness of the confidence interval: the lower the precision, the tighter the confidence interval. In Figure 4, the precisions and the corresponding quantification values obtained amplifying fungal DNA dilutions with the four assays are reported. The highest DNA dilutions move rare target samples below the lower limit of detection and outside the supported dynamic range.

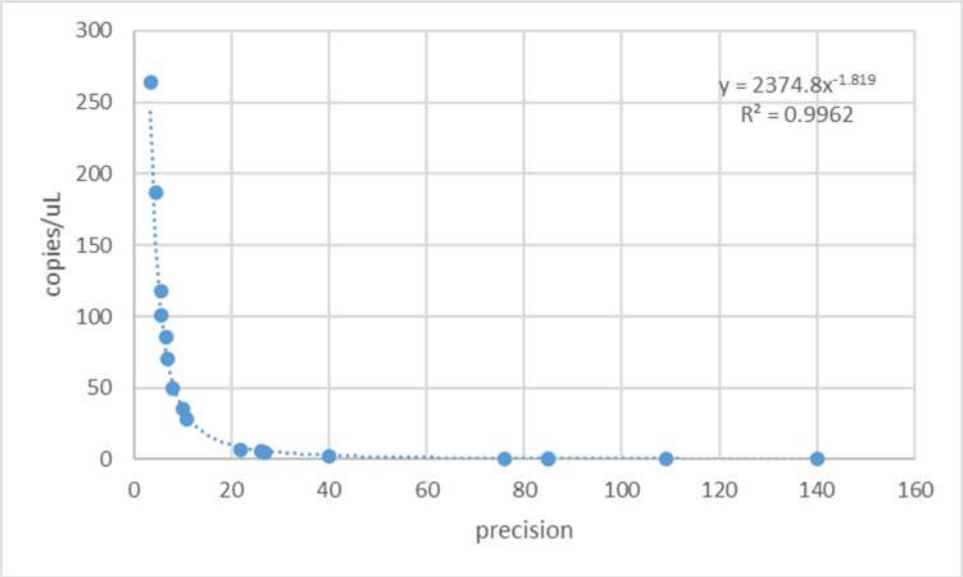

Figure 4. Relationship between precision values and copies/μL determined with the four *Fusarium* detection assays.

3.2. Plant Samples

cdPCR assays were validated on cereal samples naturally infected with mycotoxigenic fungi or spiked with fungal DNA. As previous step to cdPCR analyses, a set of durum wheat and oats samples were analyzed with the qPCR assays described in Table 2 to track fungal species. The rationale behind this preliminary step was to individuate samples naturally contaminated and samples free of fungal contamination and therefore suitable for the preparation of artificially contaminated ones. Three classes were found: i) samples in which no fungal species has been detected; ii) samples contaminated with one *Fusarium* species; iii) samples contaminated with two or more *Fusarium* species.

Starting from the data, the following two subsets of samples were further analyzed with cdPCR.

- Naturally contaminated samples, belonging to classes ii) and iii);
- Synthetic samples created by spiking plant DNA (extracted from samples found not contaminated) with fungal DNA at different concentrations.

The fungus quantifications in the two subsets of samples obtained with the qPCR and cdPCR assays are reported in Table 5.

Table 5. *Fusarium* quantification values obtained in both naturally contaminated and in spiked samples with qPCR and cdPCR technologies.

***Fusarium* Strain**	**Plant Genus**	**Naturally Contaminated Sample**	**Spiked Sample**	**pg of Fungal DNA/50 ng plant DNA (qPCR)**	**Fungal copies/µL (cdPCR)**
F. sporotrichioides	*Triticum durum*		X	360 ± 12	89 ± 7.2
F. sporotrichioides	*Triticum durum*		X	120 ± 9	50.1 ± 5.2
F. sporotrichioides	*Triticum durum*		X	61 ± 3	16.65 ± 3
F. sporotrichioides	*Triticum durum*		X	4.1 ± 0.9	2.35 ± 1.1
F. graminearum/culmorum	*Triticum durum*	X		34 ± 5	20.8 ± 3.4
F. graminearum/culmorum	*Triticum durum*	X		60 ± 2	36.35 ± 4.7
F. graminearum/culmorum	*Triticum durum*	X		66 ± 9	41.2 ± 4.9
F. graminearum/culmorum	*Triticum durum*	X		57 ± 6	27.8 ± 4.2
F. poae	*Triticum durum*	X		1.3 ± 0.2	0.08 ± 0.04
F. poae	*Triticum durum*	X		2.1 ± 0.5	0.87 ± 0.1
F. poae	*Triticum durum*	X		1.6 ± 0.6	0.91 ± 0.5
F. poae	*Triticum durum*		X	126 ± 1.5	56.7 ± 6.4
F. poae	*Triticum durum*		X	62 ± 0.9	39.2 ± 7.3
F. poae	*Triticum durum*		X	6.2 ± 0.4	4.35 ± 1.6
F. poae	*Triticum durum*		X	0.15 ± 0.1	0.40 ± 0.2
F. avenaceum	*Triticum durum*	X		1.1 ± 2.1	0.6 ± 0.46
F. avenaceum	*Triticum durum*	X		1.6 ± 0.9	0.4 ± 0.3
F. avenaceum	*Triticum durum*	X		14 ± 2.3	8.4 ± 2.2
F. avenaceum	*Triticum durum*	X		16 ± 1.9	8.3 ± 2.3
F. avenaceum	*Triticum durum*		X	300 ± 9.2	118 ± 8.5
F. avenaceum	*Triticum durum*		X	120 ± 7.5	50.1 ± 5.4
F. avenaceum	*Triticum durum*		X	46 ± 4.6	5.5 ± 1.8
F. avenaceum	*Triticum durum*		X	4.8 ± 1.8	0.89 ± 0.71
F. avenaceum	*Avena sativa*		X	286 ± 8.2	128 ± 9
F. avenaceum	*Avena sativa*		X	111 ± 5.8	55.3 ± 5.8
F. avenaceum	*Avena sativa*		X	42 ± 4.2	10.1 ± 2.4
F. avenaceum	*Avena sativa*		X	5.9 ± 1.2	3.9 ± 1.5

The contaminated plant samples belonged to *Triticum durum* and *Avena sativa* species; therefore, a further objective of our study was to develop a duplex assay, able to co-amplify both plant and fungal DNA. The rationale behind the duplex assay's development is to evaluate the impact of relevant quantity of plant DNA on the functioning of primers and probes of the fungal PCR assays. Moreover, the ratio between the quantity of *Fusarium* and plant DNA can be informative about the infection level in a sample. Two genic targets for durum wheat and oat were selected from the literature or newly developed. The Grano CO2 assay, designed on *TaHd1* gene sequence and developed by Morcia et al. [18] was used to track *Triticum* genus. A new assay (Avena dig assay) was designed on *actin1* gene sequence to track Avena genus. Grano CO2 and Avena dig assays amplification efficiencies, evaluated in qPCR, have values of 99.6 and 111%, respectively.

The compatibility of the tests to trace the fungal and plant species in cdPCR was evaluated comparing the precision values of the simplex vs duplex assays. Correlation values ranged from a minimum of 0.97 to 0.99; therefore, the assays are fully compatible and can be organized in duplex mode.

Figure 5 reports, as examples, cdPCR plots of oat DNA spiked with *F. avenaceum* DNA dilutions.

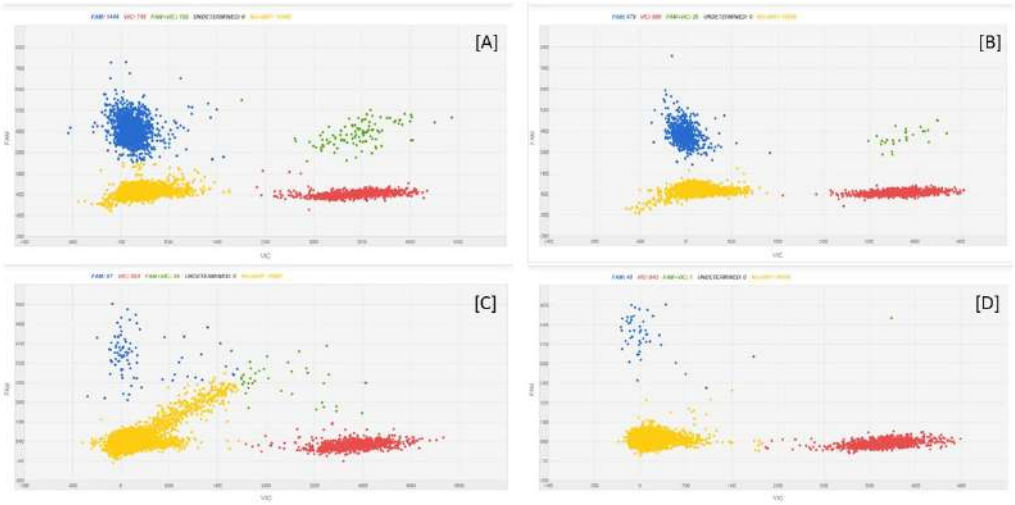

Figure 5. cdPCR amplification plots of oat DNA spiked with *F. avenaceum* DNA dilutions. [**A**] = 20 ng oat DNA + 0.25 ng *F. avenaceum* DNA; [**B**] = 20 ng oat DNA +0.1 ng *F. avenaceum* DNA; [**C**] = 20 ng oat DNA +0.01 ng *F. avenaceum* DNA; [**D**] = 20 ng oat DNA +0.001 ng *F. avenaceum*DNA. The blue dots are the PCR partitions with a positive result, indicating an amplification of the fungal target; the red dots are the partitions which are positive to the plant target; green dots are the partitions in which both the targets were amplified; and the yellow dots are the empty partitions, showing a result that was negative to the amplification of the targets.

Figure 6 reports some examples of the results obtained quantifying with cdPCR the fungal and wheat copies/uL in durum wheat samples spiked with fungal strains (Figure 6A,B) or in naturally contaminated ones (Figure 6 C,D).

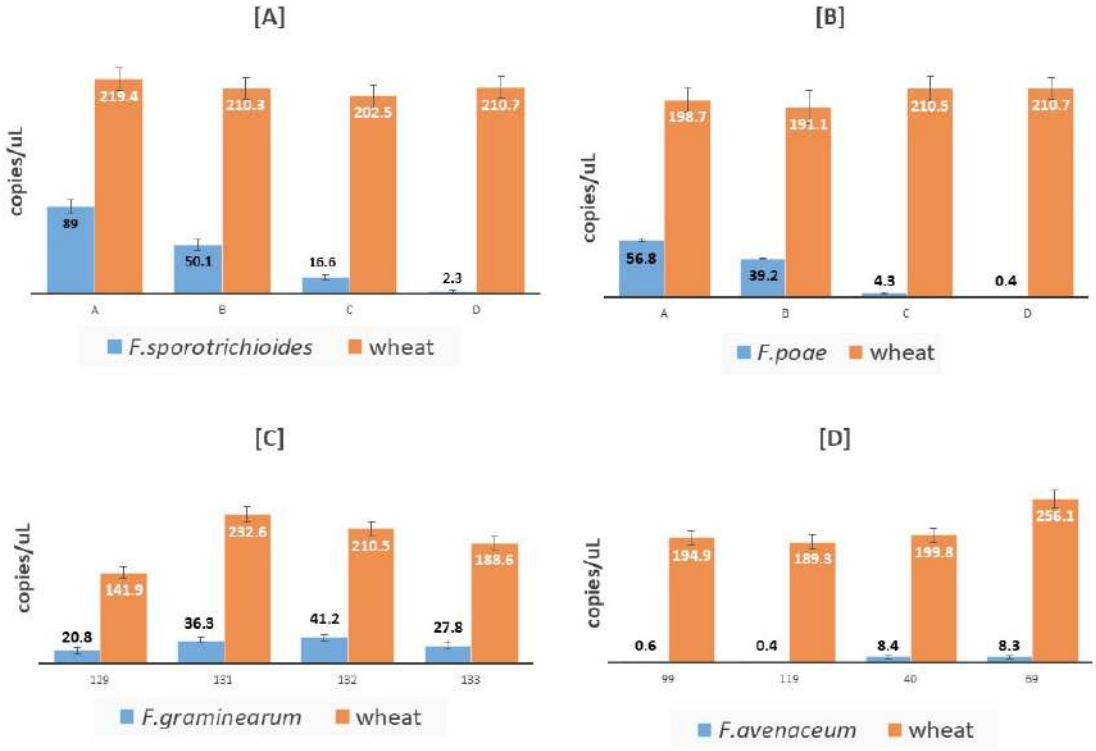

Figure 6. Mean copies/µL of fungal (blue bars) and wheat (red bars) quantified in durum wheat samples spiked with *F. sporotrichioides* [**A**] or with *F. poae* [**B**] and in durum wheat samples found naturally contaminated with *F. graminearum* [**C**] or with *F. avenaceum* [**D**].

4. Discussion

In this work we propose four cdPCR assays for detection and quantification of mycotoxigenic *Fusarium*, etiological agents of *Fusarium* Head Blight in small-grain cereals. The assays were organized as duplex assay to simultaneously quantify the fungus and the plant species. The logic behind the development of molecular tools for *Fusarium* diagnosis rely on the possibility to increase fungal control in plants. The fungal DNA can be tracked in the plant during the initial phase of infection, when visible symptoms are absent. Such early diagnosis can mitigate mycotoxin contamination problems in the harvested grains thanks to appropriate fungicidal treatments applied in the right temporal window as well as segregation of highly infected field sectors. We focused on *Fusarium* species worldwide spread in cereal cultivation areas: *F. graminearum* and *F.culmorum*, which are widely recognized as the most important DON producers in small-grain cereals [19], *F. poae* which shows a NIV chemotype although not all isolates produced NIV in vivo [20], *F. sporotrichioides*, a T-2 and HT-2 toxins producer which is frequently isolated in some temperate regions of Europe [21] and *F. avenaceum*, an enniatin and beauverin producer [22].

As already stated, several molecular assays have been recently developed for *Fusarium* diagnosis but, to the best of our knowledge, none based on a digital PCR has been proposed until now.

Our assays fill this gap, giving the chance to identify and quantify the presence of mycotoxigenic *Fusarium* in small-grain cereal samples with digital PCR technology. Such new assays can be now practically used in *Fusarium* diagnosis.

As reported by other authors [23,24], dPCR has several advantages in a comparison with qPCR.

- As the most advantageous feature, dPCR relies on absolute quantification of the target operated by dPCR; on the contrary, "*results generated from qPCR were relative to calibration curve and were not the actual number of copies in a sample itself*" [23].
- Secondarily, the high sample partitioning ensures accurate results even at very low target copy numbers as well as detection of rare targets even in a high background of non-target DNA [24],

- Lastly, dPCR is less sensitive to contaminants eventually present in the samples; complex biomolecules such as humic acid can, in fact, significantly inhibit qPCR reactions, but dPCR can overcome this lack thanks to its endpoint quantification [23].

Our cdPCR assays have a LOD ranging from 2 to 13 copies/μL; this level of sensitivity is suitable to *Fusarium* diagnosis purposes in field, for FHB control, for fungicide treatments optimization and breeding purposes. The main disadvantages we encountered, compared with qPCR, are related to the expenses of the analysis, amount of sample analyzed in a certain time. Controversial are the opinions on the commercial cost of dPCR vs qPCR assays. The processivity can be improved by multiplexing, as suggested by Demeke and Dobnik [24]; it is also related to the different instruments available on the market.

Our specific experience highlighted the necessity of specific laboratory skills for both qPCR and cdPCR as well as similar supporting instruments in the laboratory.

In conclusion, our position on the topic is that dPCR has the potential to replace qPCR in some diagnostic fields, e.g., for Genetically Modified Organisms detection [24]. With regard to microbiological routine diagnostics, the two technologies can be considered complementary, and therefore advantageously used in combination. qPCR technology, as previously stated, requires a reference standard curve for quantification, although standardized reference materials of plant pathogens are generally unavailable. Digital PCR, on the contrary, gives an absolute quantification of the molecular target as output and can, therefore, be proposed for characterization of the calibrators needed for standard curves in qPCR analyses. As suggested by other authors [25], dPCR can hypothetically be exploited for the production of calibrators.

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Chapter 2

Design of Rye-Based Foods with Enhanced Value

foods

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Article

Hierarchical Effects of Lactic Fermentation and Grain Germination on the Microbial and Metabolomic Profile of Rye Doughs

Walter Mancino [1,†], Paola Carnevali [2,†], Valeria Terzi [3], Pascual García Pérez [1,4], Leilei Zhang [1], Gianluca Giuberti [1], Lorenzo Morelli [1], Vania Patrone [1,*] and Luigi Lucini [1]

1 Department for Sustainable Food Process (DiSTAS), Università Cattolica del Sacro Cuore, 29122 Piacenza, Italy
2 R&D Food Microbiology & Molecular Biology Research Barilla G. e R. Fratelli S.p.A., 43122 Parma, Italy
3 Council for Agricultural Research and Economics, Research Centre for Genomics and Bioinformatics, 29017 Fiorenzuola d'Arda, Italy
4 Nutrition and Bromatology Group, Analytical and Food Chemistry Department, Faculty of Food Science and Technology, Universidade de Vigo, 32004 Ourense, Spain
* Correspondence: vania.patrone@unicatt.it
† These authors contributed equally to this work.

