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Role of particle size in modulating starch digestibility and textural properties in a rye bread model system

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ABSTRACT

In cereal products, the use of flour containing clusters of intact cells has been indicated as a potential strategy to decrease starch digestion. Rye possesses more uniform and thicker cell walls than wheat but its protective effect against starch digestion has not been elucidated. In this study, rye flours with three different particle sizes, large (LF) (~1700 μm), medium (MF) (~1200 μm), and small (SF) (~350 μm), were used to produce model bread. The textural properties of these breads were analysed using Textural Profile Analysis (TPA). The starch digestibility of both the flour and the bread was measured using Englyst's method, while the presence of intact cell clusters was examined using Confocal Laser Scanning Microscopy (CLSM). Additionally, the disintegration of bread digesta during simulated digestion was assessed through image analysis. CLSM micrographs revealed that bread made with MF and LF retained clusters of intact cells after processing, whereas bread made with SF showed damaged cell walls. Starch digestibility in LF and MF was lower ($p \leq 0.05$) than that in SF. Bread produced with MF and LF exhibited the least ($p \leq 0.05$) cohesive and resilient texture, disintegrated more during digestion, and exhibited higher starch digestibility ($p \leq 0.05$) than bread made with SF. These results highlight the central role of bread texture on *in vitro* starch digestibility.

1. Introduction

Rye (*Secale cereale* L.) is one of the major crops for bread production, second only to wheat (*Triticum aestivum* L.) (Deleu, Lemmens, Redant, & Delcour, 2020; Arendt and Zannini, 2013) even though it is considered a minor cereal represents 1% of total world cereal production (Arendt and Zannini, 2013). Rye has great resistance to cold temperatures and could be fruitfully bred in places with severe climates such as Germany, Poland, Russia, Denmark, and Belarus which together account for 85% of its total production (Kaur, Singh Sandhu, Singh Purewal, Kaur, & Kumar Singh, 2021; Németh & Tömösközi, 2021; Arendt and Zannini, 2013).

Rye is utilized in the production of traditional foods, such as bread, pumpernickel, and flakes for porridges, or added to traditional wheat-based bakery products to reduce the starch digestibility and the consequent glucose absorption (Deleu et al., 2020). The consumption of rye products was proven to induce a low post-prandial insulin response, prolonged glucose profile, and increased satiety compared with wheat bread, and this effect was widely observed in several randomized controlled trials (Deleu et al., 2020; Jonsson et al., 2018). Different factors seem to be responsible for the beneficial effect of rye consumption. Firstly, among common cereals used for bread production, rye is characterized by the highest dietary fiber content, ranging from 18.7-22.2% on a dry matter basis (Andersson et al., 2009). In comparison, wheat contains between 9.8–15.2% dietary fiber on a dry matter basis (Gebruers et al., 2008). In addition, the presence of bioactive compounds, such as phenolic acids, could hinder carbohydrate hydrolysis, stimulate insulin secretion, and inhibit enzymatic activity (Jonsson et al., 2018; Rosén et al., 2011). Jenkins et al. (1986) also demonstrated the key role of the firm structure of rye bread (i.e., pumpernickel) in slowing down the glycemic response. Compared to wheat, rye gluten is less prone to form a proper viscoelastic network due to the prolamin called 'secalins' which decreases the resistance to stretch, limiting the formation of an aerated voluminous bread (Arendt and Zannini, 2013). In rye bread, indeed, the structure is formed by a continuous phase of starch granules embedded in a dense fiber matrix, mainly made by

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arabinoxylans. Therefore, the peculiar structure of rye bread, i.e., dense and compact, could reduce the physical disintegration during digestion and consequentially limit starch digestion and absorption at the intestinal level (Juntunen et al., 2003). In addition, when gluten is added to rye bread, a porous aerated structure is formed, the crumb easily disintegrates during digestion and, consequentially, its glycemic and insulin response results similar to the one elicited by refined wheat bread. This confirms the central role of the compact dense structure of rye bread in decreasing its starch digestibility (Nordlund et al., 2016).

In the last 10 years, the effect of cell wall integrity was deeply researched as a fruitful strategy to decrease starch digestibility, mainly in pulses where the cell wall is thick and poorly permeable (Bhattarai et al., 2018; Dhital et al., 2016; Rovalino-Córdova et al., 2019). Intact cell walls of pulses, made with undigestible polysaccharides, were reported to modulate the starch hydrolysis limiting the diffusion of α-amylase into the cell. The inhibition of enzyme diffusion into the cells could be ascribed mainly to the synergetic effect of small pores, naturally present in the cell wall, and the adsorption of enzymes on the surface of the wall (Rovalino-Córdova et al., 2021). For what concerns cereals, the intactness of the cell wall has been reported to be efficient in decreasing the digestibility of starch in isolated cells and flour from wheat, sorghum, and barley (Bhattarai et al., 2018; Korompokis et al., 2019; Lin et al., 2020). However, when wheat coarse flour, which contains clusters of intact cells, is used to produce bread, even if intact cells were still detected through microscopical analysis, this effect of protection is lost (Korompokis et al., 2021; Tagliasco et al., 2022). Tagliasco et al. (2022) hypothesized that this may be due to the increased porosity of the cell wall during bread processing due to the thin cell wall that characterized wheat grain and the consequent solubilization of arabinoxylans and β-glucans, which could have enhanced contact between α-amylase and starch. Rye grains are known to have a thicker cell wall than wheat grains, with the wall thickness being uniform across the different parts of the starchy endosperm (Autio & Salmenkallio-Marttila, 2001).

