ALMA MATER STUDIORUM
UNIVERSITÀ DI BOLOGNA

DOTTORATO DI RICERCA IN

SCIENZE E TECNOLOGIE AGRARIE, AMBIENTALI E ALIMENTARI

Ciclo 37

Settore Concorsuale: 07/B1 - AGRONOMIA E SISTEMI COLTURALI ERBACEI ED ORTOFLORICOLI

Settore Scientifico Disciplinare: AGR/02 - AGRONOMIA E COLTIVAZIONI ERBACEE

NUTRITIONAL AND HEALTH CARE ASPECTS OF SEVERAL FLOURS WITH DIFFERENT RHEOLOGICAL PROPRIETIES

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Esame finale anno 2025

Abstract

This study conducted a three-year field trial to evaluate the composition of gluten proteins in wheat and cell proliferation before and after partial digestion. In the first year, free polyphenols in 18 ancient durum wheat flours showed higher FPC, TPC, and flavonoid levels, enhancing DPPH scavenging compared to subsequent seasons. Partial digestion resulted in high-molecular-weight (HMW) proteins breaking down into smaller peptides, with significant overall protein reductions, varying among gluten proteins and subunits. The gliadins to glutenins ratio remained high with minor fluctuations.

In the second year, bound polyphenols and flavonoids decreased, leading to lower antioxidant activity and DPPH scavenging ability. The protein profile of 16 ancient durum wheat types demonstrated minimal changes from the previous year, though overall protein levels declined without significantly impacting gluten and HMW-GS ratios. An increase in ω-gliadin coincided with a decrease in LMW-GS, influencing the gliadins/glutenins and HMW-GS/LMW-GS ratios. Following partial digestion, the second year's wheat varieties presented reduced HMW-GS and LMW-GS bands, contrasting with the first year's results. The effects of partial digestion on gliadins/glutenins and HMW-GS/LMW-GS ratios varied, often showing a decline.

In the third year, polyphenol and flavonoid levels in wheat flour remained stable, with polyphenols primarily combined and a slight increase in antioxidant activity. The protein profiles of 22 ancient wheat samples varied, mainly displaying low molecular weight glutenin subunits (LMW-GS). Notable changes in gluten protein composition were observed, including a decrease in high molecular weight glutenin subunits (HMW-GS) and ω-gliadins or reduced LMW-GS, while protein degradation post-digestion persisted. The total protein content decreased from the first to the third year but was higher than in the second year. Post-digestion protein levels fell, yet ancient wheat varieties consistently showed higher protein content than the modern variety Claudio. Ratios of gluten and its subunits, indicative of dough rheological properties (e.g., gliadin/glutenin and HMW-GS/LMW-GS), typically matched historical ranges.

Pearson correlation analysis showed that a significant positive correlation was found between the content of phenolic compounds and flavonoids and antioxidant activity. And cell viability and specific gluten protein subunits, including ω-gliadins, LMW-GS, and α/β, γ-gliadins. The comprehensive analysis of a three-year experiment revealed that that Lucana, Benedettelli, and

Guastella had the highest inhibition of L929 and Caco2 cells, with a more pronounced inhibitory effect observed on the L929 cells. Overall, this study confirmed that, while the inhibition of wheat proteins on cell proliferation varied annually, partial digestion consistently reduced the inhibitory effect of specific proteins (P2, 3R, and V1) across the three years.

Keywords: durum wheat; gluten protein; environment effect; genetic inheritance; *in vitro* digestion; cell proliferation

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1. Introduction

Evolution, domestication and diversity of wheat

Wheat (*Triticum aestivum* L.) is one of the world's most important food crops. It represents a major source of nutrients in human diets and is a major ingredient in a wide range of products such as bread, noodles, and pastries. Wheat domestication commenced with the advent of agriculture during the early Holocene, approximately 12,000 years ago, in the Fertile Crescent, which encompasses present-day Iraq, Iran, Israel, Jordan, Lebanon, Palestine, Syria, and Turkey. During this period, cereals garnered attention due to their productivity, nutritional quality, and convenient transport and storage capabilities [1-4]. The process of domestication marked an evolutionary leap from the foraging of wild cereals that had occurred over countless millennia. The transformation of wheat through domestication involved both adaptation and speciation under human influence, facilitating the establishment of sedentary communities during what is recognized as the Neolithic Revolution [5, 6]. This domestication induced various morphological, physiological, and genetic alterations, collectively referred to as domestication syndrome [5, 7]. As Neolithic agriculturalists migrated from the Fertile Crescent to Europe, Asia, and Africa, the varying environmental conditions prompted further evolutionary developments in wheat [3, 4, 8, 9]. Selective pressures originating from natural adaptation to local agro-ecological environments, along with farmer-mediated selection for desirable traits, culminated in the formation of distinct, regionally adapted populations, known as landraces [10, 11].

Wheat varieties are categorized into three classifications based on their chromosomal makeup: diploid (containing 14 chromosomes), tetraploid (consisting of 28 chromosomes), and hexaploid (having 42 chromosomes). The majority of cultivated wheat varieties are allopolyploids, which have integrated two or more sets of analogous chromosomes within a single nucleus through interspecific hybridization, followed by natural chromosome duplication or gamete reduction. This process facilitates rapid genomic changes and enhances evolutionary adaptability [12-14]. The modern-day wheat we recognize today is the culmination of millennia of crop evolution and human intervention. It is believed that the initial cultivated wheat species were Einkorn (*Triticum monococcum* L.) and Emmer (*T. turgidum ssp. dicoccum* L.) [15], both of which served as vital food sources in ancient civilizations [16]. Hybridization events between diploid species eventually led to the emergence of

tetraploid wheat. The interbreeding of *T. urartu* (2n = 2x = 14, AA) and *Aegilops speltoides* (2n = 2x = 14, SS) gave rise to emmer (*T. dicoccum*; 2n = 4x = 28, AABB) [16, 17] and durum wheat (*T. durum*; 2n = 4x = 28, AABB) [18]. *T. spelta* (2n = 6x = 42, AABBDD) is believed to have originated from a natural hybridization event between *T. turgidum* and *Ae. Tauschii* [16, 19], with the resultant hybrid being hulled due to the presence of the tenacious glume gene *Tg1* from *Ae. tauschii.* Through subsequent selection and evolutionary processes, free-threshing *T. aestivum* (2n = 6x = 42, AABBDD) emerged [16], currently accounting for approximately 95% of global wheat production[20]. Table 1 shows the classification of wheat [21].

The cultivation of wheat spans an extensive range, from Russia at 67 degrees north latitude to Argentina at 45 degrees south latitude [22]. At present, the most extensively cultivated wheat species include tetraploid (*Triticum durum*) and hexaploid *(Triticum aestivum*) variants. Tetraploid wheat, derived from durum wheat (also known as pasta wheat), is primarily cultivated in Mediterranean countries such as Italy, France, Spain, and Greece. On the other hand, hexaploid wheat, originating from soft wheat (also known as bread wheat), is the predominant variety cultivated in the majority of the world, constituting approximately 95% of global wheat production due to its superior adaptability to diverse meteorological conditions [23].

Table 1. Classification of wheat [21].

Type		Genome Formula
Diploid		
	T.urartu Tum.	AA
	T.boeoticum Boiss. spp. *aegilopoides*	AA
	spp. *thaoudar*	
	T. monococcum L.	AA
	T. sinskajae A. Pilat. and Kurk.	AA
Tetrapolid		
	T. dicoccoides (Korn) Schweinf.	AABB
	T. dicoccum (Schrank.) Schulb.	AABB
	T. paleocolchicum Men.	AABB
	T. carthlicum Ncvski	AABB
	T. turgidum L.	AABB
	T. polonicum L.	AABB
	T. durum Desf.	AABB
	T. turanicum Jakubz.	AABB
	T. araraticum Jakubz.	AAGG
	T. timopheevi Zhuk.	AAGG
	T. militinae Zhuk. and Migusch.	AAGG
Hexaploid		
	T. spelta L.	AABBDD
	T. vavilovi (Tum.) Jakubz.	AABBDD
	T. macha Dek. and Men.	AABBDD
	T. sphaerococcum Perc.	AABBDD
	T. compactum Host.	AABBDD
	T. aestivum L.	AABBDD
	T. zhukovskyi Men. and Er.	AABBGG

Structure characteristics of wheat

Wheat grains, as a type of monocarpic fruit, are referred to as caryopses. It is divided into three main segments, each structurally and chemically distinct from the others. These segments are: the germ, also known as the embryo, which is located at one end of the grain and appears as a small, yellow mound, easily distinguished from the rest of the kernel; the endosperm, which constitutes a larger portion of the grain and provides nutrition to the developing plant as the kernel matures; and the external seed coat, which contains protein cells that encase the entire kernel, protecting both the embryo and the endosperm from injury during the grain's dormant phase [24]. Notably, there are significant differences in the chemical composition of the constituents of these three parts, resulting

in a broader variation in their nutritional value [25].

Among these, the embryo is rich in proteins, lipids, and various B vitamins [26, 27]. Whole wheat flour contains bran, which is abundant in limited proteins, abundant B vitamins, trace minerals, and indigestible dietary fiber [26, 27]. Conversely, white flour is derived from the endosperm, which is rich in most of the grain's proteins, iron, carbohydrates, and various B vitamins such as riboflavin, thiamine, and niacin [26, 27]. Approximately 85% of the components in the caryopsis are carbohydrates, of which about 80% are starch, 12% are cell wall polysaccharides, and 8% are low molecular weight monosaccharides, disaccharides, and oligosaccharides, including fructans (fructooligosaccharides) [28, 29]. These components are classified as fermentable oligosaccharides, disaccharides, monosaccharides, and polyols, which are considered potential contributors to both intestinal and extraintestinal symptoms of non-celiac gluten sensitivity (NCGS) [30]. The consumption of wheat is beneficial to health. In the European Prospective Investigation into Cancer and Nutrition (EPIC) study, bread accounted for 27% of total carbohydrate intake [31]. Epidemiological research indicates that consuming dietary fiber from grains and whole grains helps prevent chronic diseases resulting from a sedentary lifestyle, such as type 2 diabetes and cardiovascular diseases [32-34].

Wheat kernels are generally elliptical, although various types of wheat exhibit kernels that can be long, flattened, slender, or spherical in shape. The length and mass of the kernels typically range from 5 to 9 mm and 35 to 50 mg, respectively. Each kernel features a crease on the lateral side, which has led to its initial association with the wheat flower. The wheat kernel (Figure 1) [25] comprises 2–3% germ, 13–17% bran, and 80–85% mealy endosperm (all components transformed into dehydrated material) [35].

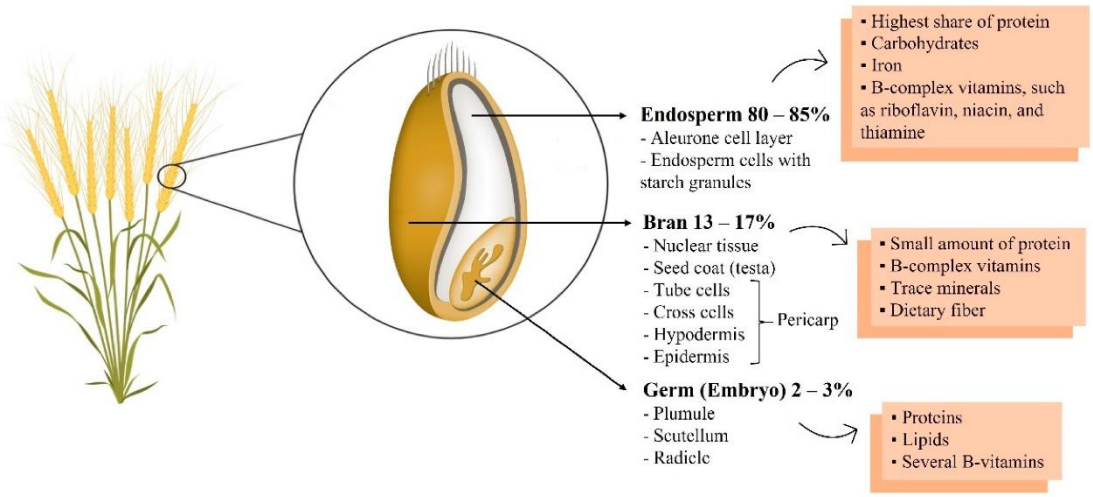


Figure 1. Wheat grain constitution.

Global yield of wheat

Grains are a primary source of food globally, occupying a crucial position in the daily diet of humans, accounting for over 50% of total daily caloric intake [36]. Specifically, the consumption of carbohydrates and proteins from wheat alone constitutes approximately 20% [37]. Wheat belongs to the Triticum family of the Gramineae family and is one of the most important food crops in the world [38], providing an important energy intake and protein source for about 35-40% of the world's population [39]. As the third largest food crop globally, wheat has a total production of 771 million tonnes, after maize and rice. However, it holds the distinction of being the largest in terms of the area harvested (in 2021; https://www.fao.org/faostat/). Primarily, wheat is utilized for human consumption, forming the basis of essential food items such as bread, pasta, pastries, couscous, bulgur, pizza, snacks, and sauces, in addition to being a key ingredient in beer and distilled spirits. Furthermore, both wheat grains and green biomass serve as valuable animal feed. The "Green Revolution" of the 1960s introduced dwarf but high-yielding wheat varieties, fundamentally altering traditional agricultural production methods and significantly increasing productivity [40]. Consequently, despite the possibility of wheat cultivation areas not necessarily expanding, global wheat production has shown a significant increasing trend over the past five decades (Food and Agriculture Organization statistics, 2019; www.fao.org). In 2020, the global annual wheat

production was approximately 780 million tons, with a consumption of around 780 million tons (https://apps.fas.usda.gov). It is projected that by the middle of this century, the world population will reach 9 billion. With the continual reduction in available arable land and the impact of global climate change, wheat production needs to increase by 70% from current levels to meet future food demand [41, 42].

Currently, wheat production grows by approximately 1.3% annually. However, to meet the anticipated demand of 9 billion people by 2050, the annual growth rate needs to increase to 2.4% [37, 41]. Production growth is constrained by various factors, including water scarcity, declining soil fertility, and the cultivation of varieties with inadequate tolerance to stress conditions such as drought and high temperatures [43]. Climate change is expected to further impact productivity [44]. Figure 2 shows the production of wheat worldwide. The top five wheat producers are China, India, Russia, USA, and France [45].

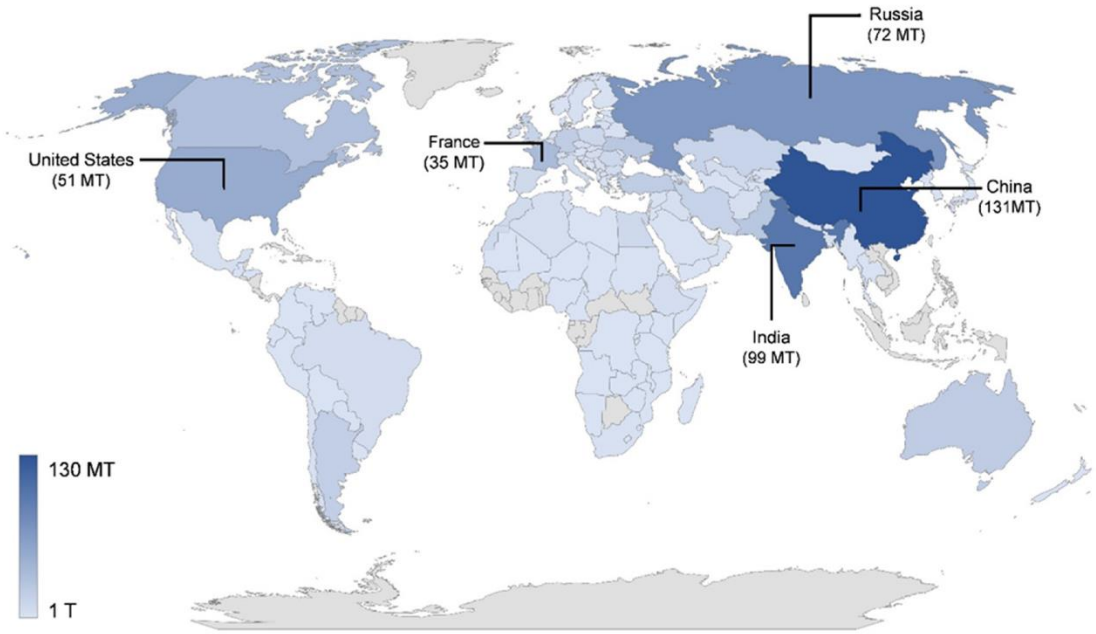


Figure 2. Worldwide wheat production. Data indicate the total wheat production in megaton (MT) in the year 2018 per country FAOSTAT [45].

Protein composition and structure of wheat

Based on the differences in protein solubility in solvents, Osborne categorized wheat proteins into

four types: albumins, globulins, gliadins, and glutenins [46]. Albumins and globulins are collectively referred to as non-gluten proteins, while gliadins and glutenins are collectively referred to as gluten proteins, constituting approximately 15-20% and 80-85% of the total protein, respectively [47]. Most non-gluten proteins have a molecular weight of less than 25 kDa, although some may range from 60-70 kDa [48]. Non-gluten proteins function as metabolic or storage proteins, comprising enzyme proteins in the cytoplasm, including α-amylase, proteinase inhibitors, regulatory enzymes, specialized synthetic enzymes, and metabolic enzymes [47]. Wheat albumins and globulins are generally considered to exist as monomers, although their specific molecular structural characteristics are not yet fully understood.

The gluten protein in wheat is a storage protein that includes gliadins and glutenins, having good physical properties and imparting elasticity to the dough while determining its viscosity. Gluten protein has low solubility in water and salt solutions due to the abundance of non-polar amino acids and proline residues [49]. Gliadins exist as spherical monomeric particles embedded in the networked polymerized glutenin, strongly affecting dough viscosity. Glutenin consists mainly of polar amino acids, and high-molecular-weight glutenin subunits (HMW-GS) and low-molecular-weight glutenin subunits (LMW-GS) can form heterogeneous polymeric structures through disulfide bonds, enhancing the elasticity of gluten [49]. Gliadins bind with glutenin monomers, intertwining to form a polymeric spatial structure, giving wheat flour its unique viscoelastic and extensibility properties (Figure 3).

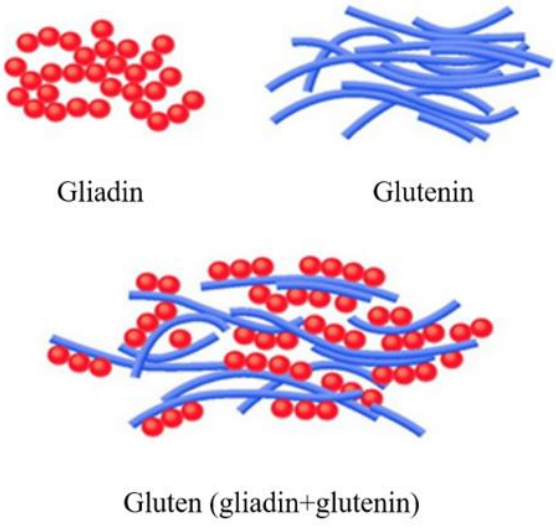


Figure 3. Structure of gluten. Spatial conformation of gliadin and glutenin with gluten formation.

The classification of gluten proteins is shown in Table 2. Gliadins are primarily monomeric proteins with a molecular weight ranging from 30 to 74 kDa. They are categorized into four groups based on their mobility at low pH in gel electrophoresis: α-, β-, γ-, and ω-gliadins [50]. α-, β-, and γ-gliadins are characterized by high cysteine and methionine residues, which facilitate the formation of intramolecular disulphide bonds, leading to a specific conformation of the polypeptide [51]. On the other hand, ω-gliadins typically lack cysteine residues. Through analysis of complete or partial amino acid sequences, amino acid compositions, and molecular weights, gliadins can be further classified into four distinct types: ω5-, ω1,2-, α/β-, and γ-gliadins [52]. The molecular weights of ω5-, ω1,2-gliadins are between 52 and 74 kDa, while α-, β- and γ-gliadins have lower molecular weights, ranging from 30 to 51 kDa. The latter approach has shown that the α- and β-gliadins are closely related and thereby they are often referred to as α-type gliadins. Glutenins are polymeric proteins with a molecular weight that can exceed 34,000 kDa [53]. They are classified into high-molecular-weight glutenins (HMW-GSs) and low-molecular-weight glutenins (LMW-GSs) based on their mobility when separated through polyacrylamide gels. In each common wheat variety, three to five different HMW-GSs can be found [45, 53].

Table 2. Characterization of gluten protein types [52, 54]. **(**GS, glutenin subunits; HMW, high molecular weight; LMW, low molecular weight; m, monomeric; MMW, medium molecular weight; MW, molecular weight; p, polymeric.)

Type	MW×10^{-3}	% of total	Form	Group	Partial amino acid composition (%)				
					Gln	Pro	Phe	Tyr	Gly
ω5-Gliadins	46–74	10–20	m	MMW	56	20	9	1	1
ω1,2-Gliadins			m	MMW	44	26	8	1	1
α/β-Gliadins	30–45	70–80	m	LMW	37	16	4	3	2
γ-Gliadins			m	LMW	35	17	5	1	3
LMW-GS	30–45		p	LMW	38	13	4	1	3
x-HMW-GS	75–120	5–10	p	HMW	37	13	0	6	19
y-HMW-GS			p	HMW	36	11	0	5	18

Modern and ancient wheat

The phrase ancient wheat typically denotes the types of wheat that were farmed by civilizations of old after they were domesticated [55]. Among the ancient wheat species that are commonly

accessible on the market are einkorn (*Triticum monococcum*), emmer (*Triticum dicoccum*), khorasan (*Triticum turgidum ssp. turanicum*) and spelt (*Triticum spelta*). Heritage wheat, a term used to refer to wheat grown between ancient wheat and modern wheat, comprises varieties selected from ancient wheat or wild wheat. Various traditional varieties of *Triticum aestivum* and *Triticum durum* have maintained their original characteristics through the years, namely *Russello, Senatore Cappelli, Timilia or Tumminia and Urria (Triticum durum),* as well as *Autonomia B, Frassineto, Gentil Rosso, Inallettabile, Maiorca, Sieve, Solina, and Verna* (*Triticum aestivum)* [56]. Ancient wheat and heritage wheat generally consist of landraces, which means they were made up of many closely related strains. In contrast, modern wheat species are made up of homogeneous strains, which have undergone significant alterations and hybridizations during the era known as the "Green Revolution". The Green Revolution represents a set of breeding initiatives aimed at boosting wheat output, serving as a defining line between ancient and heritage wheats and their modern counterparts. Its main aim was to enhance agricultural efficiency, thus elevating crop productivity to meet the escalating demands of expanding populations in developing nations [57].

Modern and Ancient wheat cultivars differ in terms of productivity, physical appearance, and biological features such as plant height, length of growth cycle, vulnerability to environmental and biological pressures, and grain quality [58]. While the modern wheat varieties, including durum wheat and common wheat, exhibit greater yield potential, they come with drawbacks when contrasted with older varieties, notably in terms of decreased resilience to environmental and biological stressors like diseases, pests, drought, high temperatures, cold weather, salinity, pollution, and lack of soil nutrients [59].

Scientific panorama still debates about benefits of ancient or heritage wheats often supposed to have superior health-promoting properties than modern cultivars. It is possible to distinguish an anti-inflammatory effect of wheat derived from antioxidant compounds, and a pro-inflammatory effect related to protein contents[56]. In particular, ancient wheat species have gained increasing attention since several studies have suggested that they could present a healthier and better nutritional profile than modern wheat, by providing more vitamins, minerals, and nutraceutical compounds. Analyses of ancient and modern wheat show that the protein content of modern bread wheat (*Triticum aestivum*) has decreased over time while the starch content increased. In addition, it was

shown that, compared to bread wheat, ancient wheat contain more protein and gluten and greater contents of many celiac disease (CD)-active epitopes[60]. Consequently, no single wheat type can be recommended as better for reducing the risks of or mitigating the severity of CD.

The discovery of gluten intolerance

Approximately 10,000 years ago, the cultivation of cereals began in the Middle East, encompassing regions along the Tigris, Euphrates, and Upper Nile rivers, with wheat and barley emerging as primary crops that gradually spread throughout Europe. Around 5000 years ago, the process of breadmaking evolved in Egypt and disseminated via Greece to Rome, eventually reaching other European regions. Wheat and rye bread became staple foods for Western populations, yet many individuals failed to adapt to this new foodstuff, lacking immune tolerance [61]. During the 1st to 2nd centuries AD, a Greek physician first described an abdominal ailment resembling CD, attributing it to partial food indigestion, advocating alleviating intestinal pressure through rest and fasting for therapeutic purposes. Archaeological findings at the Coso site revealed a young woman from the 1st century AD exhibiting clinical signs of malnutrition, including stunted growth, osteoporosis, enamel hypoplasia, and anemia, suggestive of a celiac-like disease in ancient times [62]. It wasn't until 1888 that a British physician accurately delineated the clinical syndrome of CD, defining it as a chronic malabsorption disorder occurring across all age groups, particularly in children aged 1-5 years. He advocated dietary intervention as the treatment modality in the absence of identified triggers, asserting dietary compliance as the only curative measure [63]. Consequently, various dietary regimens emerged in the ensuing decades. For instance, fat tolerance was found to surpass carbohydrate tolerance and intolerance to bread was noted. A Dutch physician observed during World War II that the scarcity of grains and bread in the Netherlands led to a reduction in the incidence of celiac disease, documenting the beneficial effects on children upon removing wheat, barley, and rye from their diet [64]. Subsequent studies elucidated the harmful effects of wheat, identifying gluten proteins as triggers for celiac disease, with a gluten-free diet successfully introduced as a standard therapeutic approach for celiac disease.

Intestinal barrier function

The intestinal barrier plays an essential role in safeguarding the organism from pathogens and potentially harmful substances originating from the external environment (Figure 4). It consists of

a mucus and epithelial layer, along with the underlying lamina propria. Immune cells, elements of the intestinal microbiota, and antimicrobial peptides are vital in preserving the integrity and functionality of the intestinal barrier [65, 66].

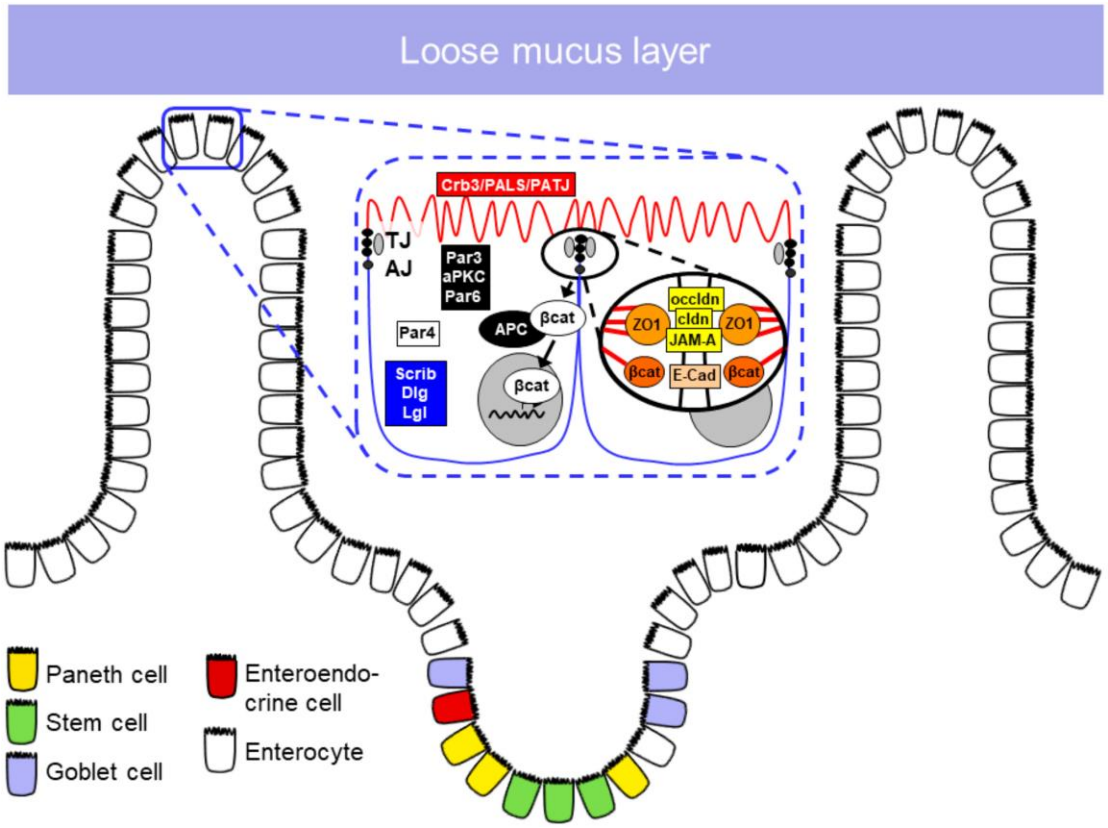


Figure 4. Mucus and epithelial barrier of the mucosa. TJ, tight junction; AJ, adherens junction; Crb3, crumbs3; PALS-1, protein associated with Lin7; PATJ, Pals1-associated tight junction protein; Par, Partition defective; aPKC, atypical protein kinase C; Scrib, scribble; Dlg, Discs large homolog-1; Lgl, Lethal giant larvae protein; β cat, β -catenin; APC, adenomatous polyposis coli protein; ZO1, zonula occludens protein-1; occldn, occludin; cldn, claudin; JAM-A, junctional adhesion molecule-A; E-Cad, e-cadherin [67].

Mucus barrier

In the gastrointestinal system, the mucus layer establishes a defensive coating over the apical surface of the intestinal epithelium, preventing adherence and subsequent infiltration by external pathogens [65]. Furthermore, this mucus layer facilitates the lubrication of food and digestive secretions, safeguarding the intestinal epithelium from potential harm [68, 69].

Mucus is a viscoelastic fluid secreted by goblet cells, primarily composed of 95% water, which acts

as a solvent for other molecules. A key component is mucins, large glycosylated proteins (like Muc2, with approximately 5200 amino acids) that are crucial for mucus formation [70, 71], as evidenced by the absence of a mucus layer in MUC2 knockout mice [72]. Additionally, lysozyme digests bacterial cell walls [73, 74], while secreted IgA immunoglobulins enhance defense against pathogens, regulate mucus microbiota, and maintain mucosal homeostasis, as their deficiency can lead to protein-losing enteropathy [75]. Growth factors like TGFβ play roles in epithelial growth, maintenance, repair, and regulation [76, 77].

Epithelial barrier

The intestinal epithelial barrier is the cellular layer covering the intestinal wall, where cells are interconnected by the apical junctional complex, sealing the paracellular space from the lumen. Epithelial cells are continuously renewed through cell division, maturation, and migration, with stem cells in the crypts giving rise to various cell types, including enterocytes, goblet cells, Paneth cells, microfold (M) cells, and tuft cells [78-80].

Epithelial cell types in the small intestine

The predominant cells are enterocytes, responsible for nutrient absorption and epithelial protection through antimicrobial protein secretion [81]. Goblet cells are the primary mucus-secreting cells, originating from crypts and migrating to the surface epithelium where they differentiate into secretory cells due to the expression of the transcription factor Math1 [82]. Paneth cells are vital for host defense against bacteria and microbiota regulation, as they produce α-defensins and support epithelial renewal by nurturing the crypt's stem cell compartment [83-86]. M cells specialize in antigen sampling, transporting antigens and microorganisms from the gut lumen to the lamina propria to engage immune cells and initiate an immune response [87]. Tuft cells survey the intestinal lumen and signal immune cells in response to injury or infection, activating the immune response [88].

Apical junctional complex

To uphold the integrity of the intestinal epithelial barrier, epithelial cells are interconnected by apical junctional protein assemblies known as tight junctions (TJs) and adherens junctions (AJs).

TJs are localized to the apical region of lateral epithelial cell membranes, serving as both a gate and a fence. They selectively regulate luminal component passage into the interepithelial space (gate

function) and restrict lateral diffusion of membrane proteins and lipids to either the apical or basolateral compartments (fence function). Key components of TJs include transmembrane proteins occludin, claudins, and junctional adhesion molecules (JAM) [89]. While occludin was the first identified component, its specific role in regulating paracellular permeability is unclear. Loss of occludin affects tricellulin localization, potentially impacting epithelial barrier integrity [90, 91]. However, studies show that occludin knockout mice maintain normal TJ structure and barrier function [92, 93], and its expression does not define the epithelial leak pathway for macromolecules [94].

Claudins are essential for TJ formation and epithelial barrier function [95, 96], also influencing cytoskeletal organization, vesicle transport, and signaling pathways with scaffold proteins like ZO-1 [97]. Changes in claudin expression are linked to homeostasis disturbances and various diseases [98-100]. The JAM family, primarily consisting of JAM-A, JAM-B, and JAM-C, is integral to TJs. Only JAM-A appears directly involved in TJ maintenance, as evidenced by its overexpression in fibroblasts lacking TJs [101], although it does not form strand-like structures. JAM-A deficient mice exhibit reduced transepithelial electrical resistance and increased permeability for 4 kDa dextrans, suggesting its role in regulating paracellular permeability [102, 103].

The AJ comprises two protein complexes for cell adhesion: the nectin–afadin and the cadherin–catenin complexes. These complexes feature an extracellular region for cell adhesion and an intracellular component for signaling and AJ dynamics, as well as cytoskeletal interactions. Cadherins, type I transmembrane proteins, interact calcium-dependently through their N-terminal domain with adjacent cadherins, while their C-terminal domain binds β-catenin, linking to the actomyosin network and EPLIN. This dynamic complex modulates various signaling pathways, with alterations contributing to tumor progression [104-107]. Nectins form a structural adhesive complex with afadin (an actin-binding protein), anchoring it to the cytoskeleton [89]. Research has shown that afadin knockout leads to AJ and TJ disorganization during embryonic ectoderm development [108], and loss of afadin delays AJ formation, highlighting the nectin–afadin complex's critical role in AJ maturation [109].