Abstract: A multi-omics approach was adopted to investigate the impact of lactic acid fermentation and seed germination on the composition and physicochemical properties of rye doughs. Doughs were prepared with either native or germinated rye flour and fermented with *Saccharomyces cerevisiae*, combined or not with a sourdough starter including *Limosilactobacillus fermentum*, *Weissella confusa* and *Weissella cibaria*. LAB fermentation significantly increased total titrable acidity and dough rise regardless of the flour used. Targeted metagenomics revealed a strong impact of germination on the bacterial community profile of sprouted rye flour. Doughs made with germinated rye displayed higher levels of *Latilactobacillus curvatus*, while native rye doughs were associated with higher proportions of *Lactoplantibacillus plantarum*. The oligosaccharide profile of rye doughs indicated a lower carbohydrate content in native doughs as compared to the sprouted counterparts. Mixed fermentation promoted a consistent decrease in both monosaccharides and low-polymerization degree (PD)-oligosaccharides, but not in high-PD carbohydrates. Untargeted metabolomic analysis showed that native and germinated rye doughs differed in the relative abundance of phenolic compounds, terpenoids, and phospholipids. Sourdough fermentation promoted the accumulation of terpenoids, phenolic compounds and proteinogenic and non-proteinogenic amino acids. Present findings offer an integrated perspective on rye dough as a multi-constituent system and on cereal-sourced bioactive compounds potentially affecting the functional properties of derived food products.

Keywords: LAB; yeast; fermentation; rye; metagenomics; dough; metabolomics; germination

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1. Introduction

Rye bread is one of the most consumed cereal-based foods in northern Europe, China, and North America [1]. In these regions, rye (*Secale cereale* L.) is a valuable crop because of its resistance towards cold temperatures and northern climates [1].

From a nutritional perspective, rye flour is gaining attention for its health-promoting potential considering its hypocholesterolemic, anti-diabetic, anti-inflammatory, and cardioprotective properties [1]. Whole grain rye is characterized by a high content of dietary fibers, such as arabinoxylans and cellulose, and bioactive compounds with antioxidant properties, such as polyphenols [2]. Driven by consumer demand for sustainable and healthier products, the utilization of rye in cereal-based, functional foods has been widely explored [3]. In this context, seed germination or sprouting has gained popularity in cereal processing as an effective practice to improve grains' nutritional quality and functional

value. Sprouting involves the activation of endogenous hydrolytic enzymes that results in augmented digestibility of cereal proteins and starch [4]. Moreover, it increases the bioavailability of simple sugars, amino acids, phenolic compounds, minerals, and certain vitamins [4]. Likewise, sourdough fermentation is a traditional process that has been shown to affect different attributes of baked goods due to metabolic activities of indigenous yeasts and lactic acid bacteria (LAB). In addition, LAB can positively affect the nutritional value of fermented cereals by increasing the content of bioactive compounds, vitamins, and minerals, and decreasing the amount of anti-nutritional factors [5]. Lactic fermentation has emerged as a promising alternative to improve gut health, preventing digestion-related issues such as gluten sensitivity, and playing a role in the detoxification of common food mycotoxins [6–8].

Previous research had shown that both germination of grains before fermentation and the type of fermentation markedly affected the structure and the potential bioactive properties of rye constituents [9,10]. However, to the best of our knowledge, no comprehensive multi-omics study has yet been made to evaluate the impact of seed sprouting and lactic acid fermentation on rye dough composition and quality. Consequently, we took advantage of targeted metagenomics to assess the evolution of the inoculated starters and their overall impact on bacterial ecology. Untargeted metabolomics analyses were applied to uncover microbial contribution to the biochemical profile of grain doughs. We sought to unravel dynamic relationships between microorganisms and food matrix components and identify potential markers of functional capacity important for developing value-added rye products. The use of advanced metagenomics techniques can be important to verify and validate the ecological success of certain starters, which can be interesting for large-spectrum industrial productions [11]. In this context, this approach may be also of interest from an industrial perspective to gain further insight into the effect of traditional technologies such as germination and fermentation on rye flour microbial, chemical, and technological multiple changes aiming to encourage the production of newly rye-based wholesome ingredients and related food products. Finally, the study of oligosaccharide profiles as a function of fermentation, can give a fundamental contribution to understanding how the substrates are modified and which carbohydrate components are present within the dough system [12].

2. Materials and Methods

2.1. Microbial Strains and Growth Conditions

Limosibacillus fermentum UC3641, *Weissella confusa* UC4052, and *Weissella cibaria* UC405, previously isolated from sorghum sourdough [13], were used as LAB starters. The strains were grown in MRS medium in anaerobic conditions, using a jar and the anaerocult P reagent (Merck, Germany) at 37 °C. Cultures were harvested by centrifugation (4000× *g* × 10 min), washed twice with sterile saline solution (0.9% NaCl), and re-suspended in 5 mL of water. *Saccharomyces cerevisiae* was a commercial, compressed fresh baker's yeast (Lessafre, Marcq-en-Barœul, France), reintegrated in water before use.

2.2. Germination of Rye Flour and Micro-Malting Process

Unprocessed commercial rye grains (SU Bendix winter rye) were subjected to a preliminary sieving step. Kernel size fractions between 2 and 2.5 mm were obtained using an Octagon 200 test sieve shaker (Endecotts Ltd., London, UK). Malting was performed on 100 g sieved seed batches with an Automatic Micromalting System (Phoenix Biosystems, Adelaide, SA, Australia) (Figure S1). The following malting cycle (144 h in total) was applied: 15-min wash at room temperature; 7-h and 15-min steep at 15 °C; 8-h rest at 19 °C; 9-h steep at 15 °C; 6-h germination at 19 °C; 30-min steep at 15 °C; 88-h and 30-min germination at 15 °C; 7-h kilning from 30 to 40 °C; 6-h kilning from 40 to 60 °C; 6-h and 30-min kilning from 60 to 70 °C; 4-h and 30-min kilning from 70 to 80 °C; and 30-min kilning at 25 °C. The chemical composition of native rye flour was: dry matter (DM): 90.2 g/100 g; total starch: 59.0 g/100 g DM; crude protein: 9.3 g/100 g DM; total

dietary fiber: 17.6 g/100 g DM, free glucose: 0.28 g/100 g DM. The chemical composition of sprouted rye flour was: DM: 93.1 g/100 g; total starch: 49.3 g/100 g DM; crude protein: 11.1 g/100 g DM; total dietary fiber: 24.1 g/100 g DM, free glucose: 5.6 g/100 g DM.

2.3. Dough Preparation and Chemical-Physical Characterization

In brief, 250 g of native or sprouted rye flours were mixed with 250 mL of tap water and 1% (*w*/*v*) of NaCl. The kneading process was preformed through a commercial kneading machine (IMETEC ZERO-GLU KM 1500, Tenacta Group Spa, Azzano S. Paolo, Italy), initially without inoculum, at machine speed 1 for 2 min. As for fermentation, three different experimental conditions were tested: (i) *S. cerevisiae* fermentation, in which *S. cerevisiae* was inoculated at a final concentration of 2% (*w*/*w*), SC; (ii) mixed fermentation, in which a mix of the three LAB strains (10^9 CFU/mL) plus *S. cerevisiae* (2% *w*/*w*) was added to the dough, LAB + SC; and (iii) spontaneous fermentation, where non-inoculated doughs were prepared by replacing the inoculum with an equal volume of plain water, considered as the control. The initial concentration of LAB in each inoculated dough was between 1.89–3.00 $\times$ 10^7 cfu/g, as expected, whereas the initial concentration of *S. cerevisiae* in inoculated doughs was 1.30–1.68x10^6 cfu/g. After inoculation, the kneading process was carried out for 5 min at machine speed 3. All doughs were maturated for 24 h; for the mixed fermentation, an initial maturation with only inoculated LAB was performed for 8 h (30 °C and 60% relative humidity), after which yeast was added and the dough was left to leaven for the remaining 16 h (35 °C and 65% relative humidity). The same temperature and humidity conditions were applied for SC and control. Dough samples were prepared in duplicate and fermentations were repeated twice. All the doughs were analyzed for pH (pH meter Hanna Edge), total titratable acidity (TTA) [14], water activity (a_w) (Aqualab Serie 4; Steroglass, Perugia, Italy), dough rise, total yeast, and LAB count. Dough rise was determined as follows: after mixing, 20 g of the dough was transferred into a graduated cylinder. The height of the dough was measured before and after fermentation, and the dough increase in volume was calculated as previously reported [15]. Yeast and LAB counts were performed in duplicate on YPD agar supplemented with chloramphenicol and MRS agar with 1% of cycloheximide, respectively.

2.4. Extraction of DNA and Full-Length 16S Metagenomics Analysis

Microbial cells were harvested from doughs as previously described [16]. Total DNA was extracted from bacterial pellets using the Fast DNA Spin Kit for Soil (MP biomedicals, Irvin, CA, USA) according to the protocol supplied. DNA quantity was determined by a Qubit fluorimeter (Life Technologies, Carlsbad, CA, USA), while DNA integrity was checked through 0.8% agarose gel electrophoresis. Samples were prepared according to the guidelines for preparing SMRTbell template for sequencing on the PacBio Sequel I System (PacBio, Menlo Park, CA, USA) at Macrogen (Seoul, Republic of Korea). The library was constructed with SMRTbell® Express Template Prep Kit 2.0 including 27F-1492R primers [17] along with barcode according to the manufacturer's instructions (Pacific Biosciences, Menlo Park, CA, USA); library purification was carried out using Ampure® PB bead (Pacific Biosciences, Menlo Park, CA, USA). Purified SMRTbell library from the pooled and barcoded samples was sequenced on a single PacBio Sequel cell using a SMRT Cell 1M v3 Tray. The resulting data were processed using the rDNATools pipeline [18]. Ambiguous reads and short reads (<1199 bp) were filtered out, and extra-long tails were trimmed according to the target size of the bacterial 16S rRNA gene. Chimeras were identified and removed using the software Vsearch v2.14.2 using the default settings. Then, a distance matrix was generated using the Unweighted/Weighted UniFrac distance, and reads were clustered using the average neighbor method. Operational Taxonomy Unit (OTU) picking was based on the de novo method; sequences that shared over 99% similarity were assigned to a single OTU. Taxonomic assignment of OTUs was obtained using QIIME2 bioinformatic pipeline v 2022.2 [19], which provides functionality for working with and visualizing

taxonomic annotations of features. OTUs were aligned with the representative sequence of the NCBI 16S Database with a similarity cutoff of 97% for species differentiation.

2.5. Oligosaccharide Semi-Quantification of Rye Doughs

The oligosaccharide semi-quantification of the different rye doughs was performed by high-performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) approach. All samples were subjected to a previous extraction, in which 1 g of sample was mixed with 10 mL of deionized water, and the resulting mixture was homogenized by a high-speed rotor (Polytron PT 1600-E) for 1 min and centrifuged at 8000× *g* for 10 min at 4 °C (Eppendorf 5810R, Hamburg, Germany). The supernatants were collected, syringe filtered (0.22 µm pore size), and transferred into vials for the subsequent analysis. The experimental conditions applied for HPAEC-PAD analysis were previously described [20]. The equipment employed consisted of a Dionex ICS-5000+ (Thermo Fisher Scientific, Waltham, MA, USA), containing an electrochemical cell with a gold working electrode combined with a pH-Ag/AgCl reference electrode as detection system. The chromatographic separation was achieved through a Dionex CarboPac PA200 column (3 × 250 mm) coupled to a guard column (3 × 50 mm), as the stationary phase (both purchased from Thermo Scientific), which provides a high-resolution separation of monosaccharides and linear oligosaccharides. The mobile phase consisted in a binary solvent system that included 100 mM NaOH (eluent A) and 1 M sodium acetate in 100 mM NaOH (eluent B). The experimental runs presented a total time of 75 min, the flow rate was adjusted at 0.4 mL min^{-1} and temperature for both column and detector compartments were set at 27 °C, following a multi-gradient elution system: 0–10 min, 98% A; 10–35 min, 55% A; 65–75 min, 98% A. The amperometric detector was set in terms of several potentials and durations as follows: E1 = 0.10 V (t1 = 0.40 s); E2 = −2.00 V (t2 = 0.01 s); E3 = 0.60 V (t3 = 0.01 s); E4 = −0.10 V (t4 = 0.06 s). The semi-quantification of oligosaccharides was achieved according to their polymerization degree (PD), with respect to individualized standards that are representative of three different well-separated structural classes: monosaccharides, low-PD oligosaccharides, and high-PD oligosaccharides. Thus, xylose was applied as the reference standard for monosaccharides (y = 3.8348x + 1.007, R^2 = 0.9918), arabinotriose was selected as the reference standard for low-PD oligosaccharides (y = 3.7960x + 1.656, R^2 = 0.9878), and arabinooctaose was chosen as the reference standard for high-PD oligosaccharides (y = 0.9963x + 0.8871, R^2 = 0.9842). The results were expressed as carbohydrate content in mg g^{-1} of sourdough. The standards and reagents employed for oligosaccharide profiling were purchased from Sigma Aldrich®, Darmstadt, Germany (xylose) and Megazyme®, Bray, Ireland (arabinotriose and arabinooctaose).

2.6. Untargeted Metabolomic Profiling of Rye Doughs

The untargeted metabolomic profiling of the different rye doughs was obtained through an ultra-high performance liquid chromatography instrument (Agilent 1200 series), presenting a binary pump and JetStream electrospray source, coupled to a quadrupole time-of-flight mass spectrometer (UHPLC/QTOF-MS; Agilent iFunnel 6550). Before the analytical determination, 1 g of each rye doughs was mixed with 10 mL of the extraction solvent MeOH:H_2O:HCOOH (80:19.9:0.1, *v*/*v*/*v*) and the extraction was performed under the same conditions described earlier [21]. All analytical conditions were set as described elsewhere [21,22]. Briefly, the injection volume was 6 µL, the chromatographic separation was achieved through an Agilent Zorbax Eclipse Plus C18 column (50 × 2.1 mm, 1.8 µm), applying an AcN:H_2O binary mobile phase with a gradient elution: 6%–94% organic phase for a 33 min run, with a flow rate of 200 µL min^{-1}. The analytical conditions for the QTOF performance were employed as follow: N_2 as sheath gas with a flow of 10 L min^{-1} at 350 °C, drying gas was applied with a flow of 8 L min^{-1} at 330 °C, nebulizer pressure was set at 60 psi, nozzle voltage at 300 V, and capillary voltage at 3.5 kV. The mass spectrometer was adjusted in positive polarity and SCAN mode, with a detection range of 100–1200 *m*/*z*, with a nominal resolution of 40,000 FWHM. Moreover, quality control pooled

samples were obtained and further analyzed through data-dependent MS/MS mode (12 precursors per cycle at 1 Hz, 50–1000 *m/z*, positive polarity), applying different collision energies: 10, 20 and 40 eV. The acquired raw data were later processed by the Agilent MassHunter Profinder v.10.0 using the "find-by-formula" algorithm, through mass and retention time alignments (5-ppm and 0.05 min tolerance, respectively). The annotation of the obtained chemical features was based on their accurate mass and isotopic patterns, given by the exact masses, relative abundance and *m/z* spacing, using the FooDB database (available at foodb.ca) to achieve their identification. Data reduction was achieved by applying the "filter-by-frequency" feature, exclusively retaining the features observed in all the replicates within the same treatment. As a result, the untargeted compound annotation obtained was in compliance with identification level 2 provided by the COSMOS Metabolomics Standard Initiative (putatively annotated compounds). To improve the confidence in the compound annotation raw data were processed by MS-DIAL software (v. 4.90), achieving the identification of chemical features through MS-MS spectral data, according to mass accuracy data, mass isotopic patterns, and spectral alignment matching. The parameters set for identification were: retention time range, 1–32 min; mass range, *m/z* 80–1200; mass tolerance, 0.05 Da. For data reduction, a filter step was performed, removing the identities that were not acquired in, at least the 80% of replicates. Finally, a score cut-off of 80% was selected to retain those compounds with the highest identification fidelity according to MS^2 level. All chemicals used for extraction and chromatographic equipment were LC-MS grade, purchased from VWR (Milan, Italy).

2.7. Statistics

A two-way analysis of variance (ANOVA) was performed to analyze the effect of starter and germination on dough general parameters using GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, CA, USA). Metagenomic data were processed using the QIIME2 v.2020.2 platform for diversity analysis of dough microbial communities. Observed species, Chao1, Shannon and Gini-Simpson indices were calculated to assess within sample diversity; sequencing depth was characterized by Good's coverage. Weighted and unweighted Unifrac distances were calculated, and Principal Coordinate Analysis (PCoA) was performed on the distance matrices to visualize community variation across samples. The OTU table was uploaded to the Microbiomeanalyst server for compositional profiling and comparative analysis, using 10% prevalence in samples for the low count filter, the default settings for other filtering and total sum scaling for data normalization [23]. To test for significance in differential bacterial taxa abundance according to starter and flour, respectively, the algorithm DeSeq2 was used [24]. The name of the samples was as follows: nDLY (native rye–Dough–LAB + SC); sDLY (sprouted rye–Dough–LAB + SC); nDY (native rye–Dough–SC); sDY (sprouted rye–Dough SC); nDCTR (native rye–Dough–Control); sDCTR (sprouted rye–Dough–Control); nF (native rye–Flour); sF (sprouted rye–Flour). For both oligosaccharide and untargeted metabolomics profiling approaches, all sourdough samples were extracted in triplicate, and two technical determinations were carried out for each replicate ($n = 6$). The results for carbohydrate content of rye sourdoughs were statistically analyzed by one-way ANOVA followed by Duncan's post hoc test, setting a significance value of $\alpha = 0.05$, using the software SPSS 25 (IBM). The Agilent Mass Profile Professional v. 15.1 software analyzed the metabolomics data as previously indicated compounds were filtered by abundance, log2-transformed, and normalized at the 75th percentile [22]. The abundance of each compound was further baselined against the median abundance among all samples. Afterwards, an unsupervised multivariate hierarchical cluster analysis (HCA) was performed to evaluate the similarities and dissimilarities of all factors as a function of their metabolic profile (Euclidean distance, Ward's linkage rule). Later, a Chemical Similarity Enrichment Analysis for Metabolomics (ChemRICH, available at chemrich.fiehnlab.ucdavis.edu) was performed to define the chemical composition of sourdoughs due to the addition of LAB. To that aim, compounds were filtered on Volcano plot and only the compounds showing a significantly different

accumulation between treatments ($p < 0.05$) and with fold-change values > 2 were considered [25]. Finally, a supervised multivariate orthogonal projection to latent structures discriminant analysis (OPLS-DA) was carried out by the SIMCA v. 16.0.2 software (Umetrics). Model quality was evaluated according to goodness-of-fit parameters (R^2X and R^2Y), and goodness-of-prediction parameter (Q^2Y). OPLS-DA predictive models were further statistically validated by cross-validation ANOVA (CV-ANOVA), and model overfitting was discarded through the development of permutation tests ($n = 100$). Such approach was followed by a variable importance in projection (VIP) analysis, providing insight into the compounds exhibiting the highest influence on the discrimination between treatments, known as VIP markers, according to their given VIP score [22].