However, until now, the impact of particle size on starch digestibility has been extensively studied in wheat. In rye, this effect has been explored in wholemeal rye bread through a human study that compared the consumption of bread made with fine or coarse flour on the apparent digestibility of macronutrients. The results showed no significant differences between coarse and fine rye bread. However, the study focused on the amounts of nutrients excreted in the feces, rather than the direct measurement of nutrient release following digestion in the small intestine (Wisker et al., 1996). Against this background, this study aimed to elucidate the effect of rye flour varying in particle size, and therefore the presence of clusters of intact cells on the starch digestibility of rye flour and a model rye bread using a simulated intestinal digestion. Traditionally, rye bread is prepared by sourdough fermentation as the cell wall degradation and solubilization of pentosans and arabinoxylans promoted by acidic conditions is essential for rye bread quality (Arendt et al., 2007). However, it has been already demonstrated that the acidity produced by sourdough fermentation could have a direct effect on decreasing starch digestibility (De Angelis et al., 2009; Németh & Tömösközi, 2021). Therefore, to rule out the effect of sourdough fermentation on starch digestibility and cell wall degradation, a simple breadmaking process with baker's yeast was used in this study. The textural quality, *in vitro* starch digestion, and physical disintegration during the digestion were further investigated to study the relationship among the integrity of cell walls, structural features of bread, and *in vitro* starch digestibility.

2. Materials and methods

2.1. Materials

Rye (*Secale cereale L.*) grain was purchased from Tibiona (Villanova Mondovi, Italy). For the confocal laser scanning microscopy, Rhodamine B R6626 (≥95 %, Sigma Aldrich) and Calcofluor White M2R (Fluorescence Bright 28, MP Biomedicals) were used as dyes. For the *in vitro* digestion, the following enzymes were used: pepsin P7000 (from porcine gastric mucosa, specific activity ≥250 units/mg solid); pancreatin P7545 (from porcine pancreas, 8 × USP); invertase I4504 (from baker's yeast, specific activity ≥300 units/mg solid); and amyloglucosidase A7095 (from *Aspergillus niger*, ≥260 U/mL) (all from Sigma Aldrich). The other chemicals and solvents utilized were of analytical grade.

2.2. Methods

2.2.1. Flour preparation

The rye was ground using a multi-mill (Alpine Hosokawa, Augsburg, Germany). The following conditions were set up: speed: 1700 rpm (28.33 HZ); power: 500 W; difference pressure: 30.0 mbar; airflow, 52 m^3/hAfter milling, two meshes were used to sieve the flour: 1800 μm and 1000 μm. The flour retained on the 1800 μm sieve was codified as large flour (LF), and the fraction between 1000 and 1800 μm was identified as medium flour (MF). The choice of flour particle sizes was based on a previous study (Tagliasco et al., 2022). To obtain a small particle size, a certain quantity of large flour was milled again following these conditions: speed: 14000 rpm (233.33 Hz); power: 600 W; pressure difference: 30.0 mbar; airflow, 52 m^3/h. This re-milled fraction was named from now on as a small flour (SF).

2.2.2. Flour characterisation

The particle size distribution (PSD) of the three different rye samples was determined by sieving the flours through 9 sieves with decreasing mesh diameters: 2500 μm, 2000 μm, 1400 μm, 1000 μm, 800 μm, 500 μm, 300 μm, 150 μm, 50 μm. A representative amount of sample (100 g) was poured inside the sieve's column placed in an electromagnetic shaker (Vibra Filtration, Barcelona, Spain), and shaken for five minutes. The analysis of PSD was repeated five times for each flour and the results were expressed as the percentage of mass particles retained on each sieve. The moisture content of the flour was measured following the official method (Method AACC 44-15.02., 1999).

Total starch content was measured following the instruction of the Total Starch assay kit purchased by Megazyme (Bray, Ireland) following the procedure of total starch content of samples containing resistant starch. Before the determination, LF and MF were re-milled using Freezer/Mill 6875D (Spex SamplePrep, USA) to produce a final particle size < 500 μm as recommended in the kit protocol. Damage starch was measured using the kit provided by Megazyme (Bray, Ireland) following the approved methodology AACC Method 76–31.01. The resulting glucose, produced after the enzymatic reaction for total starch and starch damage determination, was detected by a colourimetric method and the absorbance was measured at 510 nm using a spectrophotometer Cary 50 (Agilent Technologies, USA). The amount of protein contained in flour was determined using the Dumas method with a flash EA 1112 NC analyzer (Thermo Fisher Scientific Inc., Waltman, United States of America) following the protocol given by the manufacturer. The conversion factor for rye protein was 5.83 (Müller, 2017). Soluble and insoluble fiber was analyzed according to Megazyme kit, K-TDRF (Megazyme, Wicklow, Ireland). The medium and the large samples were previously milled to pass through a sieve with a mesh of 500 μm. Three replicates on three different days were done for all the measurements.