The function of lamina propria cells in sustaining barrier integrity

The lamina propria is a connective tissue layer beneath the intestinal epithelium, containing immune

cells like macrophages, dendritic cells, and lymphocytes that are vital for defending against harmful substances and maintaining intestinal epithelium homeostasis [110].

Dendritic cells and macrophages

Mononuclear phagocytes (macrophages and dendritic cells) reside in gut-associated lymphoid tissue and intestinal lamina propria, performing functions like phagocytosis for antigen sampling, pathogen clearance, cytokine production, and epithelial barrier maintenance [111]. Research indicates they can sample antigens from the intestinal lumen through protrusions that migrate without compromising barrier integrity, utilizing dynamic expression of tight junction proteins [112, 113]. Monocytes also maintain the epithelial barrier by producing prostaglandin E2, regulating neutrophil responses to microbial stimuli and supporting epithelial homeostasis [114, 115]. Certain macrophage subpopulations may possess regulatory properties by expressing anti-inflammatory cytokines such as interleukin-10, contributing to a tolerogenic immune response [116].

Innate lymphoid cells

Innate lymphoid cells (ILCs) are innate immune cells of lymphoid lineage classified into three groups—ILC1, ILC2, and ILC3—based on cytokine and transcription factor profiles. ILC3s promote intestinal homeostasis and T-cell tolerance while providing protection against intestinal infections through IL-22 secretion [117, 118]. However, they can also have detrimental effects in the gastrointestinal tract, being linked to Helicobacter hepaticus-induced colitis, which triggers high levels of IL-17 and interferon-γ (IFNγ) [119]. Excessive IL-22 production by ILC3s may lead to epithelial damage and intestinal injury [120].

The influence of intraepithelial lymphocytes on barrier integrity

In the epithelial layer, there exists a population of T-cells called intraepithelial lymphocytes (IELs), primarily CD3+ CD8+ T-cells, that interact with enterocytes and are positioned near antigenic material in the gut lumen, ready to prompt an immune response [121, 122]. Research by Kuhn et al. indicates that interactions between commensal gut microorganisms and IELs enhance the secretion of cytokines, improving epithelial barrier function [123]. They also found that the absence of IL-6 diminished barrier function and tight junction (TJ) protein expression, a deficiency that was remedied by transferring IL-6-expressing IELs. However, alterations in the IEL population can further compromise barrier integrity in inflammatory diseases [124, 125]. An increased IEL count

is a defining characteristic of celiac disease (CeD) and serves as a key diagnostic marker. In cases of active and refractory celiac disease (RCD), where patients do not respond to a gluten-free diet (GFD) for at least a year, IELs are activated through IL-15 upregulation, resulting in epithelial cell damage and barrier dysfunction [126-128].

Role of the luminal microbiota on barrier function

The intestinal microbiota comprises complex systems of mutualistic and commensal bacteria that enhance digestion, absorption, vitamin synthesis, and protect against pathogen overgrowth [129]. Research indicates these bacteria facilitate epithelial cell turnover in dextran sodium sulfate-treated mice, a colitis model, with depletion leading to increased mucosal injury susceptibility. Additionally, gut microbiota plays a crucial role in irritable bowel syndrome development [130], although the pathophysiological mechanisms regulating microbial balance remain poorly understood.

Gluten-related disorders (GRDs)

Cereal grains containing gluten, such as wheat, rye, and barley, have historically been staple components of diets in Western countries. However, with the increasing adoption of Western lifestyles, the consumption of these grains is also rising in Eastern countries. Against this backdrop, gluten-related disorders (GRDs) are emerging as a global epidemiological phenomenon, attracting attention from the scientific community. Numerous *in vitro* studies have substantiated the cytotoxicity of certain wheat components and their effects on intestinal permeability and immune responses. Gluten and other wheat proteins are considered primary pathogenic factors underlying GRDs [131, 132]. GRDs refer to a group of conditions that are known to be caused by the ingestion of the gluten proteins present in wheat, barley, and rye [133]. It can be classified into three different disorders: autoimmune, allergic, and neither autoimmune nor allergic (Figure 5) [134, 135]. Celiac disease (CD), dermatitis herpetiformis (DH), gluten ataxia (GA), wheat allergy (WA), and non-celiac gluten sensitivity (NCGS) are the five major GRDs that present with a wide range of clinical manifestations [133].

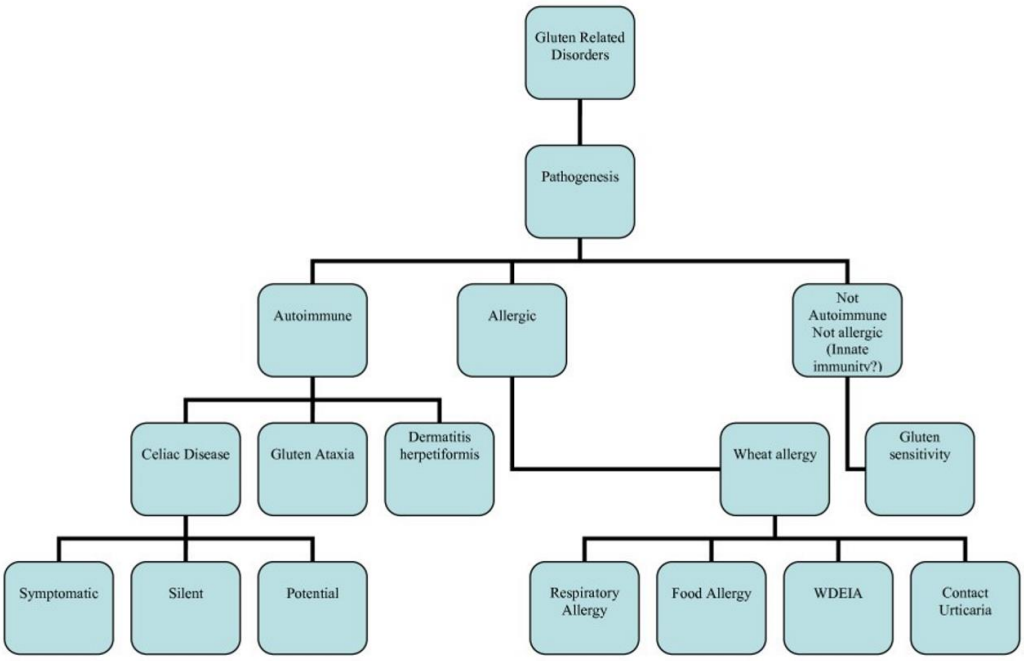


Figure 5. Gluten-related Disorders (GRDs).

Autoimmune disease

The onset of symptoms typically occurs weeks to years after the ingestion of gluten-containing products, primarily encompassing celiac disease, gluten ataxia, and dermatitis herpetiformis.

Celiac disease (CD)

Celiac disease (CD) is an autoimmune chronic intestinal disease caused by the gluten protein, which is mediated by T cells and has a genetic predisposition to develop at any age [136, 137]. Epidemiological studies estimate the worldwide prevalence of celiac disease (CD) to be approximately 1 in 100 individuals, with a significant proportion of patients remaining undiagnosed and untreated [138, 139]. The ingestion of gluten in genetically predisposed individuals carrying HLA type II DQ2/DQ8 alleles can trigger a T-cell mediated immune response against tissue transglutaminase, an enzyme of the extracellular matrix, resulting in mucosal damage and, ultimately, intestinal villous atrophy [140, 141]. Gliadins are considered the active fractions of gluten; they contain immunogenic peptides, particularly the 33-mer, and can exert a direct cytotoxic effect on cells [142, 143].

The most prevalent manifestations in both adults and children diagnosed with celiac disease (CD)

include diarrhea, which can either be chronic or episodic, abdominal pain, fatigue, abdominal distension, nausea and vomiting, constipation, bloating with gas, and weight reduction [144]. Gluten intolerance can develop at any stage of life, triggered by factors beyond gluten itself. Notable triggers may include α-interferon, gastrointestinal infections, pharmacological agents, and surgical interventions [145-147]. To diagnose CD, a comprehensive assessment involving clinical evaluations, serological assays, and duodenal biopsies is essential to identify intestinal damage attributed to the disorder [148, 149]. Patients exhibiting clinical signs of CD should undergo serological testing [150, 151]. The primary serological assay recommended for the identification of CD is IgA-tissue transglutaminase (IgA-tTG). Given that IgA deficiency occurs in 2–3% of CD patients, potentially yielding false-negative results, it is necessary to also evaluate total IgA levels [152, 153]. Additional antibody tests can include anti-deamidated gliadin (anti-DGP), anti-tissue transglutaminase (tTG), and anti-endomysial (EM) antibodies [154-157]. In cases of IgA deficiency, IgG-based antibody tests (IgG-tTG and/or IgG-DGP) should be utilized [152]. Depending on the results of the antibody tests, a duodenal biopsy is conducted to confirm the diagnosis of CD [158, 159]. According to the Marsh classification, the presence of villous atrophy, hyperplasia, and an elevated count of intraepithelial lymphocytes in conjunction with positive serological results validates the diagnosis of celiac disease [140]. HLA typing can serve as a supplementary diagnostic tool when serological and biopsy results are ambiguous and the diagnosis of CD remains uncertain [160, 161]. Determining HLA genotypes is particularly effective when combined with histological evidence, recognizing that HLA-DQ2 and HLA-DQ8 are associated with approximately 95% and 5% of CD patients, respectively [162-164].

The sole treatment currently available for celiac disease is strict adherence to a gluten-free diet (GFD). A GFD requires complete elimination of gluten and gluten-related proteins, thereby excluding wheat, rye, barley, and any products derived from these grains [153]. Consistent adherence to a GFD typically leads to an alleviation of clinical symptoms, an enhancement in bone mineral density, and an improvement in body weight and overall nutritional status [165]. With prolonged compliance to a GFD, there can be significant reconstitution of intestinal villi [166]. Nonetheless, a GFD may lead to digestive issues, such as constipation, often due to insufficient fiber intake. In many instances, maintaining a rigorous GFD is challenging because of gluten

contamination in various food items [167, 168]. Research into alternative therapies for CD is ongoing, with the most promising avenues exploring strategies to enhance intestinal permeability, detoxify gluten, or modify the immune response to gluten [149, 169].

Gluten ataxia (GA)

Gluten ataxia (GA) is a particular type of cerebellar ataxia primarily impacting Purkinje cells. It is triggered by antibodies that are released during gluten digestion, which in individuals who are sensitive and genetically predisposed, inadvertently target specific brain regions [170]. The diagnosis of GA is often bolstered by the presence of serum antibodies such as anti-tTG, anti-gliadin, and anti-TG6 (anti-transglutaminase 6). However, the optimal diagnostic strategy for patients with suspected GA is still not well-defined. One study has suggested that when considering the comprehensive gluten spectrum, the IgG anti-gliadin antibody demonstrates superior performance compared to the specific gluten ataxia marker due to its heightened sensitivity [171]. Investigations involving GA patients have revealed the presence of anti-tTG antibodies in the brain. If the serology for celiac disease (CD) returns positive, it is essential to seek confirmation of CD through an intestinal biopsy[172]. Magnetic resonance imaging (MRI) can be employed for the diagnosis of GA, with up to 60% of GA patients exhibiting signs of moderate cerebellar atrophy [173]. Adhering to a stringent gluten-free diet (GFD) is recommended as the primary treatment for individuals with GA. Additionally, research indicates that immunotherapy involving steroids and intravenous immunoglobulins (IVIG) may serve as an effective treatment modality for these patients [174].

Dermatitis herpetiformis (DH)

Dermatitis herpetiformis is a rare gluten-sensitive blistering skin disease closely associated with celiac disease, characterized by gluten-sensitive villous atrophy and the presence of circulating antibodies against both epidermal transglutaminase and tissue transglutaminase in most patients [175, 176]. Similar to patients with celiac disease, those with dermatitis herpetiformis typically possess HLA-DQ2 or HLA-DQ8 haplotypes. Both conditions are mediated by the IgA class of autoantibodies, and the diagnosis of dermatitis herpetiformis relies on the detection of granular deposits of IgA in the skin. While there is an underlying genetic predisposition for the development of dermatitis herpetiformis, environmental factors also play a significant role.

Following gluten consumption, pruritic maculopapular and vesicular lesions may develop at any age, typically appearing on the extensor surfaces of major joints, the back, buttocks, and face. Clinical diagnosis can be challenging and is currently established through frozen biopsy of perilesional skin, which is processed for direct immunofluorescence [177]. This method demonstrates IgA granular deposits localized either in the dermal papillae or along the dermo-epidermal junction44. In recent years, antibodies targeting various transglutaminases have been proposed as diagnostic tools for dermatitis herpetiformis [178-181].

Allergic disease

Wheat Allergy (WA) is an immunological response to one or more proteins present in wheat, which can manifest as either IgE-mediated, mixed IgE, or non-IgE (cell) mediated reactions, with some cases presenting solely as non-IgE (cell) mediated [182]. This condition ranks among the most prevalent food allergies in children, particularly during early developmental stages [183]. In cases of IgE-mediated allergies, exposure to wheat elicits various symptoms in sensitized individuals, including urticaria, asthma, allergic rhinitis, gastrointestinal disturbances, and exercise-induced anaphylaxis (EIA) through the activation of mast cells and basophils [184]. Conversely, non-IgE-mediated allergic responses involve lymphocytic activation, leading to eosinophilic infiltration in the upper gastrointestinal tract, which can result in eosinophilic esophagitis (EoE) and eosinophilic gastritis (EG). The management of both types necessitates wheat avoidance along with the potential use of immunomodulating agents [184].

Diagnosis of WA typically relies on skin prick tests (SPT), *in vitro* specific Immunoglobulin E (sIgE) assays, and functional assays. SPTs and *in vitro* sIgE assays are regarded as first-line diagnostic approaches for WA; however, they exhibit a low predictive value [185]. The estimated prevalence of WA is less than 0.5% within the broader population. WA is documented to be more common than other cereal allergies such as those to barley, rye, oat, and rice; however, it is less prevalent compared to milk and egg allergies in the pediatric population, as well as peanut, tree nut, and seafood allergies in older age groups [186].

Non-celiac gluten/wheat sensitivity (NCWS) disease

“Non-celiac gluten sensitivity” (NCGS) refers to a condition characterized by gastrointestinal and extra-intestinal symptoms that arise due to gluten ingestion, in the absence of any celiac-specific

antibodies, villous atrophy, or allergy-mediated responses [187]. The initial accounts of gluten sensitivity emerged in the literature during the 1980s [188]. Today, accurately determining the prevalence of NCGS remains challenging. Clinically, NCGS manifests through gastrointestinal symptoms, including abdominal pain, bloating, and changes in bowel habits, alongside systemic symptoms such as fatigue, headaches, musculoskeletal pain, mood disturbances, and dermatological issues (for instance, eczema or rashes) [187, 189, 190]. These symptoms typically correlate closely with gluten consumption and resolve following gluten elimination from the diet.

In contrast to celiac disease (CD) and wheat allergy, clinicians lack definitive serological or histopathological criteria to diagnose NCGS. The antibodies utilized to diagnose CD, specifically tissue transglutaminase (TTG) and endomysial antibodies (EMA) IgA, remain persistently negative in individuals with NCGS [191].

The pathogenesis of NCGS remains unclear, possibly stemming from innate immune responses [192]. Additionally, gluten proteins are not the sole triggers of NCGS, as other proteins such as α-amylase inhibitors [132] and certain carbohydrates may also induce NCGS. Currently, there are no specific biomarkers for diagnosing this condition, and diagnosis requires the exclusion of CD and wheat allergy, among others. Diagnosis relies on double-blind, placebo-controlled food challenge experiments for confirmation [193].

Non-Celiac Gluten Sensitivity (NCGS) should be considered for patients exhibiting signs of a gluten-related disorder. Key symptoms include symptom resolution upon gluten withdrawal. The diagnostic process involves three critical steps.

The first step is excluding Celiac Disease (CD) and Wheat Allergy (WA). The patient must follow a gluten-containing diet for 6 weeks, during which tests such as wheat-specific IgE, skin prick tests, IgA-tTG, IgG-DGP, and IgA-EMA are conducted. If CD is highly suspected, upper endoscopy for duodenal biopsy may be warranted, though not universally necessary. Should biopsy results indicate low CD probability (Marsh 0-1), the next step can proceed [194]. Testing can also be tailored based on patient symptoms, allowing clinicians to skip CD or WA testing if the initial evaluation is unremarkable.

The second step involves initiating a gluten-free diet (GFD) for a minimum of 6 weeks while monitoring symptom response through the Gastrointestinal Symptom Rating Scale (GSRS) and a

Numerical Rating Scale (NRS) [195]. A decrease in baseline GSRS scores by at least 30%, with stable other symptoms for 50% of the observation period (3 weeks), defines a positive response [195]. If no symptom improvement occurs after 6 weeks on the GFD, NCGS is excluded, and alternative diagnoses like Irritable Bowel Syndrome (IBS) should be evaluated.

The third step in confirming NCGS diagnosis for patients responding to GFD involves reintroducing GCD to rule out nocebo effects from gluten exposure in step one [196]. Patients are randomly assigned to either GFD + placebo (group x) or GFD + gluten (group y) for one week, followed by a washout period of strict GFD before crossover [195]. A 30% symptomatic improvement in group y or a 30% symptomatic decline in group x indicates a positive response; below 30% is negative [195]. This threshold, though utilized in Salerno criteria, requires scientific validation. In step three, the gluten-free placebo must closely resemble gluten so neither clinician nor patient can discern the difference. The gluten challenge dose should approximate average daily intake (10-15 g) and be easily mixed into various forms such as bread or muffins, also containing ATIs [197]. The prepared package should include 10-15 g of gluten and at least 0.3 g of ATIs, while being free of FODMAPs [195]. The proposed diagnostic algorithm for NCGS is illustrated in Figure 6.

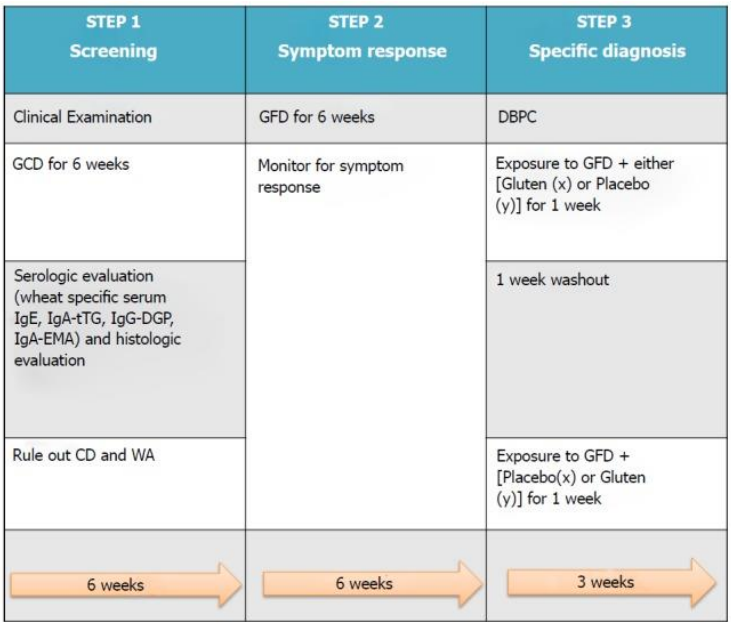


Figure 6. Proposed diagnostic algorithm for non-celiac gluten sensitivity. GCD: Gluten containing diet; GFD: Gluten free diet; CD: Celiac disease [198].

Electrophoretic techniques for cereal protein separation

The separation and identification of cereal storage proteins began with moving boundary electrophoresis, slab gels, and HPCE, evolving into high-resolution techniques such as SDS-PAGE, A-PAGE, IEF, FZCE, and 2D electrophoresis [199, 200]. In proteomics, 2D IEF×SDS-PAGE is utilized for protein separation, followed by tryptic digestion and MS peptide mapping for identification [201, 202] . Additional combinations of 2D electrophoresis have been reported in cereal proteomic studies, such as A-PAGE ×SDS-PAGE [203-205].

Single and two-dimensional gel electrophoresis techniques, encompassing both SDS-PAGE and A-PAGE, have been employed by various researchers to analyze the protein profiles of wheat, barley, rye, and oat sourced from different cereal species [206, 207]. SDS-PAGE operates on the principle of size-based separation and is extensively utilized for the qualitative assessment of all cereal proteins due to its limitations in quantification [208]. The differentiation of high molecular weight glutenin subunits (HMW-GS) in wheat proteins, as well as barley proteins, and the prolamins from oats and rice, is predominantly accomplished through SDS-PAGE, although there are dedicated protocols for low molecular weight glutenin subunits (LMW-GS) as well. For prolamins exhibiting higher hydrophobicity, such as sorghum kafirin and maize zein, the inclusion of urea within the SDS-PAGE gel enhances separative effectiveness [209]. Additionally, for substantial protein polymers such as wheat glutenins, a refined approach known as multistacking electrophoresis has been established, yielding improved separation outcomes [210].

***In vitro* digestion models in protein degradation and nutritional research**

In the study of food digestion, the *in vitro* digestion model serves as a vital research method [211]. The process of protein digestion begins in the stomach through the action of pepsin and concludes in the small intestine with the involvement of pancreatic proteases and peptidases found in the brush border membrane [212]. During digestion, proteins are hydrolyzed into (small) peptides and eventually amino acids that can then be readily assimilated by the human body. The duration and magnitude of protein hydrolysis in the gastrointestinal system can enhance the production of smaller peptides, leading to a reduced immunostimulatory response in the intestinal environment. The two-step digestion process, using pepsin and trypsin, is commonly applied to replicate the digestion that occurs in the stomach and small intestine [213]. For instance, researchers have utilized this method

to analyze the protein digestion of dry-cured ham sourced from various regions, revealing significant differences in digestibility and particle size throughout the digestion process [214].

Moreover, there is an increasing interest among scientists in investigating the composition of protein hydrolysates to more accurately evaluate their nutritional efficacy. A study centered on the *in vitro* digestion of defatted walnut meal protein revealed the presence of polypeptides with molecular weights under 3000 Da (21.66 mg/ml) and a heightened concentration of free amino acids (FAA) (8.09 mg/ml) in the hydrolysis products. Furthermore, these products demonstrated substantial angiotensin-I-converting enzyme (ACE) inhibitory activity (42.9%) and noteworthy DPPH free radical scavenging capacity [215].

In the course of wheat digestion, peptides that are resistant to proteolytic degradation and derived from gluten are produced, with some of these peptides implicated in celiac disease. *In vitro* digestion models that effectively replicate the peptide profiles generated in the human gastrointestinal system are invaluable for evaluating the pathogenic potential of wheat-derived products. Despite the numerous studies conducted on the *in vitro* digestion of protein, there remains a lack of research on the digestion process of protein extracted from durum wheat. As a whole grain, durum wheat fulfills essential health and nutritional requirements, making the characterization of its nutritional value critical for its development and application.

Objective

Wheat, a crucial global cereal crop, has been a dietary staple for centuries. There is an increasing interest in analyzing gluten proteins due to the immune reactions triggered by specific sub-fractions in susceptible individuals. The prevalence of gluten-related disorders is increasing, influenced by global dietary shifts toward Westernized and Mediterranean diets, which feature more gluten-rich foods [216]. In many regions of North Africa, the Middle East, and Asia, wheat consumption is overtaking rice [217]. The adverse effects of gluten primarily arise from immune responses, though inadequate gastrointestinal adaptations can also contribute. Advances in agricultural technology and pesticide use have led to new wheat strains, which may increase immunogenicity and the prevalence of celiac disease due to modified protein composition. Modern wheat varieties have higher gluten content due to these developments. Wholegrain wheat is a rich source of energy and essential nutrients, particularly protein, which ranges from 7% to 22%, mostly between 10% and 15% [218].

Genetic factors account for about one-third of protein content variability, while external factors such as climate, soil, and agricultural practices make up the rest [219, 220]. Additionally, gluten protein fractions and types demonstrate significant variability based on genetic and environmental influences, showing high variability even among similar wheat cultivars.

The objective of this study is to investigate the relationship between wheat gluten protein content and human health effects, with specific attention to intestinal inflammation and further identify one or more wheat germ population with higher healthy properties on human inflammation. The experiment was conducted over three consecutive growth periods to observe the differences in the digestion of various wheat proteins, using a partial digestion approach. Multiple ancient wheat varieties harvested each year were processed into pasta and subjected to digestion with pepsin for 15 minutes and pancreatin for 30 minutes, after which the total protein was extracted. Both undigested and partially digested total proteins were then used to study their effect on cell proliferation assays of cells to the intestine tissue. In particular two cell lines were used: a cell line of human enterocytes (Caco2) and a cell line of mice dermal fibroblasts (L929). Specifically, the current work focuses on a comparison of the possible inflammatory effects of gluten protein components extracted from cooked dough under "undigested" and "partially digested" conditions in multiple ancient durum wheat and modern wheat varieties. This study will provide important scientific basis for wheat variety breeding and agricultural production, helping optimize planting strategies, increase wheat yield and quality, thus contributing to food security and sustainable agricultural development.

2. Materials and methods

2.1 Chemicals and reagents

Folin–Ciocalteu (FC) reagent, gallic acid (GA), 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-triazine (TPTZ), iron(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) and iron(II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), sodium acetate, chlorhydric acid, boric acid, pepsin (P6887, 1003566234), pancreatin (P1750, 1003509078), isopropanol, acetone and Coomassie Blue were purchased from Sigma-Aldrich (Milan, Italy). Pierce Rapid Gold BCA Protein Assay Kit purchased from ThermoFisher Scientific, 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolio (MTT) assay was from Life Technologies (Carlsbad, CA, USA). Reagents for cell cultures, such as Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), L-Glutamine, Penicillin-Streptomycin were purchased from GIBCO (Waltham, MA, USA). Methanol, acetic acid, and water (HPLC-grade, Lichrosolv®) were acquired from Merck (Darmstadt, Germany). Lipopolysaccharide (LPS) were purchased from Alpha Diagnostic International, Inc. (ADI). Other reagents unmarked were of an analytical grade.

The durum wheat cultivated over three consecutive years was as follows:

First year (2019-2020): Senatore Cappelli, Russello, P1, P2, P3, P4, V1, V2, T1, T2, T3, 3R, Rhettfah, Guastella, S1, S2, S3, S4.

Second year (2020-2021): P1, V1, V2, S2, Senatore Cappelli (SC), T1, T2, T3, 3R, Russello (RI), S1, S3, S4, P2, P3, P4.

Third year (2021-2022): P1, P2, P3, P4, 3R, Russello (Ru), S1, S2, S3, S4, Lucana (LU), Pugliese (PU), Senatore Cappelli (CA), T1, T2, T3, V1, V2, Antalis (AN), Margherito (MA), Benedettelli (BE), Rhettfah (RH).

All the ancient varieties of durum wheat were cultivated in Italy. Plants were harvested at grain full ripening stage. Flours, milled using a stone mill (100% flour extraction).

2.2 Technological quality

The technological quality analyses used in the present study included total protein content, humidity and ash content of wheat flour samples. These parameters were determined on the kernel material (3 replicates per variety with 10 internal readings for each replicate) using the Infratec 1241 Grain Analyzer (FOSS, Hillerød, Denmark) based on the manufacturer's guidelines.

2.3 Polyphenols, flavonoids and anti-radical scavenging activity

Free polyphenols were extracted according to Adom et al. [221]. The residue from the free phenolic extraction was subjected to alkaline and acid hydrolysis to recover the bound phenolic compounds as reported by Mattila et al.[222]. Total polyphenol content (free and bound fractions, respectively) was measured at 765 nm according to the Folin–Ciocalteu procedure based on the method of Singleton et al. [223]. Briefly, 40 µL of diluted phenolic extract was mixed with 1.6 mL of distilled water and 100 µL of Folin–Ciocalteu reagent. After incubation for 5 min, 300 µL of aqueous sodium carbonate solution (0.2 g/mL) was added. The absorbance was measured at 765 nm after 2 h incubation in the dark. The results were expressed as mg Gallic Acid Equivalents (GAE) per 100 g fresh weight (FW).

Total flavonoid content in the free and bound fractions was measure at 510 nm, according to the method of Adom et al. [221]. Appropriate dilutions of sample extracts were reacted with sodium nitrite, followed by reaction with aluminium chloride to form a flavonoid–aluminium complex. Solution absorbance at 510 nm was immediately measured and compared to that of catechin standards. The results were expressed as mg Catechin Equivalents (CE) per 100 g fresh weight (FW).

The FRAP assay was conducted according to Benzie and Strain [224], with some modifications. The freshly prepared FRAP working solution (WS) consisted of 300 mM of acetate buffer pH 3.6, 10 mM of TPTZ, and 20 mM of $FeCl_3 \cdot 6H_2O$ (10:1:1, v:v:v). A volume of 80 µL of diluted (1:1, v/v) phenolic extracts was added to 2.4 mL of WS and incubated for 1 h in the dark. The absorbance was measured at 593 nm. Concentrations were calculated from a standard curve prepared from FeSO4 · 7H2O (0–1000 µmol/L) and the the results were expressed as mmol Fe^{2+} per 100 g of whole wheat flour.

The DPPH assay was performed following a modified procedure reported by Floegel et al. [225]. Briefly, 1 mM of DPPH solution in 80% (v/v) methanol was prepared, for which of the absorbance was adjusted to 0.650 ± 0.020 at 517 nm. Diluted phenolic extract (50 µL) was added to 2.95 mL of DPPH solution. After incubation for 30 min in the dark, the absorbance was measured at 517 nm. The results were expressed as µmol of Trolox equivalents (TE) per g of whole wheat flour.

2.4 Partial digestion of wheat proteins

After grinding the grain using a laboratory mill, the samples were digested and/or not. For each

variety, 1 g of flour was weighed, to which 700 µl of distilled water was added, thus creating a dough. Subsequently, from the latter, 4 paste-like coils of equal weight and length were obtained (Figure 7). The paste samples were dried in an oven at 40°C for 72 hours. Prior to digestion, 200mg of dried paste was cooked for 10 minutes in 80 ml of distilled water brought to the boil (Figure 8).

Figure 7. Combined 1 g of wheat flour with 700 µL of distilled water to form a dough, and then shaped it into four uniform coils of equal weight and length.

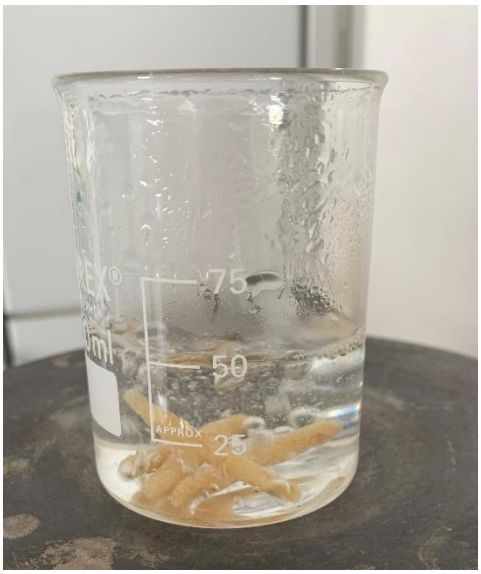


Figure 8. In 80 ml of distilled water, immersed the dried pasta and boil for 10 minutes.

Immediately after cooking, the coils of each variety were crushed using a pestle to obtain and weigh 100 mg, collected in 15 ml tubes for each condition, i.e. 'undigested protein' and 'protein digested with pepsin 15'+ pancreatin 30". In the first condition, 4 ml of 0.2N HCl was added to each sample and then thoroughly resuspended. Following this, each sample was placed in a 37°C bath for 15 minutes and, once passed, at 98°C for 5 minutes. After this time, the samples were immediately placed on ice.

Subsequently, a volume of 1M boric acid equal to 1.15 ml was added to each sample, repeating the same steps as with 0.2N HCl but with different timescales, i.e. 30 minutes in the 37°C water bath, a further 5 minutes at 98°C and, finally, storage on ice. For the second condition, i.e. 'protein digested with pepsin 15'+ pancreatin 30", initially, 4 ml of 0.2N HCl + pepsin 0.05 mg/ml was added to each sample. This solution was prepared on the spot. Immediately afterwards, the samples with 4 ml of 0.2N HCl + pepsin 0.05 mg/ml were resuspended and placed first in the water bath at 37°C for 15 minutes, then at 98°C for 5 minutes and finally on ice. Subsequently, 1.15 ml of 1M boric acid + pancreatin 0.250 mg/ml, also freshly prepared solution, was added to these same samples. Once resuspended, these samples were again subjected to a temperature of 37°C in the water bath for 30 minutes, then to 98°C for 5 minutes and finally placed on ice. At this point, all samples were centrifuged at 12,000 g for 45 minutes at 4°C. At the end of the centrifugation, the supernatant was discarded and the pellets were left to dry under a fume hood.