3. Results

3.1. General Parameters of Rye Doughs

Key technological parameters of the different doughs after fermentation are presented in Table 1. Rye doughs fermented with LAB + yeast showed total LAB counts reaching approximately 10 log CFU/g after 24-h fermentation; this value significantly exceeded the numbers of total LAB in either SC or control rye doughs, suggesting the actual growth of inoculated LAB starters. No significant difference in final LAB abundance was found between native and sprouted rye flour. A similar trend was observed for total yeast counts. Before fermentation, the pH was 6.23 ± 0.04 for doughs made with native flour and 5.88 ± 0.07 for doughs with sprouted rye flour, respectively.

Table 1. Rye dough parameters measured after 24 h of maturation.

Parameter	Rye flour	Starter			*p*-Value		
		Control	SC	LAB + SC	Starter	Germination	Starter × Germination
pH	Native	4.19 ± 0.11^{a}	4.57 ± 0.36^{b}	3.97 ± 0.10^{a}	0.0005	0.005	0.06
	Sprouted	4.57 ± 0.10^{a}	5.33 ± 0.01^{b}	4.03 ± 0.01^{a}			
TTA (%)	Native	0.39 ± 0.01^{a}	0.42 ± 0.03^{a}	0.82 ± 0.06^{bA}	<0.0001	<0.0001	<0.0001
	Sprouted	0.50 ± 0.02^{a}	0.56 ± 0.08^{a}	2.08 ± 0.05^{bB}			
a_w	Native	1.02 ± 0.002^{a}	0.94 ± 0.02^{b}	0.95 ± 0.01^{b}	0.0001	0.044	0.026
	Sprouted	0.99 ± 0.01^{a}	0.94 ± 0.01^{b}	0.96 ± 0.01^{ab}			
Dough rise (%)	Native	70 ± 4.24^{a}	143 ± 4.95^{b}	220 ± 18.38^{c}	<0.0001	0.22	0.06
	Sprouted	90 ± 4.24^{a}	130 ± 4.24^{ab}	180 ± 28.30^{b}			
Total LAB (log CFU/g)	Native	9.16 ± 0.04^{a}	6.70 ± 0.09^{bA}	10.32 ± 0.38^{c}	<0.0001	0.008	0.09
	Sprouted	8.94 ± 0.01^{a}	5.29 ± 0.01^{bB}	9.53 ± 0.12^{c}			
Total yeast (log CFU/g)	Native	6.27 ± 0.01^{a}	8.33 ± 0.10^{b}	8.24 ± 0.17^{b}	<0.0001	0.56	0.05
	Sprouted	5.47 ± 0.66^{a}	8.73 ± 0.03^{b}	8.34 ± 0.01^{b}			

Data are mean values ± SD. Statistical analysis was carried out using two-way ANOVA followed by Tukey's post hoc test. Different superscript letters (a, b, c) in a row indicate significant differences ($p < 0.05$) between starter types within each flour group; different superscript letters (A, B) in a column indicate significant differences ($p < 0.05$) between native and sprouted rye flour within each starter type group.

As expected, the addition of LAB starters caused a decrease in the pH value to about 4 both in sprouted and native rye doughs, as compared to either SC or control. However, the difference between final pH values was significant only when comparing LAB + SC vs. SC ($p = 0.002$) in sprouted rye samples. Consistently, the application of LAB starters significantly increased total acid concentration in rye doughs as compared to either yeast or control (Table 1). A two-way ANOVA revealed that there was a significant interaction between the effects of starter and germination as concerns TTA value (*p*-value interaction < 0.0001). Notably, the TTA level of LAB + SC doughs made with sprouted rye flour was higher than that of the corresponding doughs produced from native rye flour (2.08 ± 0.05 vs. 0.82 ± 0.06, *p*-value < 0.0001). The inclusion of LAB as a starter for rye flour fermentation displayed no significant impact on dough a_w. Significant differences were observed for yeast (alone or in combination with LAB) versus control in native rye flour doughs ($p = 0.0004$ and *p*-value = 0.006, respectively). Concerning dough volume, LAB

seemed to contribute to dough rise during leavening markedly. The volume increase was higher for LAB + SC vs. SC or control regardless of the type of rye flour used; differences were all statistically significant but for LAB + SC vs. SC in native rye dough (220% ± 18.87% vs. 143.3% ± 4.71%; *p*-value = 0.01). Analogously to a_w, statistical analysis indicated that the type of fermentation had the same effect in sprouted and native rye doughs (*p*-value = 0.22).

3.2. Diversity of Bacterial Communities in Rye Doughs

PacBio SMRT sequencing of the complete bacterial 16S rRNA gene resulted in 167,207 total filtered high-quality reads, with numbers ranging from 4,188 to 16,844 reads per sample (mean 10,450 reads). Clustering to 99% similarity yielded 5,657 distinct OTUs; the mean number of OTUs per sample was 353 (range 58–609; Table 2). To assess sample diversity, different indexes were calculated including Chao1, Shannon, and Gini-Simpson (Table 2).

Table 2. Number of OTUs, alpha diversity indexes, and Good's coverage of sequenced samples.

Samples *	OTUs	Chao1	Shannon	Gini-Simpson	Good's Coverage
nDLAB + SC1	217	386.5	0.733	0.132	0.991
nDLAB + SC2	348	485.8	1.486	0.296	0.987
nDSC1	194	230.1	2.414	0.610	0.993
nDSC2	236	271.3	3.108	0.696	0.984
nControl1	58	100.0	0.093	0.013	0.998
nControl2	415	449.0	3.995	0.751	0.993
sDLAB + SC1	466	602.5	2.579	0.595	0.981
sDLAB + SC2	454	565.8	2.624	0.600	0.981
sDSC1	327	438.0	1.712	0.278	0.988
sDSC2	417	533.4	2.587	0.444	0.982
sControl1	227	312.1	0.989	0.173	0.993
sControl2	305	430.4	1.199	0.205	0.989
nF1	284	300.8	3.211	0.740	0.998
nF2	504	541.1	4.947	0.884	0.996
sF1	596	737.5	5.014	0.841	0.974
sF2	609	799.0	5.028	0.794	0.967

* nDLAB + SC: native rye–dough–LAB + SC; sDLAB + SC: sprouted rye–dough–LAB + SC; nDSC: native rye–dough–SC; sDSC: sprouted rye–dough–SC; nDControl: native rye–dough–control; sDControl: sprouted rye–dough–control; nF: native rye–flour; sF: sprouted rye–flour.

No significant differences were found among dough samples in any alpha diversity indexes. As expected, flours displayed the highest bacterial diversity among all tested samples, suggesting that fermentation exerted a selection pressure on the community structure of the dough microbiota (Table 2). Overall, native rye flour and doughs tended to have a lower bacterial richness as compared to their counterparts obtained from sprouted rye. Good's coverage ranged from 97% to 99% suggesting that a high percentage of the total species was represented in each sample (Table 2). Beta diversity, based on an unweighted UniFrac distance matrix, highlighted three distinct bacterial clusters in the PCoA plot (Figure 1). Differences in microbial composition between the samples allowed a clear discrimination between flour samples and dough samples obtained by mixed fermentation, respectively. A further group, including both spontaneously fermented doughs and doughs produced by *S. cerevisiae* fermentation, could be identified.

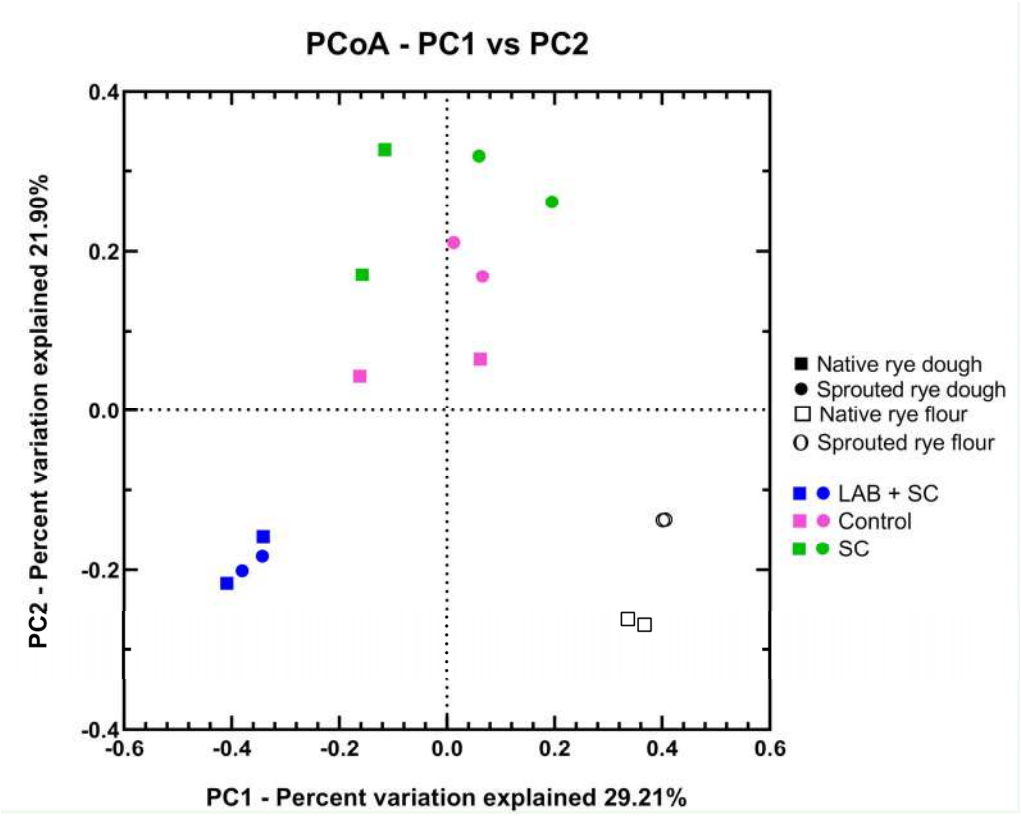

Figure 1. Beta-diversity analysis of rye dough and flour samples. PCoA plot based on unweighted UniFrac distances of microbial communities among all samples. Solid symbols represent doughs made with native rye flour (squares) and sprouted rye flour (circles); empty symbols represent native rye flour (squares) and sprouted rye flour (circles). Dough samples were colored according to the starter used for fermentation: blue = LAB + SC, green = SC, pink = no inoculated starter.

3.3. Bacterial Taxonomic Composition of Dough Samples

More than 93% of the sequences obtained by the PacBio SMRT sequencing were associated with known taxa; unclassified reads were 6.5% of the total sequences. *Weissella*, *Limosillactobacillus*, *Salmonella*, *Latilactobacillus*, and *Microcoleus* were the dominant genera in the metagenomic dataset, with an average abundance of 28.48%, 19.89%, 13.86%, 12.53%, and 6.36% of the total reads, respectively. At species level, reads were assigned to 56 different taxa, and the species with an average greater than 0.25% were 12 taxa (Figure 2). The bacterial community composition of flours differed between sprouted and native rye. *Salmonella enterica* represented more than 65% of bacterial microbiota in sprouted rye flour followed by *Microcoleus anatoxicus*, *Delftia acidovorans*, and others (Figure 2). Conversely, the most abundant bacterial species in native rye flour was the cyanobacterium *Microcoleus anatoxicus*, accounting for about 43% of the total sequences; less common species included *Pantoea agglomerans* and *Cutibacterium acnes*.

Our results indicated that, sprouted rye doughs had greater uniformity in the bacterial community structure for all the 3 fermentation conditions than the native rye counterparts. *L. fermentum* and *W. confusa/cibaria* were the only bacterial species detected in dough samples inoculated with the lactic acid starter. As expected, the two closely related *Weissella* species were indistinguishable using 16S rRNA gene sequencing. *L. fermentum* sequences greatly outnumbered those ones classified under the species pair *W. confusa*/*W. cibaria* (Figure 2), reaching 93.56 ± 4.73% in native rye doughs, and 65.26 ± 0.27% in sprouted rye doughs. Yeast-leavened doughs were enriched in cereal-sourced LAB with a different species composition based on the rye flour used. *Latilactobacillus curvatus* was dominant (relative abundance > 89%) in sprouted rye doughs, while *Lacticaseibacillus paracasei* and

Latilactobacillus graminis were found at low relative abundance. Native rye doughs harbored a bacterial community consisting of *Lactoplantibacillus plantarum* (with an average of 42.6%) followed by *Lacticaseibacillus paracasei* (relative abundance > 16%); *Lactobacillus curvatus* was detected with a relative abundance over 4%, while *W. confusa/cibaria* accounted for 38.5% of total sequences in nDY (Figure 2). Control doughs resulted in a microbiota that differed from that of other doughs and was broadly dominated by indigenous *W. confusa/cibaria* strains.

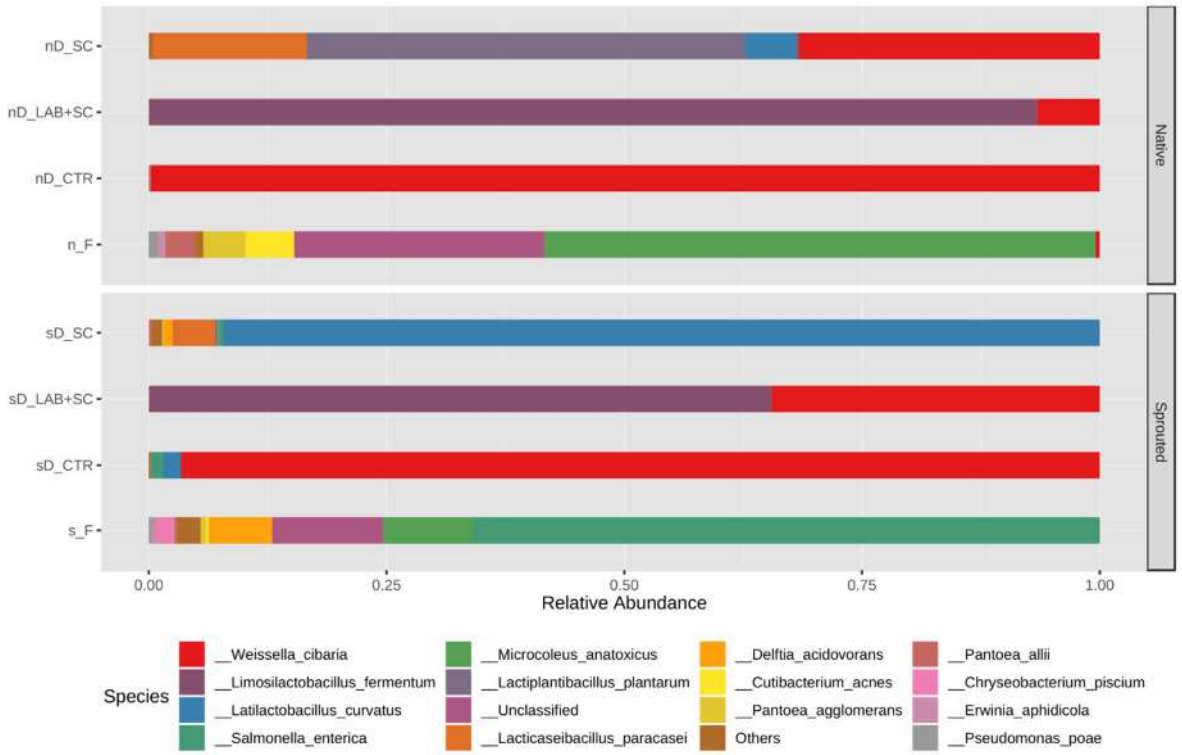

Figure 2. Relative abundance of bacterial species found in dough and flour samples. The bar plot represents the top 15 most abundant taxa among all samples identified to the species level. "Others" refers to merged species that individually showed a relative abundance below 0.25%. "Unclassified" are bacterial taxa not identified at the phylum level. The results are expressed as average of replicates for each type of dough. In the *x*-axis are reported the name of the samples as follow: sD, sprouted rye dough; nD, native rye dough; s_F, sprouted rye flour; n_F, native rye flour, CTR, dough control; LAB + SC, dough fermented with LAB + SC; SC, dough fermented with SC.

3.4. Differential Analysis of Discriminant Species between Dough Samples

Differential analysis of the abundance of microbial species revealed several features that varied significantly according to either starter or germination. When considering the impact of starter, *L. fermentum* was higher in LAB + SC samples compared to either SC or control samples, regardless of rye germination (Figure 3A,B). As for native rye doughs (Figure 3A), control samples were enriched in *W. confusa/cibaria* and *P. agglomerans* with respect to native doughs inoculated with LAB and yeast.

Moreover, the species *L. plantarum* and *L. paracasei* were more abundant in yeast-leavened samples than doughs obtained by mixed fermentation, or native spontaneous fermentation samples. Among sprouted rye doughs, spontaneously fermented samples displayed significantly higher levels of *W. confusa/cibaria* and *S. enterica* than those observed in SC samples and in LAB + SC samples, respectively. Notably, the inoculation *S. cerevisiae* alone in sprouted rye samples was associated with higher proportions of endogenous LAB species including *L. curvatus*, *L. graminis* and *L. paracasei*. In fact, these species resulted in being higher in abundance in SC samples with respect to both LAB + SC samples and

control samples. As regards the comparison between sprouted and native rye flours, *L. curvatus* was significantly higher in sprouted rye doughs as compared to native rye doughs (p-value = 0.045). Conversely, *L. plantarum* was significantly higher in native rye samples to sprouted samples (p-value = 0.045).