2.2.3. Bread model system preparation

A bread model system made up of rye flour, water, salt, and fast action yeast (*Saccharomyces cerevisiae*), as displayed in Table 1, was used to produce rye bread. The optimum of water and the mixing time to obtain the final dough consistency of 420 Brabender Units (BU) were studied with a water absorption test at 30 °C and 63 rpm using a Farinograph (Brabender GmbH & Co KG, Duisburg, Germany) following the same method previously described (Tagliasco et al., 2022). The ingredients were mixed at 20 °C with a Hobart mixer (N50, Hobart,

Table 1
Formulation of rye bread: SB = bread made with small particle size flour; MB = bread made with medium particle size flour; LB = bread made with large particle size flour. Ingredients are expressed on 100 g of flour.

	Flour (g)	Fast action yeast (g)	Salt (g)	Water (g)	Mixing time (min)	Proofing time (min)
SB	100	2	2	62	5	90
MB	100	2	2	60	60	90
LB	100	2	2	59	90	95

Woerden, The Netherlands). After the mixing step, the dough was portioned into 5 loaves weighing approximately 90 g and transferred into baking tins (Patisse, bread form 9 cm). The loaves were placed in an incubator at 30 °C and 75% humidity. *Saccharomyces cerevisiae* was used as a leavening agent instead of sourdough, commonly used for rye bread production, to rule out the effect of acid production on bread digestibility (De Angelis et al., 2009). To eliminate the effect of compositional variations on yeast metabolism, the final fermentation time for MF and LF was defined as the time needed to produce a similar amount of CO_2 as in SF in 90 min of fermentation. The CO_2 production during the fermentation was measured using a Risograph (National Manufacturing C., Lincoln, NE) and the data were collected through a Risosmart software (National Manufacturing C., Lincoln, NE). During this measurement around 65 g of dough was placed in metallic baking puns and allocated inside a proofing cabinet (model SDCC-1P/W, Koma koeltechnische Industrie B.V., Roermond, the Netherlands), at 30 °C and a pressure of 760 bar. The formulation for each particle size bread is displayed in Table 1. After the baking step, the loaves were removed from the tins and cooled down on a rack for 1 h. Three out of the 5 bread were kept in a Ziploc ® bag 17.7 × 18.8 cm (SC Johnson, United States of America) overnight to perform the quality measurements the day after. The other two bread loaves were frozen to measure the digestibility and to further observe the microstructure with confocal laser scanning microscopy. Bread production was repeated three times. Three variations in formulations were studied: a bread produced with SF (coded SB), one with MF (coded MB), and one obtained with LF (coded LB).

2.2.4. Bread quality characterization

The moisture content of around 3 g of breadcrumbs was analyzed using the quoted official method (Method AACC 44-15.02., 1999). Total starch of bread samples was analyzed following the instructions reported in the assay kit TSTA purchased by Megazyme (Bray, Ireland). The procedure followed for the analysis was the determination of the total starch content of samples containing resistant starch. The soluble and insoluble fiber was determined using the enzymatic method K-TDRF, (Megazyme, Wicklow, Ireland). For the protein determination, the Kjeldahl method was used following the manufacturer's instructions. The conversion factor for rye protein was 5.83 (Müller, 2017). The volume of each loaf was measured by the rapeseed displacement method following the official analytical protocol (Method AACC 10-05.01, 2009). The specific volume was expressed as the proportion between volume and weight of the sample (cm^3/g). The water activity of bread samples was measured at 25 °C utilizing an a_w-meter (LabMaster-aw, Novasin, Lachen, Switzerland). Briefly, around 2 g of breadcrumb was let equilibrate at 25 °C in the a_w-meter and then the a_w was measured. Representative images of whole bread and the respective slices were captured using an image acquisition cabinet (Immagini & Computer, Bareggio (Milan), Italy) which was equipped with a digital camera (EOS 550D, Canon, Milan, Italy). The textural properties of bread were measured using a TA-XT plus analyzer (Stable Micro Systems, Godalming, UK) following the method previously described (Tagliasco et al., 2022). The analyzed parameters were hardness (N), the force required to deform the food in the first bite (Lapčíková et al., 2019); cohesiveness (−), the extent to which bread deforms when compressed, and resilience (−), the ability of the sample to regain its original height (Lapčíková et al., 2019). The analysis was carried out in triplicate.