2.4.1 Extraction of protein

Once the digestion was performed, the next step was to extract the total proteins from each cooked dough sample. To this end, 50% isopropanol with 1% dithiothreitol (DTT) was added to each tube containing the pellet to obtain a final volume of 400 µl. Subsequently, the samples were sonicated for 10 seconds and immediately placed on ice for a further 15 seconds, all three times. Afterwards, the tubes were left to agitate for 30 minutes at room temperature, vortexing them for 1 minute every 10 minutes, and then centrifuged at 2000 rpm for 5 minutes. When the centrifugation was complete, the supernatant obtained for each sample was collected in a new 15 ml tube while a further 400 µl of 50% isopropanol with 1% DTT was added to each pellet, as in the previous step, and then each tube containing this solution was again subjected to centrifugation at 2000 rpm for 5 minutes. Subsequently, the second supernatant obtained was added to the corresponding supernatant collected previously. At this point, four 3.2 ml volumes of cold acetone were added to each new 15 ml tube containing the two supernatants and stored at -20°C overnight. The following day, these samples were centrifuged at 12,000 g for 45 minutes and, after this time, the supernatant was discarded while the pellet of each sample was left to dry under a hood. Finally, each pellet was resuspended in 500 µl of filtered 8M urea and transferred into a tube.

2.4.2 Protein quantification

To quantify the extracted proteins, it was necessary to perform a spectrophotometer analysis of the samples. For this purpose, the Pierce Rapid Gold BCA Protein Assay Kit was used, which elaborates the calibration curve using BSA (Bovine Serum Albumin Standard) as a standard. Next, the BCA Working Reagent (WR) was prepared by mixing 50 parts Rapid Gold BCA Reagent A with 1 part Rapid Gold BCA Reagent B (50:1 according to the kit directions). Setting up a 96-well multiwell plate, 10 µl of each standard and sample were pipetted into each well (2 replicates per sample). Immediately afterwards, 200 µl of previously vortexed WR was added to each well. The multiwell was shaken for 30 seconds and then left to incubate at 37°C for 5 minutes. Finally, using Ascent Software, a spectrophotometer reading was taken at a wavelength of 480 nm and the resulting values were analysed.

2.4.3 Protein electrophoretic profiles

The protein profiles of the cereal samples were detected by the sodium dodecyl sulfate-polyacyrlamide gel electrophoresis (SDS-PAGE) performed on 12% separating and 4% stacking Gels (ThermoFisher Scientific) in vertical electrophoretic unit. SDS-PAGE consists of an electrophoretic run on a polyacrylamide gel in the presence of sodium dodecyl sulphate and is an analytical technique that allows the analysis of protein extracts. The principle on which this electrophoretic technique is based concerns the denaturing activity of SDS, which is able to interact with proteins, separating them on the basis of molecular weight.

Through the quantification of the proteins, it is possible to proceed with the preparation of the samples, thus deriving the volume of each sample to be loaded into the gel. For the electrophoretic run, 20 µg of each sample was prepared and Loading Buffer (Boster Bio). The samples were denatured at 98°C for 5 minutes and immediately placed on ice. Once ready, the samples were pinned and loaded into the polyacrylamide gel. Afterwards, the samples were run at 100V for approximately 2 hours. Once the electrophoretic run was finished, the gel was stained with a 0.2% Coomassie Blue solution. This solution, prepared fresh each time it was used, consisted of 50 ml of filtered Coomassie Blue and 50 ml of 20% acetic acid. Then, the gel was immersed in the staining solution of blue and left for 1h in agitation at room temperature. Then, the gel was immersed in a destaining solution (30ml methanol + 10ml acetic acid + 60ml distilled water) for decolorization, which was changed repeatedly, i.e. after 10 minutes, 30 minutes and 1 h after stirring at room

temperature for at least 2 h. Finally, the gel was fixed in 2% glycerol. To study the bands on the SDS gel, a photograph was taken with the help of a white light transilluminator.

In order to compare the storage protein composition, the amounts of sub-fractions were calculated relative to the total extracted storage proteins. The ratio of gliadin on glutenin (Gliadins:Glutenins) was determined as the ratio of the amounts of extracted gliadins and glutenins. The ratio of HMW-GS to B-type LMW-GS was also determined.

2.5 Culture method and cell lines used

2.5.1 Cell culture

Caco2 (Caucasian colon adenocarcinoma) are human intestinal epithelial cells derived from colorectal adenocarcinoma. These cells are round in shape and form colonies as they grow and aggregate. They also communicate with each other and are interposed by very strong junctions, the tight junctions. L929 (mouse fibroblast cell line) are dermal fibroblasts of murine origin. Both cells grow in adhesion.

These two cell lines used were purchased from the American Type Culture Collection (ATCC). Each cell line was kept in culture at 37°C in a humidified atmosphere containing 5% CO_2 and with a specific medium, changed every two days, for each one: L929 (ATCC-CCL1) were cultured with 1X DMEM, to which 10% FBS, 1 mM L-glutamine and 1% penicillin-streptomycin were added. The Caco2 human epithelial cell line (ATCC HTB-37^{TM}), was cultured with DMEM, supplemented with 10% FBS and 1% penicillin-streptomycin.

2.5.2 Trypsinization

Cells growing in adhesion, such as L929 and Caco2, were detached from the growth plates or flasks using a proteolytic enzyme with the ability to break down cell adhesion molecules: trypsin. The latter was prepared in association with EDTA (ethylenediaminetetraacetic acid), a tetracarboxylic acid with bivalent cation chelating properties, which interfere with the action of trypsin, at the respective concentrations of 0.05% and 0.02% in PBS (Phosphate-buffered saline) devoid of calcium and magnesium.

In order to detach the cells and seed them for their experiments, the following protocol was performed: first, the medium was aspirated from the flask to proceed with a subsequent wash with PBS, which promotes the removal of bivalent cations. Next, the trypsin/EDTA solution was

administered to the cells. At this point, the flask containing the trypsin was placed at 37°C in an incubator for 3-5 minutes to promote detachment of the cells from the flask wall. Once detachment had occurred, the action of the trypsin was blocked by the addition of an equal volume of DMEM containing 10% FBS. The cell suspension was then transferred to 15 ml falcon and centrifuged at 1200 rpm for 5 minutes. Immediately afterwards, once the supernatant had been removed, the cells contained in the pellet were resuspended with their own culture medium and cell counts were performed.

2.5.3 Cell viability

In order to calculate the correct volume to reseed, i.e. the quantity containing the number of cells required for the next experiment, the cells present in the cell suspension must be counted. Cell viability was measured using the blue trypan assay. To detect viability, cells were then carefully resuspended in a 0.4% Trypan Blue (Gibco) solution and vital cells were counted using Countess®II FL (ThermoFisher Scientific, Waltham, MA, USA). The results were expressed as a viability percentage of the control.

2.5.4 Cell treatment

The preparation of the cell treatments was carried out by diluting the partially digested proteins and in DMEM at a concentration of 40 µg/ml. DMEM and urea alone were added to the cells as an untreated control, and LPS, as an inflammatory stimulus, was used as a positive control at a concentration of 1 µg/ml.

2.5.5 Cell proliferation assay (MTT)

This is a colorimetric assay that assesses the state of metabolic activity and, consequently, the viability of cells. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] is a yellow-coloured compound that is metabolised by the mitochondrial enzyme succinate dehydrogenase, which is only active in living cells. The end product of this enzymatic process is formazan, a violet-coloured salt, and the reaction is measured using a multi-well scanning spectrophotometer (Labsystems Multiskan MS Plate Reader, ThermoFisher Scientific) at a wavelength (λ) of 570 nm. High absorbance values correspond to high formazan concentrations, which in turn equate to good levels of metabolic activity. Thus, MTT is used to assess the toxicity of a given substance towards cells. In this study, the possible *in vitro* cell proliferation effect of the wheat protein fraction

constituting gluten in both the partially digested and undigested form was evaluated.

For the MTT assay, 10000 cells per well were seeded in 96 multiwells in complete medium as described above. For each treatment condition, 6 wells of cells were seeded. 24 hours after seeding, the culture medium was removed and partially digested or undigested wheat protein extracts, prepared as described in the previous section, were added. After 24 hours incubation, adding 50 µl of the MTT (1 mg/ml) solution to each well, the plates were left in an incubator at 37°C for two hours, at the end of which the MTT solution was removed and 100 µl of isopropanol was added. After shaking the plates for a few minutes, the spectrophotometric reading was taken. The experiment was repeated at least 3 times and the values, obtained from the absorbance (OD) measurement, were translated and expressed as a percentage of the control, corresponding to the value 100, in order to standardise the results.

2.6 Data analysis

Data were expressed as the mean ± standard deviation of at least three independently performed experiments. Analysis of variance (ANOVA) and Tukey's test were performed to detect differences between mean values. Signal values of protein bands in SDS-PAGE gels were quantified using ImageJ software. The proportion of gluten and its subunits were calculated from the protein bands signal values and expressed as percentage of total extractable protein signal values.

To compare differences between different groups, the results of the MTT assay were subjected to one-way analysis of variance (ANOVA). Non-parametric Kruskal-Wallis test was used when data did not satisfy normality and lognormality test. To determine the relationship between the nutritional components (phenolic and flavonoid) of wheat flour and antioxidant activity over a three-year cultivation period, as well as the correlation between total wheat protein (comprising 23 varieties) and cell proliferation, a Pearson correlation analysis was conducted. Statistical analyses were performed using GraphPad Prism Version 10.1.2.

3. Results from the first year of the experiment

3.1 Proximal composition of wheat flour

The proximate analysis of total protein content, humidity and ash content of wheat flour was shown in table 3. The protein, moisture, and ash content of the 18 wheat varieties cultivated during 2019-2020 ranged from 13.30g /100g (Senatore Cappelli) to 16.40g /100g (T1), 13.00 g/100g (Senatore

Cappelli) to 13.70 g/100g (P2), and 0.88 g/100g (P2) to 0.97 g/100g (Senatore Cappelli), respectively. Significantly different values in protein content were observed for P1, P2, P4, S1, S2, S3, S4, Russello,3R, V1, V2, T1, T2, T3 and for Senatore Cappelli among all other wheat samples ($p < 0.05$). No statistical difference was observed in moisture and ash content between all wheat varieties.

3.2 Phenolic and flavonoid content and antioxidant activity of wheat flour

Phenolic contents of the tested wheat genotypes are presented in Table 3, expressed as micromoles of gallic acid equivalent (GAE) per 100 g of grain. Free phenolic content (FPC) ranged from 83.28 GAE mg/100 g (P3) to 216.15 GAE mg/100 g (Russello). The FPC of Russello showed no statistical difference compared to the Senatore Cappelli and Guastella samples, but exhibited significant differences with all other wheat varieties. Bound phenolic content (BPC) ranged from 31.68 GAE mg/100 g (P1) to 55.36 GAE mg/100 g (Rhettfah). Significantly different values in BPC were observed for Rhettfah and for V2 (33.67 GAE mg/100 g) among all other wheat samples ($p < 0.05$). Total phenolic content (TPC) ranged from 204.10 GAE mg/100 g (P3) to 351.60 GAE mg/100 g (Russello). Significantly different values in TPC were observed for Russello and P2, P3, S2, S4, V1, V2 among all other wheat samples ($p < 0.05$). No significant differences were observed between the mean values calculated for the other samples.

Flavonoid contents of tested wheat varieties are expressed as micromoles of catechin equivalent (CE) per 100 g of grain (Table 3). Free flavonoid content (FFC) ranged from 57.51 CE mg/100 g in P3 to 131.60 CE mg/100 g in Russello. The FFC of Russello showed no statistical difference with Rhettfah (106.73 CE mg/100 g) and Senatore Cappelli (107.09 CE mg/100 g) but exhibited significant differences with the remaining 15 wheat samples. Bound flavonoid content (BFC) ranged from 31.68 CE mg/100 g in P1 to CE mg/100 g in 55.36 Rhettfah, whereas no significant differences occurred for BFC among all varieties. Total flavonoid content (TFC) ranged from 94.44 CE mg/100 g in P3 to 178.65 CE mg/100 g in Russello. Significantly different values in TFC were observed for Russello and P3 and for V2 (96.48 CE mg/100 g) among all other wheat samples ($p < 0.05$). However, no significant differences were observed between the mean values calculated for Russello and Rhettfah (162.09 CE mg/100 g) and for Senatore Cappelli (149.45 CE mg/100 g) among all other wheat samples. The mean values of TFC between rest genotypes (P1, P2, P4, S1,

S2, S3, S4,3R, V1, T1, T2, T3, and Guastella) were not statistically different.

The reducing power (FRAP assay) was evaluated in all wheat samples. The antioxidant activity values (Table 3) of the 18 cultivars determined by the FRAP assay ranged from 0.89 mmol Fe^{2+}/100 g in S1 to 1.37 mmol Fe^{2+}/100 g in Russello. And no statistical difference was observed in FRAP results between all wheat varieties.

Regarding DPPH assay, Guastella (4.01 µmol TE/g) showed the best antioxidant activity and P4 (2.21µmol TE/g) showed the worst antioxidant Activity compared to the remaining cultivars (Table 3). The DPPH values were significantly different among P2, S2, S3, Russello, Guastella and P4 ($p < 0.05$), and there was no significance among the remaining 12 wheat varieties.

Table 3. Main quality parameters of wheat varieties planted in the first year. Different letters in the same row indicate significant differences ($p < 0.05$).

Sample	FPC (GAE mg/100g)	BPC (GAE mg/100g)	TPC (GAE mg/100g)	FFC (CE mg/100g)	BFC (CE mg/100g)	TFC (CE mg/100g)	FRAP (mmol Fe^{2+}/100g)	DPPH (umol TE/g)	Protein (g/100g)	Humidity (g/100g)	Ash (g/100g)
P1	121.86±25.43^{bc}	31.68±9.35^{ab}	241.19±25.61^{abcd}	81.30±16.95^{bcde}	31.68±9.35^{a}	112.97±7.60^{def}	1.33±0.10^{a}	2.61±0.60^{ab}	15.80±1.27^{a}	13.50±0.14^{ab}	0.93±0.01^{a}
P2	96.31±1.09^{c}	36.75±5.08^{ab}	217.76±12.58^{e}	63.53±1.18^{e}	36.75±5.08^{a}	100.28±6.26^{ef}	1.35±0.07^{a}	3.70±0.06^{a}	15.90±0.42^{a}	13.70±0.00^{a}	0.88±0.01^{a}
P3	83.28±0.30^{c}	36.94±5.85^{ab}	204.10±2.58^{e}	57.51±3.44^{e}	36.94±5.85^{a}	94.44±9.29^{f}	1.34±0.00^{a}	3.54±0.28^{ab}	15.55±0.78^{ab}	13.40±0.28^{ab}	0.94±0.05^{a}
P4	119.93±13.84^{bc}	32.51±7.45^{ab}	244.59±7.44^{abcd}	72.62±6.01^{de}	32.51±7.45^{a}	105.13±1.44^{ef}	1.32±0.16^{a}	2.21±0.80^{b}	16.25±0.64^{a}	13.65±0.07^{a}	0.89±0.04^{a}
S1	105.70±38.92^{bc}	34.05±0.44^{ab}	226.52±33.87^{cde}	80.09±1.20^{bcde}	34.05±0.44^{a}	114.14±1.64^{def}	0.89±0.35^{a}	3.39±0.10^{ab}	15.90±0.28^{a}	13.25±0.07^{ab}	0.93±0.01^{a}
S2	92.96±5.84^{c}	43.37±12.95^{ab}	221.81±12.52^{e}	83.77±3.19^{bcde}	43.37±12.95^{a}	127.14±9.76^{cde}	1.20±0.09^{a}	3.86±0.09^{a}	15.90±0.14^{a}	13.40±0.28^{ab}	0.91±0.01^{a}
S3	98.82±16.76^{bc}	36.62±3.19^{ab}	224.14±10.36^{de}	85.58±1.64^{bcde}	36.62±3.19^{a}	122.20±1.54^{cdef}	1.17±0.06^{a}	3.93±0.03^{a}	16.10±0.57^{a}	13.55±0.07^{ab}	0.89±0.04^{a}
S4	87.06±7.23^{c}	48.55±15.09^{ab}	216.64±9.81^{e}	60.45±1.44^{e}	48.55±15.09^{a}	108.99±13.65^{ef}	1.31±0.03^{a}	3.61±0.26^{ab}	15.80±0.85^{a}	13.55±0.35^{ab}	0.93±0.02^{a}
Rhettfah	149.29±0.00^{abc}	55.36±0.00^{a}	291.13±0.00^{abcd}	106.73±0.00^{abc}	55.36±0.00^{a}	162.09±0.00^{ab}	1.31±0.00^{a}	3.67±0.00^{ab}	15.20±0.00^{ab}	13.20±0.00^{ab}	0.94±0.00^{a}
Russello	216.15±29.01^{a}	47.41±5.50^{ab}	351.60±20.72^{a}	131.25±0.05^{a}	47.41±5.50^{a}	178.65±5.55^{a}	1.37±0.27^{a}	3.79±0.48^{a}	16.00±0.28^{a}	13.15±0.07^{ab}	0.95±0.01^{a}
3r	113.81±22.89^{bc}	34.05±1.67^{ab}	234.82±20.94^{abcd}	81.94±8.13^{bcde}	34.05±1.67^{a}	116.00±9.80^{def}	1.01±0.21^{a}	3.51±0.21^{ab}	16.10±0.42^{a}	13.25±0.07^{ab}	0.94±0.01^{a}
V1	96.41±31.25^{c}	40.08±6.85^{ab}	218.68±34.65^{e}	79.62±17.37^{bcde}	40.08±6.85^{a}	119.70±10.52^{cdef}	1.03±0.30^{a}	3.05±0.05^{ab}	16.20±0.57^{a}	13.55±0.07^{ab}	0.92±0.01^{a}
V2	95.96±23.38^{c}	33.67±0.10^{b}	210.61±12.78^{e}	62.81±4.77^{e}	33.67±0.10^{a}	96.48±4.87^{f}	1.08±0.53^{a}	3.42±0.29^{ab}	16.25±0.35^{a}	13.45±0.07^{ab}	0.90±0.00^{a}
T1	126.39±10.35^{bc}	33.55±0.95^{ab}	241.88±19.75^{abcd}	81.26±13.93^{bcde}	33.55±0.95^{a}	114.80±14.88^{def}	1.09±0.37^{a}	3.21±0.31^{ab}	16.40±0.71^{a}	13.55±0.07^{ab}	0.91±0.05^{a}
T2	109.14±12.71^{bc}	33.58±5.46^{ab}	234.55±1.72^{abcd}	69.78±1.79^{de}	33.58±5.46^{a}	103.36±7.25^{ef}	1.05±0.20^{a}	3.15±0.79^{ab}	15.90±0.28^{a}	13.45±0.21^{ab}	0.91±0.01^{a}
T3	128.37±1.11^{bc}	44.33±2.04^{ab}	265.80±0.71^{bcde}	77.29±6.25^{cde}	44.33±2.04^{a}	121.62±4.21^{cdef}	1.09±0.34^{a}	3.15±0.42^{ab}	15.85±0.78^{a}	13.20±0.00^{ab}	0.94±0.01^{a}
Senatore Cappelli	151.96±0.00^{abc}	42.36±0.00^{ab}	292.33±0.00^{abc}	107.09±0.00^{ab}	42.36±0.00^{a}	149.45±0.00^{abc}	1.23±0.00^{a}	3.44±0.00^{ab}	13.30±0.00^{b}	13.00±0.00^{b}	0.97±0.00^{a}
Guastella	170.74±0.00^{ab}	42.72±0.00^{ab}	300.85±0.00^{ab}	96.70±0.00^{bcd}	42.72±0.00^{a}	139.42±0.00^{bcd}	1.30±0.00^{a}	4.01±0.00^{a}	14.30±0.00^{ab}	13.20±0.00^{ab}	0.95±0.00^{a}

3.3 Results of protein expression in wheat samples before and after partial digestion

To perform a qualitative comparative analysis of protein samples from ancient wheats, extracted proteins were separated by Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) and stained by colloidal Coomassie blue, as described in materials and methods section. The SDS-PAGE gel images are shown in Figure 9. It was evident from the figure that the protein characteristics of the 18 analyzed wheat varieties showed significant differences, mainly reflected in the varying protein content and types within the same wheat variety before and after partial digestion.

All wheat varieties showed multiple polypeptides bands in undigested state, mainly concentrate in 72-95kDa, 55-72kDa, 28-55kDa and <28kDa. These polypeptides represent the main components of wheat proteins, such as gluten and non-gluten. The stained gels showed that many polypeptides were located between 72-95 kDa corresponding to high molecular weight glutenin subunits (HMW-GS). The polypeptides with molecular weight between 55-72 kDa belonged of ω-Gliadins subunits with medium molecular weight (MMW). Given that low molecular weight glutenin subunits (LMW-GS) and α/β, γ-Gliadins subunits both fall within the 30-45 kDa range and overlap, the group of polypeptides with a molecular weight between 28-55 kDa were α/β-, and γ-Gliadins subunits or LMW-GS.

To be specific, in Figure 9a, for Senatore Cappelli sample, in the undigestion (ND) sample, multiple polypeptides were visible ranging from 10 kDa to 95 kDa. Upon partial digestion (D), the HMW-GS and MMW (or ω-gliadins subunits) completely disappeared, the intensity of LMW (28-55 kDa) decreased, and non-gluten (<28kDa) bands became more prominent; Russello sample had a similar trend as Senatore Cappelli, with HMW-GS and MMW-GS almost vanish, and LMW-GS polypeptide group bands reduced in intensity after digestion and an increase in the intensity of non-gluten. The only difference is that the Russello sample had lower content and quantity of polypeptides at LMW-GS (28-55 kDa) than Senatore Cappelli. The P group (P1, P2, P3, P4) samples show similar protein band changes to Senatore Cappelli and Russello, but P1 and P2 did not differ significantly between polypeptide composition and strength. In addition to P3 and P4, the ω-Gliadins subunits intensity of ND samples of both samples was much lower than that of P1 and P2 samples, and the LMW-GS polypeptides quantity and brightness of P3 and P4 were also reduced compared with the previous two. In Figure 9b, all ND samples (V1, V2, T1, T2, T3, 3R) had similar

polypeptide composition and band strength, and HMW-GS, MMW subunits, and LMW-GS could be clearly observed. After partial digestion, the changes of polypeptide composition showed the same trend as shown in Figure 9a, namely, HMW-GS and WMW subunits gradually disappeared, LMW-GS intensity decreased, and non-gluten content increased.

In Figure 9c, before digestion, all samples followed the same pattern of variation as shown in Figures 9a and 9b. After partial digestion, the intensity of HMW-GS, MMW subunits, and LMW-GS diminished or nearly disappeared, while protein components below 28 kDa gradually accumulated. The difference is that before digestion, only Guastella sample showed a clear ω-Gliadins subunits polypeptide compared with the other five samples. The polypeptide composition and strength of S1 and S4 samples were weaker than those of the other 4 samples. After partial digestion, the S3 sample developed a light band near 55kDa.

It was evident from the electrophoretic map that the undigested wheat varieties had more polypeptides in the LMW subunits. Following partial digestion, there was a significant reduction or complete disappearance of gluten proteins in all wheat samples, including HMW-GS, LMW-GS, ω-Gliadins subunits, and α/β, γ-Gliadins subunits. During the partial digestion process, these gluten proteins were progressively broken down into smaller fragments, which accumulate in the non-gluten protein range (<28 kDa). Thus, before digestion, no macroscopic differences in protein composition were detected between different ancient varieties. After partial digestion, across all samples, each wheat sample showed a consistent trend, with partial digestion leading to the degradation or disappearance of gluten and the accumulation of digested gluten in non-gluten. However, the degree of digestion and the resulting banding strength vary between wheat varieties. Some species exhibited more pronounced degradation and polypeptides accumulation than others.

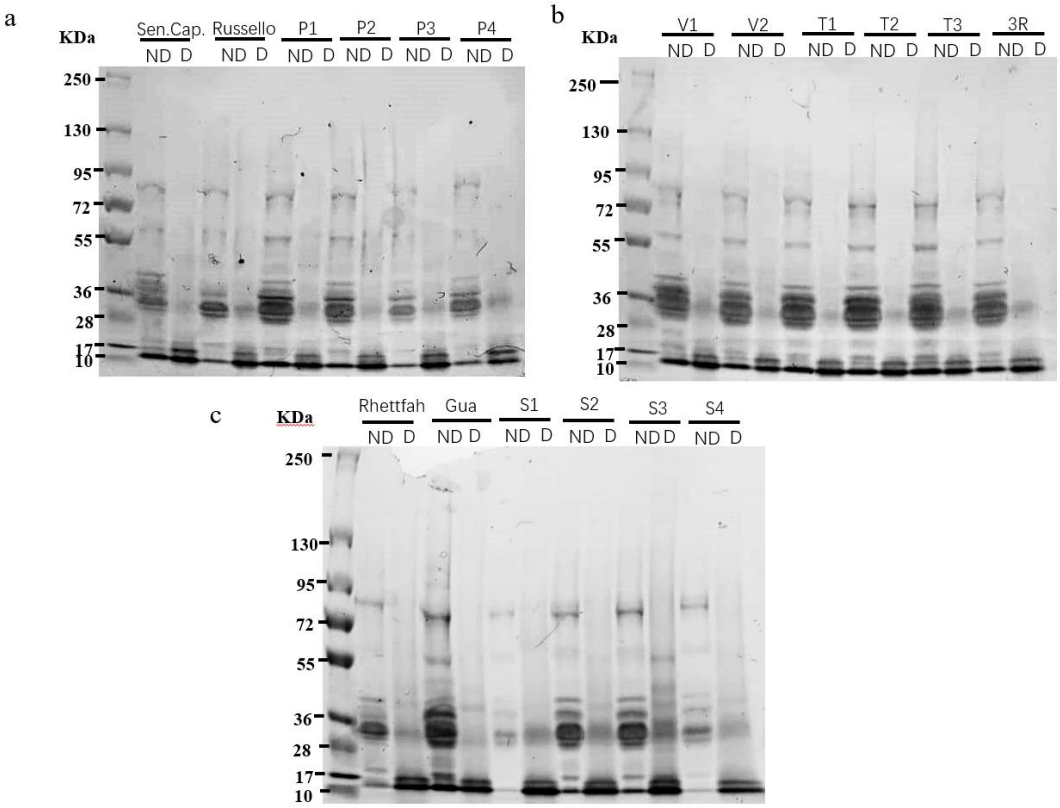


Figure 9. Photographs pictures of the SDS-PAGE gels. In the first lane of each picture was the PageRuler Plus Prestained Protein Ladder marker with the molecular weights in kDa indicated. The remaining lanes represented the protein bands of different wheat varieties before and after partial digestion. For each variety, the undigested (ND) and partially digested (D) protein bands were shown. From left to right, the lanes of: Senatore Cappelli ND and D, Russello ND and D, P1 ND and D, P2 ND and D, P3 ND and D, P4 ND and D (a); V1 ND and D, V2 ND and D, T1 ND and D, T2 ND and D, T3 ND and D, 3R ND and D (b); Rhettfah ND and D, Guastella ND and D, S1 ND and D, S2 ND and D, S3 ND and D, S4 ND and D (c).

3.4 Calculation results of the molecular weight of protein bands in wheat samples

Wheat gluten consisted of two classes of proteins, the gliadins and the glutenins [226]. The former could be subdivided into the α/β-, γ-, and ω-gliadins and the latter into the LMW and HMW glutenins. Dozens of gliadin genes were expressed in any wheat varieties, but much fewer glutenin genes were present. While the glutenins, in particular the HMW glutenins, were most crucial to the formation of high-quality dough with optimal baking properties, the gliadins contained most of the toxic fragments that caused symptoms in patients with CD [227]. The polypeptide composition of different wheat varieties after undigested and partially digested treatment is shown in Table 4.

During the the first year（2019-2020）growing season, before digestion, the molecular weight of HMW-GS of different wheat varieties ranged from 70.81 to 89.01 kDa, with the P4 variety exhibited the highest HMW-GS molecular weight (89.01 kDa) and the Guastella variety the lowest (70.81 kDa). The ω-gliadins subunits of different wheat varieties ranged from 47.74 kDa to 54.1 kDa, with the Senatore Cappelli variety had the highest molecular weight for ω-gliadins, and the T3 sample the lowest. Compared to other protein fractions, LMW-GS or α-/β- and γ-Gliadins subunits had the highest number of polypeptides and the greatest band intensity, predominantly distributed between 28 and 46.28 kDa. These subunits consist of two to four polypeptides depending on genotypes. Specifically, before digestion, the Senatore Cappelli, P1, P2, V1, V2, T1, T2, T3, 3R, and Guastella samples all exhibited 6 wheat protein characteristic bands, representing HMW-GS, ω-gliadins, and LMW-GS or α/β and γ-gliadins. The Russello, P3, P4, Rhettfah, S2, S3, and S4 samples all showed 5 wheat protein characteristic bands, lacking one LMW subunit band compared to the varieties with six bands. The S3 wheat sample displayed only 3 protein bands, missing the ω-gliadins and LMW bands found in other varieties. After partial digestion, only the S3 sample showed a prominent ω-gliadin subunit band, while no distinct bands were observed in the other varieties. In summary, partial digestion led to the weakening and disappearance of wheat protein bands, with variations in band intensity and number observed among different varieties.

Table 4. Presence of gluten in different wheat varieties produced in the first year of experiment (ND: undigested, D: partially digested).

Wheat varieties						
Band (kDa)	Senatore Cappelli ND	Russello ND	P1 ND	P2 ND	P3 ND	P4 ND
1	85.14	80.72	77.21	77.21	80.72	89.01
2	54.10	53.14	49.49	50.83	39.14	41.16
3	42.21	34.23	39.14	39.14	33.66	35.40
4	37.22	30.44	34.52	33.66	30.19	32.01
5	32.28	28.23	29.94	29.69	28.00	29.20
6	29.94		28.00	28.00		
Band (kDa)	V1 ND	V2 ND	T1 ND	T2 ND	T3 ND	3R ND
1	80.43	79.74	77.01	71.21	72.46	79.74
2	52.99	49.86	48.58	46.92	47.74	51.18
3	42.79	41.47	38.94	38.94	40.19	40.19
4	38.04	36.29	35.44	35.44	36.29	36.29
5	34.62	32.00	31.01	31.01	31.01	32.25
6	30.53	28.67	28.00	28.00	28.00	28.00
Band (kDa)	Rhettfah ND	Guastella ND	S1 ND	S2 ND	S3 ND	S4 ND
1	78.75	70.81	72.99	73.55	72.44	75.82
2	44.38	48.07	32.38	43.65	44.01	46.28
3	38.49	43.28	29.11	38.17	38.17	38.82
4	32.83	38.82		32.28	32.83	32.55
5	29.94	32.83		28.00	28.23	29.69
6		28.95				
Wheat varieties						
Band (kDa)	S3 D					
1	49.17					

3.5 Results of the total protein signal in wheat samples

To investigate the differences in total protein among various wheat varieties, ImageJ software was used for quantitative analysis of protein signal values from SDS-PAGE images. The total protein signals of different wheat samples from the first year (2019-2020) growing season are shown in Figure 10. From the figure, it can be observed that, except for the S1 sample, all wheat varieties typically exhibited higher protein signal values in the undigested state, indicating their protein content in the original state. In the partially digested state, only the S1 variety showed an increasing trend in total protein signal value, while the total protein signal values of all other samples displayed varying degrees of decrease. This reflects the extent of protein degradation during the digestion process among different wheat varieties.

Despite common trends, different wheat varieties exhibited specificity in total protein signal values. To better understand the differences in wheat protein under different treatments, we ranked the total protein signal values of various wheat varieties before and after digestion, as shown in Figure 11. In the undigested samples (Figure 11a), the ranking order of total protein signal values was: Guastella > T3 > T2 > P1 > T1 > S3 > 3R > P2 > V1 > V2 > S2 > Rhettfah > Senatore Cappelli > Russello > P4 > P3 > S4 > S1. The highest total protein signal value is observed in the Guastella variety (662,072 pixels), while the lowest is in the S1 variety (90,046 pixels), with a difference of 7.35-fold. Among the T group samples, T3 has the highest total protein signal value and T1 the lowest. For the P group samples, P1 has the highest total protein signal value and P3 the lowest. Among the S group wheat varieties, S3 has the highest total protein signal value and S1 the lowest. Finally, in the V group wheat varieties, V1 has a higher total protein signal value than V2.

After partial digestion (Figure 11b), the ranking of total protein signal values is: S3 > S1 > S2 > Rhettfah > P2 > 3R > Guastella > P1 > Russello > P3 > P4 > S4 > Senatore Cappelli > V1 > T3 > T2 > T1 > V2. The highest total protein signal value is observed in the S3 variety (414,831 pixels), while the lowest is in the V2 variety (191,303 pixels), with a difference of 2.17-fold. The trend in total protein signal values among the T group samples remained the same as in the undigested group, with T3 having the highest and T1 the lowest total protein signal value. For the P group samples, P2 has the highest total protein signal value and P4 the lowest. Among the S group wheat varieties, S3 has the highest total protein signal value and S2 the lowest. After digestion, V1 still has a higher

total protein signal value than V2. The total protein signal value decreased the most in Guastella and the least in S4. T3, P1, V2 and S3 were the most decreased total protein in each wheat group varieties.

In summary, the total protein signal values in undigested samples are generally higher than in partially digested samples (except for S1). Partial digestion significantly reduced total protein signal values, indicating that proteins were partially degraded during the digestion process.