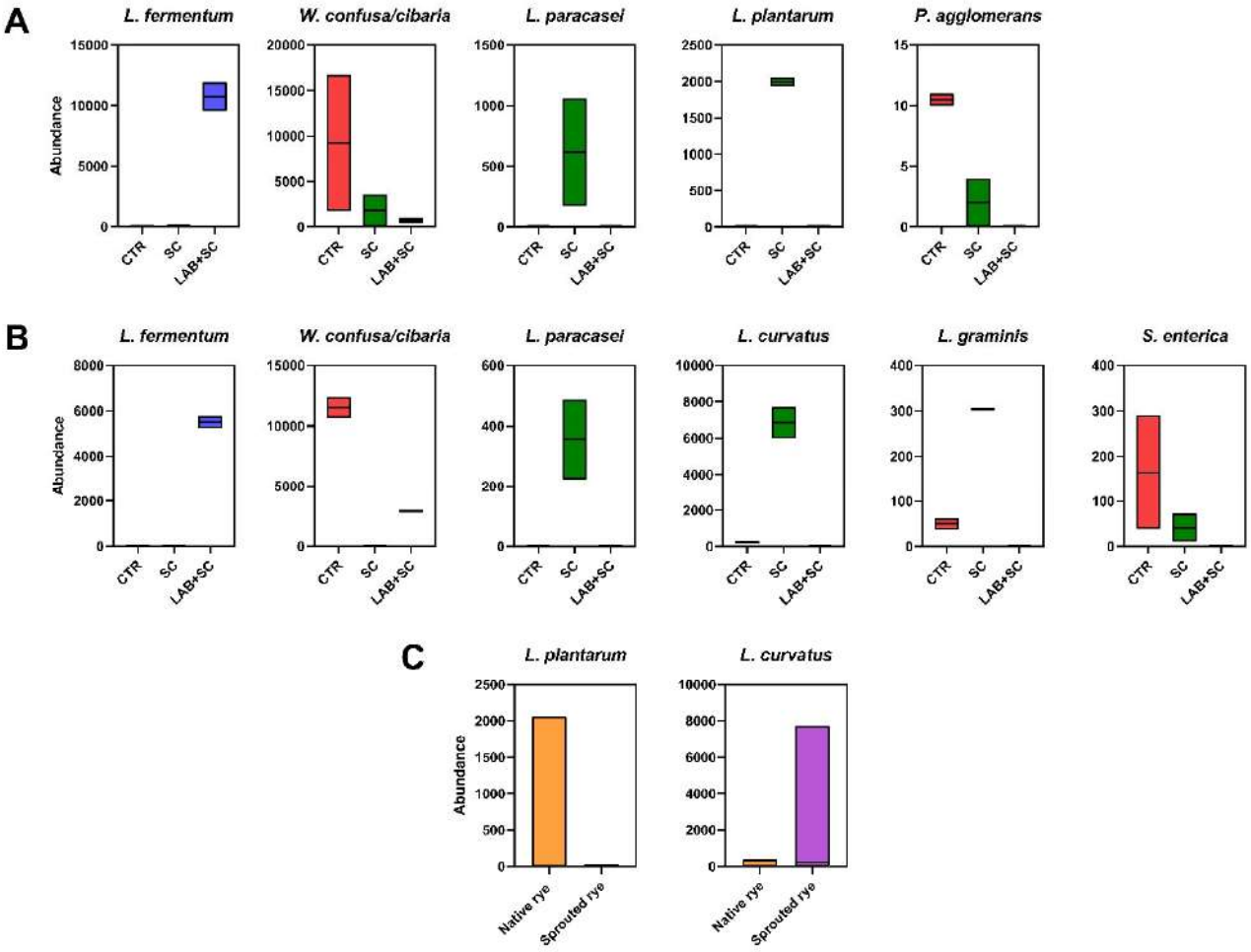

Figure 3. Differential abundance analysis of bacterial species among rye doughs. Box and whiskers plot indicate the proportion of differentially abundant taxa between starters used for fermentation in native rye doughs (**A**) and in sprouted rye doughs (**B**), and between native and sprouted rye doughs (**C**). Only significant species (FDR < 0.05) detected by DESeq2 are reported in the figure. Boxes represent the minimum and maximum of abundance values of replicates for each condition; the line in the box is the median.

3.5. Oligosaccharide Semi-Quantification of Rye Sourdoughs

The oligosaccharide semi-quantification of rye flour doughs is shown in Figure 4. In general, germination played a critical role on carbohydrate compositions since native rye doughs exhibited a lower carbohydrate content to the sprouted counterparts. In parallel, adding fermentation starters played a significant role on the carbohydrate content of rye doughs (Figure 4).

Thus, for native rye doughs, SC-mediated fermentation promoted a significant decrease of both monosaccharides and low-PD oligosaccharides that were further significantly decreased in the LAB + SC fermentation by whereas high-PD carbohydrates were not affected (Figure 4A). In total, the combination of LAB with SC led to a harsh carbohydrate content reduction of 55.5% with respect to control. In contrast, the fermentation of sprouted rye doughs caused only a significant decrease in the carbohydrate content in the case of mixed fermentation, as SC fermentation did not promote any significant difference in

comparison to control (Figure 4B). As a result, concerning total carbohydrate content, the LAB + SC treatment led to a 22.8% decrease with respect to control, suggesting a lower impact of fermentation than that observed for native-derived dough. Concerning the different carbohydrates, the high-PD oligosaccharides content was not affected by the type of fermentation, whereas the contents of both monosaccharides and low-PD oligosaccharides was significantly decreased after the LAB + SC combined fermentation (Figure 4B).

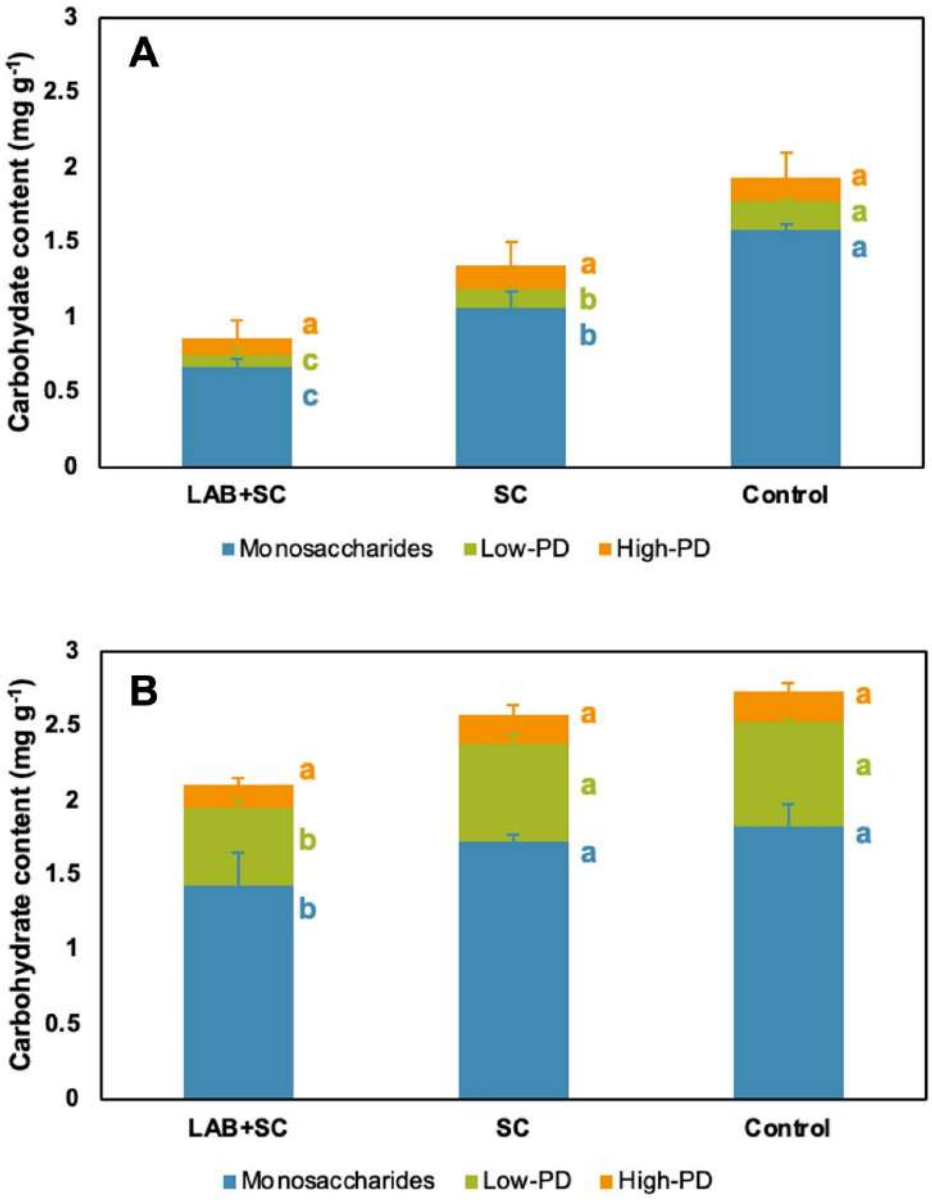

Figure 4. HPAEC-PAD carbohydrate content of rye sourdoughs. Carbohydrate content of native rye doughs (**A**). Carbohydrate content of sprouted rye doughs (**B**). Results are expressed as carbohydrate content (mg g-1) referred as xylose, arabinotriose, and arabinoctaose equivalents for monosaccharides, low-polymerization degree oligosaccharides (low-PD), and high-polymerization degree oligosaccharides (high-PD) contents, respectively (**B**). Vertical bars indicate standard deviation ($n = 6$). Different letters indicate statistically significant differences ($p < 0.05$) among treatments.

3.6. Untargeted UHPLC/QTOF-MS Metabolomic Profile of Rye Doughs

Rye doughs were subjected to metabolic profiling via UHPLC/QTOF-MS, providing 1909 annotated chemical features (Table S1). From these annotated compounds, 158 were identified according to their MS^2 spectral features (Table S2). The effect of grain germination

and the addition of starters on the metabolic profile of rye doughs were evaluated by an unsupervised multivariate hierarchical cluster analysis (HCA), and the results are displayed in Figure 5. Among the factors involved in this study, germination was the most prevalent factor affecting the metabolome of samples since it ruled the establishment of two major clusters. Secondarily, within both clusters, fermentation starters played a significant role, providing three subclusters according to the different experimental conditions involved in dough production: non-inoculated, SC, and LAB + SC (Figure 5).

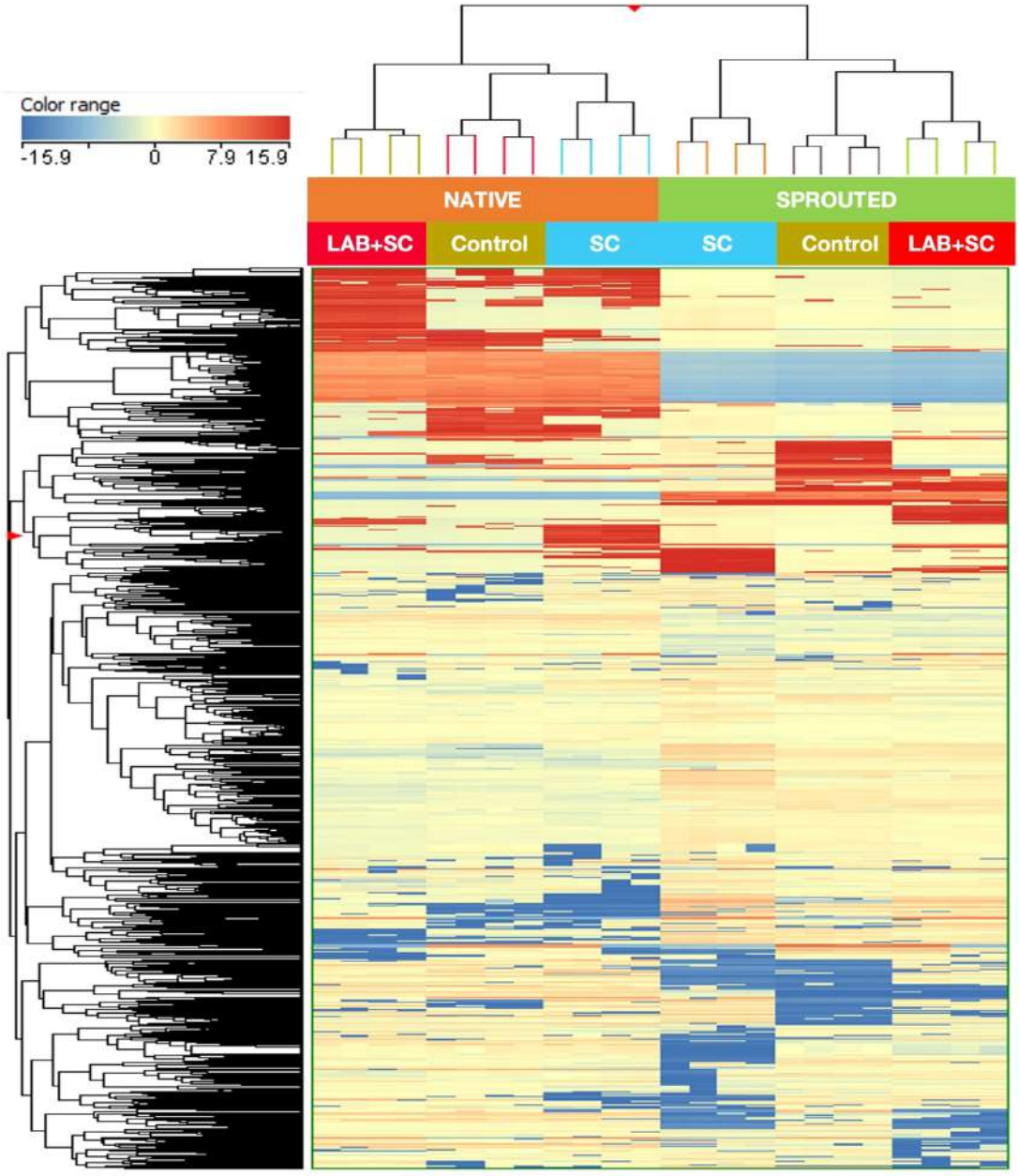

Figure 5. Unsupervised hierarchical cluster analysis on the untargeted metabolic profiling of rye dough. The fold change values, represented by a color range, for each compound were calculated with respect to the median of all samples, and further used to obtain a fold change-based heatmap, according to Ward's algorithm (Euclidean distance). The factors involved in clustering were NATIVE and SPROUTED, for native and sprouted-derived rye sourdough, respectively; and control, SC, and LAB + SC, for unfermented, yeast-fermented and yeast and LAB-combined fermented rye sourdough, respectively.

Due to the heterogeneous metabolic profile of fermented rye doughs, an additional supervised multivariate orthogonal projection to latent structures discriminant analysis (OPLS-DA) was performed. It was followed by a variable importance in projection (VIP) analysis, with the aim of discriminating the effect of germination and starters on the metabolome of these matrices, providing insight on the metabolic markers mostly involved in such discrimination (VIP markers). The Figure 6 shows the OPLS-DA models and the proportion of VIP markers according to their chemical class for the discrimination between germination conditions (Figure 6A,B, respectively), fermentation starters on native rye-derived doughs (Figure 6C,D, respectively), and fermentative starters on sprouted rye-derived doughs (Figure 6E,F, respectively).

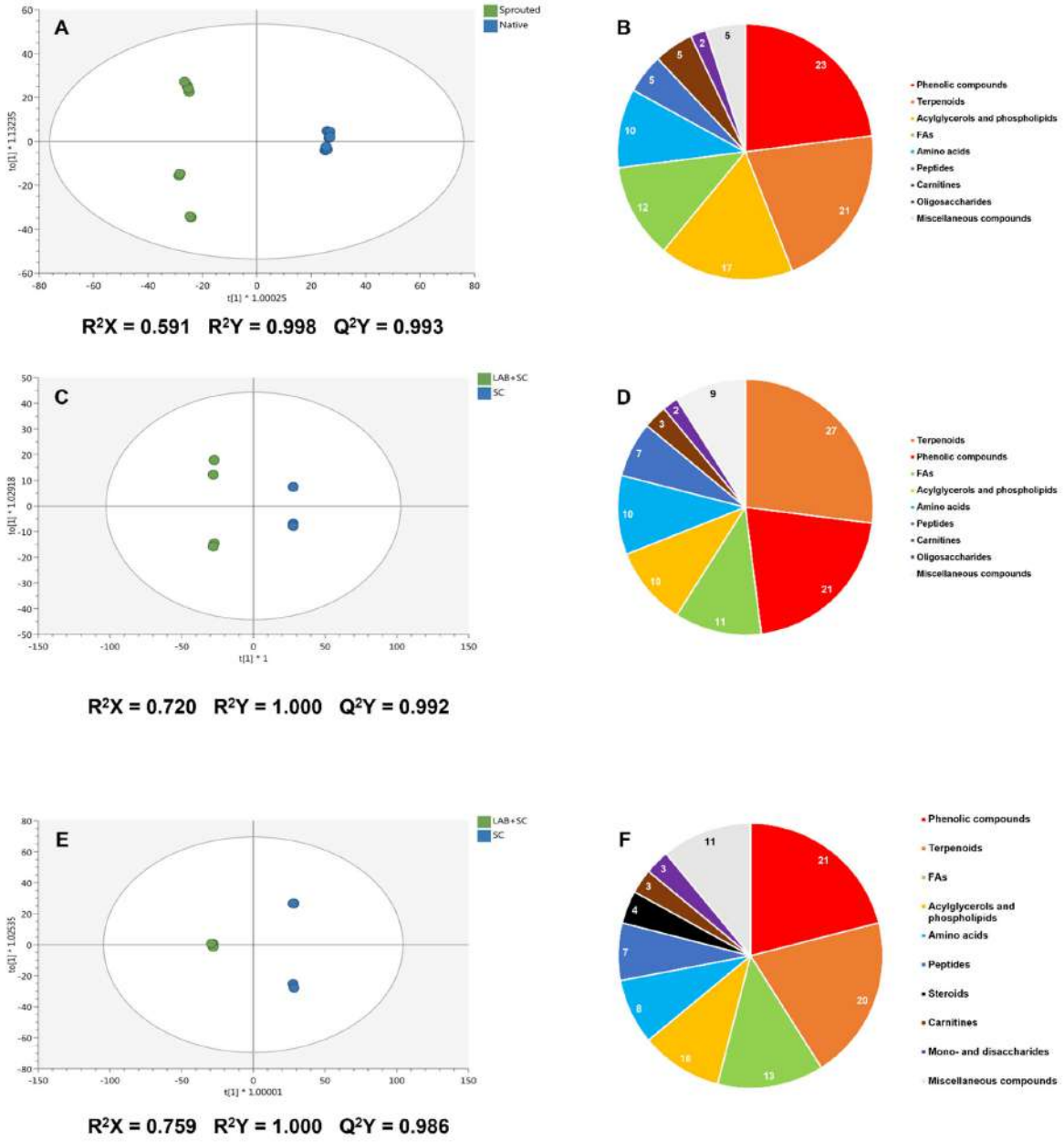

Figure 6. Orthogonal projection to latent structures discriminant analysis and variable importance in projection analysis of rye doughs. The OPLS models were combined with the proportion of VIP markers on the discrimination of metabolomic effects caused by germination conditions (**A**,**B**), fermentation starters on native rye doughs (**C**,**D**), fermentation starters on sprouted rye doughs (**E**,**F**).