2.2.5. In vitro starch digestibility of flour and bread

The digestibility of rye flour and bread was determined according to *in vitro* Englyst's method (Englyst et al., 2018). Briefly, 2 g of sample was weighed, the flour as it was, and the bread was cut into cubes of about 5 × 5 × 5 mm. The *in vitro* digestion procedure is divided into two phases (i.e., gastric one lasting 30 min and intestinal one lasting 120 min). In the first phase, the samples were mixed with 10 mL of guar gum solution (0.05 M HCl) with pepsin (≥ 250 units/mg solid) and were shaken for 30 min at 37 °C and 180 rpm. During the intestinal phase, 5 mL of enzyme solution containing pancreatin (8 × USP), invertase (≥ 300 units/mg solid), and amyloglucosidase (≥ 260 U/mL), have been added to the digesta together with 10 mL of 0.25 M sodium acetate buffer (37 °C) and 5 marbles. The samples were kept at 37 °C for 120 min and agitated at 180 rpm. At minutes 20 and 120, 100 μL of digesta were sampled and the enzymatic reaction was stopped with the addition of 4 mL of ethanol 96%. Later samples were centrifuged at 14,000 rpm, 50 μL was sampled, and let react for 20 min at 50 °C with 1.5 mL of GOPOD reagent (Megazyme, Bray, Ireland). The spectrophotometer Cary 60 UV – Visible (Agilent Technologies, USA) was used to measure the absorbance at 510 nm.

The data obtained from Englyst's digestion were used to calculate the rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS). RDS represents the amount of glucose released in the first 20 min of digestion multiplied by 0.9 to convert the glucose detected into a starch amount. SDS represents the quantity of glucose released between minutes 20 and 120. RS represents the fraction of starch that was not digested after 120 min and was calculated as the difference between the total starch (TS) and the digested starch (RDS + SDS). Total starch content was measured following the kit protocol Total Starch assay (Megazyme, Bray, Ireland). The data obtained, RDS, SDS and RS were expressed in grams of starch per 100 g of total starch on a wet basis. The total starch content was measured 6 times for each bread and the *in vitro* digestibility was performed 12 times for each sample.

2.2.6. The disintegration of the sample during in vitro digestion

The disintegration of the samples during the *in vitro* digestion was studied by image analysis, measuring the particle size of the digesta over time. Briefly, 2 g of each bread sample was prepared as described in the previous paragraph (2.2.5) and subjected to *in vitro* digestion. The particle size of the digesta was measured after the gastric phase (T0) and at T20 and T120 of the intestinal phase. At these time points, the total digesta was diluted with 40 mL of water, placed in a flat plastic container (20.3 cm × 30.5 cm × 5.1 cm), and gently spread with a spatula. The plastic container was placed on a flatbed scanner CanoScan 9000 F MarkII (Canon Europa, Amstelveen, the Netherlands) to capture the digesta images. The collected images were processed by ImageJ (ImageJ, Bethesda, Maryland, USA) following the method described by Chen et al., (2021). The captured images were transformed in 8-bit and adjusted for brightness/contrast. A threshold of 50–255 was used to obtain a binary picture. For every image, the average area of digesta particles (expressed in mm^2) was measured to determine the size of the digesta particles. Particles smaller than 0.015 mm^2 were excluded from the data analysis to eliminate any interference with the background. The areas (mm^2) of the digesta particles obtained from the image analysis were then categorized in 7 intervals mm^2; **1**: < 0.12; **2**: ≥ 0.12, < 0.3; **3**: ≥ 0.3, < 0.6; **4**: ≥ 0.6, < 0.9; **5**: ≥ 0.9, < 1.2; **6**: ≥ 1.2, < 1.5; **7**: ≥ 1.5 to show the particle size distribution in the different phase of *in vitro* digestion (Suo et al., 2021). All measurements were done in quadruplicate.

2.2.7. Confocal laser scanning microscopy (CLSM)

Confocal laser scanning microscopy Stellaris 5 (Leica Microsystem CMS GmbH Wetzler, Germany) was used to detect the cell wall integrity

in each particle size in rye flour and bread. The software Leica Application Suite X (LAS X) (Leica Microsystem CMS GmbH Wetzler, Germany) was used to process and analyze the micrographs. For flour, around 2 g of sample was weighed in a glass tube and stained with around 2 mL of a solution containing 0.005% Rhodamine B (RB) and 0.01% Calcofluor white (CFW) for one hour. The cell walls, consisting mainly of arabinoxylan and β-glucan, were stained in blue with CFW, and at the same time, the proteins were stained in red/purple color with RB (Rovalino-Córdova et al., 2019). After that, the flour was placed on a glass slide, elevated with a spacer, and analyzed. Regarding bread, it was defrosted 16 h before the measurement and carefully cut with a razor blade (Personna, Verona, Italy) to obtain slices 1 mm thick and stained in the same conditions already explained for flour. RB was excited at 543 nm with He-Ne laser and CFW at 405 nm with argon laser. The pictures were captured at two levels of magnification (i.e., 10x or 20x) and a depth of 8 bits.