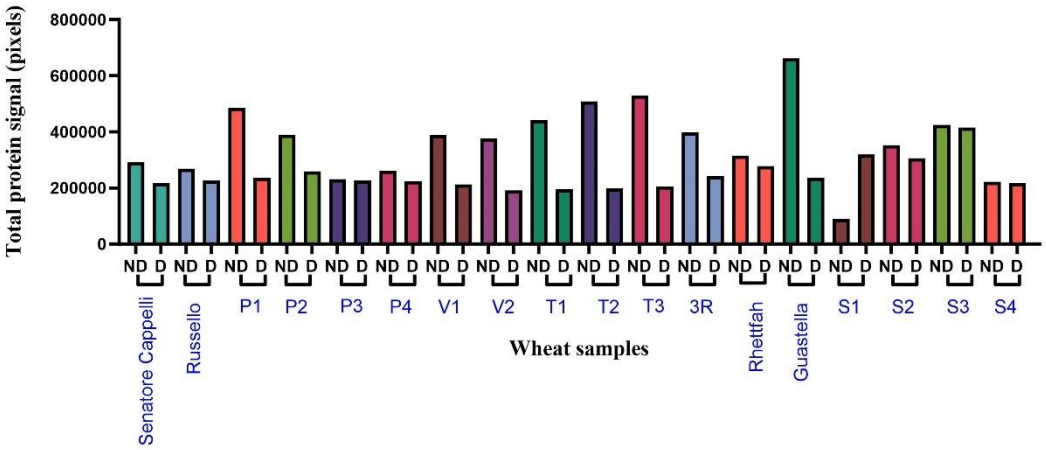
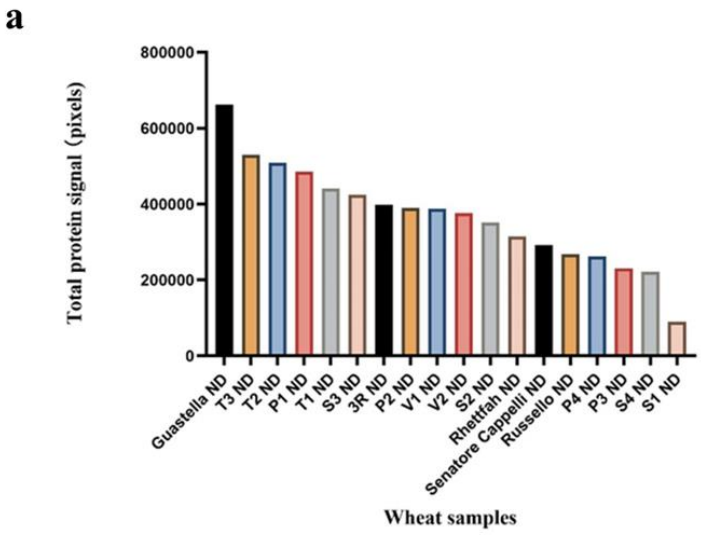


Figure 10. Total protein signal values of different wheat samples before and after partial digestion in the first year of the experiment. For each variety, the undigested (ND) and partially digested (D) total protein signal values were shown.



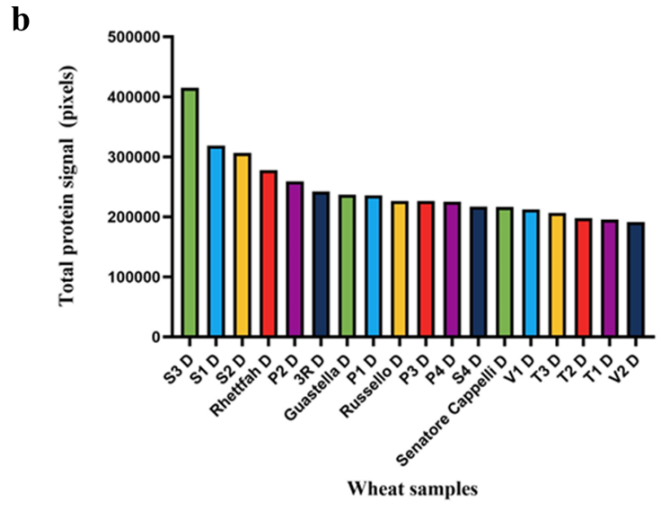


Figure 11. Ranking of total protein signal values of different wheat varieties before and after digestion in the first year experiment. (a): undigested (ND) samples; (b): partially digested (D) samples.

3.6 Proportions of gluten protein types analysis results

Figure 12 shows the influence of species on gluten and gluten subunits types relative to total proteins before digestion. In general, gluten is the major component of total protein content (61.20 %–83.99 %), and among the types of gluten subunits, α/β, γ-Gliadins and LMW-GS (41.24 %–57.67 %) are the predominant type, followed by HMW-GS (6.58 %–12.74 %), ω-Gliadins (2.74 %–10.69 %) belong to the minor types. Wheat gluten proteins – the major group of grain-storage proteins found in the starchy endosperm – are responsible for the unique properties of wheat exploited in bread making. High protein and gluten content or medium protein content with strong gluten is needed for optimal baking properties [228]. Before digestion, there is significant variation in gluten content among different wheat varieties. S1 has the highest gluten content at 83.99%, while S4 has the lowest at 61.20%, indicating substantial differences in the gluten protein composition across wheat types. The HMW-GS content ranged from 6.58% (V2) to 12.74% (S3), suggesting a relatively small variation in the structural proteins that affect dough strength and elasticity. LMW-GS influences dough strength and elasticity, while α/β and γ-Gliadins impact dough extensibility and viscosity, thereby affecting the texture of the final product [229]. There was a significant difference in the proportions of LMW-GS and α/β, γ-Gliadins among different varieties. S2 had the highest proportion at 57.67%, while P3 has the lowest at 41.24%. ω-Gliadins, which are present in the smallest amounts, range from 10.69% (P3) to 2.74% (S4). Given that ω-Gliadins are known for their

role in celiac disease, the variation in ω-Gliadins content also reflects differences in immunogenic potential.

Specific analysis within sample groups reveals the following gluten content rankings: for the S group, S1 > S2 > S3 > S4; for the P group, P2 > P4 > P1 > P3; for the T group, T2 > T1 > T3; and for the V group, V1 > V2. Regarding HMW-GS content, the rankings are: S3 > S1 > S2 > S4; P2 > P3 > P4 > P1; T2 > T3 > T1; and V1 > V2. For the α/β, γ-Gliadins and LMW-GS content, the rankings are: S2 > S1 > S3＞S4; T2 > T1 > T3; V1 > V2; and P1 > P4 > P2 > P3. The ω-Gliadins content rankings are: P3 > P2 > P1 > P4; T3 > T1 > T2; V2 > V1; and S2 > S1 > S3 > S4. Overall, most wheat varieties exhibited high gluten content, a critical factor in determining the baking quality of wheat flour. The significant differences in the proportions of gluten subunits, including HMW-GS, LMW-GS, α/β, γ-Gliadins, and ω-Gliadins, among different wheat varieties indicate genetic diversity and potential variations in end-use quality.

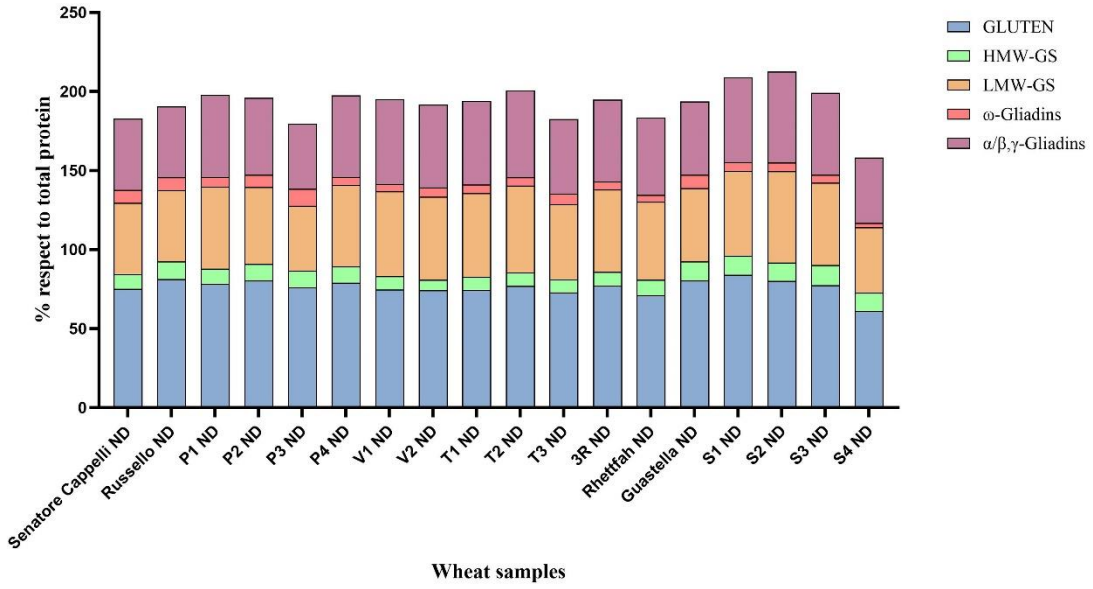


Figure 12. Proportion of gluten subunits to total protein in undigested (ND) wheat samples in the first year of the experiment. All gluten subunits proportion were reported as percentage of the total protein.

After partial digestion, gluten remains the main component of the total protein content. Among the types of gluten subunits, α/β, γ-Gliadins, and LMW-GS remain predominant, followed by HMW-GS and ω-Gliadins. The content of gluten and its subunits after partial digestion is shown in Figure 13. Following partial digestion, the gluten content ranged from 32.34% (T1) to 71.85% (S3). The

proportions of gluten subunits, specifically HMW-GS, LMW-GS, α/β, γ-Gliadins, and ω-Gliadins, ranged from 2.52% (3R) to 8.44% (P1), 18.80% (T2) to 42.21% (S3), and 1.79% (P4) to 11.87% (S3), respectively.

A detailed analysis of the digested sample group reveals the following order of gluten content for each sample set: for the S group, the gluten content is ranked as S3 > S4 > S2 > S1; for the P group, it is P1 > P2 > P3 > P4; for the T group, it is T3 > T2 > T1; and for the V group, it is V1 > V2. Regarding HMW-GS content, the proportions for each sample set are as follows: S3 > S4 > S2 > S1; P1 > P2 > P4 > P3; T3 > T1 > T2; and V2 > V1. For α/β, γ-Gliadins, and LMW-GS content, the proportions among different sample sets are: S3 > S4 > S2 > S1; T3 > T1 > T2; V1 > V2; and P1 > P3 > P4 > P2. For ω-Gliadins content, the ranking is: P2 > P1 > P3 > P4; T3 > T1 > T2; V2 > V1; and S3 > S4 > S2 > S1. After digestion, the content of wheat gluten decreased the most, ranging from 5.70% (S3) to 42.23% (T1), with S4 showing almost no change. Among the gluten subunits, LMW-GS and α/β, γ-Gliadins exhibited the highest reduction, ranging from 2.35% (S4) to 36.16% (T2), followed by HMW-GS, which decreased by 0.96% (V2) to 8.91% (S1). The lowest reduction was observed in ω-Gliadins, ranging from 0.82% (V1) to 5.55% (P3), ω-Gliadins signal values of S1, P1, P2, S2, Rhettfah, S4 and S3 increased slightly after digestion. Therefore, partial digestion reduces the total gluten content and its subunits in most wheat samples, but the extent of reduction varies among different wheat varieties.

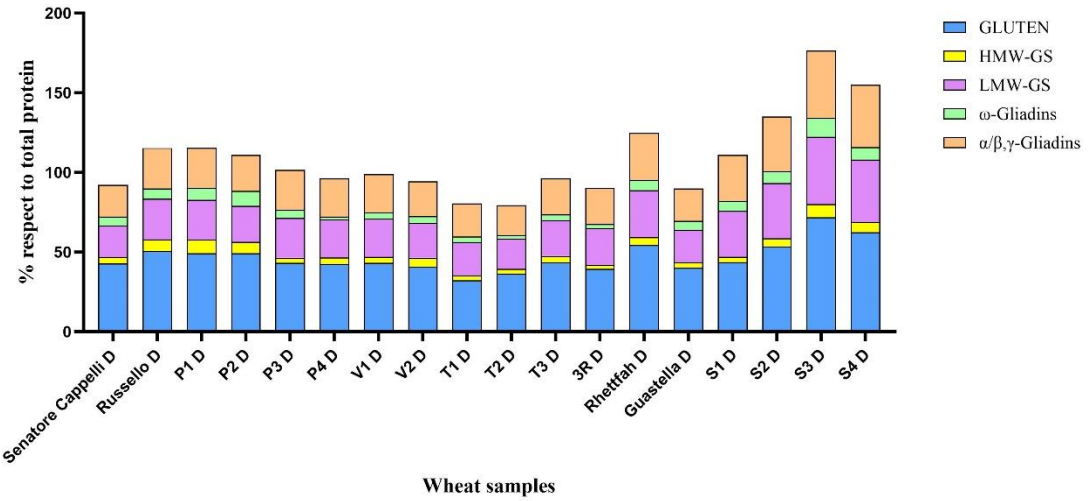


Figure 13. Proportion of gluten subunits to total protein in partially digested (D)

wheat samples in the first year of the experiment. All gluten subunits proportion were reported as percentage of the total protein.

3.7 Gliadins/Glutenins and HMW-GS/LMW-GS results

Gluten gives dough its unique properties by forming interchain disulfide bonds and hydrogen bonds between glutamine residues in gluten proteins. This viscoelastic network contributes to the elasticity and viscosity of the dough, making it versatile for various applications in the food industry [228]. The strength of gluten indicates its viscosity and elasticity, portraying the proteins' capacity in forming a consistent and durable network, influencing its appropriateness for final production [230]. Despite comparable protein levels, gluten strength varies based on the kinds of glutenin and gliadin proteins present, genetically fixed, along with their proportion, the ratio of high molecular weight (HMW) to low molecular weight (LMW) glutenin subunits (GS), and the quantity of unextractable polymeric proteins (UPP) [230, 231]. To emphasize the importance of the ratio of gliadins/glutenins and HMW-GS/LMW-GS on the baking quality and rheological properties of wheat varieties, we have summarized the results of different wheat varieties before and after partial digestion in Table 5.

Before digestion, the Gliadins/Glutenins ratios of different wheat samples ranged from 0.83 (S4) to 1.01 (P3). The ratios for each sample series were as follows: S series: S2 (0.91) > S1 (0.90) > S3 (0.88) > S4 (0.83); P series: P3 (1.01) > P2 (0.95) > P1 (0.94) > P4 (0.91); T series: T3 (0.97) > T1 (0.95) = T2 (0.95); V series: V2 (0.99) > V1 (0.94). After partial digestion, the Gliadins/Glutenins ratios of different wheat samples ranged from 0.92 (P4) to 1.09 (S1). The ratios for each sample series also showed different changes: S series: S1 (1.09) > S3 (1.07) > S2 (1.05) > S4 (1.03); P series: P2 (1.07) = P3 (1.07) > P1 (0.97) > P4 (0.92); T series: T1 (1.02) > T3 (0.99) > T2 (0.96); V series: V1 (1.01) > V2 (0.95).

The HMW-GS/LMW-GS ratios exhibited subtle differences before and after digestion. Before digestion, the HMW-GS/LMW-GS ratios ranged from 0.13 (V2) to 0.28 (S4). The ratio for each sample series was as follows: S group: 0.20 (S2) to 0.28 (S4); P group: 0.18 (P1) to 0.25 (P3); T group: 0.15 (T2) to 0.17 (T3); V group: 0.13 (V2) to 0.15 (V1). After digestion, the HMW-GS/LMW-GS ratios ranged from 0.11 (3R) to 0.34 (P1). The ratios for each sample series were as follows: S group: 0.12 (S2) to 0.20 (S3); P group: 0.13 (P3) to 0.34 (P1); T group: 0.15 (T1) to 0.18

(T3); V group: 0.16 (V1) to 0.26 (V2).

By comparing the changes in the two ratios before and after digestion, we found that for Gliadins/Glutenins, all wheat samples showed an increasing trend after digestion except for the V2 variety. Regarding HMW-GS/LMW-GS, seven varieties (Russello, P1, P2, V1, V2, T2, T3) exhibited an increasing trend, while the rest of the wheat samples exhibited a decreasing trend. Additionally, there were varying degrees of change in Gliadins/Glutenins and HMW-GS/LMW-GS within the wheat groups before and after digestion. For example, in the Gliadins/Glutenins ratio, the S series showed an increase after digestion, with S1 reaching the highest value of 1.09. The P series also showed an increasing trend, with P2 and P3 reaching 1.07. The T series exhibited minor fluctuations, with T1 increasing to 1.02. The V series showed slight changes, with V1 increasing to 1.01. For HMW-GS/LMW-GS ratio, the S series showed a decrease or remained stable, with S1 and S4 showing the most significant declines. In the P series, P1 and P2 increased, while P3 and P4 decreased, with P1 showing the most significant increase and P3 the largest decrease. The T series ratios remained relatively stable, with minor increases or decreases. In the V series, V2 increased to 0.26, while V1 remained relatively stable.

Overall, except for V2, the Gliadins/Glutenins ratio in the remaining 17 wheat samples showed a slight increase after partial digestion. The HMW-GS/LMW-GS ratio exhibited both increasing and decreasing trends across all wheat samples.

Table 5. The Gliadins/Glutenins and HMW-GS/LMW-GS of different wheat samples before and after partial digestion in the first year of the experiment (ND: undigested, D: partially digested).

Sample	Gliadins/Glutenins		HMW-GS/LMW-GS	
	ND	D	ND	D
Senatore Cappelli	0.98	1.06	0.21	0.20
Russello	0.95	0.97	0.25	0.29
P1	0.94	0.97	0.18	0.34
P2	0.95	1.07	0.21	0.32
P3	1.01	1.07	0.25	0.13
P4	0.91	0.92	0.20	0.17
V1	0.94	1.01	0.15	0.16
V2	0.99	0.95	0.13	0.26
T1	0.95	1.02	0.16	0.15
T2	0.95	0.96	0.15	0.16
T3	0.97	0.99	0.17	0.18
3R	0.94	1.01	0.16	0.11
Rhettfah	0.91	1.04	0.20	0.16
Guastella	0.94	1.08	0.26	0.18
S1	0.90	1.09	0.23	0.12
S2	0.91	1.05	0.20	0.15
S3	0.88	1.07	0.25	0.20
S4	0.83	1.03	0.28	0.17

3.8 Effects of wheat proteins on L929 proliferation

The proliferation effects of different wheat proteins were evaluated by MTT assay. Figures 14 shows the first year L929 cell viability expressed as a relative percentage compared with the untreated control cells. All wheat proteins had lower L929 cell viability than the control cells when undigested (Figure 14a). And viability of L929 cells was highest in R3(74.73%) and lowest in P4(28.23%). After digestion, the viability of L929 cells was the highest in P3 (103.86%) and the lowest in Rhettfah (36.53%), except for the P1 and P3 varieties, all other varieties exhibited lower L929 cell viability than the control cells. Before digestion (Figure 14a), compared to the control group, 11 ancient wheat varieties (P1, P3, P4, S1, S2, S3, S4, Rhettfah, Russello, Senatore Cappelli) and the modern wheat variety Claudio showed significant differences, while the remaining varieties did not show significant differences. This indicated that the native wheat proteins of these 11 ancient wheat varieties exhibited a stronger inhibitory effect on L929 cell proliferation compared to other ancient wheat varieties. After partial digestion (Figure 14b), only the P4, Rhettfah, Russello, V2, Senatore

Cappelli, Guastella, and Claudio varieties showed significant differences, suggesting that the wheat proteins of these varieties had stronger inhibitory effect on the proliferation of L929 cells than the other 12 ancient wheat varieties. Furthermore, digestion affected the proliferation effects of wheat proteins on the cells. After partial digestion (Figure 14b), a small amount of wheat protein inhibited the proliferation of L929 cells, such as Russello, V2, and Guastella. Conversely, some wheat proteins promoted the proliferation of L929 cells, such as P1, P3, S1, S2, S3, S4, V1, Senatore Cappelli, and Claudio. P4 and Rhettfah retain the same proliferation effects on L929 cell after digestion as before digestion. The remaining ancient wheat samples showed no difference on L929 cell proliferation effect before and after digestion.

The impact of different wheat series on L929 cell proliferation varied before and after digestion. Only the T group wheat proteins (T1, T2, T3) showed no significant effect on L929 cell viability both before and after digestion. In the P group samples, partial digestion had no significant effect on P2, and P4 maintained the same significant difference. However, the viability of L929 cells treated with P1 and P3 decreased after digestion, indicating that partial digestion enhanced the ability of P1 and P3 proteins to promote L929 cell proliferation, P2 and P4, however, had no significant effect on L929 cell proliferation. All wheat proteins in S group promoted the proliferation of L929 cell. Among the V group wheats, after digestion, V1 promoted L929 cell proliferation, while V2 had the opposite effect. Additionally, before digestion, the modern variety Claudio exhibited a stronger inhibitory effect on L929 cell proliferation compared to the ancient varieties P2, 3R, V2, T1, T2, T3, and Guastella. The remaining ancient varieties, however, showed a stronger inhibitory effect on L929 cell proliferation than the modern variety Claudio. After digestion, the proteins from P4, Rhettfah, Russello, V2, and Guastella exhibited a stronger inhibitory effect on L929 cell proliferation compared to the modern variety Claudio, while the remaining ancient wheat proteins showed a weaker inhibitory effect than Claudio.

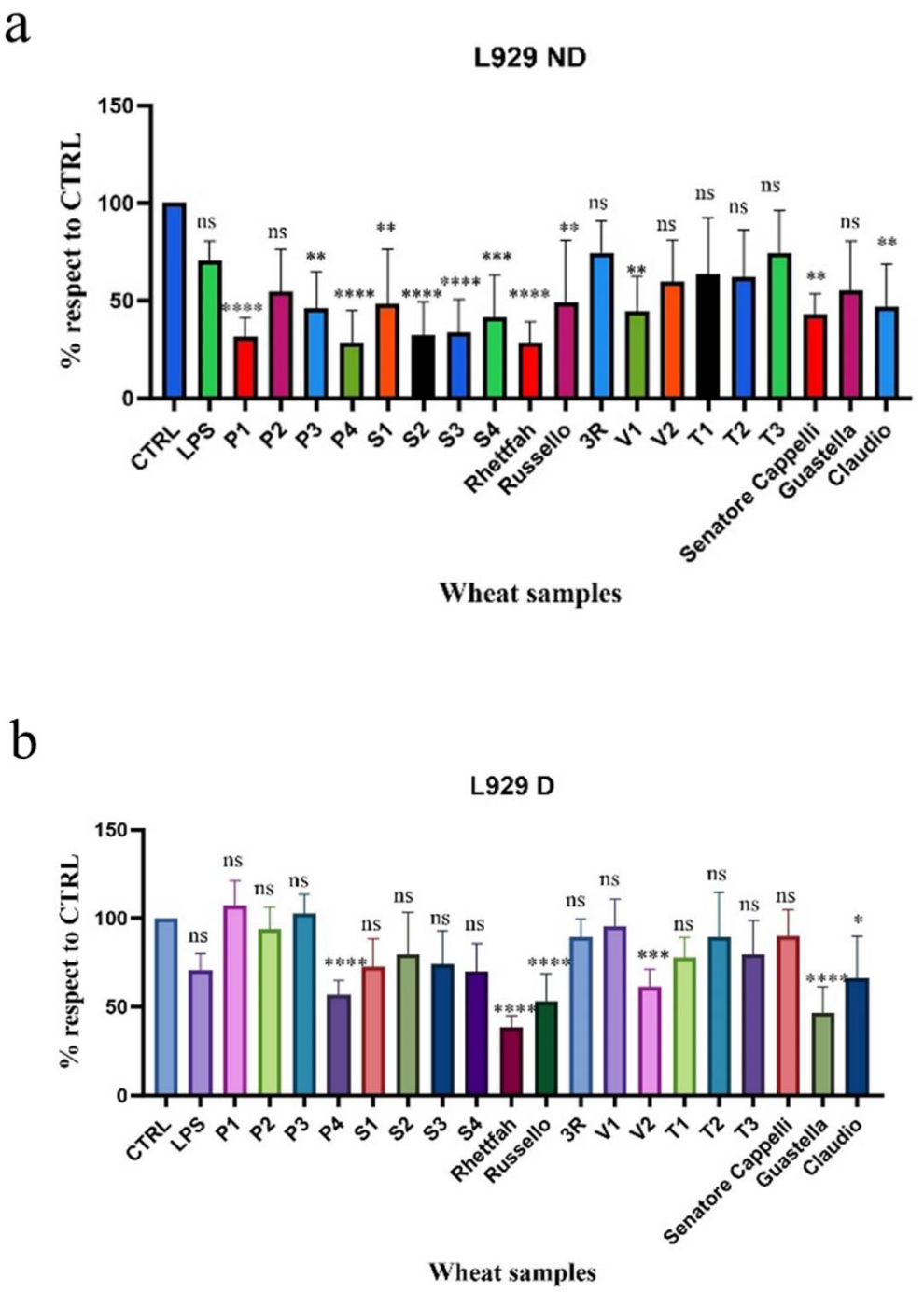


Figure 14. The effects of undigested (a) and partially digested (b) wheat proteins on the proliferation of L929 cells. All treatments were compared to the untreated control (CTRL). Significant differences between treatments were reported as follows: * $0.01 < p < 0.05$, ** $0.001 < p < 0.01$, *** $0.0001 < p < 0.001$, **** $p < 0.0001$. ND: undigested, D: partially digested.

3.9 Effects of wheat proteins on Caco2 proliferation

Figure 15 illustrates the relative percentage of Caco2 cell viability in the first year compared to the untreated control cells. The results showed that the viability of Caco2 cells before and after digestion was significantly lower compared to the control cells. Before digestion (Figure 15a), out of the 7 ancient wheat varieties P2, P4, Rhettfah, Russello, Senatore Cappelli, Guastella, and the modern wheat Claudio, exhibited significant differences compared to the control group. The differences among the remaining varieties were not significant, indicating that these seven ancient wheat varieties had a stronger inhibitory effect on Caco2 cell proliferation compared to other ancient wheat varieties. And viability of Caco2 cells was highest in T2 (99.09%) and lowest in Senatore Cappelli (54.55%). After partial digestion (Figure 15b), the viability of Caco2 cells was the highest in P2 (97.26%) and the lowest in Guastella (42.84%), only P4, Rhettfah, Russello, V2, T1, T3, Guastella, and Claudio varieties showed significant differences, this indicated that the wheat proteins from these varieties exhibited a stronger inhibitory effect on Caco2 cell proliferation compared to the other 11 ancient wheat varieties. Moreover, digestion affected the proliferation effect of wheat proteins to Caco2 cells. After partial digestion (Figure 15b), a small amount of wheat proteins promoted the proliferation of Caco2 cells, for example P1 and Senatore Cappelli; while some wheat proteins had a opposite effect, for example P4, Rhettfah, V2, T1, T3, Guastella, and Claudio. Russello retain the same proliferation effects on Caco2 cell after digestion as before digestion. The remaining ancient wheat samples had no effect on Caco2 cells proliferation before and after digestion.

The impact of different wheat varieties on Caco2 cells proliferation before and after digestion varies. Only the S-group wheat proteins (S1, S2, S3, S4) showed no significant effects on the viability of Caco2 cells before and after digestion. Among the P-group varieties, after partial digestion, P2 and P3 showed no significant differences, but the viability of Caco2 cells increased after treatment with P1 and decreased after treatment with P4, it indicated that partial digestion promoted the proliferation of Caco2 cells by P1 protein, while inhibiting the proliferation by P4 protein. In the V-group wheat, only V2 decreased the viability of Caco2 cells after digestion, with no effect on V1. Apart from T2, the inhibitory effect of T1 and T3 on the proliferation of Caco2 cells in T group was enhanced. Furthermore, before digestion, except for P1, Rhettfah, and Senatore Cappelli proteins, which showed an inhibitory effect on Caco2 cell proliferation equal to or greater than the modern

variety Claudio, the inhibitory effects of other ancient wheat proteins were lower than that of Claudio. After digestion, except for P4, Rhettfah, V2, T3, and Guastella showing similar significance to Claudio, it indicated that the inhibitory effects of P4, Rhettfah, V2, T3, and Guastella proteins on Caco2 cell proliferation were comparable to the modern variety Claudio, while the inhibitory effects of other ancient wheat proteins were lower than those of Claudio.

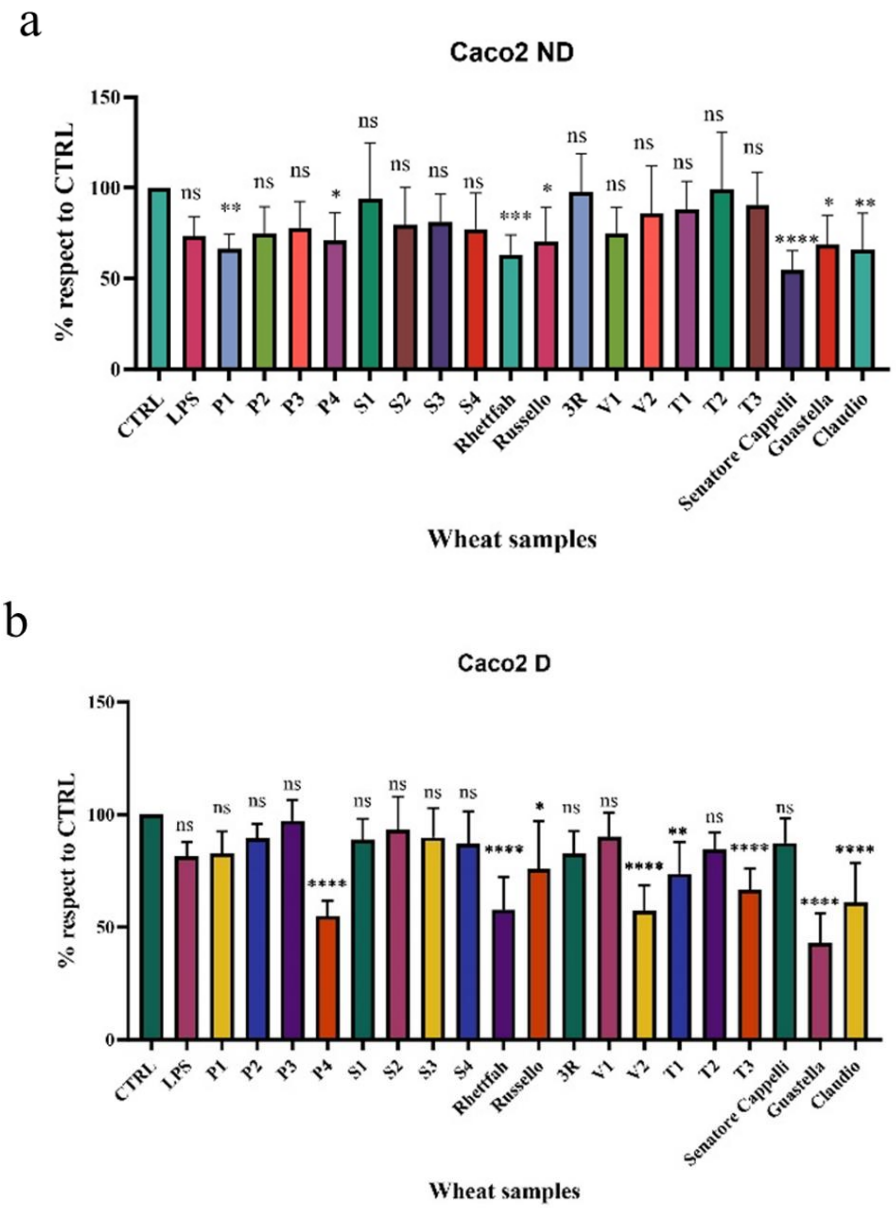


Figure 15. The effects of undigested (a) and partially digested (b) wheat proteins on the proliferation of Caco2 cells. All treatments were compared to the untreated control (CTRL). Significant differences between treatments were reported as follows: * $0.01 < p < 0.05$, ** $0.001 < p < 0.01$, *** $0.0001 < p < 0.001$, **** $p < 0.0001$. ND: undigested, D: partially digested.

4. The results of the second year of the experiment

4.1 Proximal composition of wheat flour

The proximate analysis of total protein content, humidity and ash content of wheat flour was shown in table 5. The protein, moisture, and ash content of the 16 wheat varieties cultivated during the first year ranged from 14.88g /100g (P4) to 15.62g /100g (P2), 12.92 g/100g (T2) to 13.03 g/100g, and 0.81 g/100g (T1) to 0.98 g/100g, respectively. No statistical difference was observed in protein, moisture and ash content between all wheat varieties.

4.2 Phenolic and flavonoid content and antioxidant activity of wheat flour

Phenolic contents of the tested wheat genotypes are presented in Table 6, expressed as micromoles of gallic acid equivalent (GAE) per 100 g of grain. The FPC, BPC and TPC of all the samples ranged from 62.48 GAE mg/100 g (V1) to 91.72 GAE mg/100 g (Russello), 90.55 GAE mg/100 g (S4) to 113.57 GAE mg/100 g (T1), 167.12 GAE mg/100 g (P3) to 197.87 GAE mg/100 g (Russello), respectively. However, no statistical difference was observed in FPC, BPC and TPC results between all wheat varieties.

Flavonoid contents of tested wheat varieties are expressed as micromoles of catechin equivalent (CE) per 100 g of grain (Table 6). The FFC, BFC and TFC of all the samples ranged from 36.21 CE mg/100 g in T3 to 50.12 CE mg/100 g in P4, 23.89 CE mg/100 g in S1 to 36.97 CE mg/100 g in T2, 54.50 CE mg/100 g in S4 to 80.60 CE mg/100 g in P3. Like the significance of polyphenol content, there was no statistical difference observed in FFC and BFC results between all wheat varieties. Significantly different values only in TFC were observed for P3 and S4 (54.50 CE mg/100 g) among all other wheat samples ($p < 0.05$).