Moreover, the full list of VIP markers associated with all models, together with their VIP score, logFC values, and chemical class are provided in Tables S4–S6, respectively. In all cases, the obtained OPLS-DA models showed high-quality parameters in terms of goodness-of-fit, given by R^2X and R^2Y coefficients, and predictability, given by the Q^2Y coefficient ($Q^2Y > 0.5$; Figure 6). Concerning germination, the OPLS-DA model spotted a definitive role of this factor on the metabolome of rye doughs, indicating a clear discrimination between native rye- and sprouted rye-derived doughs (Figure 6A). Phenolic compounds, terpenoids, and phospholipids (Figure 6B) predominantly represent the VIP markers provided for the discrimination between native and sprouted-derived doughs. In general, and regarding the logFC values (Table S3), sprouted rye-derived doughs exhibited an enhanced accumulation of phenolic compounds, including flavonoids like mulberrin, zapotinin, and isoferreirin (logFC = 13.5), and phenolic acids, mostly represented by spermidine esters, and resorcinols. In the case of terpenoids, triterpenoid acids like 3-benzoyloxy-6-oxo-12-ursen-28-oic acid and 2, 3, 23-triacetylsericic acid (log FC = 13.5) were accumulated in sprouted rye-derived doughs (Table S3). To a lesser extent, amino acids like His and Phe derivatives and oligopeptides, as well as glucosinolates were found to be differentially up accumulated in sprouted rye doughs, providing evidence on the metabolic richness of this matrix. Conversely, the accumulation of lipid metabolites did not follow a clear pattern. Metabolites like docosan-1-ol, phosphatidylethanolamines, and 3-hydroxy-9-hexadecenoylcarnitine exhibited a decrease in native rye doughs (log FC = −9.4; Table S3), whereas saturated fatty acids 22-hydroxydocosanoate and 10-hydroxymyristic acid methyl ester and some di-glycerides were mostly measured in sprouted rye doughs (log FC = 13.5; Table S3). As germination played such a discriminant effect on the metabolome of rye sourdoughs, two additional OPLS models were performed to evaluate the impact of starters on either native rye or sprouted rye doughs (Figure 6C,E, respectively). In both cases, a clear discrimination between SC-fermented doughs and LAB + SC-fermented doughs was obtained, and phenolic compounds, terpenoids, and lipid metabolites were mostly identified as VIP markers (Figure 6D,F, respectively). Firstly, in the case of native rye doughs, the inoculation of LAB promoted a general up-regulation of the metabolome, since 54% of VIP markers possessed logFC = 8.6, and only 17% of markers were found to be down-accumulated as compared to SC-fermented doughs (Table S4). Thus, terpenoids were generally accumulated, ranging from triterpenoids and sesquiterpenoids to monoterpenoids, including sterols and carotenoids (Table S4). Likewise, phenolic compounds were all up-accumulated due to LAB addition, involving flavonoids, phenolic acids like *p*-coumaroyl derivatives, and spermine and putrescine esters, stilbenes, coumarins and lignans (Table S4). In parallel, amino acids were also selected as VIP markers accumulated in LAB + SC fermented doughs, represented by both proteinogenic amino acids, such as Gln (logFC = 3.3), Gly (logFC = 3.4), and Cys derivatives (log FC = 8.6), and non-proteinogenic amino acids, like ornithine (logFC = 2.0; Table S4). Peptides were found to be accumulated as a result of LAB inclusion (log FC = 2.3–8.6; Table S4) as well, suggesting an intense proteolytic activity. In contrast, lipid metabolites showed an unclear pattern of accumulation between LAB + SC fermentation and SC fermentation, as given by log FC values. Notably, lysophospholipids were mainly accumulated in LAB + SC-fermented doughs (log FC = 2.0–8.6), whereas fatty acids were heterogeneously detected (Table S4). A similar trend was observed for the inclusion of LAB as fermentation starters on the metabolome of sprouted rye doughs, as indicated by the corresponding OPLS-DA (Figure 6E). A metabolic stimulation was shown by the inclusion of LAB, with 76% of VIP markers up regulated in LAB + SC-fermented matrices to those fermented exclusively with SC (Table S5). Again, phenolic compounds constituted the class with the highest contribution to VIP markers, followed by terpenoids and lipid metabolites (Figure 6F). Considering phenolic compounds, lignans were the compounds presenting the highest accumulation (log FC = 10.0 for schidigeragenin B and clusin, Table S5), together with ferulic, caffeic acid esters (log FC = 10.0), whereas flavonoids presented much lower log FC values (Table S5). Considering terpenoids, LAB inclusion promoted the accumulation of high-isoprene subunits terpenoids, including

steroids, triterpenoids and sesquiterpenoids (log FC = 10.0, Table S5), whereas the accumulation of mono- and diterpenoids was reduced (log FC < −3.3). In parallel, in the case of amino acids and peptides, the accumulation and down-accumulation did not follow a clear pattern. While saturated fatty acids, especially octadecanoic acids (log FC = −12.8), and sulfur-containing compounds, like 1-methoxyspirobrassinin (log FC = −12.8), were harshly down accumulated upon the addition of sourdough LAB starters, lysophospholipids were found accumulated upon the inclusion of LAB (log FC = 3.7–10.0; Table S5).

4. Discussion

This study explored the microbial, chemical, and technological profiles of rye doughs made with either native or sprouted flour and fermented with *S.cerevisiae* in combination or not with selected LAB starters. Two complementary approaches were applied to assess the metabolic profiling of rye doughs after fermentation. Firstly, the carbohydrate profile of rye doughs was assessed, and results indicate that grain germination and LAB fermentation played a significant role in the composition of these constituents. The carbohydrate content of sprouted rye doughs was significantly higher than that of native rye doughs, due to the induction of hydrolytic enzymes during seed germination, which includes amylases, pentosanases and glucanases [26]. Due to hydrolytic activity, insoluble fiber is mainly converted into soluble sugars, such as monosaccharides, that were spotted as the major sugar constituents of rye doughs in this work. Notably, VIP analysis indicated that characteristic oligosaccharides of sprouted rye doughs were maltopentaose and maltotetraose, functional maltodextrins potentially involved in glycemic control response and enterocyte differentiation [27]. Considering the fermentation starters, including LAB promoted a significant decrease in carbohydrate content in terms of monosaccharides and low-PD oligosaccharides. This can be explained considering the heterofermentative metabolism of sourdough LAB, which relies on the activity of a wide range of catabolic enzymes [7]. Analysis of technological parameters revealed important features of experimental doughs connected to the evolution of bacterial ecology during fermentation and the interplay between starter inoculation and germination. As expected, the application of sourdough LAB starters led to a substantial reduction of pH, especially in comparison to yeast-leavened doughs, as a result of LAB extensive exploitation of carbohydrates for organic acids biosynthesis [28,29]. Indeed, the germination-related enzymatic breakdown of carbohydrates into simple sugars can boost fermentative metabolism by sourdough LAB resulting in the accumulation of organic acids [9,30]. Interestingly, LAB + SC fermentation was also associated to a greater dough rise as compared to either SC or control. Heterofermentative LAB activity can affect dough leavening through the production of CO_2 [31]. In mixed LAB + SC samples, metagenomics analysis highlighted a strong dominance of inoculated *L. fermentum* over *Weissella* strains at the end of the fermentation. On the other hand, endogenous *W. confusa/cibaria* was the predominant taxon in control samples. The latter finding is not surprising since several studies identified *Weissella* alone, or in combination with other LAB, as the dominant bacterial genus in rye sourdough after 24 h fermentation which may include or not refreshments [31–35]. Indeed, the ecological fitness of sourdough microorganisms is largely dependent on the interplay between strain-specific traits and process conditions including temperature, pH, dough hydration, fermentation time, and type of flour [36,37]. All these parameters can contribute to affect the growth rate of organisms, their competitiveness in sourdough fermentation and eventually their impact on product quality. It is thus presumably to suppose that a long fermentation at elevated temperature (i.e., 35 °C) as applied in the present study selected for *L. fermentum* owing to the thermophilic behavior and high acid resistance of this *Limosilactobacillus* species [38]. Consistent with this hypothesis, the cultivable microbiota of sourdoughs fermented at 37 °C was constituted by *L. fermentum* strains exclusively [39]. Notably, the VIP analysis on the metabolomics profile of doughs revealed that in both native and sprouted rye doughs there was an accumulation of mannitol when LAB were added as starters. Conversion of fructose to mannitol by heterofermentative LAB has been reported in sourdough fermentations [39].

Consistent with this, *L. fermentum* UC3641 has in its genome two Open Reading Frames (ORFs) encoding for a NAD(P)H-dependent mannitol/alcohol dehydrogenase [13].

In addition to oligosaccharide semi-quantification, a metabolomics approach was employed to investigate the overall effect of germination and fermentation on the metabolome of rye sourdoughs. The unsupervised HCA analysis of doughs revealed that germination of rye grains played a major role on the metabolic profile than fermentation, which was also supported by the results from the OPLS-DA models. Germination has been previously assessed as a physiological process in which phytohormones can play a critical role on the development and metabolome of rye grains, which may affect further processing, including fermentation [26]. Furthermore, germinated grains show a high biosynthetic potential and promote the activity of hydrolytic enzymes that lead to structural modifications [9], which could reflect in greater accessibility or diversity of fermentative bacteria. We assessed the presence of LAB species that could be differentially associated to either native or sprouted rye flour regardless of the fermentation conditions. Metagenomic data revealed that the species *L. plantarum* was typical of the microbiota of native rye doughs, whereas *L. curvatus* was significantly higher in doughs made with sprouted rye. As for *L. plantarum,* this species is known to metabolize a wide range of different carbohydrates of varying complexity, thanks to its rich repertoire of lytic enzymes [40]. Furthermore, among the significant compounds responsible for the discrimination between native- and sprouted-derived rye doughs obtained from OPLS-DA model, several compounds were spotted as VIP markers, especially primary metabolites as amino acids, peptides, and lipid metabolites.

Concerning phenolics, the accumulation of flavonoids and phenolic acids was mostly modulated by fermentation, with yeast as the sole fermenting agent or in combination with LAB, which agrees with the previous study by Katina et al. [9] who reported increased levels of phenolic compounds after fermentation, especially in germinated rye. In parallel, sourdough fermentation contributed to increase significantly the content of total phenolic compounds, especially phenolic acids and alkylresorcinols, because of the pH reduction caused during fermentation [7]. Such compounds were found in this work as discriminant metabolites associated to LAB fermentation, as it is the case of feruloyl, caffeoyl, and coumaroyl derivatives, thus being in line with the results provided by other authors [10]. Ferulic and *p*-coumaric acids are the most prevalent phenolics attributed to rye, reaching a proportion of about the 95% of total phenolic compounds [41]. Notably, all the LAB starters used in our experimental conditions presented in their genomes ORFs encoding for esterases, phenolic acid decarboxylases and phenolic acid transferases [13], suggesting the possible involvement of these enzymes in the conversion of *p*-coumaric acid and ferulic acid in their esterified derivatives. It is important to note that phenolic acids have been previously reported in their esterified forms with diverse biogenic amines [10] as reflected by our results with spermine, spermidine, and putrescine. Moreover, the same authors reported an increase in the accumulation of flavonoids due to mixed fermentation, agreeing with present findings. Overall, the polyphenols enrichment associated with LAB + SC fermentation may suggest an enhancement of the nutritional value of rye doughs, given the biological activities of these compounds as multifaceted bioactive compounds, acting as antioxidant, anti-inflammatory, antitumor and antimicrobial agents, among other health-promoting properties [42].

In the case of terpenoids, little is known about the effect of fermentation on biosynthesis of these compounds in rye sourdough [43] that were widely identified during the current research as triterpenoids. Nevertheless, the presence of terpenoids may improve the shelf life and safety of these matrices, due to their associated antibacterial properties [44]. Concerning protein-derived metabolites, both germination and fermentation played a critical role on the catabolites determined in rye doughs, which agrees with the existing literature. Germination can increase the total proteolytic activity in rye whereas acidification mediated by both LAB and yeasts in sourdough fermentation triggers cereal protease activity by shifting the dough pH to the optimum of aspartic proteases, which represent the major proteases in rye and wheat [45]. Even more important for proteolysis is

the activity of strain-dependent intracellular peptidases of sourdough lactobacilli, which enhances the accumulation of amino acids in fermented doughs providing key sources of nitrogen for yeast growth [40,45]. Thus, all these factors contribute to the plethora of free amino acids and peptides spotted in this work. The amount and type of peptides and amino acids occurring in cereal doughs mostly account for the overall quality of bread in that many of these compounds act as taste-active components and flavor precursors. However, as a result of the proteolytic activity of sourdough starters, non-proteinogenic amino acids, like citrulline or ornithine, were previously spotted [10] as well as in this case. *L. fermentum* is among the *Lactobacillus* species that can convert arginine to ornithine via the arginine-deiminase (ADI) pathway [46]. Notably both native and sprouted rye doughs fermented by sourdough LAB were enriched in γ-glutamyl dipeptides such as γ-glutamylglutamic acid and γ-l-glutamyl-l-pipecolic acid in the present study. Besides being naturally presented in certain foods, the synthesis of γ-glutamyl dipeptides may occur in fermented foods via microbial γ-glutamyl transpeptidases and γ-glutamyl cysteine synthetases. Formation of γ-glutamyl dipeptides in sourdough fermented by *Limosilactobacillus reuteri* was attributed to strain-specific biosynthetic capabilities and consistently improved the sensory attributes of the resulting bread [47]. Lipid metabolites play a minor role on the composition of rye sourdoughs motivated by the low-fat content of rye flour [7]. According to our results, the accumulation of lipid metabolites did not show a clear pattern, regardless of the germination and fermentation conditions, with the exception of lysophospholipids, which were harshly accumulated as a result of mixed fermentation. This finding could be explained by the activity of hydrolytic enzymes, such as lipases and phospholipases acting on di- and tryacylglycerides, which were found to be heterogeneously accumulated during fermentation. These enzymes could be sourced from rye flour as well as sourdough starter microorganisms. Nevertheless, the results reported by Koistinen et al. [10] on the untargeted metabolomic profile of rye sourdoughs indicate that fermentation promoted the accumulation of phosphatidylcholines, whereas oxidized fatty acids were found to be down-accumulated. Remarkably, in the present study, a higher level of hydroxy fatty acids was detected in doughs fermented with sourdough LAB as compared to *S. cerevisiae* alone. It is known that hydratases by sourdough lactobacilli can convert oleic acid, linoleic acid, and linolenic acid to hydroxy fatty acids.

5. Conclusions

The results of the present study provide a comprehensive view of multiple compositional changes induced by germination and lactic fermentation in cereal flour, which may have implications for the nutritional value, sensory attributes, and functional characteristics of rye bakery products. Fermentation by selected sourdough lactic acid bacteria in addition to baker's yeast resulted in lower levels of simple sugars and increased levels of mannitol in the dough system, and could thus represent a relevant strategy to reduce sugar in baked goods. Grain germination promoted the accumulation of maltooligosaccharides, a class of molecules displaying several potential biological capabilities. Overall, the combination of rye germination with the combined fermentation of *S. cerevisiae* and LAB promoted the accumulation of nutritionally important metabolites, such as polyphenols, terpenoids, hydroxy fatty acids, and peptides, which also contribute to the enhancement of the technological and sensorial properties associated with rye flour. Indeed, the integrated information provided by metagenomics and untargeted metabolomics offered new insights into the impact of processing technologies on dough quality, which can guide the design and development of novel, health-promoting rye foods.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods12050998/s1, Figure S1: Automated system for malting rye grains, Table S1: Dataset of annotated compounds by UHPLC/QTOF-MS analysis, Table S2: Dataset of annotated compounds by UHPLC/QTOF-MS analysis, according to their MS2 spectral features, Table S3: List of VIP markers associated with the OPLS-DA model discriminating between germination conditions of rye doughs (sprouted vs. native), Table S4: List of VIP markers associated

with the OPLS-DA model discriminating between starters of native rye doughs (SC+LAB vs. SC), Table S5: List of VIP markers associated with the OPLS-DA model discriminating between starters of sprouted rye doughs (SC+LAB vs. SC).

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Data Availability Statement: Raw sequences of full 16S rRNA gene profiling are accessible through SRA study accession number PRJNA850138, with biosample accession numbers from SAMN29160520 to SAMN29160535.