2.3. Statistical analysis

The data were expressed as mean ± standard deviation (SD) and subjected to statistical analysis by IBM SPSS Statistics for Windows version 23.0 (IBM Corp., Armonk, N.Y., USA). The statistical method used to determine the significance differences between the analyzed samples was a One-way ANOVA ($p \leq 0.05$) and a post-hoc Tukey's test ($p \leq 0.05$) was performed to identify which sample was different from the others. The chi-square test ($p \leq 0.05$) was used to assess the statistical differences between the particle size distribution of the digesta of SB, MB and LB, for each time point. The analysis was performed in the XLSTAT version 2021.1 software.

3. Results

3.1. Flour characterization

The particle size distribution of the three flour samples is displayed in Fig. 1. SF, obtained by double milling LF, had a particle distribution ranging from 0 to 500 μm, with a peak at 150 μm. MF ranged between 800 μm to 2000 μm, and 70% of the particles were bigger than 1000 μm and smaller than 1400 μm. LF, instead, presented 80% of the particles between 1400 μm and 2000 μm. The proximate composition of the flour fractions is shown in Table 2. LF, MF, and SF were not different in terms of protein, total starch content and soluble and insoluble dietary fiber. However, the moisture content of SF was 17 % and 18 % lower than that of MF and LF, respectively. Damaged starch damage was detected only in SF since it underwent a double milling process.

Table 2
Protein, total and damaged starch, moisture content, and soluble and insoluble dietary fiber of rye flour with different particle sizes.

Sample	protein content (g/100 g)*	total starch (g/ 100 g)	damaged starch (g/ 100 g)	moisture content (g/100 g)	soluble fiber (g/100 g)	insoluble fiber (g/ 100 g)
SF	7.94 ± 0.18[a]	51.58 ± 3.32[a]	0.70	9.95 ± 0.33[a]	3.72 ± 0.34[a]	11.83 ± 0.40[a]
MF	7.99 ± 1.11[a]	49.07 ± 4.22[a]	n.d.	11.97 ± 0.48[b]	3.21 ± 0.54[a]	12.84 ± 0.97[a]
LF	8.04 ± 2.64[a]	46.36 ± 0.74[a]	n.d.	12.12 ± 0.32[b]	3.18 ± 0.84[a]	11.99 ± 1.87[a]

Values are reported as % of flour's fresh weight and expressed as mean ± SD. Mean values (n = 9) within a column with different letters were significantly different ($p \leq 0.05$; Tukey's test). *Protein conversion factor = 5.83; "n.d.": not detected; SF = small particle size flour; MF = medium particle size flour; LF = large particle size flour.

3.2. Bread characterization

The characteristics of bread made with different particle sizes and their textural properties are shown in Tables 3 and 4, respectively. The production of bread was standardized to have the same consistency after the mixing step and the same amount of CO_2 produced during the leavening. However, significant differences in bread properties were found among the three samples. The moisture content of MB was 3 % and 1.4 % higher than that of SB and LB, respectively, whereas no difference was observed between SB and LB (Table 3). The water activity showed a different pattern, the lowest value was found for SB (a_w = 0.95), whereas MB and LB (a_w = 0.96) were not significantly different (Table 4). Water activity was well related to moisture content, which is logical for this set of samples. No significant differences ($p > 0.05$) were found among the three samples (Table 3) concerning the total starch, soluble and insoluble dietary fibre. The protein content instead increased, increasing the particle size of the bread. Moreover, the volume of bread decreased with increasing particle size. Indeed, the volume of SB was statistically higher ($p \leq 0.05$) than the other samples, whereas

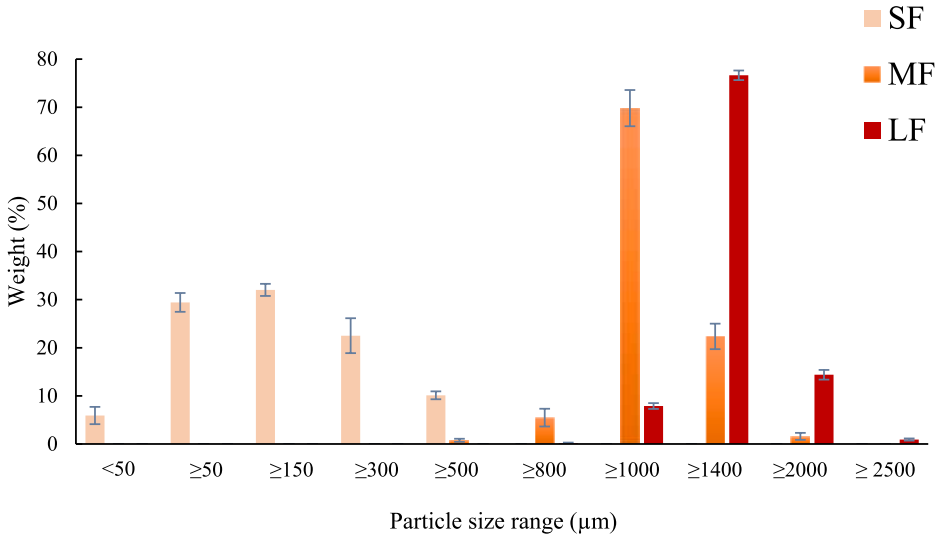

Fig. 1. Particle size distribution (PSD) of rye flours. SF = small particle size flour; MF = medium particle size flour; LF = large particle size flour. Results are expressed as mean particle weight retained on each sieve % ± SD: 2500 μm, 2000 μm, 1400 μm, 1000 μm, 800 μm, 500 μm, 300 μm, 150 μm, 50 μm.