The reducing power (FRAP assay) was evaluated in all wheat samples. The antioxidant activity values (Table 6) of the 16 cultivars determined by the FRAP assay ranged from 0.91 mmol Fe^{2+}/100 g in S1 to 1.05 mmol Fe^{2+}/100 g in P4. Regarding DPPH assay, T1 (2.96 µmol TE/g) showed the best antioxidant activity and T2 (1.91 µmol TE/g) showed the worst antioxidant Activity compared to the remaining cultivars (Table 6). And no statistical difference was observed both in FRAP and DPPH results among all wheat varieties.

Table 6. Main quality parameters of wheat varieties planted in the second year. Different letters in the same row indicate significant differences ($p < 0.05$).

Sample	FPC (GAE mg/100g)	BPC (GAE mg/100g)	TPC (GAE mg/100g)	FFC (CE mg/100g)	BFC (CE mg/100g)	TFC (CE mg/100g)	FRAP (mmol Fe^{2+}/100g)	DPPH (umol TE/g)	Protein (g/100g)	Humidity (g/100g)	Ash (g/100g)
P1	70.67±12.19^{a}	107.93±39.02^{a}	178.60±48.31^{a}	44.16±9.92^{a}	25.75±3.31^{a}	69.91±11.30^{ab}	1.04±0.12^{a}	2.02±0.28^{a}	15.62±1.81^{a}	12.93±0.12^{a}	0.97±0.02^{a}
P2	74.70±19.39^{a}	108.57±12.83^{a}	183.28±30.70^{a}	40.72±10.26^{a}	23.94±5.05^{a}	64.66±8.86^{ab}	0.99±0.15^{a}	2.62±0.34^{a}	14.92±1.45^{a}	13.02±0.15^{a}	0.97±0.03^{a}
P3	66.77±22.70^{a}	100.35±13.13^{a}	167.12±25.53^{a}	44.31±6.67^{a}	36.29±8.86^{a}	80.60±5.84^{a}	1.01±0.10^{a}	2.58±0.33^{a}	15.03±1.52^{a}	13.03±0.12^{a}	0.97±0.02^{a}
P4	73.95±17.74^{a}	110.35±16.24^{a}	184.30±8.60^{a}	50.12±9.85^{a}	28.16±5.91^{a}	78.28±12.79^{ab}	1.05±0.17^{a}	2.53±0.79^{a}	14.88±1.37^{a}	12.98±0.08^{a}	0.96±0.03^{a}
3R	73.20±14.62^{a}	105.71±9.90^{a}	178.90±22.51^{a}	45.13±4.42^{a}	23.87±6.67^{a}	69.00±7.06^{ab}	0.98±0.10^{a}	2.53±0.54^{a}	15.00±1.44^{a}	13.00±0.14^{a}	0.96±0.03^{a}
RUSSELLO IBLEO	91.72±15.79^{a}	106.15±10.11^{a}	197.87±11.02^{a}	49.29±15.93^{a}	26.64±6.18^{a}	75.93±21.91^{ab}	1.01±0.08^{a}	2.28±0.14^{a}	15.50±1.10^{a}	13.00±0.08^{a}	0.95±0.03^{a}
S1	76.64±17.37^{a}	103.05±18.37^{a}	179.69±16.52^{a}	42.08±7.82^{a}	23.89±5.07^{a}	65.97±7.45^{ab}	0.91±0.21^{a}	2.83±0.34^{a}	15.02±1.47^{a}	13.03±0.12^{a}	0.96±0.02^{a}
S2	79.56±20.21^{a}	104.28±2.08^{a}	183.84±21.81^{a}	37.91±3.61^{a}	30.42±6.26^{a}	68.33±9.30^{ab}	1.01±0.09^{a}	2.25±0.26^{a}	15.08±1.54^{a}	13.00±0.13^{a}	0.95±0.04^{a}
S3	63.19±17.86^{a}	105.73±13.03^{a}	168.91±26.52^{a}	43.92±7.56^{a}	32.95±6.83^{a}	76.86±7.58^{ab}	0.97±0.19^{a}	2.34±0.69^{a}	15.03±1.51^{a}	13.03±0.16^{a}	0.96±0.02^{a}
S4	84.34±13.75^{a}	90.55±10.79^{a}	174.89±18.19^{a}	30.08±8.74^{a}	24.42±5.93^{a}	54.50±14.50^{b}	1.01±0.05^{a}	2.74±0.33^{a}	15.13±1.62^{a}	12.98±0.18^{a}	0.98±0.02^{a}
SENATORE CAPPELLI	79.40±28.88^{a}	106.78±26.04^{a}	186.18±53.53^{a}	38.34±13.46^{a}	30.64±6.97^{a}	68.98±19.31^{ab}	1.04±0.25^{a}	2.74±0.69^{a}	15.48±1.40^{a}	12.95±0.18^{a}	0.98±0.05^{a}
T1	68.73±10.59^{a}	113.57±12.85^{a}	182.30±22.13^{a}	40.26±5.31^{a}	28.28±6.86^{a}	68.54±7.68^{ab}	0.91±0.27^{a}	2.96±0.40^{a}	15.03±1.62^{a}	13.02±0.19^{a}	0.81±0.35^{a}
T2	79.40±13.43^{a}	104.06±9.58^{a}	183.46±13.30^{a}	37.76±10.21^{a}	36.97±6.74^{a}	74.73±8.11^{ab}	0.97±0.09^{a}	1.91±0.26^{a}	15.28±1.55^{a}	12.92±0.08^{a}	0.98±0.02^{a}
T3	77.94±24.27^{a}	111.02±20.93^{a}	188.96±23.23^{a}	36.21±12.29^{a}	25.42±4.50^{a}	61.63±12.37^{ab}	0.96±0.14^{a}	2.15±0.38^{a}	15.32±1.75^{a}	12.98±0.13^{a}	0.97±0.02^{a}
V1	62.48±4.93^{a}	104.95±17.59^{a}	167.42±16.91^{a}	47.03±4.69^{a}	29.47±3.33^{a}	76.50±3.86^{ab}	1.04±0.12^{a}	2.51±1.00^{a}	15.00±1.49^{a}	13.03±0.14^{a}	0.96±0.02^{a}
V2	83.90±23.02^{a}	112.54±18.38^{a}	196.44±35.27^{a}	40.62±18.59^{a}	33.36±13.17^{a}	73.98±21.61^{ab}	1.01±0.22^{a}	2.43±0.34^{a}	15.15±1.57^{a}	12.93±0.16^{a}	0.97±0.02^{a}

4.3 Results of protein expression in wheat samples before and after partial digestion

The SDS-PAGE gel images are shown in Figure 16. The figure illustrated that the differences in the 16 ancient wheat proteins were predominantly attributed to the various treatment methods employed, specifically the undigested and partially digested treatments. These methods lead to variations in both the protein content and types of wheat. In the undigested state, all wheat varieties exhibited multiple polypeptide bands, representing the main components of wheat proteins, which were concentrated in the ranges of 72-95 kDa, 72-55 kDa, and 36-55 kDa, corresponding to the wheat gluten subunits: HMW-GS, ω-gliadin subunits, and α/β, γ-gliadin subunits (also LMW-GS), respectively.

Specifically, in Figure 16a, none of the undigested (ND) samples exhibited HMW-GS polypeptides. In the P1 sample, multiple polypeptides ranging from 36 kDa to 95 kDa were visible. Upon partial digestion (D), HMW-GS and MMW (ω-gliadin subunits) completely disappeared, the intensity of LMW (36-55 kDa) decreased, no significant polypeptide bands were observed, and no accumulation was seen in the non-gluten protein region. Samples V1, V2, S1, S2, and SC showed a similar trend to P1, with HMW-GS and MMW-GS almost disappearing, a reduced intensity of LMW-GS polypeptide bands post-digestion, no visible polypeptide bands, and no significant differences in the protein composition of different wheat varieties. In Figure 16b, all ND samples (T1, T2, T3, 3R, RI) had similar polypeptide compositions and band intensities, clearly showing HMW-GS, MMW subunits, and LMW-GS. After partial digestion, the trend of polypeptide composition changes differed from Figure 16a; weaker intensity polypeptides were still observable in HMW-GS and LMW-GS in all samples. In Figure 16c, before and after digestion, the composition and protein variation patterns of all wheat proteins were similar to those in Figure 16b, with HMW-GS, MMW subunits, and LMW-GS visible before digestion and HMW-GS and LMW-GS polypeptides still observable after partial digestion.

The electrophoretic profiles showed that the wheat varieties planted in the second year had minor differences in protein composition among different varieties, with low molecular weight gluten subunits still being the primary components of wheat gluten. After partial digestion, gluten proteins in all wheat samples significantly decreased or completely disappeared, including HMW-GS, LMW-GS, ω-gliadin subunits, and α/β, γ-gliadin subunits. However, after partial digestion, the polypeptide content of different varieties was slightly different, with some samples retaining HMW and LMW

polypeptides. MMW subunits were almost unobservable.

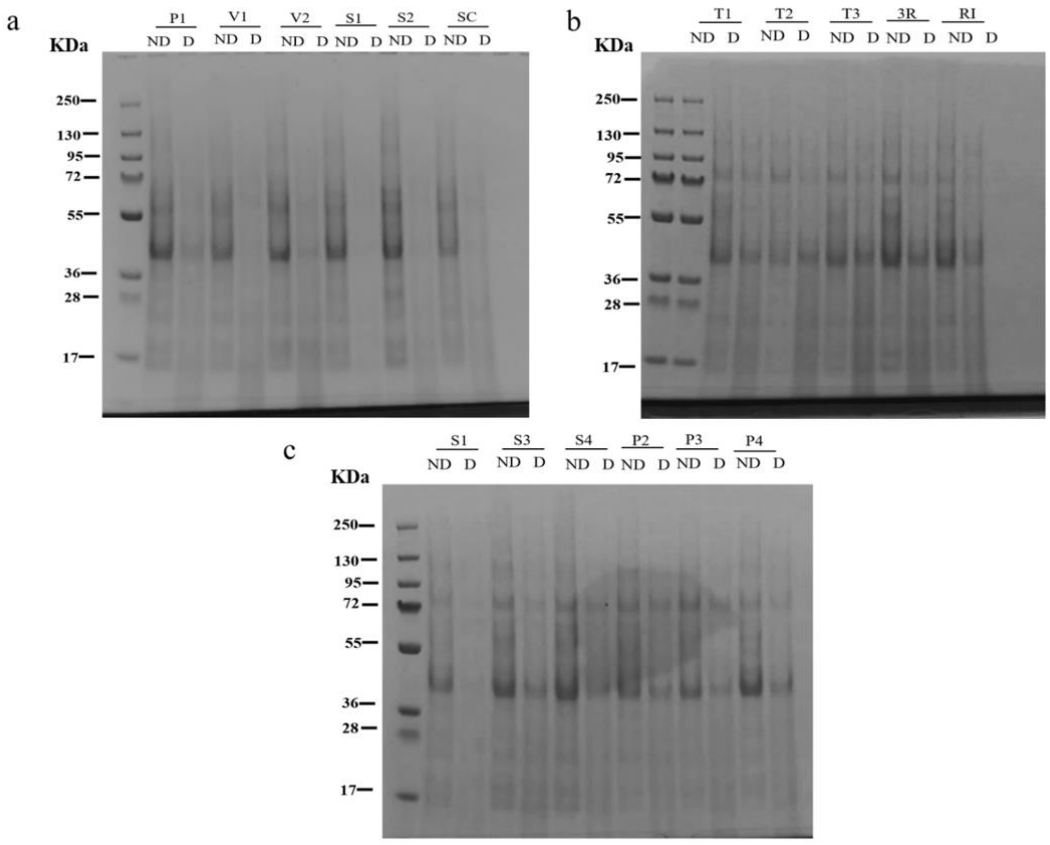


Figure 16. Photographs pictures of the SDS-PAGE gels. In the first lane of each picture was the PageRuler Plus Prestained Protein Ladder marker with the molecular weights in kDa indicated. The remaining lanes represented the protein bands of different wheat varieties before and after partial digestion. For each variety, the undigested (ND) and partially digested (D) protein bands were shown. From left to right, the lanes of: P1 ND and D, V1ND and D, V2 ND and D, S1 ND and D, S2 ND and D, SC ND and D (a); T1 ND and D, T2 ND and D, T3 ND and D, 3R ND and D, RI ND and D (b); S1 ND and D, S3 ND and D, S4 ND and D, P2 ND and D, P3 ND and D, P4 ND and D (c).And SC is Senatore Cappelli, RI is Russello, the same below.

4.4 Calculation results of the molecular weight of protein bands in wheat samples

Table 7 shows the polypeptide composition of wheat proteins before and after digestion in the second year of planting. In the second year planting year, before digestion, the molecular weights of HMW-GS in different wheat varieties ranged from 86.16 kDa to 93.05 kDa, with the S1 variety having the highest HMW-GS molecular weight (93.05 kDa) and the P2 variety the lowest (86.16

kDa). The molecular weights of ω-gliadins in different wheat varieties ranged from 55.74 kDa to 68.69 kDa, with the S3 variety having the largest ω-gliadins molecular weight and the V2 sample the smallest. LMW-GS had the highest band intensity compared to other protein components, primarily distributed between 39.31 kDa (S4) and 43.13 kDa (T1).

Specifically, before digestion, the P1, V1, V2, S1, S2, and SC samples all exhibited three typical bands, including two light ω-gliadins bands and one dark LMW-GS or α/β, γ-gliadins band. The T1 and T3 samples had one polypeptide band each in the HMW-GS, ω-gliadins, and LMW-GS regions, while T2, compared to T1 and T3, lacked one ω-gliadins subunit band. The 3R and RI samples, besides having one light HMW-GS band and one dark LMW-GS band, also exhibited two light ω-gliadins bands. The protein band composition in the S3, S4, P2, P3, and P4 samples was consistent with that of the 3R and RI samples, each having one light HMW-GS band, two light ω-gliadins bands, and one dark LMW-GS band. After digestion, the number of polypeptides changed across samples. The P1, V1, V2, S1, S2, and SC samples showed no significant polypeptide bands. The T1 sample, after digestion, still exhibited one light HMW-GS band and one light LMW-GS band. The samples showing the same trend in band changes as T1 included S3, S4, P2, P3, and P4. The T2, T3, 3R, and RI samples retained consistent polypeptide numbers after digestion, each showing only one light band in the LMW-GS region.

In summary, before digestion, the LMW-GS polypeptide band exhibited the greatest intensity. The differences in protein bands were mainly due to the absence of HMW-GS polypeptide bands and slight variations in the number of ω-gliadins bands. After partial digestion, the intensity and presence of protein bands in all wheat varieties weakened or disappeared, with differences in the intensity and number of protein bands among the different varieties.

Table 7. Presence of gluten in different wheat varieties produced in the second year of experiment (ND: undigested, D: partially digested).

Wheat varieties						
Band (kDa)	P1 ND	V1ND	V2 ND	S1 ND	S2 ND	Senatore Cappelli ND
1	64.76	64.76	62.95	63.20	64.49	64.76
2	57.11	57.11	55.74	55.74	56.19	57.11
3	41.30	41.64	39.98	41.47	41.64	42.15
Band (kDa)	T1 ND	T2 ND	T3 ND	3R ND	Russello ND	S3 ND
1	92.93	90.53	90.20	89.53	90.20	90.57
2	68.21	42.50	63.31	68.20	67.19	68.69
3	43.13		41.71	61.00	63.08	63.09
4				41.10	41.25	41.01
Band (kDa)	S4 ND	P2 ND	P3 ND	P4 ND		
1	87.16	86.16	87.83	87.16		
2	67.56	66.28	67.86	68.44		
3	61.65	57.09	59.33	57.31		
4	39.31	39.76	39.61	40.69		
Wheat varieties						
Band (kDa)	T1 D	T2 D	T3 D	3R D	Russello D	S3 D
1	91.89	42.50	42.02	41.56	42.34	41.56
2	43.78					
Band (kDa)	S4 D	P2 D	P3 D	P4 D		
1	85.50	86.82	87.16	88.17		
2	39.92	39.31	40.69	40.69		

4.5 Results of the total protein signal in wheat samples

Figure 17 shows the total protein signals of different wheat samples in the second year of experiment. It could be observed from the figure that all wheat varieties exhibited higher protein signal values in the undigested state. After partial digestion, the total protein signal values of all samples displayed varying degrees of decline, with the largest decrease observed in S1 and the smallest in T2. The total protein signal values of different wheat varieties before and after digestion were ranked to better understand the differences in wheat proteins under different treatments, as shown in Figure 18.

Before digestion, the order of total protein signal values of different wheat varieties was as follows: S4 > 3R > S3 > T1 > Russello > T3 > P2 > S2 > P4 > P1 > P3 > S1 > V2 > T2 > V1 > Senatore Cappelli (Figure 18a). The variety with the highest total protein signal value was S4 (87256 pixels), and the lowest was Senatore Cappelli (36605 pixels), with a 2.38-fold difference between them. Among the S group samples, S4 had the highest total protein signal value, and S1 had the lowest. In the T group samples, T1 had the highest total protein signal value, and T2 had the lowest. Among the P group wheat varieties, P2 had the highest total protein signal value, and P3 had the lowest. Lastly, in the V group wheat varieties, V2 had a higher total protein signal value than V1.

After partial digestion, the order of total protein signal values of wheat varieties was as follows: T3 > 3R > T1 > Russello > S4 > S3 > P2 > P3 > P4 > T2 > V2 > P1 > V1 > S1 > Senatore Cappelli > S2 (Figure 18b). The variety with the highest total protein signal value was T3 (65234 pixels), and the lowest was S2 (11870 pixels), with a 5.50-fold difference between them. The trends in total protein signal values varied among the different series of wheat after digestion. In the T group samples, T3 had the highest total protein signal value, and T2 had the lowest. Among the S group wheat varieties, S4 had the highest total protein signal value, and S2 had the lowest. In the P group samples, P2 had the highest total protein signal value, and P1 had the lowest. After digestion, in the V group wheat varieties, V2 still had a higher total protein signal value than V1.

In summary, the total protein signal values of wheat exhibited a general declining trend after partial digestion, with different wheat varieties showing varying degrees of decrease.

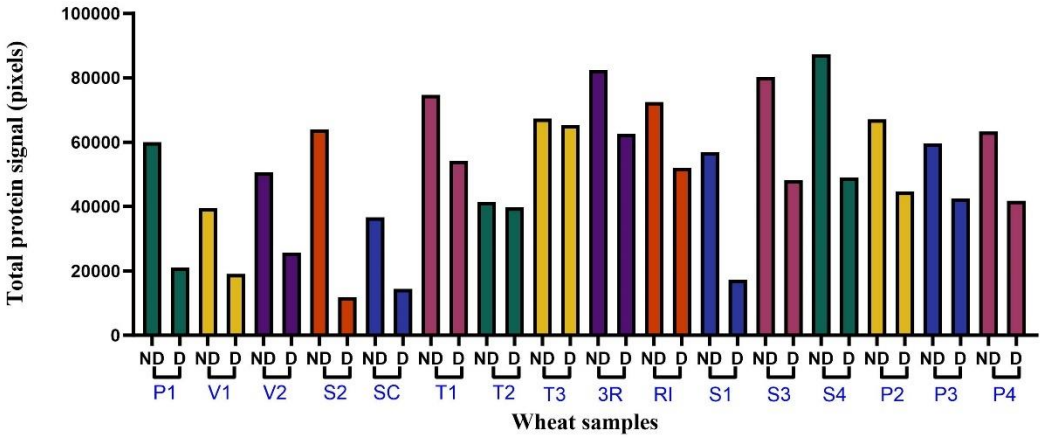
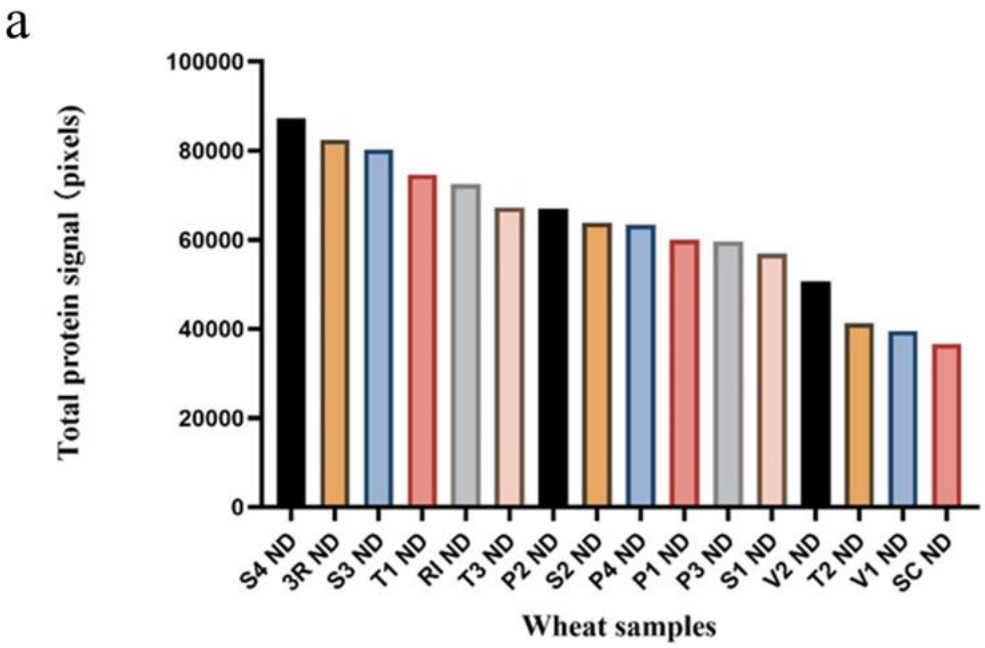


Figure 17. Total protein signal values of different wheat samples before and after partial digestion in the second year of the experiment. For each variety, the undigested (ND) and partially digested (D) total protein signal values were shown.



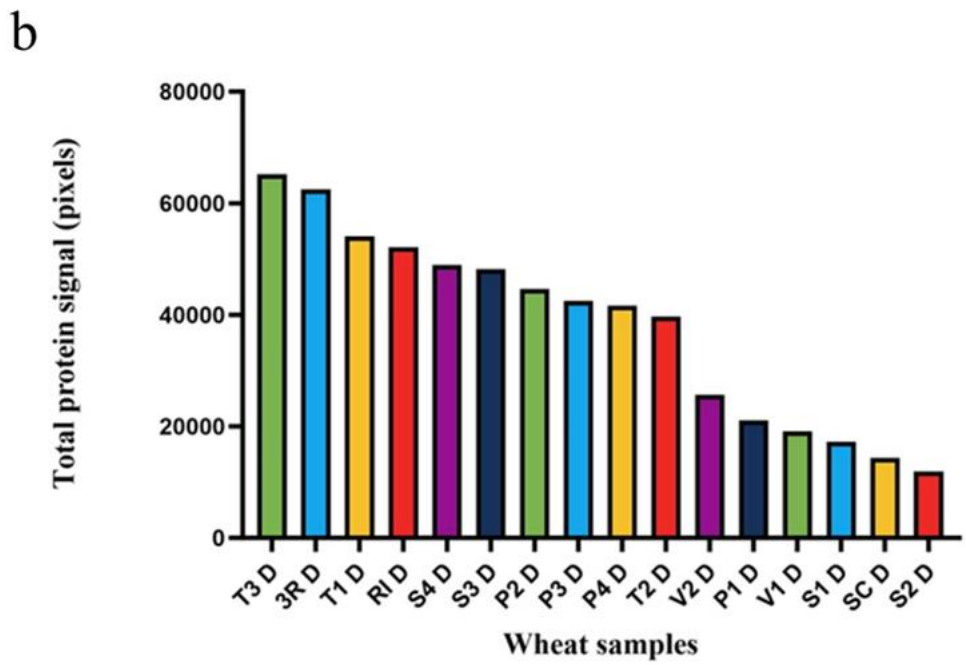


Figure 18. Ranking of total protein signal values of different wheat varieties before and after digestion in the second year experiment. (a): undigested (ND) samples; (b): partially digested (D) samples.

4.6 Proportions of gluten protein types analysis results

Figure 19 shows the influence of species on gluten and gluten subunit types relative to total proteins before digestion. Before digestion, all wheat varieties exhibited high gluten content, with P2 (88.38%) having the highest gluten content and T3 (74.51%) the lowest. The HMW-GS content ranged from 19.02% (P3) to 6.70% (V1). The ω-gliadins content varied between 27.63% (Senatore Cappelli) and 15.89% (S1). The LMW-GS and α/β, γ-gliadins content was the same, with P4 (38.41%) having the highest proportion and T2 (29.45%) the lowest.

Analysis of gluten subunit content in different sample groups revealed that, for gluten content, the ranking among the P group samples was P2 > P4 > P3 > P1; among the S group samples, it was S4 > S3 > S2 > S1; among the V group samples, it was V1 > V2; and among the T group samples, it was T2 > T1 > T3. For HMW-GS content, the proportions in each sample group were as follows: P3 > P2 > P4 > P1; T2 > T1 > T3; S1 > S3 > S4 > S2; V2 > V1. For α/β, γ-gliadins and LMW-GS content, the proportions were: P4 > P1 > P2 > P3; S2 > S1 > S3 > S4; V1 > V2; T3 > T1 > T2. For ω-gliadins content, the ranking was: V2 > V1; S2 > S3 > S4 > S1; P1 > P3 > P2 > P4; T1 > T2 > T3.

Overall, gluten was the major component of wheat proteins. Compared to HMW-GS and ω-gliadins, LMW-GS or α/β, γ-gliadins remained the predominant components of gluten subunits, with the highest proportions.

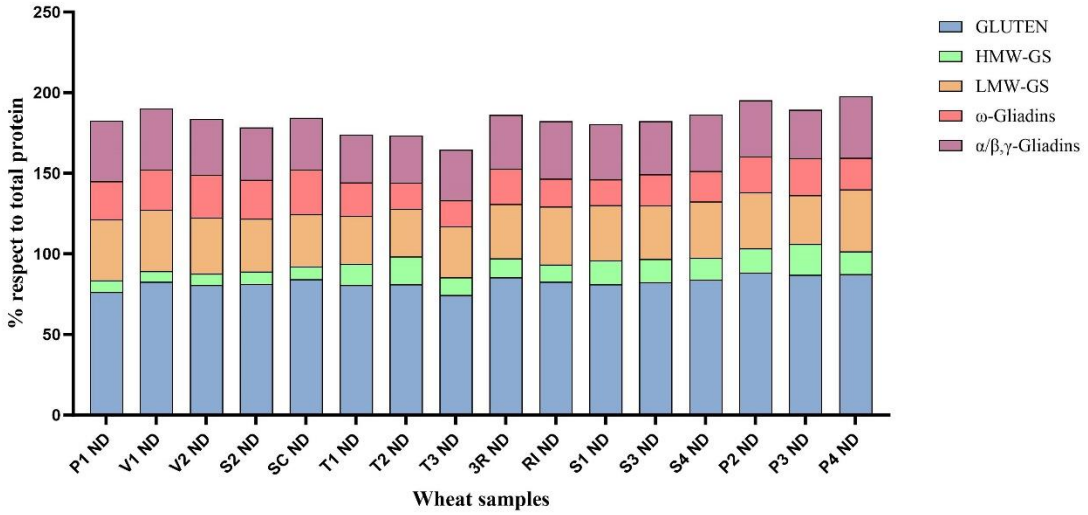


Figure 19. Proportion of gluten subunits to total protein in undigested (ND) wheat samples in the second year of the experiment. All gluten subunits proportion were reported as percentage of the total protein.

After partial digestion, gluten remained the main component of the total protein content. Among the gluten subunits, α/β, γ-gliadins, and LMW-GS were still predominant, followed by HMW-GS and ω-gliadins. The content of gluten and its subunits after partial digestion is shown in Figure 20. After partial digestion, gluten content ranged from 48.67% (V1) to 86.75% (T2). The proportions of gluten subunits, including HMW-GS, LMW-GS or α/β, γ-gliadins, and ω-gliadins, were 4.12% (V2) to 17.17% (P3), 17.99% (V1) to 43.04% (T2), and 9.41% (3R) to 18.78% (Senatore Cappelli), respectively.

A detailed analysis of the digested sample groups revealed the following rankings for gluten content: for the T group samples, T2 > T3 > T1; for the S group samples, S4 > S3 > S1 > S2; for the P group samples, P2 > P3 > P4 > P1; and for the V group samples, V2 > V1. For HMW-GS content, the proportions were P3 > P4 > P2 > P1; S1 > S4 > S3 > S2; T2 > T1 > T3; and V2 > V1. For α/β, γ-gliadins and LMW-GS content, the proportions were T2 > T3 > T1; S4 > S3 > S1 > S2; P1 > P4 > P2 > P3; and V2 > V1. For ω-gliadins content, the rankings were P2 > P1 > P3 > P4; S4 > S2 > S3 >

S1; V2 > V1; and T2 > T3 > T1. Therefore, the total gluten content and its subunits in wheat samples exhibited varying degrees of decrease after partial digestion.

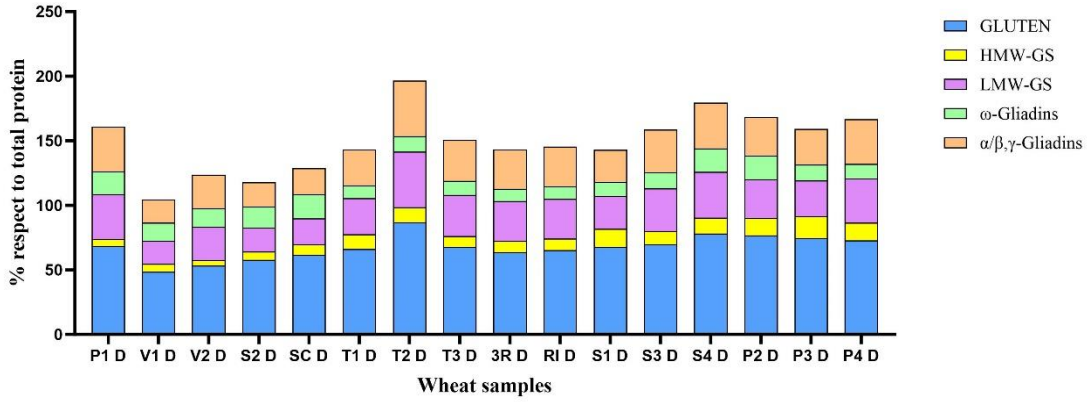


Figure 20. Proportion of gluten subunits to total protein in partially digested (D) wheat samples in the second year of the experiment. All gluten subunits proportion were reported as percentage of the total protein.

4.7 Gliadins/Glutenins and HMW-GS/LMW-GS results

Table 8 summarizes the Gliadins/Glutenins and HMW-GS/LMW-GS ratios before and after digestion of wheat proteins from the second year season. The Gliadins/Glutenins ratios of different wheat varieties fluctuated significantly before and after digestion. Before digestion, the Gliadins/Glutenins ratios ranged from 0.54 (T2) to 1.49 (Senatore Cappelli). The ratios for each sample series were as follows: V series: V2 (1.46) > V1 (1.41); S series: S2 (1.40) > S4 (1.11) > S3 (1.09) > S1 (0.71); P series: P1 (1.36) > P2 (1.14) > P4 (1.10) > P3 (1.08); T series: T1 (1.18) > T3 (0.89) > T2 (0.54). After partial digestion, the Gliadins/Glutenins ratios ranged from 0.89 (P3) to 1.41 (S2). The ratios for each sample series were as follows: S series: S2 (1.41) > S3 (1.16) > S4 (1.12) > S1 (0.95); V series: V2 (1.33) = V1 (1.33); P series: P1 (1.29) > P2 (1.11) > P4 (0.95) > P3 (0.89); T series: T1 (0.96) > T3 (0.90) > T2 (0.89). The HMW-GS/LMW-GS ratios exhibited smaller differences before and after digestion. Before digestion, the HMW-GS/LMW-GS ratios ranged from 0.18 (V1) to 0.63 (P3). The ratios for each sample series were as follows: P series: P1 (0.2) to 0.63 (P3); T series: 0.34 (T3) to 0.55 (T2); S series: 0.24 (S2) to 0.43 (S3); V series: 0.18 (V1) to 0.21 (V2). After digestion, the HMW-GS/LMW-GS ratios ranged from 0.16 (V2) to 0.62 (P3). The ratios

for each sample series were as follows: P series: 0.17 (P1) to 0.62 (P3); S series: 0.31 (S3) to 0.56 (S1); T series: 0.26 (T3) to 0.41 (T1); V series: 0.16 (V2) to 0.33 (V1).

Comparing the changes in ratios before and after digestion, it was found that for Gliadins/Glutenins, seven varieties (S2, T2, T3, Russello, S1, S3, S4) showed an increasing trend after digestion, while the remaining varieties showed a decreasing trend. For HMW-GS/LMW-GS, six varieties (V1, S2, Senatore Cappelli, S1, P2, P4) showed an increasing trend, while the remaining varieties showed a decreasing trend. Additionally, the Gliadins/Glutenins and HMW-GS/LMW-GS ratios exhibited varying changes within the wheat groups before and after digestion. For the Gliadins/Glutenins ratio, the S series showed an increasing trend after digestion, while the T series samples (except T1) also showed an increasing trend. T1 exhibited the largest decrease, and T2 showed the largest increase. The P and V series exhibited varying degrees of increase. Regarding the HMW-GS/LMW-GS ratio, P2 and P4 in the P series increased, while P1 and P3 decreased, with P4 showing the largest increase and P1 the largest decrease. In the V series, V1 increased to 0.33, while V2 decreased. In the S series, S1 and S2 increased, while S3 and S4 decreased, with S1 showing the largest increase and S3 the largest decrease. The T series showed a decreasing trend, with T2 showing the largest decrease.

Overall, the ratios of Gliadins/Glutenins and HMW-GS/LMW-GS exhibited varying degrees of increase or decrease after partial digestion, but most samples showed a decrease in these ratios after digestion.