Conflicts of Interest: Paola Carnevali is an employee in Barilla G. e R. Fratelli S.p.A. The company provided the commericial rye flour that has been exploited for experimental analysis.

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Chapter 3

Design of Triticale-Based Foods with Enhanced Value

foods

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Article

Combining Native and Malted Triticale Flours in Biscuits: Nutritional and Technological Implications

Isabella Piazza [1,*], Paola Carnevali [2], Nadia Faccini [3], Marina Baronchelli [3], Valeria Terzi [3], Caterina Morcia [3], Roberta Ghizzoni [3], Vania Patrone [4], Lorenzo Morelli [4], Mariasole Cervini [4] and Gianluca Giuberti [4]

[1] Centre BIOGEST-SITEIA, Department of Life Science, University of Study of Modena and Reggio Emilia, Via Amendola, n. 2, 42122 Reggio Emilia, Italy
[2] R&D Food Microbiology & Molecular Biology Research, Barilla G. e R. Fratelli S.p.A., 43122 Parma, Italy; paola.carnevali@barilla.com
[3] Research Centre for Genomics and Bioinformatics, Council for Agricultural Research and Economics, 29017 Fiorenzuola d'Arda, Italy; nadia.faccini@crea.gov.it (N.F.); marina.baronchelli@crea.gov.it (M.B.); valeria.terzi@crea.gov.it (V.T.); caterina.morcia@crea.gov.it (C.M.); roberta.ghizzoni@crea.gov.it (R.G.)
[4] Department for Sustainable Food Process (DiSTAS), Università Cattolica del Sacro Cuore, 29122 Piacenza, Italy; vania.patrone@unicatt.it (V.P.); lorenzo.morelli@unicatt.it (L.M.); mariasole.cervini@unicatt.it (M.C.); gianluca.giuberti@unicatt.it (G.G.)
* Correspondence: isabella.piazza@unimore.it

Abstract: Triticale-based biscuits were formulated with increasing substitution levels (i.e., 0, 25, 50, 75, and 100% *w*/*w*) of malted triticale flour (MTF). The products were analyzed for technological and nutritional characteristics, including the evaluation of the in vitro starch digestion. The results indicated that the substitution of triticale flour with MTF increased ($p < 0.05$) the total dietary fiber and ash contents. Total starch decreased ($p < 0.05$) when the level of MTF increased in the formulation, causing an increase in reducing sugars and an increase in the starch hydrolysis index and in the in vitro predicted glycemic index (pGI). The hardness and spread ratio values of biscuits decreased ($p < 0.05$) with increasing levels of MTF in the recipe. The lightness of doughs and biscuits decreased ($p < 0.05$) with increasing MTF levels. Overall, MTF could be used to formulate biscuits with higher dietary fiber content than native triticale flour and a medium to high in vitro glycemic index value as a function of the substitution level.

Keywords: biscuits; malted triticale flour; dietary fiber; texture; in vitro digestion

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1. Introduction

Triticale (*Triticosecale wittmack*) is a wheat/rye hybrid grain with a worldwide production that has consistently increased during the last two decades, reaching about 17 million tonnes in 2014 [1]. Triticale was traditionally used as animal feed and for biofuel production; however, the growing demand for food resources and the current consumer trend of trying novel products has led to an increased interest in food production [2]. In addition, triticale could be an important crop to ensure food security due to its tolerance to drought, disease, more acid soils, low susceptibility to biotic stresses, and high grain yield even in marginal environments [3,4].

From a nutritional standpoint, the chemical composition of triticale is more similar to wheat than rye due to its genome proportions [5]. Accordingly, wheat, rye, and triticale flours contained similar total protein contents but different protein fractions and amino acid composition [6]. In addition, triticale total starch content (i.e., 63.3–68.8 g/100 g dry matter) is comparable to wheat and rye; however, the ratio of amylose to amylopectin can vary considerably [5]. Considering the dietary fiber content, triticale has a high amount of soluble fraction, especially water-extractable arabinoxylans [5,6].

Several studies have been conducted to formulate triticale-based foods in recent years. Most works were focused on the development of triticale flour dough suitable for

breadmaking [7–9]. The results indicated that triticale was characterized by a low gluten quantity and quality, and triticale typically exhibits low falling number and lower dough stability and dough development time than wheat [7–9]. Contrarily, other studies reported that triticale flour was better suited for unleavened baked products, including biscuits and crackers [10,11]. For instance, an improvement in the spread ratio of biscuits made by blending triticale with wheat flour has been reported [10–13].

Other than the use of native flour, triticale is a promising cereal for malting and brewing owing to its high levels of α-amylase and proteolytic enzymes, which allow a short soaking time and a quick malting process [3]. During the malting process, which involves soaking, germination, and drying, several physicochemical changes can occur that can positively affect the grain's chemical and nutritional composition regarding macro- and micro-nutrients and bioactive compounds [14]. Through the milling of the malted grains, malt flour is produced, which can often be added to wheat flour in adequate amounts to improve the technological and sensorial properties of bread [14]. In addition, previous studies indicated that malted sorghum flour can be used in place of up to 60% (*w/w*) of wheat flour for the preparation of nutritionally enhanced biscuits without changes in texture, crispiness, appearance, and overall acceptability [4,14].

Given the optimal nutritional value of malted flours, the good attitude of triticale for the malting process, the suitability of triticale flour for biscuits preparation, and the trend in the food market to formulate baked products with unconventional and under-exploited flours, this study aimes to formulate novel biscuits made only with triticale flour and malted triticale flour (MTF) in different ratios. To our knowledge, this is the first study in which triticale flour (native or malted) was exclusively employed in biscuit formulation. Exploring the potential of triticale flour as the base ingredient in biscuits could spawn consumer and stakeholder interest to seek out cereal-based products made from cereal grains other than common wheat cultivars. Indeed, different studies dealing with the formulation of biscuits produced from malted flours are currently present in the literature but using the following cereals: wheat, barley, buckwheat, oat, sorghum, and millet [4,10,11,14–16]. In most of these works, biscuits are made with composite flours containing malted and native flours from the same cereal and blended in different ratios with other grains (mainly wheat) and pulses. In this work, to better explore the suitability of triticale and MTF in biscuits, products were analyzed in terms of technological and nutritional attributes, including the evaluation of the in vitro starch digestion.

2. Materials and Methods

2.1. Raw Materials, Recipes, and Baking Conditions

To obtain triticale malt, native triticale seeds (Etere variety) were put into an automatic Micromalting System (Phoenix Biosystems, Adelaide, South Australia) as described in Gianinetti et al. [17]. The malting process lasted for six days. The durations of the malting cycles with the corresponding temperatures are shown in Table 1.

After the process, rootlets and coleoptiles were removed manually. Native and malted triticale grains were milled (final fineness < 300 µm; Knife Mill Grindomix GM 200, Retsch, Germany) with pre-cutting at a revolution speed of 4000 min^{-1} for 10 s, followed by fine-grinding at a revolution speed of 10,000 min^{-1} for 20 s.

The composite flours for biscuit formulation were prepared by replacing native triticale flour (TF) with malted triticale flour (MTF) at 0, 25, 50, 75, and 100% *w/w*. Biscuits prepared with 100% TF were used as control. Biscuits were made using the following ingredients: 240 g of composite flour, 120 g of whole eggs, 60 g of skimmed milk, 50 g of creamed butter, and 1 g of salt. The ingredients were mixed and manually kneaded for 5 min, and the resulting doughs were kept at 4 °C for 1 h. Then, the doughs were sheeted to a 5 mm thickness with a rolling pin and cut into circular shapes of 3 cm in diameter. Biscuits were baked (180 °C for 15 ± 2 min) in a household oven (RKK 66130, Rex International, Mestrino, Italy). For each recipe, three different biscuit batches were produced.

Table 1. Malting stages, duration, initial temperature (Ti), and final temperature (Tf), are expressed as °C.

Malting Stage	Hours	Minutes	Ti	Tf
Cleaning		15		
Steeping 1	7	15	15	15
Germination 1	8		19	19
Steeping 2	9		15	15
Germination 2	6		19	19
Steeping 3		30	15	15
Germination 3	39	30	15	15
Germination 4	24		15	15
Germination 5	25		15	15
Kilning 1	7		30	40
Kilning 2	6		40	60
Kilning 3	6	30	60	70
Kilning 4	4	30	70	80
Kilning 5		30	25	25

2.2. Physical Characteristics of Doughs and Biscuits

The surface color of the doughs and the biscuits were measured using a Minolta CR410 Chroma Meter (Konica Minolta Co., Tokyo, Japan) based on CIELAB system color space *L**, *a**, *b** values, with reference to the D65 standard illuminant and a visual angle of 10. The parameter *L** represents the lightness of the sample, whereas *a** (degree of redness) and *b** (degree of yellowness) are chromatic components [18,19].

The thickness and diameter of biscuits were evaluated by using a Vernier caliper (on average 20 readings for each thesis). The ratio of diameter/thickness was used to calculate the spread ratio.

2.3. Chemical Composition of Samples

The biscuits were finely ground (final fineness < 300 µm; Knife Mill Grindomix GM 200, Retsch, Germany), and the moisture was measured gravimetrically using the air oven method (method 930.15) [20]. The water activity (a_w) was determined using the water activity meter Aqualab 4TE (Meter Food, Munich, Germany). Ash (method 942.05), crude protein (method 976.05), crude lipid (method 954.02 without acid hydrolysis) total starch (method 996.11), and the content of D-Fructose and D-Glucose (method 985.09) were considered [20]. The total dietary fiber (TDF) content was assessed enzymatically (Megazyme assay kit K-INTDF 02/15; Neogen, Lansing, MI, USA) on flours and biscuits.

2.4. Texture Evaluation of Biscuits

A Texture Analyser TexVol TVT 6700 (Perten Instruments, Hägersten, Sweden) with a cylinder probe of 45 mm diameter was used. The instrument was calibrated before the measurements were performed with the following settings: the height of the sample was 15 mm, the probe starting distance was 5 mm, the probe speed was 3 mm/s, the trigger force was 50 g, and the load cell was 5–10 kg. The maximum force recorded during the first compression stroke detected the firmness (g), and the adhesiveness (g) was measured by the work required to overcome the sticky forces between the sample and the probe. The second compression stroke determined the force B (g), the cohesiveness was calculated by the ratio force B/firmness, and the springiness (m) was obtained by the distance of the detected height (firmness) during the second compression divided by the original compression distance [21]. In addition, gumminess (g), conceived as the energy required to disintegrate a semisolid food to make it ready for swallowing (firmness × cohesiveness), and the chewiness (g), the energy needed to chew a solid food until it is ready for swallowing (firmness × cohesiveness × springiness), were measured [21]. For each batch, 15 biscuits were tested.

2.5. In Vitro Starch Digestion

A 2-step (i.e., gastric and pancreatic phases) static in vitro starch digestion procedure was used [22]. Briefly, samples were inserted in 50 mL tubes containing glass balls and pre-treated with a 0.05 M HCl solution (5 mL) containing pepsin (5 mg/mL; Sigma P-7000, Sigma-Aldrich® Co., Milan, Italy) for 30 min at 37 °C. The pH was then adjusted to 5.2 by adding 0.1 M sodium acetate buffer before the addition of 5 mL of an enzyme mixture with an amylase activity of about 7000 U/mL given by pancreatin (about 7500 FIP-U/g; Merck 7130, Merck KGaA, Darmstadt, Germany), amyloglucosidase (about 300 U/mL; Sigma A-7095, Sigma-Aldrich® Co., Milan, Italy), and invertase (about 300 U/g; Sigma I-4504, Sigma-Aldrich® Co., Milan, Italy) [22]. Aliquots (0.5 mL) were taken from each tube at 0 (before the addition of the enzyme mixture simulating the pancreatic phase), 30, 60, 120, and 180 min after the addition; absolute ethanol was added, and the amount of released glucose was determined colorimetrically (GODPOD 4058, Giesse Diagnostic snc, Rome, Italy). A blank was also included. A factor of 0.9 was used to convert mono to polysaccharide. The in vitro predicted glycemic index (pGI) was calculated as reported by Giuberti et al. (2015) [22]. For each treatment, samples were analyzed in triplicate.

2.6. Statistical Analyses

Data are presented as the mean values ± standard deviation of at least three replicates. The analysis of variance (One-way ANOVA) with a post hoc Tukey test at $p < 0.05$ using IMB SPSS Statistics software (Version 25) was used for data comparison.

3. Results

3.1. Chemical Composition of Native and Malted Grains

The total starch content of the malted grains was significantly ($p < 0.05$) lower than that of the native grains (Table 2). On the contrary, similar crude protein and ash contents were reported comparing native versus malted grains. The TDF content increased from 11.1 to 17.3 g/100 g of flour comparing native to malted triticale flour, respectively.

Table 2. Chemical composition of native and malted Etere triticale flours (g/100 g flours).

Triticale	Crude Protein	Total Starch	Ash	TDF
Native	12.3 ± 0.49 ^{a}	58.4 ± 4.04 ^{a}	2.6 ± 0.02 ^{a}	11.1 ± 2.21 ^{b}
Malted	13.7 ± 0.18 ^{a}	52.1 ± 1.92 ^{b}	1.7 ± 0.01 ^{a}	18.3 ± 3.01 ^{a}

Mean values with different superscripts within column are significantly different at ($p < 0.05$). Data are presented as the mean values ± standard deviation of at least three replicates. TDF: total dietary fiber.

3.2. Physical Characteristics of Doughs and Biscuits

The color of the dough and biscuits was affected ($p < 0.05$) by increasing levels of MTF in the recipe (Table 3 and Figure 1). In doughs, the L^* value decreased ($p < 0.05$) from 44.82 (control) to 41.01 (100% MTF), thus indicating a darkening of the products following MTF inclusion. In addition, the b^* values showed a similar trend, decreasing from 14.36 (control) to 12.37 (100% MTF), while for the a^* values, only the 25% MTF reported a difference ($p < 0.05$) to the control and the other MTF dough samples, showing the highest value (7.16; $p < 0.05$). Similarly, all the MTF-containing biscuits were darker ($p < 0.05$) than the control (i.e., 48.36), with the darkest sample being the 75% MTF (41.31; $p < 0.05$). In addition, control biscuits were characterized by the lowest b^* value ($p < 0.05$), whereas the 75% MTF biscuits had the greatest a^* values when compared to the other samples ($p < 0.05$) (Figure 1). In addition, color changes, in terms of lower L^* and b^* values and greater a^* values, were obtained by comparing doughs and biscuits at the same inclusion level of MFT in the recipe, due to the baking of the products.

Table 3. Physical and chemical characteristics of triticale-based doughs.

	Control	25% MTF	50% MTF	75% MTF	100% MTF
L^*	44.82 ± 0.63 ^{a}	45.43 ± 0.75 ^{a}	43.22 ± 0.93 ^{b}	42.38 ± 0.89 ^{b}	41.01 ± 1.02 ^{c}
a^*	6.66 ± 0.25 ^{b}	7.16 ± 0.29 ^{a}	6.93 ± 0.24 ^{b}	6.81 ± 0.19 ^{b}	6.60 ± 0.18 ^{b}
b^*	14.36 ± 0.62 ^{a}	14.04 ± 0.48 ^{a}	13.37 ± 0.51 ^{b}	13.38 ± 0.45 ^{b}	12.37 ± 0.63 ^{c}
Moisture ^{1}	44.76 ± 0.01 ^{a}	43.78 ± 0.01 ^{a}	44.68 ± 0.02 ^{a}	44.99 ± 0.02 ^{a}	41.22 ± 0.02 ^{b}
a_w	0.973 ± 0.002 ^{a}	0.959± 0.001 ^{b}	0.946 ± 0.004 ^{b}	0.944 ± 0.001 ^{b}	0.925 ± 0.005 ^{c}

Control: dough prepared with native 100% triticale flour (TF). 25% MTF: dough prepared by replacing 25 g/100 g *w/w* of TF with malted triticale flour (MTF). 50% MTF: dough prepared by replacing 50 g/100 g *w/w* of TF with MTF. 75% MTF: dough prepared by replacing 75 g/100 g *w/w* of MF with MTF. 100 MTF: dough prepared with 100% MTF. Mean values with different superscripts within rows significantly differ ($p < 0.05$). The analysis of variance (One-way ANOVA) with a post hoc Tukey test was used. Data are presented as the mean values ± standard deviation of at least three replicates. ^{1} g water/100 g food.

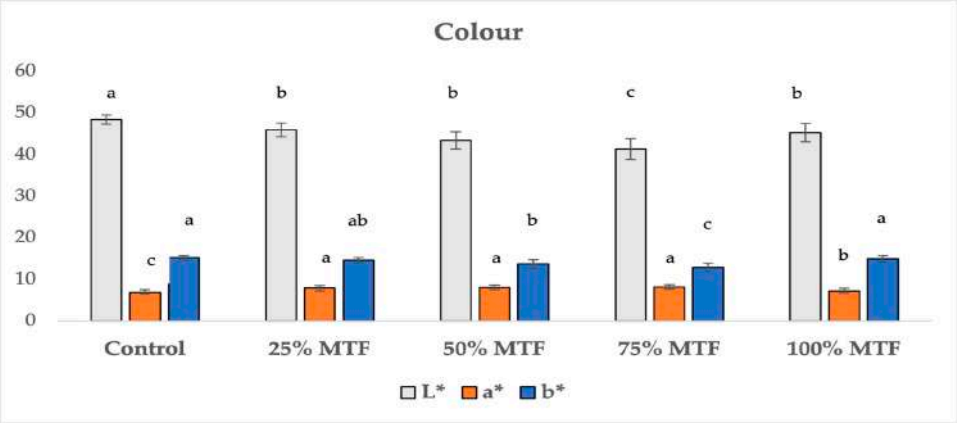

Figure 1. L^*, a^*, and b^* color values of triticale-based biscuits. Control: biscuits prepared with 100% native triticale flour (TF). 25% MTF: biscuits prepared by replacing 25 g/100 g *w/w* of TF with malted triticale flour (MTF). 50% MTF: biscuits prepared by replacing 50 g/100 g *w/w* of TF with MTF. 75% MTF: biscuits prepared by replacing 75 g/100 g *w/w* of MF with MTF. 100 MTF: biscuits prepared with 100% MTF. Different superscript letters in the same parameter are significantly different at the $p < 0.05$ level. The analysis of variance (One-way ANOVA) with a post hoc Tukey test was used. Data are presented as the mean values ± standard deviation of at least three replicates. Details of biscuits prepared with native and malted triticale flours are available in Supplementary Figure S1.