Table 3
Moisture content, total starch, soluble and insoluble dietary fiber and protein of rye bread samples produced with small particle size flour, medium particle size flour, and large particle size flour.

Sample	moisture content (g/100 g)	total starch (g/100 g)	soluble fiber (g/100 g)	insoluble fiber (g/100 g)	protein (g/100 g)
SB	41.8 ± 0.6^{b}	33.3 ± 2.1^{a}	2.66 ± 0.58^{a}	7.74 ± 0.07^{a}	4.73 ± 0.21^{a}
MB	43.1 ± 0.5^{a}	31.7 ± 2.7^{a}	2.16 ± 0.62^{a}	8.11 ± 0.05^{a}	4.96 ± 0.18^{ab}
LB	42.5 ± 0.5^{b}	30.3 ± 0.5^{a}	2.12 ± 0.22a	8.59 ± 0.60^{a}	5.26 ± 0.33^{b}

Values are reported on fresh weight and expressed as mean ± SD. Mean values (n = 9) within a column with different letters were significantly different ($p \leq 0.05$; Tukey's test). SB = bread made with small particle size flour; MB = bread made with medium particle size flour; LB = bread made with large particle size flour.

Table 4
Water activity, specific volume, hardness, cohesiveness and resilience of rye bread samples produced with small particle size flour, medium particle size flour, and large particle size flour.

Sample	water activity	specific volume (cm^3/g)	hardness (N)	cohesiveness	resilience
SB	0.95 ± 0.00^{b}	1.27 ± 0.04^{a}	50.3 ± 4.2^{b}	0.57 ± 0.03^{a}	0.26 ± 0.02^{a}
MB	0.96 ± 0.00^{a}	0.98 ± 0.04^{b}	61.8 ± 8.9^{a}	0.45 ± 0.03^{b}	0.22 ± 0.02^{b}
LB	0.96 ± 0.00^{a}	1.01 ± 0.04^{b}	58.3 ± 5.3^{a}	0.38 ± 0.05^{c}	0.17 ± 0.03^{c}

Values are expressed as mean ± SD. Mean values (n = 9) within a column with different letters were significantly different ($p \leq 0.05$; Tukey's test). SB = bread made with small particle size flour; MB = bread made with medium particle size flour; LB = bread made with large particle size flour.

there was no significant difference between MB and LB. The values of hardness decreased when the specific volume increased (Table 4). SB showed the lowest value (50.3 N), instead, MB and LB had similar hardness (around 60 N). Cohesiveness and resilience decreased with the increase of the particle size. Representative images of the entire bread loaf and slices were shown in Fig. 2, and they clearly displayed the difference in structure among the three bread samples prepared with increasing particle sizes. The crumb of SB was quite uniform, compact, and cohesive, however, the crumb of MB and LB appeared fragile, non-homogeneous, and easy to break. Moreover, in MB and LB the flour particles were still visible, and they were not well embedded in the dough structure as in SB.

3.3. Starch digestibility

The results of *in vitro* starch digestion of rye flour and rye bread are shown in Fig. 3. Regarding the flour starch digestibility, RDS, SDS, and RS values of SF were significantly different ($p \leq 0.05$) from those of MF and LF, which were not significantly different. MF and LF had a significantly ($p \leq 0.05$) lower value of RDS and SDS than SF, whereas SF showed the lowest value for RS (2.1 ± 1.5). Significant differences were found for SDS when the flours were used for breadmaking. In SB, SDS was 46% lower than that in MB and 33 % lower than that in LB, instead LB and MB were not significantly different. Moreover, RS in bread had an opposite trend compared to that found in flour, being SB the bread with a significantly ($p \leq 0.05$) higher value of RS (26.1 ± 10.5) than other bread samples. No difference, instead, was found in RDS among bread samples.

3.4. Disintegration of bread sample during in vitro digestion

To understand how bread samples were disintegrated during the *in vitro* digestion, the images of the total simulated digesta were captured after the gastric phase (T0) (Fig. 4 panel a), at 20 min (T20) (Fig. 4 panel b), and the end of the intestinal phase (T120) (Fig. 4 panel c) and analyzed through image analysis to measure the area of digesta particles. The mean area of the particles decreased from T0 to T120, for all three samples, but the magnitude of these differences changed among the three bread loaves made with increased flour particle size. As shown in Fig. 4a, at T0, the digesta from sample SB showed a normal distribution with a peak in the interval around 0.9–1.2 mm^2. The digesta particles of MB, instead, were largely distributed among 7 intervals, with a peak around 0.9–1.2 mm^2. For the LB, two populations were observed, the first peaking in the interval between 0.12–0.3 mm^2 and the second one at 0.9–1.2 mm^2. At T20 (Fig. 4 panel b), instead, the particles of SB were significantly bigger than those of both LB and MB. Seventy per cent of the SB particles were around 0.6–0.9 mm^2, instead, 50% of particles of MB and LB had a size smaller than 0.3 mm^2. At the end of the *in vitro* digestion (Fig. 4 panel c), 80% of SB and MB particles were smaller than 0.12 mm^2, instead, for LB, 30% of them ranged between 0.12 and 0.3 mm^2, probably due to cell clusters, bigger in LB than SB and MB.