Table 8. The Gliadins/Glutenins and HMW-GS/LMW-GS of different wheat samples before and after partial digestion in the second year of the experiment (ND: undigested, D: partially digested).

Sample	Gliadins/Glutenins		HMW-GS/LMW-GS	
	ND	D	ND	D
P1	1.36	1.29	0.20	0.17
V1	1.41	1.33	0.18	0.33
V2	1.46	1.33	0.21	0.16
S2	1.40	1.41	0.24	0.34
SC	1.49	1.39	0.25	0.39
T1	1.18	0.96	0.44	0.41
T2	0.54	0.90	0.59	0.27
T3	0.89	1.15	0.34	0.26
3R	1.00	0.97	0.36	0.29
Russello	0.88	1.23	0.30	0.29
S1	0.71	0.95	0.43	0.56
S3	1.09	1.16	0.43	0.31
S4	1.11	1.12	0.38	0.34
P2	1.14	1.11	0.43	0.45
P3	1.08	0.89	0.63	0.62
P4	1.10	0.95	0.37	0.39

4.8 Effects of wheat proteins on L929 proliferation

Figures 21 shows the second year L929 cell viability expressed as a relative percentage compared with the untreated control cells. Before and after digestion, all samples exhibited lower viability in L929 cells compared to control cells. Prior to digestion (Figure 21a), the viability of L929 cells was highest in V1 (81.28%) and lowest in P3(46.45%), except for S3, T1, and T3, all ancient wheat varieties showed significant differences compared to the control group. This indicated that S3, T1 and T3 had lower inhibitory effects on the proliferation of L929 cells than other varieties. After partial digestion (Figure 21b), the viability of L929 cells was the highest in V2 (83.62%) and the lowest in T3(54.20%), except for V2, S3, and P1, all ancient wheat varieties exhibited significant differences, suggesting that V2, S3, and P1 had lower inhibitory effects on the proliferation of L929 cells post-digestion compared to the other varieties. Additionally, digestion affected the effects of wheat proteins on cells proliferation. Post-digestion, the wheat varieties that inhibited L929 cells proliferation included V1, S1, S2, T1, T2, T3, and Russello. The varieties that promoted the proliferation of L929 cells were V2, Senatore Cappelli, S4, P1, P3, and P4. Moreover, S3 had no effect on L929 cell proliferation, and 3R and P2 maintain the same L929 cell proliferation effects

after digestion.

Different wheat varieties showed varied effects on L929 cell proliferation before and after digestion. The partially digested wheat proteins from different series had different impacts on L929 cell proliferation. After partial digestion, V1 in the V series inhibited the proliferation of L929 cells, while V2 did the opposite. In S series, S1 and S2 inhibited L929 cell proliferation, S4 promoted it, and S3 had no significant difference. All varieties in the T series inhibited the proliferation of L929 cells. In the P series, P1, P3, and P4 promoted L929 cell proliferation, while P2 exhibited the same proliferation effect as before digestion.

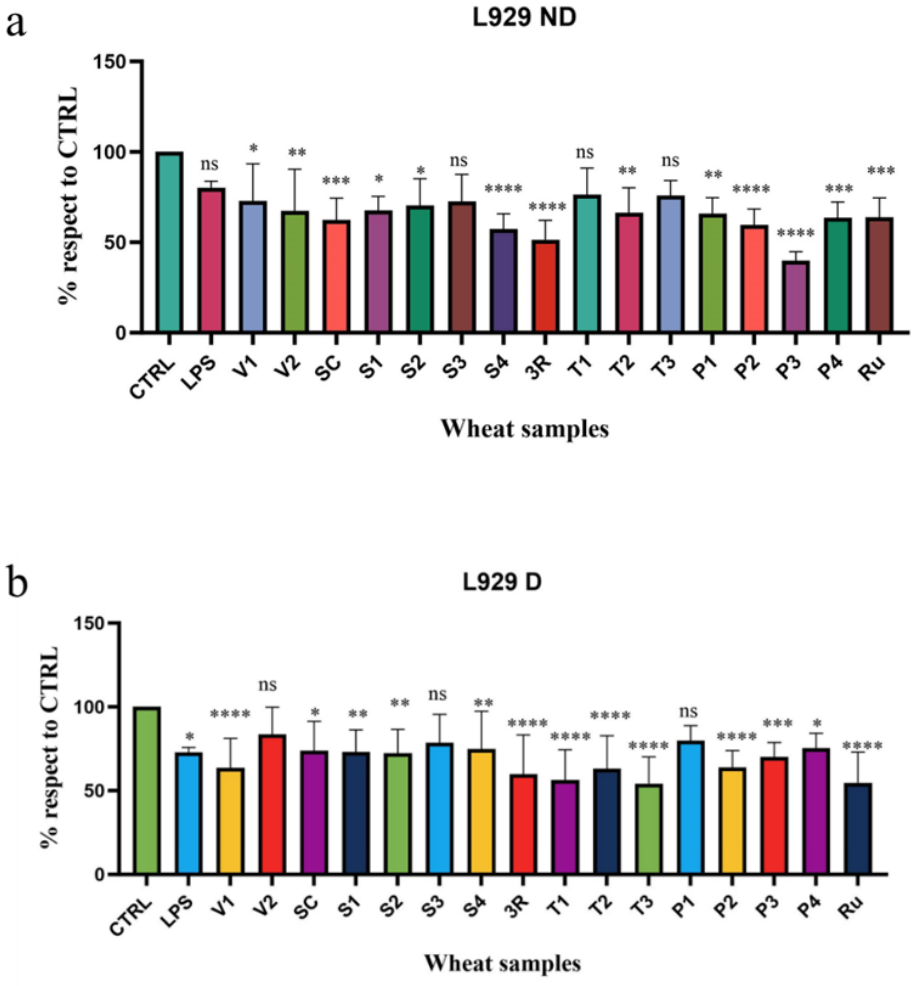


Figure 21. The effects of undigested (a) and partially digested (b) wheat proteins on the proliferation of L929 cells. All treatments were compared to the untreated control (CTRL). Significant differences between treatments were reported as follows: * $0.01 < p < 0.05$, ** $0.001 < p < 0.01$, *** $0.0001 < p < 0.001$, **** $p < 0.0001$. ND: undigested, D: partially digested.

4.9 Effects of wheat proteins on Caco2 proliferation

Figures 22 shows the relative percentage of Caco2 cell viability in the second year compared to the untreated control cells. The results showed that the survival rate of Caco2 cells was significantly lower compared to control cells both before and after digestion. Before digestion (Figure 22a), the viability of Caco2 cells was highest in P1 (84.33%) and lowest in Russello (59.84%). 11 ancient wheat varieties (V1, V2, Senatore Cappelli, S1, S4, 3R, T2, P2, P3, P4, Russello) exhibited significant differences compared to the control group, while the other varieties did not show significant differences. This indicated that the original proteins of these 11 ancient wheat varieties exhibited a stronger inhibitory effect on Caco2 cell proliferation compared to other ancient varieties. After partial digestion (Figure 22b), the viability of Caco2 cells was the highest in P4 (89.37%) and the lowest in V1 (54.24%), varieties V1, Senatore Cappelli, S2, S4, 3R, T1, T2, T3, P2, and Russello showed significant differences, indicating that the inhibitory effect of these varieties on Caco2 cell proliferation was higher than that of other 6 ancient wheat varieties.

Additionally, digestion affected the proliferation effects of wheat proteins on Caco2 cells. After partial digestion, a small number of wheat proteins promoted the proliferation of Caco2 cells, such as V2, S1, S4, 3R, P2, P3, and P4. However, the effects of some wheat proteins were opposite, including V1, Senatore Cappelli, S2, T1, T3, and Russello. Only T2 did not change the proliferation effect of Caco2 before and after digestion. and S3 and P1 had no effect on Caco2 cell proliferation. Different wheat varieties had different proliferation effects on Caco2 cells before and after digestion. After partial digestion, In the V series, V1 inhibited Caco2 cell proliferation, while V2 had the opposite effect. In the S series, S2 inhibited Caco2 cell proliferation, while S1 and S4 promoted cell proliferation, S3 showed no significant difference. In the T series, T1 and T3 inhibited Cco2 cell proliferation, while the cell proliferation effect of T2 remained unchanged. In the P series, P2, P3 and P4 all promoted the proliferation of Caco2 cells, but P1 had no significant difference.

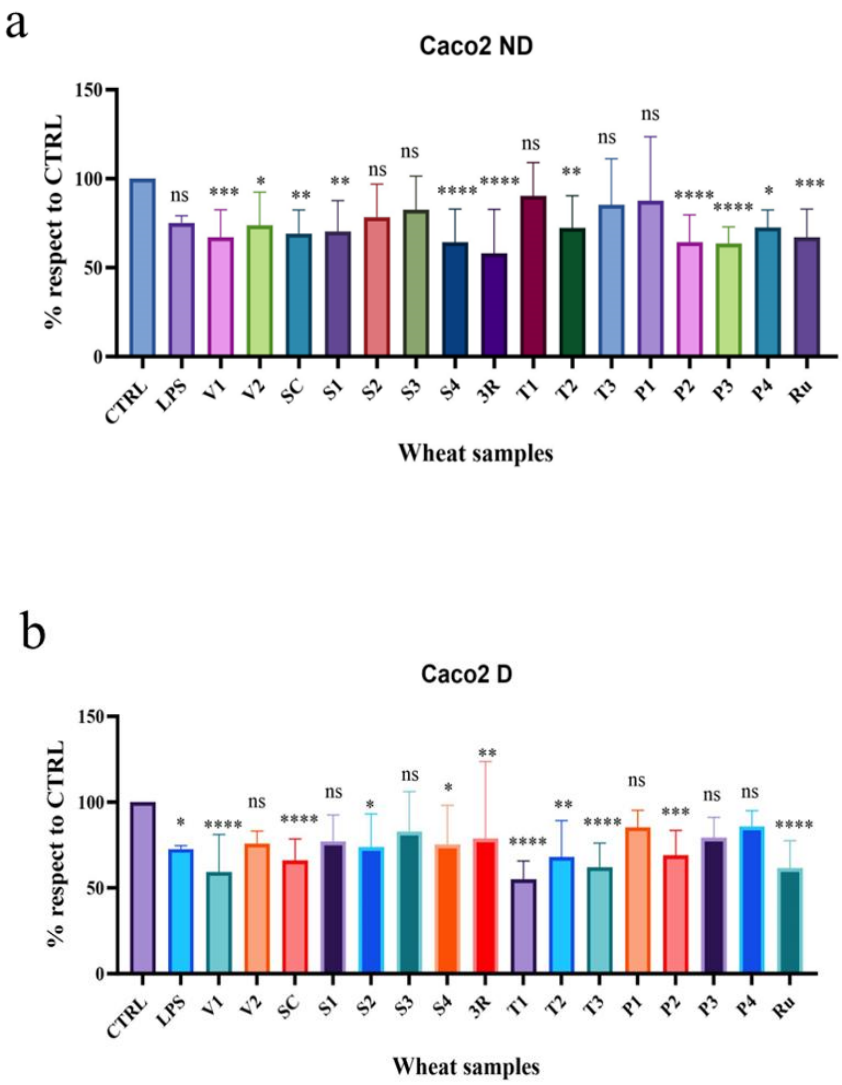


Figure 22. The effects of undigested (a) and partially digested (b) wheat proteins on the proliferation of Caco2 cells. All treatments were compared to the untreated control (CTRL). Significant differences between treatments were reported as follows: * $0.01 < p < 0.05$, ** $0.001 < p < 0.01$, *** $0.0001 < p < 0.001$, **** $p < 0.0001$. ND: undigested, D: partially digested.

5. The results of the third year of the experiment

5.1 Phenolic and flavonoid content and antioxidant activity of wheat flour

Phenolic contents of the tested wheat genotypes are presented in Table 9, expressed as micromoles of gallic acid equivalent (GAE) per 100 g of grain. The FPC, BPC and TPC of all the samples ranged from 8.33 GAE mg/100 g (P2) to 25.39 GAE mg/100 g (S1), 88.55 GAE mg/100 g (Lucana) to 125.52 GAE mg/100 g (Senatore Cappelli), 157.69 GAE mg/100 g (Lucana) to 216.15 GAE mg/100 g (Rhettfah), respectively. However, no statistical difference was observed in FPC and BPC results between all wheat varieties. Significantly different values only in TPC were observed for Rhettfah and Lucana among all other wheat samples ($p < 0.05$).

Flavonoid contents of tested wheat varieties are expressed as micromoles of catechin equivalent (CE) per 100 g of grain (Table 9). The FFC of all the samples ranged from 52.70 CE mg/100 g in P1 to 90.17 CE mg/100 g in Rhettfah. Significantly different values in FCC were observed for Rhettfah and P1, Russello, S4, V1 Benedettelli among all other wheat samples ($p < 0.05$). The BFC of all the samples ranged from 28.49 CE mg/100 g in S3 to 40.86 CE mg/100 g in Margherito, however, no statistical difference was observed in BFC results between all wheat varieties. And TFC of all the samples ranged from 82.81 CE mg/100 g in P1 to 128.88 CE mg/100 g in Rhettfah, significantly different values only in TFC were observed for Rhettfah and P1 among all other wheat samples ($p < 0.05$).

The antioxidant activity values of the four cultivars determined by the FRAP assay ranged from 0.81 mmol Fe^{2+}/100 g in Benedettelli to 1.10 mmol Fe^{2+}/100 g in Pugliese. And no statistical difference was observed in FRAP results between all wheat varieties (Table 9).

Regarding DPPH assay, Rhettfah (3.69 µmol TE/g) showed the best antioxidant activity and Russello (2.46 µmol TE/g) showed the worst antioxidant Activity compared to the remaining cultivars (Table 9). The DPPH values were significantly different among Rhettfah and Russello ($p < 0.05$), and there was no statistical difference among the rest 20 wheat varieties.

Table 9. Main quality parameters of wheat varieties planted in in the third year. Different letters in the same row indicate significant differences ($p < 0.05$).

Sample	FPC (GAE mg/100g)	BPC (GAE mg/100g)	TPC (GAE mg/100g)	FFC (CE mg/100g)	BFC (CE mg/100g)	TFC (CE mg/100g)	FRAP (mmol Fe^{2+}/100g)	DPPH (umol TE/g)
P1	12.68±5.17^{a}	111.91±20.76^{a}	193.31±27.26^{ab}	52.70±15.49^{c}	30.11±5.23^{a}	82.81±16.83^{b}	0.86±0.08^{a}	2.55±0.48^{ab}
P2	8.33±3.40^{a}	111.24±23.97^{a}	189.17±27.53^{ab}	69.72±9.35^{abc}	32.00±2.42^{a}	101.72±11.50^{ab}	0.95±0.14^{a}	2.92±0.44^{ab}
P3	12.03±4.91^{a}	119.87±22.01^{a}	210.21±25.38^{ab}	73.65±9.08^{abc}	36.07±7.62^{a}	109.72±5.60^{ab}	0.99±0.21^{a}	3.10±0.55^{ab}
P4	15.31±6.25^{a}	111.87±12.34^{a}	198.03±9.77^{ab}	68.61±5.93^{abc}	30.10±4.81^{a}	98.72±6.76^{ab}	0.94±0.12^{a}	2.88±0.34^{ab}
3R	14.47±5.91^{a}	111.49±19.27^{a}	204.48±29.07^{ab}	67.54±25.86^{abc}	34.62±8.85^{a}	102.15±33.39^{ab}	0.85±0.18^{a}	2.83±0.66^{ab}
RUSSELLO IBLEO	12.59±5.14^{a}	103.48±12.91^{a}	198.62±17.82^{ab}	56.75±6.92^{bc}	34.66±7.16^{a}	91.42±11.54^{ab}	0.88±0.21^{a}	2.46±0.81^{b}
S1	25.39±10.37^{a}	115.41±11.04^{a}	200.73±24.81^{ab}	73.69±7.19^{abc}	28.68±6.02^{a}	102.37±11.28^{ab}	1.00±0.16^{a}	2.91±0.50^{ab}
S2	25.10±10.25^{a}	117.05±31.94^{a}	195.87±47.43^{ab}	63.70±10.42^{abc}	31.74±10.04^{a}	95.44±18.31^{ab}	0.92±0.19^{a}	2.93±0.42^{ab}
S3	10.99±4.49^{a}	107.95±21.81^{a}	204.73±16.77^{ab}	73.94±12.82^{abc}	28.49±9.77^{a}	102.43±19.40^{ab}	0.95±0.15^{a}	2.64±0.60^{ab}
S4	15.12±6.17^{a}	107.99±18.15^{a}	196.43±8.63^{ab}	54.16±11.66^{c}	34.94±11.73^{a}	89.10±12.16^{ab}	0.88±0.13^{a}	2.78±0.27^{ab}
SARAGOLLA LUCANA	11.98±5.99^{a}	88.55±14.67^{a}	157.69±16.25^{b}	67.35±7.77^{abc}	34.99±10.09^{a}	102.33±12.69^{ab}	0.87±0.15^{a}	2.67±0.34^{ab}
SARAGOLLA PUGLIESE	8.57±4.28^{a}	104.60±15.96^{a}	207.49±9.04^{ab}	84.56±11.05^{ab}	33.73±5.88^{a}	118.29±12.59^{ab}	1.10±0.16^{a}	3.45±0.54^{ab}
SENATORE CAPPELLI	19.08±7.79^{a}	125.52±17.48^{a}	205.66±27.03^{ab}	68.90±12.30^{abc}	38.45±6.85^{a}	107.35±14.72^{ab}	0.96±0.12^{a}	3.09±0.60^{ab}
T1	10.31±4.21^{a}	115.28±18.20^{a}	196.39±16.93^{ab}	70.96±11.32^{abc}	32.02±6.51^{a}	102.98±12.24^{ab}	0.94±0.14^{a}	2.99±0.23^{ab}
T2	10.11±4.13^{a}	103.15±6.75^{a}	190.41±15.31^{ab}	73.27±12.95^{abc}	28.75±5.60^{a}	102.02±16.67^{ab}	0.88±0.13^{a}	2.70±0.45^{ab}
T3	12.23±4.99^{a}	107.99±17.70^{a}	185.73±18.59^{ab}	67.35±13.39^{abc}	31.16±6.86^{a}	98.50±18.62^{ab}	0.86±0.11^{a}	2.55±0.42^{ab}
V1	8.43±3.44^{a}	110.52±15.84^{a}	181.35±21.76^{ab}	59.10±13.71^{bc}	32.91±10.79^{a}	92.01±21.65^{ab}	0.82±0.14^{a}	2.82±0.31^{ab}
V2	24.53±10.01^{a}	121.22±25.61^{a}	211.10±42.92^{ab}	77.24±20.41^{abc}	35.76±16.30^{a}	113.00±36.46^{ab}	0.92±0.25^{a}	3.02±0.45^{ab}
VAR. ANTALIS	11.32±4.62^{a}	111.40±16.19^{a}	186.32±21.92^{ab}	69.06±10.32^{abc}	32.33±10.23^{a}	101.38±19.38^{ab}	0.91±0.21^{a}	2.89±0.67^{ab}

VAR. MARGHERITO	13.97±5.70[a]	119.87±16.70[a]	205.58±8.47[ab]	61.29±8.34[abc]	40.86±11.52[a]	102.15±14.94[ab]	1.02±0.14[a]	3.19±0.72[ab]
MIX BENEDETTELLI	15.55±11.00[a]	100.24±19.13[a]	191.12±3.58[ab]	57.17±7.13[bc]	30.64±6.05[a]	87.81±13.18[ab]	0.81±0.14[a]	2.77±0.44[ab]
RHETTFAH	8.58±6.07[a]	123.12±20.02[a]	216.15±11.44[a]	90.17±3.50[a]	38.71±3.77[a]	128.88±7.27[a]	0.88±0.16[a]	3.69±0.21[a]

5.2 Results of protein expression in wheat samples before and after partial digestion

In the undigested state, the wheat proteins of 22 ancient wheat varieties and the modern variety Claudio exhibited multiple polypeptide bands. These peptides or polypeptide groups represented the main components of wheat proteins, concentrated at 72-95 kDa, 55-72 kDa, 28-55 kDa, and <28 kDa, corresponding to gluten subunits: HMW-GS, ω-gliadins, α/β- and γ-gliadins (also LMW-GS), and non-gluten proteins, respectively.

Specifically, in Figure 23a, weak HMW-GS polypeptide bands between 28 kDa and 95 kDa were observed in all undigested (ND) samples. After partial digestion (D), the intensity of gluten subunits decreased in all varieties. Except for P1, which did not show significant HMW-GS bands, other varieties (P2, P3, P4, 3R) displayed faint HMW-GS and ω-gliadins bands. The intensity of LMW-GS (36-55 kDa) decreased markedly, and some accumulation was noted in the non-gluten protein region. In Figure 23b, no significant HMW-GS and ω-gliadins bands were observed in any wheat proteins before and after digestion. However, the intensity of LMW-GS polypeptide bands weakened after digestion, with notable accumulation in the non-gluten protein region. In Figure 23c, Lucana and Pugliese wheat proteins showed only a decrease in ω-gliadins and LMW-GS before and after digestion, without HMW-GS bands. Senatore Cappelli was unique, showing distinct polypeptide bands only in the LMW-GS region before and after digestion. T1, T2, and T3 followed the pattern in Figure 23a, with reduced gluten subunit intensity after digestion but still faint HMW-GS and ω-gliadins bands, and strong LMW-GS bands with some accumulation in the non-gluten protein region. In Figure 23d, the patterns of V1 and V2 wheat proteins were consistent with Senatore Cappelli in Figure 23c, showing weakened polypeptide bands only in the LMW-GS region. The remaining samples (Antalis, Margherito, Benedettelli, Rhettfah, Claudio) followed the patterns of Lucana and Pugliese in Figure 23c, with weakened bands in the ω-gliadins and LMW-GS, and accumulation of non-gluten proteins in some samples.

Electrophoretic profiles indicated substantial differences in gluten composition among wheat varieties grown in the third year. HMW-GS subunits were present only in the P group, 3R, and T group wheat proteins, whereas S group, Senatore Cappelli, and V group wheat proteins exhibited only LMW-GS subunits. Other varieties comprised ω-gliadins and LMW-GS. After digestion, all varieties showed reduced gluten protein intensity, with accumulation of non-gluten proteins in some samples.

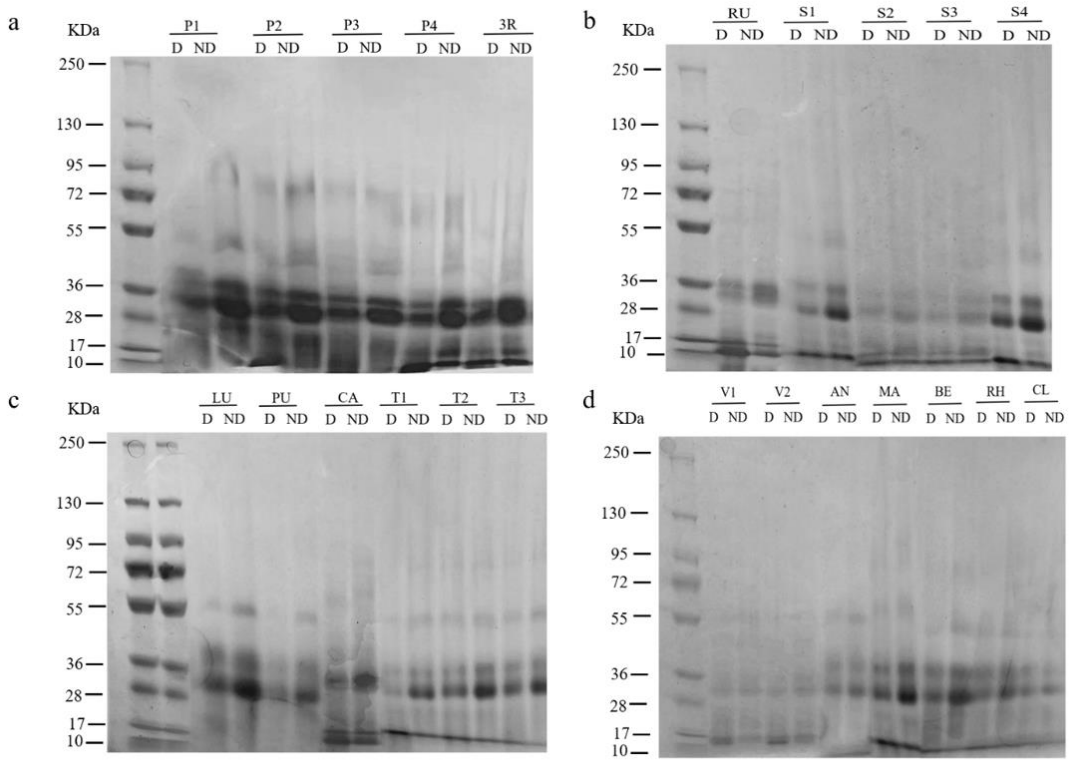


Figure 23. Photographs pictures of the SDS-PAGE gels. In the first lane of each picture was the PageRuler Plus Prestained Protein Ladder marker with the molecular weights in kDa indicated. The remaining lanes represented the protein bands of different wheat varieties before and after partial digestion. For each variety, the undigested (ND) and partially digested (D) protein bands were shown. From left to right, the lanes of: P1 D and ND, P2 D and ND, P3 D and ND, P4 D and ND, 3R D and ND (a); RU D and ND, S1 D and ND, S2 D and ND, S3 D and ND, S4 D and ND (b); LU D and ND, PU D and ND, CA D and ND, T1 D and ND, T2 D and ND, T3 D and ND (c); V1 D and ND, V2 D and ND, AN D and ND, MA D and ND, BE D and ND, RH D and ND, CL D and ND (d). LU is Lucana, PU is Pugliese, CA is Senatore Cappelli, AN is Antalis, MA is Margherito, BE is Benedettelli, RH is Rhettfah, and Cl is Claudio, the same below.

5.3 Calculation results of the molecular weight of protein bands in wheat samples

Table 10 presents the polypeptide composition of wheat proteins before and after digestion for the third year of planting. During the third year growing season, before digestion, the molecular weight of HMW-GS in different wheat varieties ranged from 71.39 kDa (3R) to 103.08 kDa (T3). The molecular weight of ω-gliadins ranged from 47.54 kDa (3R) to 57.68 kDa (MA). LMW-GS had the greatest band intensity compared to other protein components, primarily distributed between 28.06

kDa (P4) and 40.76 kDa (P1). After digestion, the molecular weight of HMW-GS in different wheat varieties ranged from 65.95 kDa (3R) to 96.16 kDa (T3), and the molecular weight of ω-gliadins ranged from 47.87 kDa (P3) to 56.84 kDa (MA). The band intensity of LMW-GS remained the highest, primarily distributed between 28.06 kDa (P4) and 42.16 kDa (S1).

Specifically, before digestion, the P group and T group each had four typical bands, including one faint HMW-GS band, one faint ω-gliadins band, and two intense LMW-GS or α/β and γ-gliadins bands. The Russello sample had only one intense LMW-GS band. The S group and V group each had only two faint LMW-GS bands. Other varieties exhibited three typical bands, including one faint ω-gliadins band and two intense LMW-GS bands. After digestion, the number of polypeptides in each sample did not change significantly. Except for the absence of HMW-GS in the P1 sample, the protein composition and band number in other samples remained consistent with the pre-digestion state. However, the intensity of gluten proteins weakened after digestion, with differences in the intensity and number of protein bands among different varieties.

Table 10. Presence of gluten in different wheat varieties produced in the third year of experiment (ND: undigested, D: partially digested).

Wheat varieties								
Band (kDa)	P1 ND	P2 ND	P3 ND	P4 ND	3R ND	Russello ND	S1 ND	S2 ND
1	93.39	84.51	85.69	73.89	71.39	34.66	41.99	37.94
2	53.08	52.36	47.87	48.37	47.54		34.71	32.00
3	40.76	36.62	34.09	34.91	33.96			
4	32.38	29.08	28.17	28.06	29.90			
Band (kDa)	S3 ND	S4 ND	Lucana ND	Pugliese ND	Senatore Cappelli ND	T1 ND	T2 ND	T3 ND
1	37.03	36.29	52.33	48.17	31.26	85.24	90.53	103.08
2	30.98	33.06	38.73	37.04		48.19	48.41	52.14
3			30.86	30.36		34.27	34.80	37.24
4						29.92	30.39	31.34
Band (kDa)	V1 ND	V2 ND	Antalis ND	Margherito ND	Benedettelli ND	Rhettfah ND	Claudio ND	
1	34.45	35.33	53.10	57.68	50.09	49.36	48.41	
2	31.35	31.35	35.85	34.99	34.43	34.15	34.15	
3			28.69	28.46	28.11	28.46	29.04	
Wheat varieties								
Band (kDa)	P1 D	P2 D	P3 D	P4 D	3R D	Russello D	S1 D	S2 D
1	55.71	85.69	83.93	72.13	65.95	38.47	42.16	40.00
2	39.51	52.54	47.87	48.20	47.71	32.74	35.13	33.46
3	34.09	37.95	35.19	32.50	33.82			
4		30.26	28.85	28.06	28.74			
Band (kDa)	S3 D	S4 D	Lucana D	Pugliese D	Senatore Cappelli D	T1 D	T2 D	T3 D
1	37.33	36.88	54.67	50.33	31.26	83.28	90.11	96.16
2	31.49	32.13	40.66	37.95		47.97	48.86	50.01
3			32.53	30.99		34.06	34.80	35.67
						29.56	29.74	30.67
Band (kDa)	V1 D	V2 D	Antalis D	Margherito D	Benedettelli D	Rhettfah D	Claudio D	
1	35.33	34.67	53.36	56.84	50.33	49.12	48.65	
2	30.96	30.96	36.89	35.28	34.71	34.15	34.15	
3			29.76	28.57	28.11	28.11	29.16	

5.4 Results of the total protein signal in wheat samples

Figure 24 displays the total protein signals of different wheat samples in the third year of experiment. It was observed that all wheat varieties exhibited high protein signal values in the undigested state. After partial digestion, the total protein signal values of all samples showed varying degrees of decline, with the greatest decrease observed in P2 and the smallest in Antalis. The total protein signal values of different wheat varieties before and after digestion were ranked and normalized against the Claudio, as shown in Figure 25. Notably, both before and after digestion, the total protein signal values of the 22 ancient wheat varieties were higher than those of the modern wheat Claudio.

Before digestion (Figure 25a), the highest total protein signal value was observed in Senatore Cappelli (425,391 pixels), and the lowest in Antalis (88,392 pixels), with a 4.81-fold difference between them. Among the S group samples, S3 had the highest total protein signal value and S1 the lowest. Within the P group, P3 had the highest total protein signal value and P4 the lowest. In the T group, T2 had the highest total protein signal value and T3 the lowest. Finally, in the V group, V2 had a higher total protein signal value than V1. After partial digestion (Figure 25b), Senatore Cappelli had the highest total protein signal value (342,477 pixels) and Pugliese the lowest (58,098 pixels), with a 5.89-fold difference between them. The trend in total protein signal values varied among different wheat series after digestion. In the S group, S4 had the highest total protein signal value and S1 the lowest. Within the P group, P1 had the highest total protein signal value and P2 the lowest. In the T group, T2 had the highest total protein signal value and T1 the lowest. In the V group, V1 had a higher total protein signal value than V2.

In conclusion, the total protein signal of wheat always decreased after partial digestion, and the extent of the decrease varied among different wheat varieties. However, the total signal values of ancient wheat varieties were consistently higher than those of the modern variety Claudio, both before and after digestion.

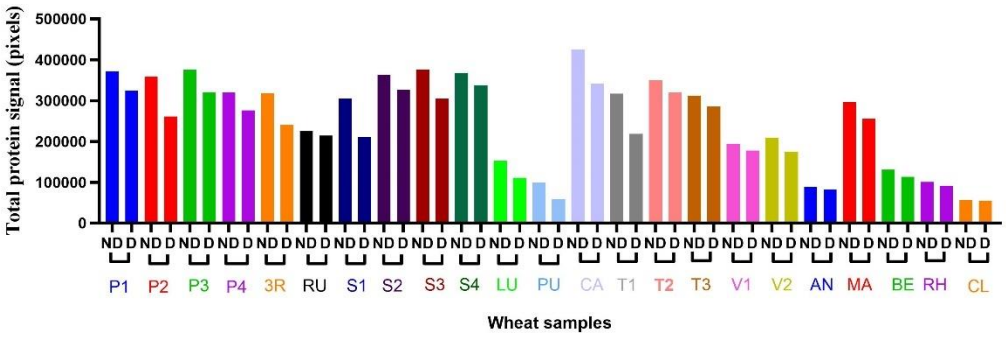


Figure 24. Total protein signal values of different wheat samples before and after partial digestion in the third year of the experiment. For each variety, the undigested (ND) and partially digested (D) total protein signal values were shown.