Biscuits were similar in diameter (Table 4), but different ($p < 0.05$) thickness and spread ratio values were recorded among the different formulations. All the MTF-containing samples were characterized by a higher thickness than the control, with the 100% MTF showing the highest value (1.55; $p < 0.05$). Consequently, different spread ratio values were recorded; the spread ratio of all the MTF-containing samples were lower than the control ($p < 0.05$; Table 4).

Table 4. Physical characteristics of triticale-based biscuits.

	Control	25% MTF	50% MTF	75% MTF	100% MTF
Diameter (mm)	3.28 ± 0.10 ^{a}	3.45 ± 0.09 ^{a}	3.47 ± 0.08 ^{a}	3.56 ± 0.06 ^{a}	3.54 ± 0.07 ^{a}
Thickness (mm)	1.38 ± 0.11 ^{c}	1.47 ± 0.11 ^{b}	1.51 ± 0.08 ^{b}	1.48 ± 0.11 ^{b}	1.55 ± 0.16 ^{a}
Spread ratio (D/T)	2.46 ± 0.20 ^{a}	2.36 ± 0.19 ^{b}	2.29 ± 0.12 ^{c}	2.39 ± 0.17 ^{b}	2.31 ± 0.21 ^{c}

Control: biscuits prepared with native 100% triticale flour (TF). 25% MTF: biscuits prepared by replacing 25 g/100 g *w/w* of TF with malted triticale flour (MTF). 50% MTF: biscuits prepared by replacing 50 g/100 g *w/w* of TF with MTF. 75% MTF: biscuits prepared by replacing 75 g/100 g *w/w* of MF with MTF. 100 MTF: biscuits prepared with 100% MTF. Mean values with different superscripts within rows are significantly different at ($p < 0.05$). The analysis of variance (One-way ANOVA) with a post hoc Tukey test was used. Data are presented as the mean values ± standard deviation of at least three replicates.

3.3. Chemical Composition of Doughs and Biscuits

Control and MTF-containing doughs reported a similar moisture content (Table 5); only the 100% MTF dough was characterized by a lowest moisture content ($p < 0.05$; 41.2 g water/100 g product). In contrast, the biscuits' moisture content increased with the addition of MTF in the recipe. Biscuits with 50 and 100% MTF had a higher moisture content compared to the control and to products with lower levels of MTF, being 17.03 g and 18.89 g water/100 g food, respectively ($p < 0.05$). The incorporation of MTF significantly ($p < 0.05$) decreased the a_w of doughs (Table 4) and biscuits (Table 5). In addition, the total starch content of biscuits decreased ($p < 0.05$) as the inclusion level of MTF increased in the recipe, ranging from 49.51% to 36.15% of dry matter, respectively, for the control and 100% MTF biscuits. The glucose and fructose content ($p < 0.05$) increased from 0.31% (control) to 2.80% (100% MTF) and from 0.24% (control) to 1.29% g/100 d dry matter (100% MTF), respectively. Similar results ($p < 0.05$) were reported considering the crude protein content of samples. With regards to the ash content, 75 and 100% MTF biscuits showed the highest values ($p < 0.05$), having 1.61–1.62% dry matter. The TDF of biscuits increased ($p < 0.05$) as the substitution levels of MTF increased from 25 to 100% *w/w*. In particular, 100% MTF biscuits approximately reached values that were three times higher than the value of the control sample. The dietary fiber content ranged from 3.49% to 9.92% dry matter for the control and biscuits formulated with 100% MTF, respectively.

Table 5. Chemical composition (g/100 g dry matter), in vitro starch hydrolysis index (HI), and in vitro predicted glycemic index (pGI) of biscuits.

	Control	25% MTF	50% MTF	75% MTF	100% MTF
Moisture [1]	14.22 ± 0.02 ^{c}	14.23 ± 0.01 ^{c}	18.35 ± 0.04 ^{a}	17.03 ± 0.00 ^{b}	18.89 ± 0.04 ^{a}
a_w	0.859 ± 0.012 ^{a}	0.859 ± 0.006 ^{a}	0.806 ± 0.012 ^{b}	0.801 ± 0.022 ^{b}	0.759 ± 0.003 ^{c}
Crude protein	12.44 ± 0.47 ^{b}	13.09 ± 1.02 ^{a}	13.56 ± 0.37 ^{a}	13.53 ± 0.58 ^{a}	13.84 ± 0.42 ^{a}
Crude lipid ^{ns}	16.72 ± 0.06	16.11 ± 0.17	16.47 ± 0.15	16.53 ± 0.02	15.74 ± 0.05
Total starch	49.51 ± 1.64 ^{a}	46.52 ± 1.25 ^{b}	40.45 ± 1.94 ^{c}	37.28 ± 2.14 ^{d}	36.15 ± 1.87 ^{d}
Glucose	0.31 ± 0.00 ^{d}	1.05 ± 0.01 ^{c}	1.23 ± 0.02 ^{c}	1.72 ± 0.03 ^{b}	2.80 ± 0.07 ^{a}
Fructose	0.24 ± 0.00 ^{d}	0.51 ± 0.00 ^{c}	0.59 ± 0.01 ^{c}	0.87 ± 0.01 ^{b}	1.29 ± 0.01 ^{a}
Ash	1.59 ± 0.07 ^{b}	1.59 ± 0.00 ^{b}	1.60 ± 0.01 ^{ab}	1.61 ± 0.00 ^{a}	1.62 ± 0.00 ^{a}
Total dietary fiber	3.49 ± 0.57 ^{e}	5.09 ± 1.11 ^{d}	7.21 ± 0.98 ^{c}	9.54 ± 1.02 ^{b}	9.92 ± 1.16 ^{a}
Starch HI [2]	59.50 ± 2.56 ^{d}	62.51 ± 2.45 ^{d}	78.89 ± 2.04 ^{c}	88.31 ± 3.01 ^{b}	104.43 ± 3.33 ^{a}
pGI	59.49 ± 2.99 ^{d}	62.08 ± 2.54 ^{d}	76.20 ± 2.33 ^{c}	84.32 ± 1.44 ^{b}	98.22 ± 2.23 ^{a}

Control: biscuits prepared with 100% native triticale flour (TF). 25% MTF: biscuits prepared by replacing 25 g/100 g *w/w* of TF with MTF. 50% MTF: biscuits prepared by replacing 50 g/100 g of TF with MTF. 75% MTF: biscuits prepared by replacing 75 g/100 g of MF with MTF. 100 MTF: biscuits prepared with 100% malted triticale flour (MTF). Mean values with different superscripts within lines are significantly different at ($p < 0.05$). ns superscript within one line denotes means were not significantly different ($p \geq 0.05$). The analysis of variance (One-way ANOVA) with a post hoc Tukey test was used. Data are presented as the mean values ± standard deviation of at least three replicates. [1] g water/100 g food. [2] Calculated using white wheat bread as reference (HI = 100 by definition) [20].

The in vitro starch hydrolysis index (starch HI) and the predicted glycemic index (pGI) ($p < 0.05$) increased with increasing levels of MTF in the formulation. The highest starch HI and pGI occurred in 100% MTF biscuits, reaching 104.43 and 98.22, respectively, while the control and the 25% MTF showed the lowest results: 59.50 and 62.51 for the starch HI and 59.49 and 62.08 for the pGI value, respectively (Table 5).

3.4. Texture Evaluation of Biscuits

Increasing the level of MTF in the biscuit recipe contributed to modifying the texture of the final products (Table 6). In particular, firmness, force B, chewiness, and gumminess decreased ($p < 0.05$) with increasing levels of MTF in the recipe, with the lowest values being recorded for 100% MTF samples ($p < 0.05$). The 25% MTF biscuits showed similar values to the control in firmness, chewiness, and gumminess; however, they reported significantly ($p < 0.05$) higher force B than the control and the highest springiness value ($p < 0.05$). Similar cohesiveness values were reported among the different formulations.

Table 6. Texture evaluation of triticale-based biscuits. All values are reported in grams (g).

	Control	25% MTF	50% MTF	75% MTF	100% MTF
Firmness	21,483.87 ± 3987.87 ^{a}	20,913.87 ± 2119.96 ^{a}	16,309.29 ± 1991.21 ^{b}	16,068.56 ± 1863.12 ^{b}	15,543.27 ± 1835.50 ^{c}
Force B	18,700.20 ± 3606.08 ^{a}	17,995.66 ± 1876.98 ^{b}	13,899.94 ± 1695.89 ^{c}	13,611.72 ± 1591.88 ^{c}	13,337.45 ± 1596.17 ^{c}
Springiness	0.89 ± 0.16 ^{a}	0.97 ± 0.06 ^{c}	0.91 ± 0.11 ^{b}	0.87 ± 0.14 ^{a}	0.94 ± 0.08 ^{b}
Cohesiveness ^{ns}	0.5 ± 0.04 ^{a}	0.5 ± 0.02 ^{a}	0.5 ± 0.01 ^{a}	0.5 ± 0.01 ^{a}	0.5 ± 0.03 ^{a}
Chewiness	10,741.60 ± 2458.83 ^{a}	10,672.80 ± 1193.46 ^{a}	8010.70 ± 1142.59 ^{b}	7965.28 ± 1112.44 ^{b}	7921.18 ± 1147.62 ^{c}
Gumminess	10,750.13 ± 2460.51 ^{a}	10,671.20 ± 1192.92 ^{a}	8008.00 ± 1138.10 ^{b}	7962.56 ± 1111.79 ^{b}	7914.36 ± 1146.52 ^{b}

Control: biscuits prepared with 100% native triticale flour (TF). 25% MTF: biscuits prepared by replacing 25 g/100 g *w/w* of TF with MTF. 50% MTF: biscuits prepared by replacing 50 g/100 g of TF with MTF. 75% MTF: biscuits prepared by replacing 75 g/100 g of MF with MTF. 100 MTF: biscuits prepared with 100% malted triticale flour (MTF). Mean values with different superscripts within rows are significantly different at ($p < 0.05$). ns superscript within one row denotes means were not significantly different ($p \geq 0.05$). The analysis of variance (One-way ANOVA) with a post hoc Tukey test was used. Data are presented as the mean values ± standard deviation of at least three replicates.

4. Discussion

Nowadays, the interest in formulating baked products with under-exploited flour is increasing, driven by consumers' demand for healthier food products [1]. In this regard, germination has been identified as an inexpensive and effective green technology to improve the quality of cereal and legume grains by enhancing nutrient content and digestibility and reducing the levels of antinutrients [14,15,19,23–27]. The effect of germination on nutrient contents has been widely studied; however, very little information is found in the literature about the effects of germination/malting on triticale composition and physicochemical properties necessary to know possible food applications [1,19].

The MTF incorporation into the biscuit recipe contributed to changes in several physio–chemical characteristics. As expected, the increase in MTF level caused doughs and biscuits to darken and brown, in line with previous findings [28]. This mainly occurs because of the increase in small molecules produced by the enzymatic degradation of starch and protein during germination. The small molecules primarily involved are reducing sugars and amino acids, which, during baking, can react, originating the Maillard reaction, a range of reactions that lead to the formation of brown nitrogenous polymers and co-polymers known as melanoidins [28–30]. In addition, the color changes in biscuits can also be attributed to the caramelization of reducing sugars during cooking [29,31].

The diameter of biscuits was not influenced by the addition of MTF. However, as MTF substitution level increased in the recipe, the thickness increased, probably due to the more intense indigenous yeast activity in the presence of free sugars [30]. Consequently, biscuits with higher MTF amounts obtained lower spread ratio values. The biscuit spread ratio represents the ratio of diameter to height. Thus, the effects of free sugars on the diameter (sugar dissolution) and height (inhibiting gluten development) are combined into a single parameter [27]. During malting, enzymatic degradation of starch and protein in flours to smaller sugars and peptides may occur. As a result, the hydrophilic nature of the biscuits can be increased, thus contributing to the decrease in the spread factor. Higher spread ratio values are considered an important quality attribute of biscuits because of their relationship with texture, bite, and overall mouthfeel [27]. The lowering in the spread ratio value can also be related to MTF containing more water-absorbing constituents like fiber and protein, as already reported [27–29]. Comparable results were reported in biscuits containing different malted flours [28,29].

Data indicated that the MTF inclusion in the formulation increased the moisture content of products. Overall, dry biscuits should have a moisture content lower than 5 g/100 g of product after baking and generally an a_w of 0.4 [27]. The substitution of native triticale flour with increasing levels of MTF increased biscuits' moisture content in the study of Chung et al. [32]. In addition, Karimzadeghan et al. [33] observed that the moisture content of the samples containing triticale significantly increased due to the high mineral and fiber content of triticale flour compared to wheat flour. On the contrary, the decrease in a_w following MTF increasing inclusion levels might be related to the binding

of water to smaller molecules broken by enzymes during germination [15,30]. However, considering both the relatively high moisture and a_w levels in the experimental biscuits, shelf-life studies are strongly warranted to evaluate the microbiological stability of the newly developed products. In addition, different baking conditions should be tested, aiming to reduce moisture and a_w levels in these products.

Biscuits with increasing levels of MTF presented enhanced nutritional characteristics, owing, in part, to the increase in TDF and protein and to the decrease in total starch content. Several studies reported increased TDF in different germinated cereals at different germination times and temperatures [15,19,28,32]. This increase could be due to the solubilization of the relevant macromolecules, the cleavage of intermolecular bonds, and the breakdown of protein structures [34]. The increase in TDF can partly be explained by the loss of compounds such as starch due to respiration and the synthesis of new polysaccharides during germination, which can cause changes in the cell wall matrix [15,28,30,34]. In terms of TDF, biscuits may therefore be ordered as follows: 100% MTF > 75% MTF > 50% MTF > 25% MTF > control. Dietary fiber exerts several benefits to human health and wellbeing. Plenty of studies have suggested that higher consumption of dietary fiber is beneficial for a variety of health outcomes, including, but not limited to, the prevention of arteriosclerosis, protection against colon cancer, lower concentrations of serum inflammatory biomarkers, and a lower risk of coronary heart disease [1,8,14,19]. Consequently, the use of MTF could be considered a valuable strategy to formulate baked goods that might promote a higher fiber intake as part of a healthy diet.

Ash content significantly increased starting from 75% MTF; this measure is an indication of the mineral's constituents present in the food. Several authors observed increased mineral content (Fe, Zn, Ca, Se) after grain germination [28,30,34]. This occurs for the activation of phytase, which hydrolyses phytic acid during germination, making minerals more bioavailable [15,30].

Adding MTF to native triticale flour in the biscuit recipe resulted in a slightly higher protein content of all MTF-containing biscuits to the control, with no differences among the different inclusion levels. This suggests that MTF could contain small amounts of amino acids synthesized during germination, which were added to the intact proteins of native flour, thus providing a higher protein content. Analogously, Chauhan et al. [35] observed increased protein content in germinated amaranth flour, and Aluge et al. [36] found that protein content increased with increasing malted sorghum flour substitution. Authors indicated that the increase in protein content in germinated/malted flours could be related to the synthesis of enzymes, which might have resulted in the production of some amino acids during protein synthesis.

As expected, the use of MTF determined a decrease in total starch and an increase in reducing sugars in triticale-based biscuits. According to Baranzelli et al. [37], 24 h for germination is insufficient to activate the amylolytic enzymes because their maximum hydrolysis activity is between 48 and 72 h. In this study, a total of 96 h of germination was employed. Our findings agree with previous findings, in which glucose and fructose contents increased considerably throughout the malting process [15,19,28,32].

Food with low starch HI and pGI values would promote slow and moderate postprandial glucose and insulin responses; thus, these foods can be more desirable for diabetic patients as well as for healthy individuals [38]. However, results obtained from the in vitro digestion indicated that the addition of higher levels of MTF in biscuits determined an increase in the starch HI and the pGI, and hence an enhancement of the overall in vitro starch digestibility. This may be somewhat undesirable, since current nutritional guidelines encourage the consumption of carbohydrate-rich foods with slowly digestible starch properties to promote good health [38]. The increase in HI and pGI following MTF inclusion in biscuit recipe can be attributed to the lower total starch content in MTF biscuits and the higher content of glucose. The present findings agree with the study of Yang et al. [34], showing that germination enhanced the starch digestibility of different cereal flours. A downside of germination can be that the inherent starch structure is degraded by the action of the enzyme hydrolysis, making it easier for starch to be degraded by amylase

enzymes [15]. In addition, germination can also promote the activity of α-amylase and deactivate some amylase inhibitors [15,34]. The pGI is based on the in vitro release of glucose following carbohydrate hydrolysis. Hence, it is strongly linked with the content of glucose and starch morphology. In the present study, biscuits with MTF replacement above 25% obtained high pGI indices (pGI > 70), whereas the control and biscuits with 25% MTF showed medium values (56 < pGI < 69) [38]. On the contrary, Molinari et al. [29] showed that malted tartary cookies presented lower HI and pGI compared to native tartary flour cookies because of the higher content of dietary fiber and resistant starch. Even Cornejo et al. [39] found that the HI as well as the pGI of bread were significantly reduced with the germination of brown rice. Present in vitro results indicated that the structural attributes of products (i.e., hardness) can also contribute to dictate the in vitro breakdown pattern of starch. It has been widely reported that samples with similar composition may also be digested in vitro at different rates and extents depending on their structural attributes [22,30,39]. However, discrepancies among studies can be related to the different chemical composition of products; the different germination, malting, and baking conditions employed; and differences in the in vitro systems used. This suggests the need for harmonization of the in vitro starch digestion systems to make data from different studies more comparable. In addition, the enhanced in vitro starch digestibility of MTF-containing biscuits should be carefully considered to optimize the use of MTF in biscuit formulation. Nevertheless, these results based on in vitro studies clearly warrant further in vivo studies.