3.5. Confocal laser scanning microscopy

CLSM was used to capture images of rye flour and bread, as shown in Fig. 5. The samples were stained to simultaneously visualize the cell wall (in blue) and protein (in red). The cell walls in sample SF appeared to be extensively broken into small pieces (Fig. 5A), likely because of double milling. Instead, a significant amount of intact cell clusters was observed in samples MF and LF (Fig. 5B and C). A similar pattern was observed in bread samples. Cell walls appeared to have completely lost their integrity in sample SB (5D), whereas big clusters of intact cells were still detectable in samples MB and LB (5E and 5F). The shape and the size of the cells seemed not to have been radically affected by bread processing.

4. Discussion

Maintaining the integrity of the cell wall, such as by increasing the flour particle size, has been proposed as a promising strategy to reduce starch digestibility in wheat flour and porridge. This approach, however, does not seem efficient in modulating starch digestibility in bread. It was hypothesized that the steps for producing bread, such as mixing, fermentation and baking, could increase the porosity of the cell wall, which in turn increased the accessibility of enzymes to starch granules (Korompokis et al., 2021; Tagliasco et al., 2022). However, to the best of our knowledge, this effect was mainly studied in wheat, even if rye is characterized by a thicker cell wall than wheat grain (Autio & Salmenkallio-Marttila, 2001). In the present study, the effect of three different particle sizes on the digestibility and textural quality of rye flour and bread produced therefrom was investigated.

CLSM images revealed the presence of large clusters of intact cells in MF and LF, and their integrity was maintained during bread processing, i.e., MB and LB. Instead, the cell walls in SF and SB were largely damaged due to the harsh milling conditions. This is also confirmed by the level of damaged starch in small flour, not detected in medium and large flour. The starch digestibility of rye flour decreased with the increase in flour particle size. The effect of particle size on cell wall integrity and starch digestibility has been previously observed in legumes (Bhattarai et al., 2017; Dhital et al., 2016; Rovalino-Córdova et al., 2019) and cereals like wheat, sorghum and barley (Bhattarai et al., 2018; Korompokis et al., 2019; Lin et al., 2020). In LF, starch is mostly protected by intact cell walls, which restricts the α-amylase enzyme diffusivity inside the particles and acts as a barrier limiting its contact with starch. In contrast, the physical hindrance is lost once the structure

Fig. 2. Representative images of the whole bread samples (A, B, C) and the respective slices (D, E, F) of SB = bread made with small particle size flour; MB = bread made with medium particle size flour; LB = bread made with large particle size flour.

of the grain is damaged by the milling process to transform it into fine flour so that starch can be easily digested (Bhattarai et al., 2018; Korompokis et al., 2019; Tagliasco et al., 2022).

In bread, instead, the results obtained on starch digestibility were quite unexpected. RDS, which represents the amount of starch digested after 20 min, was not significantly different among the three bread samples. Instead, SDS was significantly lower in SB than in MB and LB. Moreover, SB contained the highest amount of RS among the three analyzed breads. Overall, the extent of starch digestibility in bread made with SF was lower than in bread made with bigger particle sizes and, therefore, with clusters of intact cells. The physical hindrance effect in MF and LF was lost in the corresponding bread, despite intact cell walls being clearly detected in the CLSM images. A similar behavior was recently observed when coarse wheat flours were processed into bread (Korompokis et al., 2021; Tagliasco et al., 2022). These authors showed that during bread processing the cell wall was not damaged, but its porosity might have increased due to the change in molecular weight of arabinoxylans. This might have been occurred also in bread produced from rye grain, even though rye was reported to have a thicker primary cell wall than wheat grain (Comino et al., 2014). It is possible to hypothesize that the fiber solubilization and the resulting increase in cell wall porosity might explain the increase in starch digestibility in bread compared to the flours but this cannot completely explain the differences in starch digestibility in SB compared to MB and LB. As reported by several authors, during bread processing, arabinoxylan hydrolysis reduced the proportion of high-molecular-weight extractable arabinoxylans and increased the proportion of low-molecular-weight arabinoxylans compared to rye flour (Cyran & Dynkowska, 2014; Andersson et al., 2009). Moreover, during the baking process, water-extractable arabinoxylan increased and water-unextractable arabinoxylans decreased (Cyran & Dynkowska, 2014). Nevertheless, the doughs made with MF and LF required up to 90 min of mixing to achieve the desired consistency of 420 BU. This extensive mixing might have increased further the cell wall porosity but might have also increased hydration of the grain and starch swelling, which in turn enhanced enzyme mobility and subsequently improved starch accessibility. This effect was limited in the dough made with SF which reached the same consistency with only 5 min of mixing. Moreover, the prolonged mixing time might have enhanced the activity of endogenous enzymes (Andersson et al., 2008). Nevertheless, the differences in SDS and RS content of the three bread samples could also be attributed to the distinct texture of these rye bread loaves. Unlike in durum wheat, rye proteins cannot form a three-dimensional structure and a stable viscoelastic network capable of holding gas during fermentation. Rye proteins are less prone to form intermolecular disulphide bonds and the presence of pentosan hinders the formation of a strong network (Beck et al., 2011).