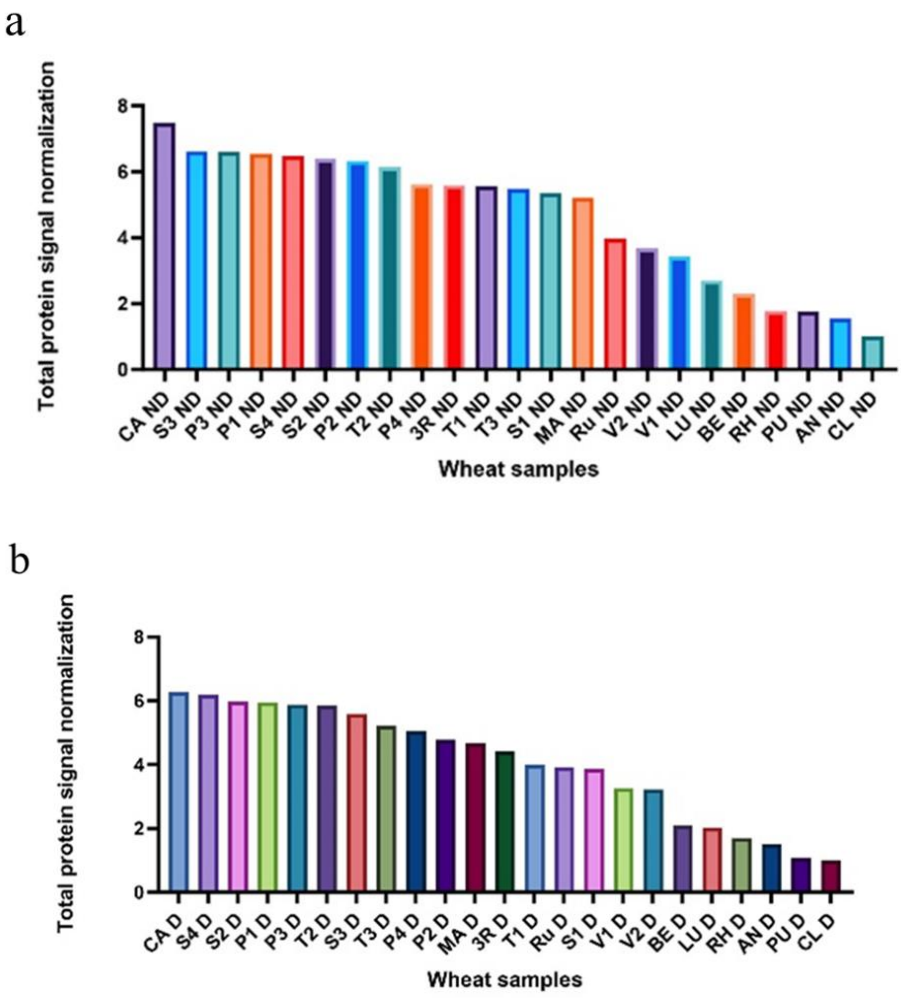


Figure 25. The ranking and normalization of total protein signal values before and after digestion for different wheat varieties in the third year of the experiment. All data had been normalized with respect to the Claudio variety as the baseline. (a): undigested (ND) samples; (b): partially digested (D) samples.

5.5 Proportions of gluten protein types analysis results

Figure 26 shows the influence of species on gluten and gluten subunits types relative to total proteins before digestion in the third year of experiment. Before digestion, all wheat varieties exhibited high gluten content, with T3 showing the highest gluten content at 85.38%, and S2 showing the lowest at 64.90%. The HMW-GS content ranged from 3.08% (Lucana) to 14.77% (P2). The ω-Gliadins content varied between 7.2% (Margherito) and 16.07% (V1). The LMW-GS and α/β, γ-Gliadins had identical content, with Lucana having the highest proportion at 54.64%, and V1 the lowest at 17.63%. Analysis of glutenin subunit content among different wheat groups revealed the following rankings for gluten content: within the T group, T3 > T2 > T1; within the P group, P1 > P2 > P4 > P3; within the S group, S4 > S1 > S3 > S2; and within the V group, V1 > V2. For HMW-GS content, the rankings were: P2 > P1 > P4 > P3; T3 > T1 > T2; S4 > S1 > S2 > S3; and V1 > V2. For α/β, γ-Gliadins and LMW-GS content, the rankings were: T2 > T3 > T1; P1 > P4 > P3 > P2; S4 > S1 > S2 > S3; and V2 > V1. Regarding ω-Gliadins content, the rankings were: V1 > V2; P2 > P4 > P1 > P3; T3 > T1 > T2; and S2 > S3 > S4. Overall, glutenins constituted the primary component of wheat proteins, with LMW-GS or α/β, γ-Gliadins being the major subunits, followed by ω-Gliadins, and HMW-GS representing the smallest proportion.

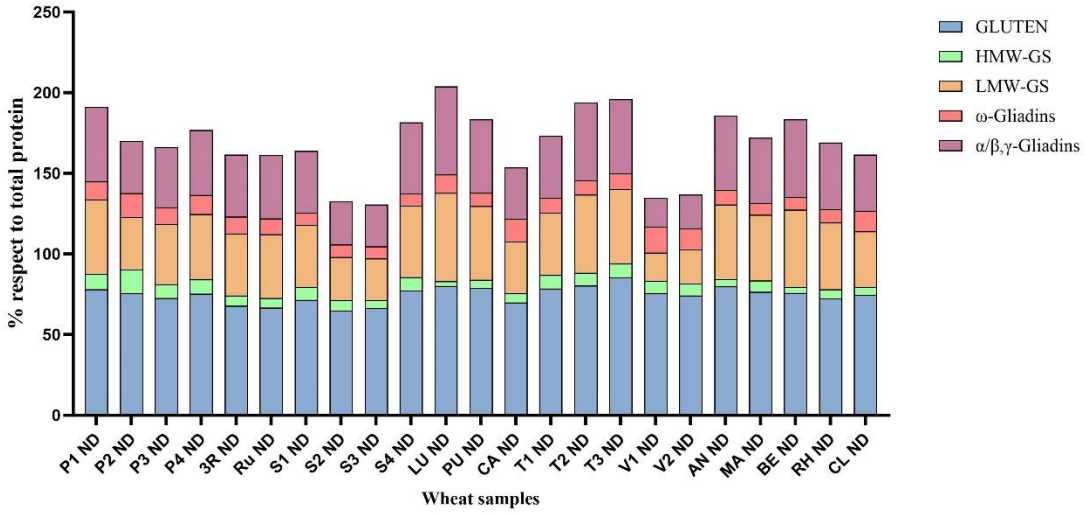


Figure 26. Proportion of gluten subunits to total protein in undigested (ND) wheat samples in the third year of the experiment. All gluten subunits proportion were reported as percentage of the total protein.

After partial digestion, gluten remained the main component of the total protein content, with α/β, γ-Gliadins, and LMW-GS still being the predominant subunits, followed by HMW-GS and ω-Gliadins. The gluten and its subunit contents after partial digestion are shown in Figure 27. Following partial digestion, the gluten content ranged from 85% (Claudio) to 55.26% (Senatore Cappelli). The proportions of gluten subunits, specifically HMW-GS, LMW-GS or α/β, γ-Gliadins, and ω-Gliadins, ranged from 2.17% (Lucana) to 11.69% (P2), 18.97% (V1) to 50.32% (Lucana), and 5.37% (P1) to 13.33% (V1), respectively. A detailed analysis of the digested sample groups revealed the following rankings for gluten content: within the V group, V2 > V1; within the T group, T3 > T2 > T1; within the P group, P1 > P2 > P3 > P4; and within the S group, S4 > S1 > S2 > S3. For HMW-GS content, the rankings were: P2 > P3 > P4 > P1; S1 > S4 > S2 > S3; T1 > T3 > T2; and V2 > V1. Regarding α/β, γ-Gliadins or LMW-GS content, the rankings were: P1 > P4 > P2 > P3; T3 > T2 > T1; S4 > S3 > S1 > S2; and V2 > V1. For ω-Gliadins content, the rankings were: P2 > P1 > P3 > P4; T3 > T2 > T1; S4 > S1 > S3 > S2; and V2 > V1. Thus, the total gluten content and its subunit levels in wheat samples consistently showed varying degrees of decrease following partial digestion.

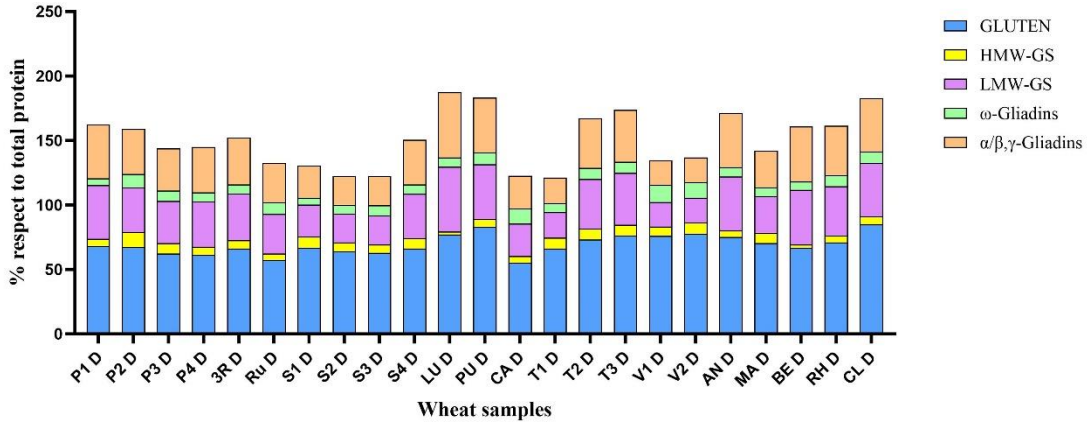


Figure 27. Proportion of gluten subunits to total protein in partially digested (D) wheat samples in the third year of the experiment. All gluten subunits proportion were reported as percentage of the total protein.

5.6 Gliadins/Glutenins and HMW-GS/LMW-GS results

Table 11 summarizes the Gliadins/Glutenins and HMW-GS/LMW-GS ratios before and after digestion of wheat proteins from the third year growing season. The Gliadins/Glutenins ratio of

different wheat varieties showed minimal fluctuation before and after digestion. Prior to digestion, the Gliadins/Glutenins ratio ranged from 0.98 (S4) to 1.34 (V1). The ratios for each sample series were as follows: V series: V1 (1.34) > V2 (1.18); S series: S3 (1.09) > S2 (1.04) > S1 (1.00) > S4 (0.98); P series: P4 (1.05) > P3 (1.04) > P1 (1.03) > P2 (1.00); T series: T1 (1.02) = T3 (1.02) > T2 (1.01). After partial digestion, the Gliadins/Glutenins ratio ranged from 0.91 (S1) to 1.24 (V1). The ratios for each series after digestion were as follows: V series: V1 (1.24) > V2 (1.13); S series: S3 (1.05) > S2 (1.00) > S4 (0.98) > S1 (0.91); P series: P4 (1.01) > P1 (1.00) > P3 (0.99) > P2 (0.97); T series: T2 (1.00) = T3 (1.00) > T1 (0.95). The differences in HMW-GS/LMW-GS ratios before and after digestion were relatively small. Before digestion, the HMW-GS/LMW-GS ratio ranged from 0.06 (Lucana) to 0.45 (P2). The ratios for each sample series were as follows: P group: 0.21 (P1) to 0.45 (P2); V group: 0.36 (V2) to 0.43 (V1); T group: 0.16 (T2) to 0.21 (T1); S group: 0.19 (S3/S4) to 0.24 (S2). After digestion, the HMW-GS/LMW-GS ratio ranged from 0.04 (LU) to 0.45 (V2). The post-digestion ratios for each series were as follows: V group: 0.38 (V1) to 0.45 (V2); T group: 0.21 (T2/T3) to 0.42 (T1); S group: 0.23 (S4) to 0.35 (S1); P group: 0.13 (P1) to 0.34 (P2).

By comparing the changes in the ratios before and after digestion, it was found that for Gliadins/Glutenins, there was a slight increase after digestion in three varieties: Russello, Pugliese, and Rhettfah. The ratios remained unchanged for S4, Senatore Cappelli, and Benedettelli, while all other wheat varieties showed a slight decreasing trend. For the HMW-GS/LMW-GS ratio, a slight decrease was observed in six varieties: P1, P2, P4, Lucana, V1, and Benedettelli, while the remaining wheat varieties exhibited an increasing trend. Additionally, the Gliadins/Glutenins and HMW-GS/LMW-GS ratios within the wheat groups also experienced varying degrees of change before and after digestion. For instance, in the Gliadins/Glutenins ratio, the P, T, and V series showed a decreasing trend after digestion, while in the S series, except for S4 which remained unchanged, the others showed a decrease. Among all varieties, Russello and Rhettfah had the greatest increase, while Claudio exhibited the largest decrease. Regarding the HMW-GS/LMW-GS ratio, the P series showed a decrease in P1, P2, and P4, while P3 increased. Both the S and T series saw increases, while in the V series, V1 decreased and V2 increased. Across all varieties, T1 showed the greatest increase, and P2 had the largest decrease.

In summary, a comparison of the Gliadins/Glutenins and HMW-GS/LMW-GS ratios before and

after digestion revealed slight increases or decreases. Most varieties exhibited a decrease in the Gliadins/Glutenins ratio after digestion, while the HMW-GS/LMW-GS ratio generally showed a slight increase across most varieties.

Table 11. The Gliadins/Glutenins and HMW-GS/LMW-GS of different wheat samples before and after partial digestion in the third year of the experiment (ND: undigested, D: partially digested).

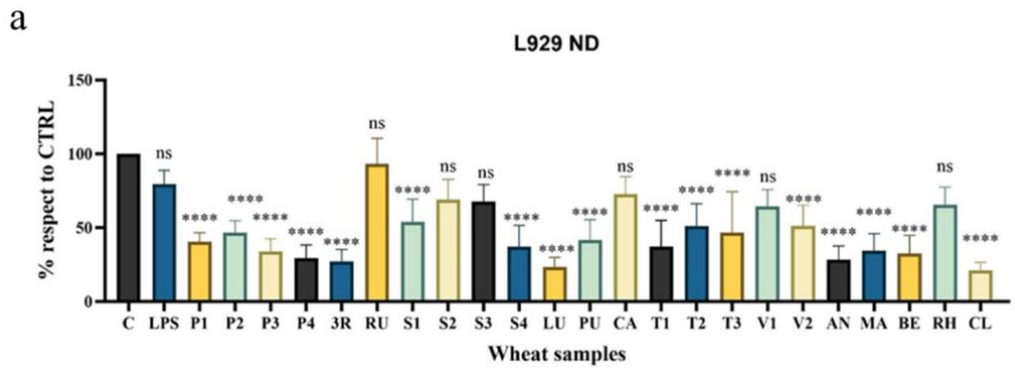
Sample	Gliadins/Glutenins		HMW-GS/LMW-GS	
	ND	D	ND	D
P1	1.03	1.00	0.21	0.13
P2	1.00	0.97	0.45	0.34
P3	1.04	0.99	0.22	0.25
P4	1.05	1.01	0.22	0.19
3R	1.09	1.01	0.16	0.18
Russello	1.08	1.11	0.15	0.16
S1	1.00	0.91	0.21	0.35
S2	1.04	1.00	0.24	0.31
S3	1.09	1.05	0.19	0.28
S4	0.98	0.98	0.19	0.23
Lucana	1.14	1.10	0.06	0.04
Pugliese	1.06	1.07	0.11	0.14
Senatore Cappelli	1.22	1.22	0.18	0.20
T1	1.02	0.95	0.21	0.42
T2	1.01	1.00	0.16	0.21
T3	1.02	1.00	0.19	0.21
V1	1.34	1.24	0.43	0.38
V2	1.18	1.13	0.36	0.45
Antalis	1.09	1.06	0.10	0.12
Margherito	1.00	0.97	0.17	0.28
Benedettelli	1.08	1.09	0.07	0.06
Rhettfah	1.06	1.07	0.14	0.14
Claudio	1.20	1.05	0.14	0.15

5.7 Effects of wheat proteins on L929 proliferation

Figure 28 illustrates the relative percentage of L929 cell viability in the third year of experiment compared to untreated control cells. Both before and after digestion, all samples exhibited lower viability in L929 cells than the control. Before digestion (Figure 28a), the highest L929 cell viability was observed in Russello (93.16%), and the lowest in Claudio (20.92%). Compared to the control group, there were no significant differences in Russello, S2, S3, Senatore Cappelli, V1, and Rhettfah, while the other ancient varieties showed significant differences. This indicated that Russello, S2, S3, Senatore Cappelli, V1, and Rhettfah exhibited a lower inhibitory effect on the proliferation of L929 cells compared to other varieties. After digestion (Figure 28b), the highest L929 cell viability was observed in P2 (80.60%), and the lowest in Claudio (19.93%). No significant differences were

found in P2, S2, Senatore Cappelli, V1, and V2 compared to the control group, whereas the other ancient varieties showed significant differences, the inhibitory effects of P2, S2, Senatore Cappelli, V1, and V2 on the proliferation of L929 cells were comparatively lower than those of other varieties. Moreover, digestion influenced the proliferation effects of wheat proteins on cells. After partial digestion (Figure 28b), the wheat varieties that enhanced the inhibitory effect on L929 cell proliferation include Russello, S3, and Rhettfah. In contrast, P2 and V2 facilitated the proliferation of L929 cells.-Additionally, S2, Claudio, and V1 showed no statistically significant effects on the proliferation of L929 cells before and after digestion, while P1, P3, P4, 3R, S1, S4, Lucana, Pugliese, T1, T2, T3, Antalis, Margherito, Benedettelli, and Claudio maintained the same proliferation effect on L929 cells post-digestion.

The impact of digestion on L929 cell proliferation varied among different series of wheat varieties. After partial digestion, in the P group, only P2 promoted the proliferation of L929 cells, while P1, P3, and P4 retained the same proliferation effects as before digestion. In the S group, S1 and S4 maintained the same proliferation effects, S2 showed no significant difference, and S3 inhibited the proliferation of 1929 cells. In the T group, all varieties maintained the same proliferation effects as before digestion. In the V group, V1 showed no significant difference, while V2 promoted the proliferation of L929 cells.



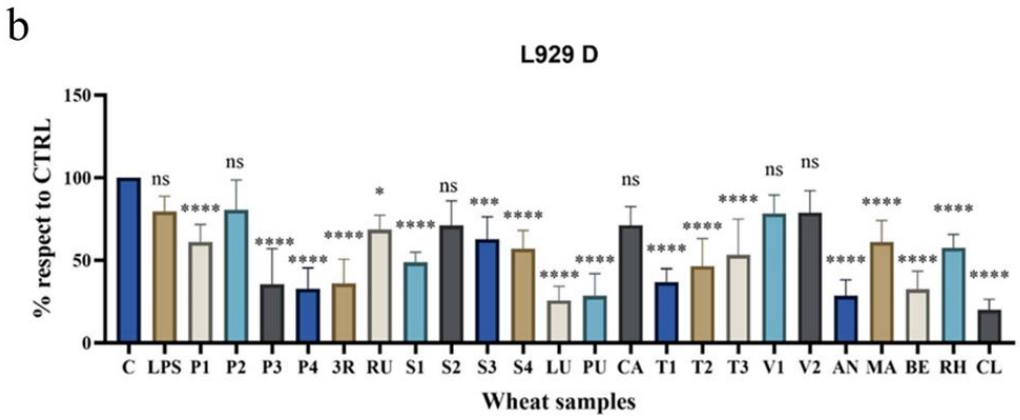


Figure 28. he effects of undigested (a) and partially digested (b) wheat proteins on the proliferation of L929 cells. All treatments were compared to the untreated control (CTRL). Significant differences between treatments were reported as follows: * $0.01 < p < 0.05$, ** $0.001 < p < 0.01$, *** $0.0001 < p < 0.001$, **** $p < 0.0001$. ND: undigested, D: partially digested.

5.9 Effects of wheat proteins on Caco2 proliferation

Figure 29 presents the relative percentage of Caco2 cell viability in the third year of experiment compared to untreated control cells. The results indicated that before digestion (Figure 29a), Caco2 cell viability was lower than that of the control cells. The highest Caco2 cell viability was observed in S3 (94.30%), and the lowest in P4 (41.88%). There were no significant differences in Russello, S2, S3, and T2, while the other ancient varieties showed significant differences. This indicates that, compared to the control group, the Russello, S2, S3, and T2 varieties exhibited a lower inhibitory effect on the proliferation of Caco2 cells. After partial digestion (Figure 29b), only P2 exhibited higher Caco2 cell viability than the control group, while the viability in all other varieties was lower than that of the control cells. The highest Caco2 cell viability was observed in P2 (107.60%), and the lowest in Benedettelli (53.99%). There were no significant differences in P2, S2, Claudio, V1, and V2, whereas the other ancient varieties showed significant differences, indicating that P2, S2, Claudio, V1, and V2 had a lower inhibitory effect on the proliferation of Caco2 cells compared to other wheat varieties. Moreover, digestion influenced the proliferation effects of wheat proteins on cells. After partial digestion, a small portion of wheat proteins promoted the proliferation of Caco2 cells, including P1, P2, 3R, V1, and V2. In contrast, other wheat proteins inhibited the proliferation of Caco2 cells, such as Russello, S3, Senatore Cappelli, and T2. The proliferation effects of P3, P4, S1, S4, Lucana, Pugliese, T1, T3, Antalis, Margherito, Benedettelli, Rhettfah, and Claudio remained

unchanged before and after digestion, while the S2 sample had no impact on Caco2 cell proliferation. The impact of digestion on the proliferation effects of wheat varieties on Caco2 cells also varied across different series. After partial digestion, in the P group, only P1 and P2 promoted the proliferation of Caco2 cells, while P3 and P4 retained the same proliferation effects as before digestion. In the S group, S1 and S4 maintained the same proliferation effects, S2 showed no significant difference, while S3 inhibited the proliferation of Caco2 cells. In the T group, T1 and T3 maintained the same proliferation effects as before digestion, however, the inhibitory effect of T2 on the proliferation of Caco2 cells was enhanced. In the V group, both V1 and V2 reduced their inhibitory effects on the proliferation of Caco2 cells.

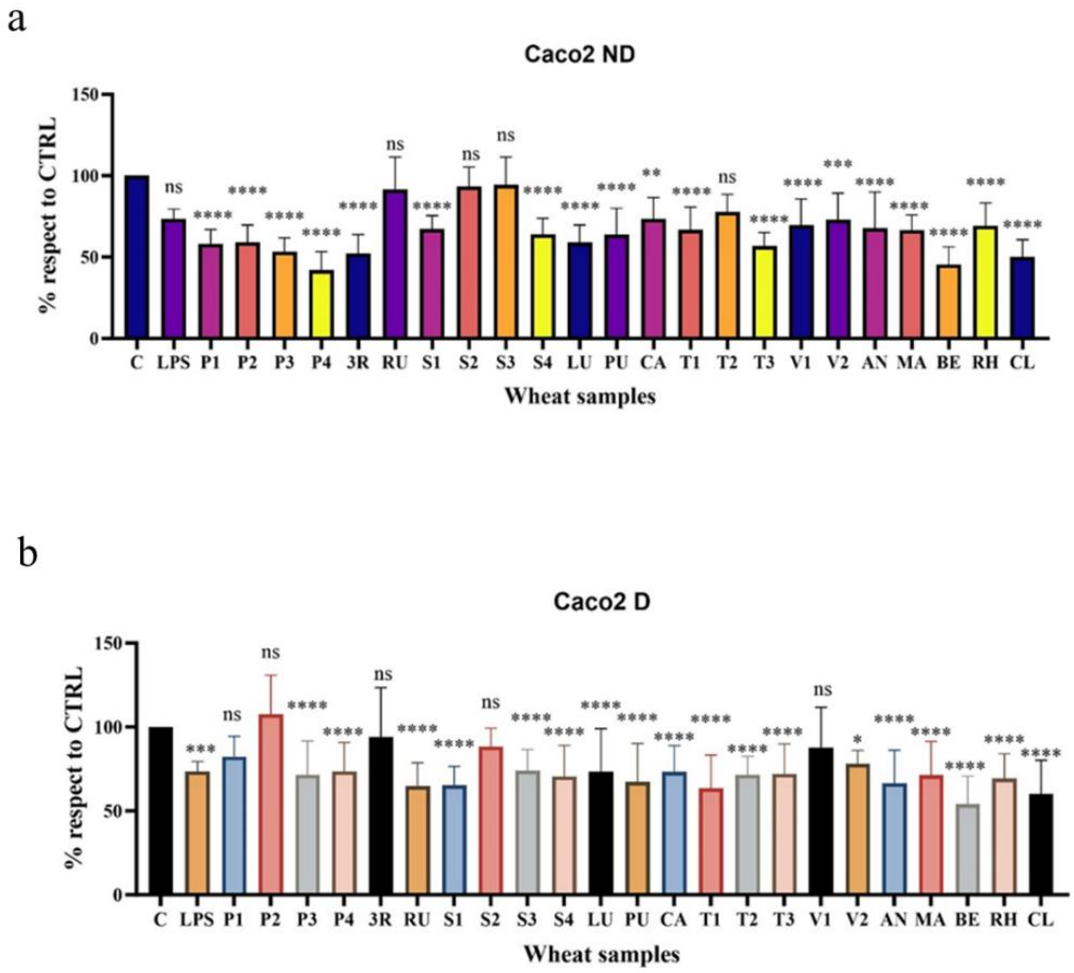


Figure 29. The effects of undigested (a) and partially digested (b) wheat proteins on the proliferation of Caco2 cells. All treatments were compared to the untreated control (CTRL). Significant differences between treatments were reported as follows: * $0.01 < p < 0.05$, ** $0.001 < p < 0.01$, *** $0.0001 < p < 0.001$, **** $p < 0.0001$. ND: undigested, D: partially digested.

6. The antioxidant capacity and anti-proliferative effects of wheat

6.1 The relationship between phenolic and flavonoid content and antioxidant capacity in wheat flour during a three-year cultivation period.

The correlation coefficients (r) between phenolic and flavonoid content and antioxidant capacity are summarized in Table 12. Phenolic compounds (including FPC, BPC, and TPC) and flavonoids (FFC, BFC, and TFC) in wheat flour were significantly correlated with antioxidant activity (FRAP and DPPH). According to the results of the Pearson correlation analysis, all types of phenolic compounds and flavonoids showed a significant positive correlation with antioxidant activity, and the correlations were consistent.

Table 12. The correlation coefficient (r) between phenolic and flavonoid content and antioxidant capacity.

	FPC	BPC	TPC	FFC	BFC	TFC	FRAP	DPPH
FPC	1							
BPC	.280**	1						
TPC	.867**	.722**	1					
FFC	.540**	.267**	.528**	1				
BFC	.286**	.336**	.381**	.414**	1			
TFC	.534**	.331**	.558**	.949**	.681**	1		
FRAP	.351**	.267**	.392**	.194**	.259**	.246**	1	
DPPH	.343**	.278**	.392**	.466**	.409**	.518**	.350**	1

Note: **: The correlation is significant at the 0.01 level (two-tailed).

6.2 Relationship between total wheat protein content, gluten composition, and cell proliferation during 3-year planting period

The correlation coefficients (r) between the total protein abundance of wheat samples grown over three consecutive years, the proportion of each gluten component, and cell antiproliferation are summarized in Table 13 (before digestion) and Table 14 (after partial digestion).

Before digestion, the total protein signal values exhibited significant correlations with multiple protein components. Specifically, the total protein signal was significantly negatively correlated with gluten (r = -0.409) and ω-Gliadins (r = -0.691), and significantly positively correlated with LMW-GS and α/β, γ-Gliadins (r = 0.454). However, it did not show a significant correlation with HMW-GS, L929 viability, or Caco2 viability. Furthermore, gluten showed positive correlations with

all groups of gliadin components, being significantly positively correlated with HMW-GS (r = 0.480) and ω-Gliadins (r = 0.515), and weakly correlated with LMW-GS and α/β, γ-Gliadins (r = 0.06). The L929 cell viability was significantly positively correlated with ω-Gliadins (r = 0.445) and significantly negatively correlated with LMW-GS and α/β, γ-Gliadins (r = -0.422). In contrast, Caco2 cell viability was only significantly positively correlated with L929 cell viability (r = 0.619).

Table 13. The correlation coefficient (r) between total protein abundance, the proportion of gluten components, and cell proliferation activity before wheat digestion.

	total protein signal	GLUTEN	HMW-GS	LMW-GS	ω-Gliadins	α/β, γ-Gliadins	L929 viability	Caco2 viability
total protein signal	1.00							
GLUTEN	-.409**	1.00						
HMW-GS	-0.16	.480**	1.00					
LMW-GS	.454**	0.06	-0.15	1.00				
ω-Gliadins	-.691**	.515**	.273*	-.632**	1.00			
α/β, γ-Gliadin	.454**	0.06	-0.15	1.000**	-.632**	1.00		
L929 viability	-0.21	-0.01	0.05	-.422**	.445**	-.422**	1.00	
Caco2 viability	0.11	-0.16	-0.03	0.03	-0.05	0.03	.619**	1.00

Note: *: The correlation is significant at the 0.05 level (two-tailed); **: The correlation is significant at the 0.01 level (two-tailed).

After partial digestion, the total protein signal values were significantly positively correlated with Caco2 cell viability (r = 0.276) and significantly negatively correlated with gluten (r = -0.316), HMW-GS (r = -0.33), and ω-Gliadins (r = -0.556). Furthermore, gluten was significantly positively correlated with all its components but was significantly negatively correlated with L929 cell viability (r = -0.406). Post-digestion, L929 cell viability showed a significant negative correlation with gluten, LMW-GS, and α/β, γ-Gliadins. In contrast, Caco2 cell viability was significantly positively correlated with both the total protein signal and L929 cell viability.

Table 14. The correlation coefficient (r) between the total protein abundance, the proportion of gluten components, and cell proliferation activity after partial digestion.

	total protein signal	GLUTEN	HMW-GS	LMW-GS	ω-Gliadins	α/β, γ-Gliadins	L929 viability	Caco2 viability
total protein signal	1.00							
GLUTEN	-.316*	1.00						
HMW-GS	-.330*	.570**	1.00					
LMW-GS	-0.03	.635**	0.09	1.00				
ω-Gliadins	-.556**	.528**	.543**	0.07	1.00			
α/β, γ-Gliadins	-0.03	.635**	0.09	1.000**	0.07	1.00		
L929 viability	0.00	-.406**	0.02	-.478**	0.10	-.478**	1.00	
Caco2 viability	.276*	-0.05	0.09	0.02	0.03	0.02	.556**	1.00

Note: *: The correlation is significant at the 0.05 level (two-tailed); **: The correlation is significant at the 0.01 level (two-tailed).

6.1 Proliferation of l929 and caco2 cells by all wheat proteins over three years

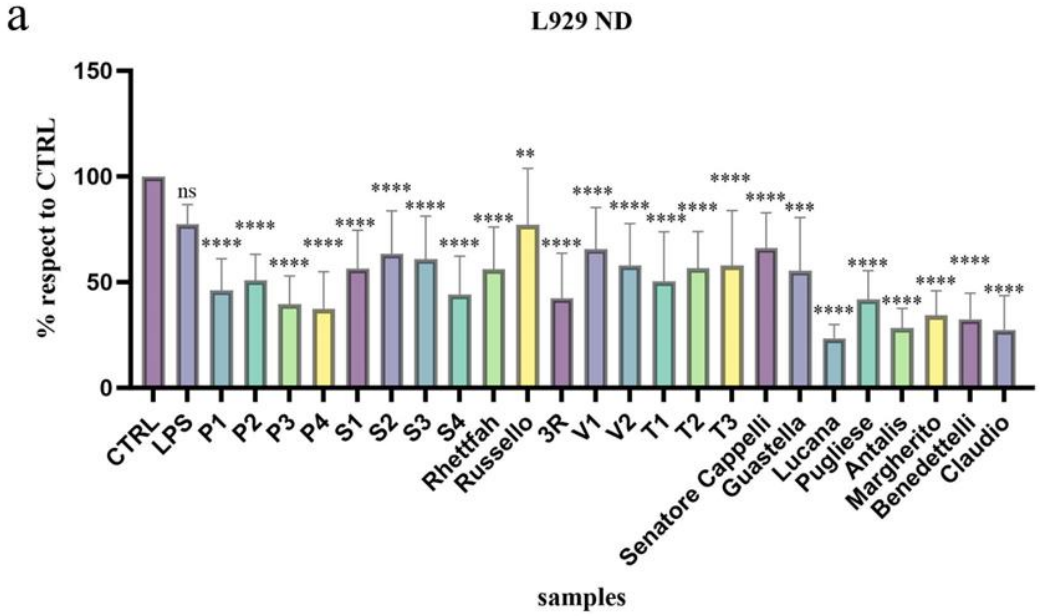
Figures 30 and 31 summarize the effects of all wheat proteins on the proliferation of L929 and Caco2 cells over three years. The MTT assay data analysis revealed a negative correlation between the proliferation of L929 and Caco2 cells and wheat proteins both before and after digestion, as the survival rates of L929 and Caco2 cells treated with wheat proteins were lower than those of the control group.

In the analysis of L929 cells, notable variations in survival rates were recorded across all wheat proteins both before and after digestion. Before digestion, Russello displayed the minimal inhibitory effect on L929 cell proliferation, restricting growth by only 22.96%, whereas Lucana demonstrated the most substantial inhibition, curtailing 76.56% of cell proliferation. After partial digestion, the impact of the various wheat proteins on L929 cell proliferation changed, with P2 indicating the least inhibition at 21.37%, while Lucana maintained the highest level of inhibition, suppressing 74.21% of cell proliferation.

The growth patterns of Caco2 cells exhibited similarities to those of L929 cells. Prior to digestion, all wheat proteins, with the exception of S2 and S3, significantly influenced the survival rates of Caco2 cells. S2 and S3 demonstrated minimal inhibitory effects, suppressing cell proliferation by 11.81% and 12.90%, respectively, whereas Benedettelli exhibited the highest level of inhibition, curtailing 54.51% of cell proliferation. Following partial digestion, P2 presented the least inhibitory effect on Caco2 cells, limiting cell proliferation by merely 5.55%, while Guastella displayed the

most significant inhibition, reducing proliferation by 57.16%. It is noteworthy that Lucana manifested the strongest inhibitory action on L929 cells both before and after digestion, whereas the proliferation of Caco2 cells was predominantly impacted by Benedettelli (prior to digestion) and Guastella (following partial digestion).