Regarding texture results, MTF-containing biscuits became softer with the increasing substitution of MTF. Similar results were obtained by Chung et al. [32] for cookies containing germinated brown rice flour and by Chauhan et al. [35] for cookies made with germinated amaranth flour. The decrease in hardness could be attributed to the formation of a weaker matrix in biscuits caused by the structural degradation of protein and starch during germination. In addition, this decrease might be related to the increasing dietary fiber content following the incorporation of MTF, as well as to the higher level of water in the products. Sozer et al. [40] observed that the cookie's firmness decreased with the highest contents of fiber incorporation. Because of the decrease in firmness, biscuits with the highest MTF levels showed a reduction in the second compression (force B), in gumminess, in chewiness, and an increase in springiness. However, the latter parameter did not increase linearly with MTF inclusion levels, as 25% of MTF in biscuit formulation obtained the highest value, while 75% of MTF resulted in the lowest value. All the samples showed higher springiness values compared with the optimal values reported in the literature, showing that the optimal range is in the range of 0.05–0.72 [27].

5. Conclusions

This study reveals that triticale and malted triticale flours can be used in biscuit formulation. Increasing the incorporation of malted triticale flour contributed to enhancing the nutritional value of triticale-based products by mainly increasing the fiber content. From a nutritional standpoint, starting from the threshold of 50% of malted triticale flour substitution, biscuits could benefit from the claim of "high fiber content", with the total dietary fiber content being >6 g/100 g [41]. Changes occurred considering the technological parameters since a decrease in firmness, chewiness, gumminess, and spread ratio was reported. However, the lowering in these texture values can be tailored for specific consumers who have chewing problems, such as the elderly population. Considering the in vitro starch digestion, chemical modifications following the malting process of triticale flour influenced the in vitro digestibility of starch by increasing the in vitro starch hydrolysis index. This aspect should be carefully considered and better investigated through in vivo trials. Data indicated that substituting native with malted triticale flour could contribute to formulating food products with attractive nutritional properties without drastically influencing technological parameters. However, further in vivo studies and a shelf-life evaluation are needed to better explore the potential of native and malted triticale flour in biscuit food formulation. In addition, since malting can promote the development of more desirable

aromas and flavors, a sensory evaluation of products is warranted. Lastly, future studies are needed to optimize the malting protocol to obtain the highest total dietary fiber or protein contents to improve the nutritional profile of malted triticale flour for different food applications.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods12183418/s1, Figure S1: Details of biscuits prepared with native and malted triticale flours.

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CONCLUSIONS

At global level, the challenges in the food sector are closely connected with critical issues to be solved such as the protection of the planet, the climate change, the sustainability of food production and of natural resources.

Advances in biotechnologies promise to be one of the major leaps in human scientific understanding in this century, offering new products and processes that may help to address some of the world's most pressing challenges, whilst maintaining high levels of environmental, food and consumer safety.

Biotech solutions for the Agri-Food sector encompass a range of modern techniques and tools, which can help with understanding the fundamental mechanisms of the basis of life (https://cordis.europa.eu/).

In this broad context, it has been decided to consider research studies on cereals.

The first part was focused on Molecular Identification of Durum Wheat, in terms of varieties and species, and on Quality and Safety assessment of wheat and pasta chain, this because I am working in Barilla Company that is the world leader in pasta production.

The second part was dedicated to design of cereal-based foods with enhanced value: Rye and Triticale may be of interest for their nutritional, technological and environmental sustainability implications.

Here are the research studies done during Ph.D.

Set up Molecular Toolkit for Wheat and pasta chain

The world is changing, but the importance of food safety remains.

Discover the latest trends and innovations in molecular DNA-based tool is a crucial challenge.

- ***Digital PCR: What Relevance to Plant Studies?***

In this review, the major applications of digitalPCR (dPCR) are presented, developed so far in the field of plant science. As it is reported in literature, dPCR assays are mainly used for tracking genetically modified plant components, pathogenic and non-pathogenic microorganisms (7%), and plant species (35%), the other applications had regarded studies on structural and functional genetics.

The benefits of digital PCR include absolute target quantification without standards or reference curves, high tolerance to PCR inhibitors, high precision to detect small changes, high sensitivity, low limit of detection and high reproducibility. DPCR is an advanced molecular technique where the sample is partitioned into many individual reaction wells so that either zero, one or more target molecules might be present in each reaction. After partitioning, the reactions undergo end-point PCR cycling, and partitions are analysed for the presence (positive reaction) or absence (negative reaction) of a fluorescent signal. The software for dPCR applies the statistical calculation of the Poisson distribution to the data obtained and expresses the result in number of target present in the sample. The peculiar feature of dPCR is the absolute quantification of nucleic acids, so has lower detection limit and higher precision than qPCR. Finally, it is an advanced molecular tool which provides reliable results in short time (3h), at low costs, and it is easy to use. In the plant science sector, the use of digital PCR is still a young but resulted very promising technique, because dPCR fit for our purposes oriented on authenticity, together with the possibility of tracing significant aspects both from the point of view of quality and food safety, topics of relevance for both producers and consumers. For these reasons, DNA-based method has proved to be effective, economical and, suitable to test various points along agri-food pasta supply chains.

A toolkit that gathers several DNA-based assays has been developed including:

- qPCR and dPCR assays for *Triticum* species quantification

- qPCR and SNP based screening method for durum wheat varietal identification
- cdPCR and Point-of-care methods for the traceability of specific variety
- cdPCR and Point-of -care methods for fast diagnosis of mycotoxigenic Fusaria

To develop, to validate and to apply molecular traceability dedicated to the pasta and wheat-derived products, a cooperation has been established between public and private research bodies, CREA-GB, CREA-DC, WUR, ITT, Barilla, and COOP IT (GDO).

DNA-based assays have been developed that can answer several important questions for the durum wheat and pasta supply chain such as the presence of soft wheat in pasta, such as the varieties of wheat that which are the characteristic of the product and the possible presence of microbial criticalities. Listed below are the studies carried out and published.

- ***The innovative DNA-based method to detect soft wheat***

Among the most important factors in the pasta supply chain, there is the possible presence of soft wheat: in Italy, by law, the presence of soft wheat in semolina and pasta must be <3% according to Legge 580/67. Italian pasta can only be produced from durum wheat, which due to the quality and quantity of gluten for a cooking al dente, a feature that has made our pasta iconic in the world.

The collaborative research work has produced an effective response the need to quantify the presence of different species of wheat and to assure the legal compliance. An innovative system, based on the dPCR assay, which makes possible to identify and quantify with high precision and low cost the presence of cereal species other than durum wheat in semolina and pasta.

- ***DigitalPCR and Point-of-care: two ways for detection wheat varieties based on Durum wheat target variety private point mutation***

Since the food quality can also depend on the use of specific varieties, work has been done to develop molecular tool that answer to this need. Two different and complementary approaches have been successfully developed: the one, based on

digitalPCR, an high-precision laboratory method that reports whether the desired variety is present in the sample and in what percentage, and the other is a Point-Of-Care system, a simplified approach that can also be applied where and when you need a quick response. A single nucleotide polymorphism (SNP) was identified as a reliable marker for wheat varietal discrimination, and a rapid test for easy and clear identification of specific wheat varieties was developed, too. Notably, an approach based on the loop-mediated isothermal amplification reaction (LAMP) as an SNP discrimination tool, in combination with naked-eye visualization of the results, was designed and optimized. Our assay was proven to be effective in the detection of adulterated products, including both substitution and mixing with different crop varieties.

- ***A chip digital PCR (cdPCR) assay has been developed to detect and quantify percentages of hulless (i.e., common and durum wheat) and hulled (i.e., einkorn, emmer and spelt) wheats in grains, flour, and foods (pasta, bakery products, bread, baby foods).***

The samples tested were commercially available. The assay can be used along production chains, from raw materials to final food products. Of couse this method offers a new important possibility for the authenticity's identification at species level.

- ***digitalPCR and Point-of-care to assess Fusarium issues***

Among DNA-based technologies for *Fusarium* detection, qPCR (single and multiplex assays) is currently the most applied method. However, pathogen diagnostics is now enforced by dPCR, that provides ultrasensitive and absolute nucleic acid quantification. In our work, a panel of chip digitalPCR assays was developed to quantify *Fusarium graminearum, F. culmorum, F. sporotrichioides*, *F. poae* and *F. avenaceum*. Most of the species associated with Fusarium Head Blight (FHB), in advantageous environmental conditions, invade the ear of the cereals and produce toxic secondary metabolites mycotoxins and, therefore, compromises not only the yield but also the grain safety and quality due to the accumulation of mycotoxins in infected kernels. Depending on species and chemotypes, *Fusarium* can produce trichothecenes A include highly toxic

mycotoxins T-2 and HT-2, while type B trichothecenes include, among others, deoxynivalenol (DON), nivalenol (NIV) and acetyl-NIV. All these fungal secondary metabolites can cause a wide range of diseases as well as death in humans and animals. Diagnostic tools, able to track *Fusarium* species even in the early stages of infection, can contribute to mycotoxins' risk control and mitigation. For a better evaluation of infection level in plants, duplex assays were developed, able to co-amplify both plant and fungal DNA. To the best of our knowledge, this is the first study directed to the application of digital PCR to *Fusarium* diagnosis in plants.

The technological transfer of wheat and pasta molecular toolkit has been completed, the results were disseminated at many international events. The methods were included in protocols. These studies proved how it is crucial to invest in Advanced Research and Innovation and the importance of adopting a multidisciplinary approach safety and quality assessment included to further promote pasta, the Italian iconic food competitive in global food market.

Design of Rye-Based Foods with Enhanced Value

Rye (*Secale cereale* L.) is the only cross-pollinating small-grain cereal. Rye (*Secale cereale* L.) has been cultivated in Europe for over 1000 Before the Common Era and it is widely grown, especially in areas of Northern Europe, and North America, where the soil and temperature are unfavourable for cultivation other cereals. Today, an increase in rye production offers a viable option for the European Green Deal to transform the EU into a sustainable, resource-efficient, and competitive bioeconomy that ensures no net emissions of greenhouse gases by 2050.

Rye grains are distinguished by having one of the greatest levels of fiber content as compared to the other cereals, B vitamins, vitamin E. Rye whole grains are rich source of various phytochemicals such as phenolic acids, lignans and alkylresorcinols.

Rye bread is one of the most consumed cereal-based foods in northern Europe, China, and North America. Consumers increasingly prefer whole grains for their nutritional benefits, including gut-friendly metabolism and health-promoting properties attributed to some bioactive compounds such as flavonoids, phytoestrogens, phenolic compounds, and carotenoids.

- ***Hierarchical Effects of Lactic Fermentation and Grain Germination on the Microbial and Metabolomic Profile of Rye Doughs***

From a nutritional perspective, rye whole grain flour is gaining attention for its health-promoting potential considering its hypocholesterolemic, anti-diabetic, anti-inflammatory, and cardio-protective properties. Whole grain rye is characterized by a high content of dietary fibers, such as arabinoxylans and cellulose, and bioactive compounds with antioxidant properties, such as polyphenols.

Driven by consumer demand for sustainable and healthier products, the utilization of rye in cereal-based foods has been widely explored.

The use of sourdough fermentation is increasingly being re-considered as a sustainable bioprocessing to improve sensorial, functional, and nutritional attributes of baked goods. While huge progress has recently been made in advanced molecular tools that explain the ecology of sourdough microbiota, less is known about how phytochemicals in whole grain are affected by microbial metabolism and food processing.

In this context, seed germination or sprouting has gained popularity in cereal processing as an effective practice to improve grains' nutritional and functional quality. Sprouting involves the activation of endogenous hydrolytic enzymes that increase the digestibility of cereal proteins and starch.

The aim of this study was to evaluate the influence of selected and/or sourdough-related Lactic Acid Bacteria (LAB) fermentation on the chemical composition of rye flour in combination with seed sprouting. The approach to combine these two traditional technologies has given excellent results, a synergic effect was demonstrated.

In germinated grain rye the content of Total Dietary Fiber (TDF), Soluble Fiber (SF), Insoluble fiber (IF), glucose, and fructose, resulted improved. Start from germinated rye grains, a rye flour has been obtained and fermented. In rye sourdough an improvement of prebiotics properties, an accumulation of bioactive contents was observed and finally, the content of monosaccharide decreased. Whole Grain rye doughs and sprouted doughs were prepared with the addiction of sourdough starters including *Limosilactobacillus fermentum*, *Weissella confusa and W. cibaria* (strains coming by UCSC Collection), and *Saccharomyces cerevisiae* as commercial and compressed fresh baker's yeast.

Total DNA was extracted from bacterial pellets and quantified (Operational Taxonomy Unit OTU sequences shared over 99% similarity). OTUs were aligned with the representative sequence of the NCBI 16S Database.

UHPLC/QTOF-MS (Ultra-High-Performance Liquid Chromatography coupled to a Quadrupole Time-Of-Flight Mass Spectrometer) was used to assess metabolic profile: the results indicated that, sprouted rye doughs had greater uniformity in the bacterial community structure than the native rye counterparts. *Limosilactobacillus fermentum* is prevalent in native rye doughs, but not in sprouted rye doughs.

HPAE-PAD (High Performance Anion-Exchange Chromatography With-Pulsed Amperometric Detection) was applied for detection of carbohydrate content in rye sourdough showed a decrease in monosaccharides with respect to native rye dough.

A graphical plot (Variable Importance Plot) ranks the importance of each variable /feature, revealed an increase in the mannitol content of rye doughs when LAB were added, suggesting an interesting implication for sugar-reduced product applications: an important nutritional topic for bakery products. Concurrently, LAB metabolism promoted a general up-regulation of the dough system metabolome, leading to the accumulation of a few bioactive molecules, that includes well-known precursors of antioxidant and aromatic compounds, which may potentially contribute to enhance health benefits and organoleptic properties of derived breads.

Among lipid metabolites, doughs fermented with sourdough LAB displayed a significantly higher amount of lysophospholipids that can contrast staling in bread.

Overall, these results shed light on LAB fermentation-induced modifications of content, properties, and biological availability of rye grain constituents, providing insight to guide the development of new healthy and nutritious rye-based products.

In conclusion, in rye malted sourdough resulted in lower levels of simple sugars and increased levels of mannitol in dough: it might represent a relevant strategy to reduce sugar in baked goods. Sprouted rye dough promoted the accumulation of maltopentaose and maltotetraose, functional maltodextrins potentially involved in glycaemic control response and enterocyte differentiation.

Finally, metagenomic data were crucial to assess microbial ecology in doughs:

Lactoplantibacillus plantarum was typical of native rye, *Latilactobacillus curvatus* was significantly higher of sprouted rye. Untargeted metabolomics analyses were applied to discover microbial contribution to the biochemical profile of grain doughs, particularly the bioactive compounds.

Concurrently, LAB metabolism promoted a general up-regulation of the dough system metabolome, leading to the accumulation of a few bioactive molecules, including terpenoids, flavonoids, phenolic acids like p-coumaroyl derivatives, stilbenes, coumarins and lignans. This group includes well-known precursors of antioxidant and aromatic compounds, which may potentially contribute to enhance health benefits and organoleptic properties of derived breads.

Overall, these results shed light on LAB fermentation-induced modifications of content, properties, and biological availability of rye grain constituents, providing insight to guide the development of new healthy and nutritious rye-based products.

Design of Triticale-Based Foods with Enhanced Value

- ***Combining Native and Malted Triticale Flours in Biscuits: Nutritional and Technological Implications***

Triticale (*Triticosecale wittmack*) is wheat/rye hybrid grain with a worldwide production that has consistently increased during the last decades. Triticale was traditionally used as animal feed and for biofuel production; however, the growing demand for food resources and the current consumer trend of trying novel products has led to an increased interesting food production. In addition, triticale could be an important crop to ensure food security due to its tolerance to drought, disease, more acid soils, low susceptibility to biotic stresses, and high grain yield even in marginal environments.

Other than the use of native flour, triticale is a promising cereal for malting owing to its high levels of α-amylase and proteolytic enzymes, which allow a quick malting process. During the malting process several physicochemical changes can occur that can positively affect the grain's chemical and nutritional composition regarding macro-and micro-nutrients and bioactive compounds.

Given the optimal nutritional value of malted flours, the good attitude of triticale for the malting process, the suitability of triticale flour for biscuits preparation, and the trend in the food market to formulate baked products with unconventional and under- exploited flours, this study aims to formulate novel biscuits made only with triticale flour and malted triticale flour (MTF) in different ratios. To our knowledge, this is the first study in which triticale flour (native or malted) was exclusively employed in biscuit formulation by exploring the potential of triticale flour as ingredient in biscuits.

In this work, to better explore the suitability of triticale and MTF in biscuits, products were analysed in terms of technological and nutritional attributes, including the evaluation of the in vitro starch digestion. Nowadays, the interest in formulating baked

products with under-exploited flour is increasing, driven by consumers' demand for healthier food products.

Some results of relevance are presented below. The in vitro starch hydrolysis index (starch HI) and the predicted glycaemic index (pGI) ($p < 0.05$) increased with increasing levels of MTF in the formulation, biscuits the 25% MTF showed the lowest predicted Glycaemic Index: 59.50 and 62.51. In this regard, germination has been identified as an inexpensive and effective green technology to improve the quality of cereal grains by enhancing nutrient content and digestibility. Very little information is found in the literature about the effects of germination/malting on triticale composition and physicochemical properties necessary to know possible food applications. The MTF incorporation into the biscuit recipe contributed to changes in several physio– chemical characteristics, such as brown colour, originating the Maillard reaction reactions that lead to the formation of brown nitrogenous known as melanoidins.

However, further *in vitro* studies and a shelf-life evaluation are needed to explore the potential of native and malted triticale flour in bakery products combined with definition of sensorial profile that would be expected once the process and the product have been fine-tuned.