When comparing the proliferation rates of L929 and Caco2 cells before and after digestion, it was observed that wheat proteins could inhibit L929 cell growth by approximately 70% and Caco2 cell growth by about 50%. This finding indicates a more pronounced inhibitory effect of wheat proteins on L929 cells in contrast to Caco2 cells. Furthermore, both before and after digestion, Claudio demonstrated comparable proliferation effects on L929 and Caco2 cells in relation to the highest inhibition observed in ancient wheat varieties, suggesting that the inhibition associated with the modern variety Claudio is similar to that of many ancient wheat varieties. Analyzing the variations in cell proliferation pre- and post-digestion, P2, 3R, and V1 showed a reduction in inhibitory effects toward L929 cells, while Russello increased inhibition. P1 and P2 were noted to diminish inhibitory effects on Caco2 cells, whereas S2, S3, and Guastella intensified inhibition. The impacts of other wheat varieties on both cell types remained consistent before and after digestion.



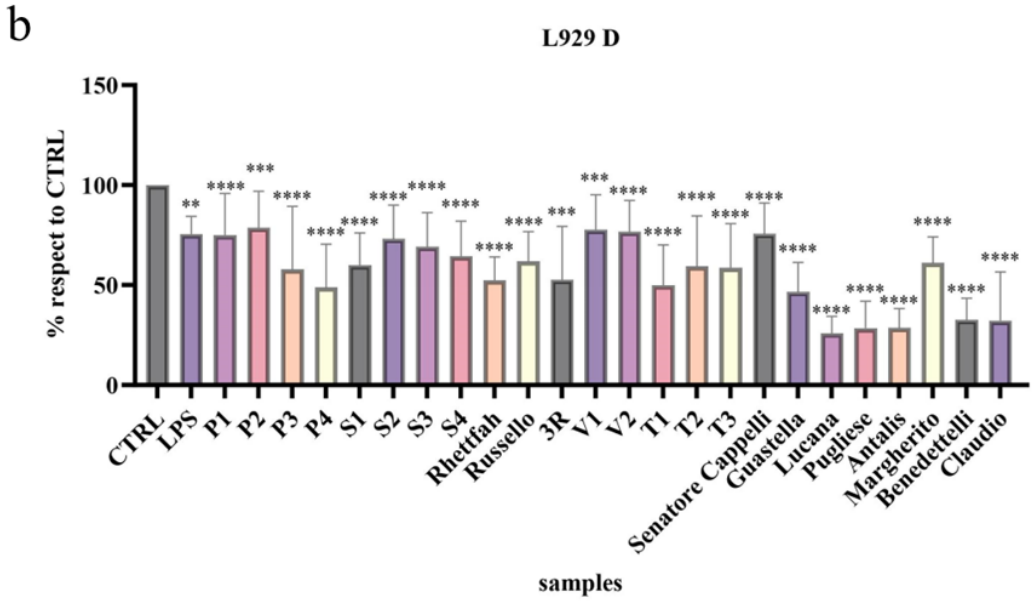


Figure 30. The effects of undigested (a) and partially digested (b) wheat proteins on the proliferation of L929 cells during 3 years planting period. All treatments were compared to the untreated control (CTRL). Significant differences between treatments were reported as follows: * $0.01 < p < 0.05$, ** $0.001 < p < 0.01$, *** $0.0001 < p < 0.001$, **** $p < 0.0001$. ND: undigested, D: partially digested.

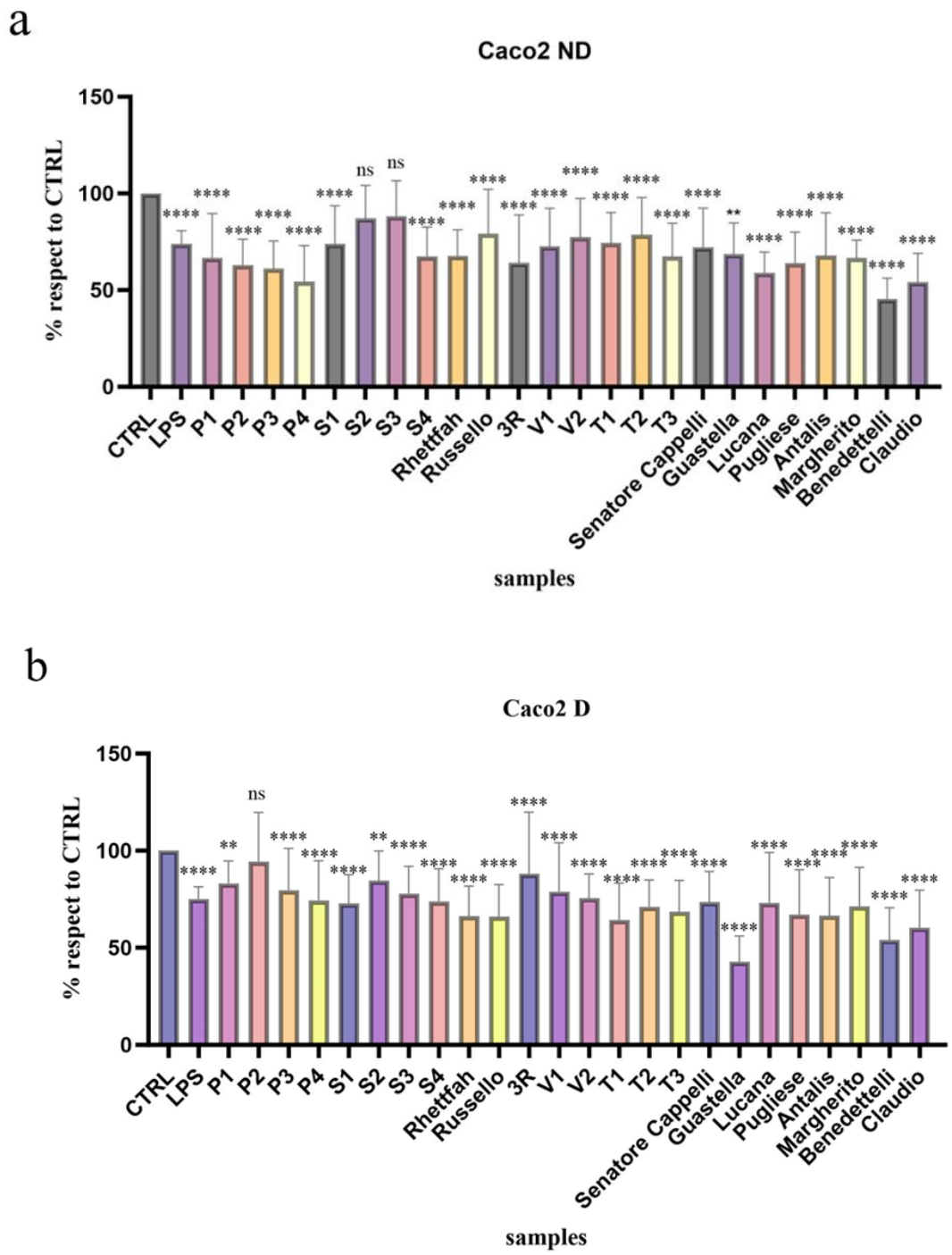


Figure 31. The effects of undigested (a) and partially digested (b) wheat proteins on the proliferation of Caco2 cells during 3 years planting period. All treatments were compared to the untreated control (CTRL). Significant differences between treatments were reported as follows: * $0.01 < p < 0.05$, ** $0.001 < p < 0.01$, *** $0.0001 < p < 0.001$, **** $p < 0.0001$. ND: undigested, D: partially digested.

7. Discussion

To investigate the alterations in gluten quality and composition throughout the breeding of durum wheat in Italy during the 20th century, a comparison was made between various ancient Italian durum wheat genotypes, which were cultivated over a three-year field trial, and contemporary Italian durum wheat genotypes. We examined the quality parameters of wheat flour harvested annually, primarily including protein, moisture, ash content, polyphenol and flavonoid content, as well as antioxidant activity. The analysis of gluten proteins, extracted from cooked dough, was carried out using SDS-PAGE under both undigested and partially digested scenarios. Variations in the composition and expression of gluten protein subunits were identified, and *in vitro* tests assessed the proliferation effects of all wheat varieties on cells.

7.1 Quality parameters, nutritional composition and antioxidant activity of wheat flour

The quality parameters, nutritional composition, and antioxidant activity of multiple wheat varieties were evaluated over three growing seasons. Overall, the results for protein, moisture, and ash content from the first and second year seasons were consistent with previous studies [232, 233], and no significant differences were observed for most traits studied, indicating that continuous cultivation had little impact on wheat quality parameters.

The assessment of phenolic content in whole grains revealed differences among the various ancient wheat genotypes studied each year. Previous research has investigated the levels of free and bound phenols in various common wheat varieties [234, 235]. In our study, significant differences were observed in the average polyphenol content (including FPC, BPC, and TPC) and flavonoid content (including FFC, BFC, and TFC) among some varieties in the first year of the experiment. However, the results from the second and third years indicated that the average variations in polyphenol and flavonoid content among wheat varieties were relatively small. Moore et al. [236] measured the total phenolic content of the bran of 20 different wheat genotypes. The phenolic content ranged from 2700 to 3500 µg GAE/g. Yu et al. [237] reported the phenolics content in wheat flour to be from 177 to 257 µg GAE/g. These values have a good comparable with our measurements. The researchers also found total phenolic content is closely associated with wheat genotype [236, 238]. Based on the range of polyphenol content in each year, the FPC and TPC levels in the first year season were higher than in the other two years, while the BPC range was lower. Although previous

studies have reported that phenols in wheat grains primarily exist in bound forms [234], our results indicated that in the first year season, phenols were predominantly present in free forms, whereas in the following two years, they were primarily in bound forms. This means that over the three growing seasons, FPC levels showed a continuous decline, while BPC levels exhibited a constant increase. For FFC, BFC, and TFC, the content ranges in the first year were always higher than in the other two years, with FFC levels consistently higher than BFC, indicating that polyphenol and flavonoid contents varied by wheat variety, and continuous cultivation affected their levels in wheat.

Both genetic makeup and environmental factors have been shown to influence the phenolic composition of wheat grains. Prior research indicated notable variations in polyphenol levels among different wheat cultivars, implying the specificity of this trait to genotype. Additionally, comparisons of wheat varieties cultivated in diverse locations revealed that environmental factors and cultivation practices can significantly impact the biosynthesis and accumulation of phenolic compounds [239-242]. Further research is essential to enhance our understanding of the interplay between genotype and environmental conditions, to identify wheat varieties that, under specific cultivation parameters, can yield wheat-based food products with enhanced nutraceutical benefits.

Over the three years, the antioxidant activity results of various wheat varieties showed no significant differences in FRAP values among the wheat varieties, while only minor differences were observed in DPPH values between a small subset of samples. Additionally, based on the range of DPPH results for each year, the DPPH scavenging ability in the first year and third years was slightly higher than in the second year. These findings suggest that continuous cultivation had little impact on the antioxidant activity of different wheat varieties. Previous literature has indicated that the antioxidant and chelating capacities are dependent on the wheat genotype and the surrounding environmental conditions [243-245]. In the present analysis of wheat varieties, a significant positive correlation was found between the content of phenolic compounds and flavonoids and antioxidant activity. As earlier research has indicated [246], these phenomena may be attributed not only to the quantitative presence of phenolic compounds but also to the unique phenolic profile specific to each wheat cultivar. While the findings of this study were based on three consecutive years of cultivation, they highlighted the necessity for further investigation to enhance our understanding of the influence of genotype and, potentially, environmental factors on the antioxidant properties of wheat.

7.2 Changes in protein composition and band patterns before and after digestion

This three-year analysis of wheat protein reveals significant changes in its composition and digestion patterns. Native wheat proteins exhibit considerable diversity, particularly in low molecular weight glutenin subunits (LMW-GS) and gliadins (α-/β- and γ-Gliadins). The first year findings indicated that the post-digestion protein profile aligns with Zhang et al.'s [247] *in vitro* study on green wheat proteins. High molecular weight glutenin subunits (HMW-GS) and ω-Gliadins underwent near complete digestion, while LMW-GS and α/β, γ-Gliadins were partially digested. An overall increase in non-gluten protein content was noted after digestion. Prior to digestion, molecular weight ranges of HMW-GS, ω-Gliadins, and α/β, γ-Gliadins were consistent with previous studies [54, 248]. The undigested proteins generally displayed 5-6 prominent glutenin bands, except for S1 with only 3, while LMW-GS or α/β, γ-Gliadins comprised 2-4 polypeptides. Post-digestion, distinct protein bands were largely absent, although strong staining appeared in the LMW-GS area, supporting findings by Žilić et al. [249] on the molecular weight and polypeptide similarities among glutenin subunits. The analysis revealed that partial digestion weakened or removed wheat protein bands with intensity and quantity variations among genotypes, highlighting differences in protein profiles. Zhang et al. [247] noted that most pepsin digestion products exceed 2000 Da, while trypsin products are between 0.5 kDa-2 kDa. Our results showed that molecular weights of partially digested products clustered below 25 kDa, suggesting a mix of pepsin and trypsin-digested products. It remains unclear if gluten proteins and subunits were mainly digested by pepsin or trypsin. Thus, SDS-PAGE analysis of various ancient wheat proteins, including undigested and partially digested samples, revealed a similar degradation pattern, indicating that higher molecular weight proteins fragmented into smaller peptides during digestion, consistent across all tested wheat varieties.

In the second year, there was a notable decline in HMW-GS and LMW-GS in specific wheat varieties compared to the first year, accompanied by a decrease in total protein bands. Although ω-Gliadins bands increased in the second year, gluten proteins generally diminished post-digestion, particularly with ω-Gliadins, which completely vanished. Unlike the first year, residual HMW-GS and LMW-GS polypeptide bands were detectable in various varieties after digestion in the second year. Additionally, the first year exhibited non-gluten protein accumulation post-digestion, a trend

not observed in the second year, suggesting that the protein composition and intensity in the second year's wheat were reduced.

In the third year, the wheat retained diverse protein types, with variability in protein band intensity and quantity across varieties. LMW-GS subunits remained predominant, while similar protein digestion patterns were noted, characterized by decreased glutenin subunits and some non-gluten protein accumulation. In this year, 2-4 typical glutenin bands were present, but HMW-GS and ω-Gliadins were absent, with a reduction in LMW-GS. HMW-GS subunits were restricted to specific groups, while other varieties primarily contained ω-Gliadins and LMW-GS. In contrast to prior years, the third year's protein composition showed minimal variation in peptide number and intensity pre- and post-digestion.

7.3 Differences in total protein, gluten, and their subunit contents

The study conducted in the first year revealed notable variations in total protein, gluten, and subunit contents among 18 ancient durum wheat varieties pre- and post-digestion. The total protein and gluten composition establish the dough's functional properties including elasticity and viscosity, thus influencing the final product's quality [250, 251]. Initially, total protein signal values before digestion varied from 90,046 to 662,072, with Guastella being the highest and S1 being the lowest (7.35 times lower). Post-digestion, S1 had the highest total protein signal value, indicating a general reduction in protein content across varieties post-digestion consistent with previous findings on protein degradation. The gluten proteins critically define the rheological characteristics of the dough [252]. In the examined period, many varieties, including S1, Russello, Guastella, P2, and S2, showed high gluten content (exceeding 80%). Varieties with balanced gluten subunits are favored for optimal baking quality. High HMW-GS and LMW-GS levels in varieties S3 and S2 suggest superior dough strength and elasticity, whereas P3's high ω-Gliadins may pose risks for gluten-sensitive individuals. Gluten protein content trends aligned with earlier studies, emphasizing its significance in dough extensibility and elasticity [253]. S1 demonstrated exceptional gluten quality pre-digestion due to notable total protein content, which increased post-digestion as protein degraded into polypeptides. S3 exhibited the highest gluten content after digestion, showcasing varied enzyme consumption rates across varieties. The observed decline in total gluten and subunit content across all samples highlights the impact of digestion on wheat protein profiles.

Gluten, LMW-GS, and α/β, γ-Gliadins are the main components, with their content decreasing more significantly than HMW-GS and ω-Gliadins. The T1 variety had the highest gluten reduction at 42.23%, while S3 had the lowest at 5.7%, and S4 showed almost no change. T2 had the highest reduction in low molecular weight proteins at 36.16%, with S4 having the lowest at 2.35%, due to T1 and T2's higher susceptibility to enzymatic degradation. This highlights significant structural and compositional differences among wheat varieties [254].

In the second year, total protein signal values ranged from 36605 pixels to 87256 pixels, highest in S4 and lowest in Senatore Cappelli. There were significant differences in total wheat protein content prior to digestion due to the year, with all values in the second year lower than in the first, revealing considerable variations among wheat varieties. The overall reduction in total protein intensity in the second year resulted in lower post-digestion signal values compared to the first year, although generally, post-digestion values were lower than pre-digestion values, except for S1 in the first year. This suggests gradual degradation of wheat protein during partial digestion. Despite a significant drop in total protein across varieties in the second year, gluten protein and HMW-GS levels remained relatively stable compared to the first year. Conversely, ω-Gliadins proportions increased while LMW-GS (and α/β, γ-Gliadins) proportions decreased. This indicates that total protein content is year-dependent, with limited impact on gluten and HMW-GS proportions, likely due to simultaneous reductions in total protein alongside gluten and HMW-GS. Notably, the number of ω-Gliadins bands rose in the second year, while LMW-GS bands decreased significantly, leading to a higher proportion of ω-Gliadins and a substantial decrease in LMW-GS relative to the first year.

Wheat grain protein content is affected by genetic, environmental, and management factors, particularly nitrogen (N) fertilization, which is the key management strategy [255] [256]. N is typically applied as a basal before sowing and a topdressing during growth, mainly at jointing and booting stages, to optimize yield and protein levels [257]. Early to mid-vegetative N topdressing enhances yield potential, while applications during reproductive stages boost protein synthesis [258]. Specifically, splitting N applications at booting or later increases grain protein and improves bread quality [259]. Applying N at spike formation also strengthens gluten in quick-maturing cultivars in cooler areas [260, 261]. Cultivation year significantly influences protein content due to climatic variations and nitrogen application, aligning with Graziano's [262] finding that durum wheat quality

relies on genetic and environmental factors over a 2-year cycle.

In the third year, total protein signal values before digestion ranged from 88,392 pixels (Antalis) to 425,391 pixels (Senatore Cappelli), a 4.81-fold difference. Generally, total protein values decreased after partial digestion, but ancient wheat varieties consistently surpassed the modern Claudio variety. Protein abundance was lower than in the first year yet higher than in the second. Compared to the first year, there was a decrease in HMW-GS and ω-gliadins, with all varieties showing reduced LMW-GS. In contrast to the second year, LMW-GS intensity increased in the third year across all varieties. Significant evidence linked pasta quality to gluten protein composition [231, 263], which could be influenced by genetic [264-266] or environmental factors [267-270], elucidating the three-year variations in wheat protein composition. The gluten content of the third year (64.90%–85.38%) closely mirrored the first year (61.20%–83.99%) but fell short of the second year (74.51%–88.38%). Changes in HMW-GS and ω-gliadins paralleled gluten content, peaking in the second year (6.70%–19.02% and 15.89%–27.63%, respectively). The proportion of LMW-GS or α/β, γ-gliadins in the third year (17.63%–54.64%) fell between the first and second years, with LMW-GS or α/β, γ-gliadins being predominant each year. Previous studies indicated that reduced rainfall during grain-filling correlated with heightened expression of HMW-GS and ω-gliadins [271], suggesting that the second year's increases were attributable to lower rainfall during this phase. Across the three years, partial digestion typically diminished total protein abundance and the ratios of gluten and its subunits, although this reduction varied by wheat variety.

7.4 Relationship of Gliadins/Glutenins and HMW-GS/LMW-GS ratio with dough rheological properties and bread-making quality

The processing capability of wheat flour into various foods is primarily influenced by gliadins and glutenins [272], which make up 63–90% of the total grain proteins [273]. Their unique viscoelastic characteristics determine the bread-making quality of the flour [274]. Gliadins contribute to gluten's cohesiveness and extensibility, while glutenins enhance elasticity and strength [275]. A higher gliadin content correlates with increased extensibility and resistance to extension [54], indicating that the ratio of gliadins to glutenins is crucial for dough strength and extensibility [276, 277].

In the first year, the Gliadins/Glutenins ratios before and after digestion varied from 0.83 (S4) to 1.01 (P3) and 0.92 (P4) to 1.09 (S1), respectively. The S4 variety demonstrated the highest elasticity

and strength, whereas the P3 sample provided superior extensibility. Our Gliadins/Glutenins ratios surpassed the 0.59 to 0.84 range found by Stehno et al. [278] for ten Czech bread wheat varieties but aligned with the 0.49 to 1.01 range reported by Žilić et al. [249] for Serbian wheat. This difference is attributed to a higher degradation rate of HMW-GS relative to ω-Gliadins during digestion, leading to more significant degradation of glutenins and a consequent increase in the ratio. Žilić et al. [249] classified wheat varieties with the highest ratios (0.97 and 1.01), such as ZP Zlatna and Apache, as Grade A, high-quality bread-making varieties. Therefore, the 18 ancient wheat varieties analyzed could also be considered high-quality options for bread-making.

Glutenins, particularly high molecular weight (HMW) and low molecular weight (LMW) glutenins are critical for gluten elasticity and strength in bread-making. Higher gliadin content correlates with reduced extensibility [54]. Reported HMW-GS/LMW-GS ratios for wheat glutenin range from 0.18 to 0.74 [47], similar to ratios of 0.13 to 0.28 and 0.11 to 0.34 observed in the second year study before and after digestion. Optimal HMW-GS/LMW-GS ratios for durum wheat varieties fall between 0.15 and 0.25, with values exceeding 0.30 weakening dough strength [279]. In the second year study, only P1 and P2 exhibited increased ratios (0.34 and 0.32) post-digestion, indicating that partial digestion could compromise dough strength. Therefore, high protein content does not guarantee superior Gliadins/Glutenins or HMW-GS/LMW-GS ratio. These observations align with existing literature [267, 280-282], showing the minimal impact of digestion on these ratios.

By comparing the Gliadins/Glutenins and HMW-GS/LMW-GS ratios before and after digestion across two years, it was observed that both rheological characteristic ratios improved in the second year. This was primarily due to a significant reduction in total protein content across all ancient wheat samples, a relatively stable HMW-GS proportion, an increase in ω-gliadins, and a notable decrease in LMW-GS. Consequently, the Gliadins/Glutenins and HMW-GS/LMW-GS ratios increased. Koga et al. [283] indicated that gluten protein composition and the glutenin to gliadin and HMW-GS to LMW-GS ratios were affected by climatic factors, particularly desiccation. The accumulation of gluten proteins involves complex spatial and temporal regulation, shaped by environmental and abiotic signals [284]. Environmental conditions alter gluten fractions, with high temperatures influencing yield by changing grain filling duration and rate [285]. Under high temperatures, gene transcription occurs earlier, but the transcript accumulation period is shorter

[286]. Elevated temperatures impact gliadin gene transcription, modifying the gliadin to glutenin ratio and, consequently, dough properties [287]. Studies consistently show that extremely high temperatures, low relative humidity, and elevated grain nitrogen during grain filling reduce dough strength and gluten quality [284, 288, 289]. In the second-year study, total protein levels of all ancient wheat varieties declined, indicating diminished protein quality and content, which compromises gluten strength and affects protein composition and flour functionality. This functionality is affected by factors including cultivar, protein content, grain hardness, growing conditions, and crop season [290].

Analyzing the ratios of key dough rheological properties pre- and post-digestion over three years, it was noted that the ratios of Gliadins/Glutenins and HMW-GS/LMW-GS in the third year fell between those of the first two years, with minimal differences. This variation is likely correlated with total protein content in the third year, which was intermediate to the prior years. The accumulation of gluten proteins is intricate, influenced by climatic conditions, especially dryness. A longer grain filling duration at lower temperatures significantly affects the composition of gluten proteins, despite having a lesser impact on total protein accumulation per grain [283]. Changes in gluten composition may affect dough properties, although this remains speculative due to the numerous factors in protein network formation during bread-making. Overall, optimal bread-making performance relies on a precise balance of elasticity and viscosity [291].

7.5 Effects of different wheat proteins on the viability of L929 and Caco2 cells before and after digestion

In our research, we assessed the impact of various wheat proteins on the proliferation of L929 and Caco2 cells through the MTT assay to evaluate cell proliferation and alterations during digestion. The results in the first year indicated that most undigested wheat proteins significantly decreased the viability of both cell types, confirming the cell inhibitory effects, consistent with prior studies observing similar inhibitory impacts on cell proliferation [292]. After digestion, the effects of certain wheat proteins on cell proliferation changed, revealing the significant impact of the digestive process on their bioactivity. In the L929 assay, wheat proteins from P1 and P3 varieties showed reduced inhibition post-digestion, potentially due to enzyme-induced structural alterations that lowered their binding affinity to cellular receptors [293, 294]. A parallel pattern emerged in the

Caco2 assay, where proteins from P1 and Senatore Cappelli displayed diminished inhibition after digestion. The digestion process modifies the inhibition of wheat proteins by changing their structure and revealing new active sites [247, 295].

The wheat series also influenced L929 and Caco2 cell responses differently. Notably, proteins from the T group (T1, T2, T3) had negligible effects on L929 viability before and after digestion, whereas the T1 and T3 types increased Caco2 cell inhibition post-digestion. This variation may stem from the differing cell type reactivity to wheat proteins. Previous work underscored that digestion could alter the functional activity of wheat proteins, affecting their spatial and secondary structures [294] and resulting in inconsistent bioactivities among digestion products across cell types. For Caco2 cells, the S group (S1, S2, S3, S4) proteins similarly did not influence cell proliferation, likely due to their structural stability resisting breakdown during digestion. Conversely, several P and T group varieties exhibited notable cell viability alterations post-digestion, likely due to specific proteins' varying sensitivity to digestion. In comparison to the modern variety Claudio, many ancient wheat varieties demonstrated equal or greater inhibition in undigested L929 cells. However, only three ancient varieties matched or exceeded Claudio's inhibitory effect in Caco2 cells, highlighting a greater efficacy of ancient wheat proteins in inhibiting L929 proliferation. After digestion, most ancient wheat proteins showed reduced inhibitory relative to Claudio, except for the P4, Rhettfah, Russello (L929), V2, T3 (Caco2), and Guastella varieties.

The experiments conducted in the first and second years demonstrated that all wheat proteins prior to digestion were negatively correlated with cell proliferation. In the first year, P1 and P3 promoted the proliferation of L929 cells after partial digestion, while other proteins continued to inhibit both L929 and Caco2 cells, indicating differing inhibitory effects. Before digestion, P4 and Senatore Cappelli exhibited the strongest inhibitory effects on L929 and Caco2 cells, respectively. After digestion, Rhettfah and Guastella showed the greatest inhibitory effects. On the contrary, in the second year, P3 and Russello exhibited the greatest inhibitory effects on the two cell lines before digestion, while T3 and V1 demonstrated the highest inhibition post-digestion. This indicated that different wheat varieties had varying effects on the proliferation of L929 and Caco2 cells, and that digestion altered the influence of wheat proteins on cell proliferation [296]. Partially digested ancient wheat proteins in first year showed reduced inhibition for L929 (P1, P3, S1, S2, S3, S4, V1,

Senatore Cappelli) and Caco2 (P1, Senatore Cappelli). In the second year, 6 types (V2, Senatore Cappelli, S4, P1, P3, P4) and 7 types (V2, S1, S4, 3R, P2, P3, P4) of ancient wheat proteins reduced inhibition to L929 and Caco2 cells, respectively. Overall, the inhibitory effects of ancient wheat proteins on L929 cells decreased over the second year, contrasting with their effects on Caco2 cells, while partial digestion consistently reduced wheat protein inhibition across both experimental years [297].

The cellular proliferation results from the third year mirrored those of the previous two years. Nearly all wheat proteins led to reduced survival rates for L929 and Caco2 cells compared to the control group, although various wheat varieties exhibited different effects on cell growth inhibition. In the third year, before digestion, Claudio and P4 exhibited the strongest inhibitory effects on L929 and Caco2 cells, respectively. After digestion, Claudio and Benedettelli showed the greatest inhibitory effects. This deviated from prior years and emphasized that the inhibitory effects of wheat proteins on cells fluctuated annually. A three-year comprehensive analysis revealed that, for L929 cells, Lucana exhibited the strongest inhibitory effect on proliferation; whereas for Caco2 cells, the most significant impacts were observed with the Benedettelli (pre-digestion) and Guastella (partially digested) varieties. Pearson correlation analysis highlighted key factors influencing cell proliferation: L929 cell viability pre-digestion had a significant positive correlation with ω-Gliadins and a negative correlation with LMW-GS and α/β, γ-Gliadins. Post-digestion, L929 cell viability negatively correlated with GLUTEN, LMW-GS, and α/β, γ-Gliadins. Meanwhile, Caco2 cell viability was consistently positively correlated with L929 cell viability or total protein signals, suggesting that gluten and its subunits might affect cell survival rates.

Across the three years of experiments, wheat proteins demonstrated greater inhibitory effects toward L929 cells compared to Caco2 cells. P2, 3R, and V1 reduced inhibitory effects on L929 cells, while Russello heightened it. P1 and P2 lessened inhibition to Caco2 cells, whereas S2, S3, and Guastella increased it. Overall, partial digestion effectively decreased the inhibitory effects of certain wheat proteins on cell proliferation, confirming findings from the previous two years.

8. Conclusion

This study evaluated the impact of different ancient durum wheat varieties on cell proliferation following partial digestion of wheat protein over three growing seasons. Analysis showed

significant variations in protein composition, digestibility, and proliferation before and after digestion.

In the first year, the quality parameters of wheat flour, such as protein, moisture, and ash content, were consistent with the results of previous studies. Polyphenols were present in free form, with FPC and TPC levels higher than in the other two seasons, while BPC levels were lower. The range of flavonoid content was higher than in the other two years. Antioxidant activity, as measured by FRAP values, showed no significant differences, but DPPH values exhibited stronger scavenging ability. HMW proteins were degraded into smaller peptides during digestion across all varieties. Total protein content decreased significantly post-digestion, showing degradation variability in gluten proteins. The gliadin to glutenin ratio remained consistently higher pre- and post-digestion, suggesting these 18 ancient varieties are potential high-quality bread-making cultivars. Digestion had minimal impact on gliadin/glutenin and HMW-GS/LMW-GS ratios. Before digestion, P4 and Senatore Cappelli exhibited the strongest inhibitory effects on L929 and Caco2 cells, respectively. After digestion, Rhettfah and Guastella showed the greatest inhibitory effects.

During the second year, wheat flour quality parameters were stable. Polyphenols and flavonoids decreased compared to the previous year, with polyphenols mainly in bound forms. Antioxidant activity weakened, reflected by a slight decline in DPPH scavenging ability. Variations in protein composition among wheat varieties were observed compared to the first year. There was a reduction in the number and intensity of protein and gluten subunit bands. While total protein content decreased, gluten and HMW-GS proportions remained stable; ω-Gliadins increased significantly, and LMW-GS decreased notably, raising the Gliadins/Glutenins and HMW-GS/LMW-GS ratios. Following partial digestion, the second-year wheat varieties showed reduced HMW-GS and LMW-GS polypeptide bands, a change not noted in the first year. The gliadin/glutenin and HMW-GS/LMW-GS ratios varied after digestion, with most samples showing a downward trend. P3 and Russello exhibited the strongest inhibitory effects on L929 and Caco2 cells, respectively. After digestion, T3 and V1 showed the greatest inhibitory effects.

In the third year, there was minimal variation in polyphenol and flavonoid contents in wheat flour, predominantly in bound forms. Antioxidant activity slightly increased, yet changes remained minimal, with DPPH scavenging ability comparable to the first year season. Significant changes

from the previous years included the loss of HMW-GS and ω-Gliadins, along with reduced LMW-GS in many samples, while post-digestion protein degradation persisted. Protein levels in the third year were lower than the first year but higher than the second, with a consistent decline after digestion. Ancient wheat varieties consistently exhibited higher protein signals compared to the modern variety Claudio. The proportions of gluten subunits and rheological ratios like Gliadins/Glutenins and HMW-GS/LMW-GS generally aligned with previous years. Before digestion, Claudio and P4 exhibited the strongest inhibitory effects on L929 and Caco2 cells, respectively. After digestion, Claudio and Benedettelli showed the greatest inhibitory effects.

Pearson correlation analysis showed that a significant positive correlation was found between the content of phenolic compounds and flavonoids and antioxidant activity. And cell viability and specific gluten protein subunits, including ω-gliadins, LMW-GS, and α/β, γ-gliadins. The comprehensive analysis of a three-year experiment revealed that that Lucana, Benedettelli, and Guastella had the highest inhibition to L929 and Caco2 cells, with a more pronounced inhibitory effect observed on the L929 cells. Overall, this study confirmed that, while the inhibition of wheat proteins on cell proliferation varied annually, partial digestion consistently reduced the inhibitory effect of specific proteins (P2, 3R, and V1) across the three years. This experiment indicated that ancient wheat proteins generally inhibited cell proliferation, however, the effect was diminished in specific varieties due to partial digestion. This highlighted the variability in wheat protein effects influenced by genetic and environmental factors, alongside the potential of partial digestion to modulate protein inflammatory effects.

Overall, this research highlighted that partial digestion affects the electrophoretic profile, protein composition, and cell proliferation of ancient wheat proteins, underscoring the need to consider wheat protein sources and processing in dietary assessments. Future studies should target specific peptides associated with cell inhibition and strategize to mitigate health risks from wheat consumption, crucial for enhancing wheat's health benefits through selective breeding and processing.

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