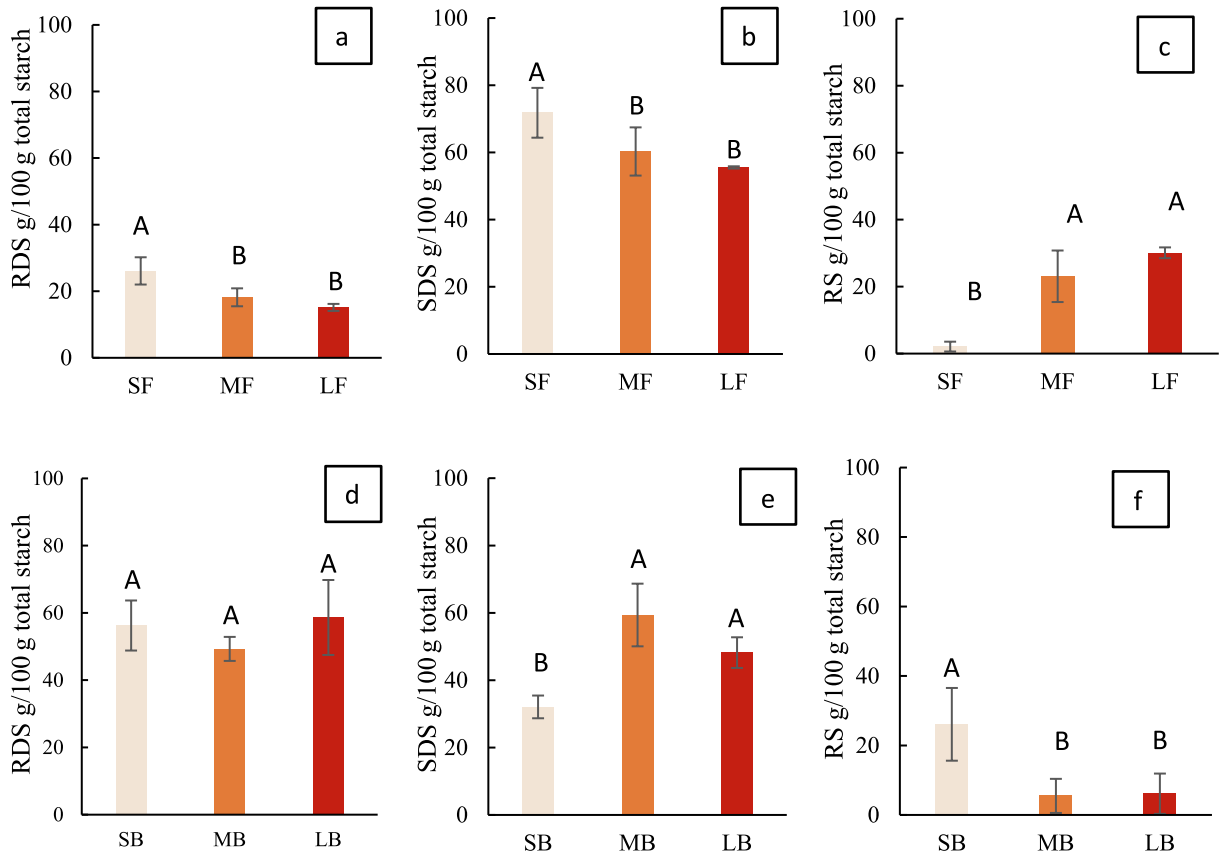

Fig. 3. Rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) of rye flours with different particle sizes (panels a, b, and c) and of rye bread made with flours with different particle sizes (panels d, e and f). SF = small particle size flour; MF = medium particle size flour; LF = large particle size flour; SB = bread made with small particle size flour; MB = bread made with medium particle size flour; LB = bread made with large particle size flour. Results are expressed as % of the total starch. Columns sharing the same letter were not significantly different ($p \leq 0.05$; Tukey's test) (n = 9).

Hence, in rye bread, where the structure is mainly formed by a continuous phase of fiber, mainly arabinoxylans, connected with proteins that surround the starch granules, the use of a large flour particle size (> 1000 μm) decreased drastically the overall bread quality (Döring et al., 2015). Indeed, MB and LB exhibited statistically greater hardness compared to SB, and consequently, a lower specific volume. As reported by Renzetti and Jurgens (2016), crumb hardness is an exponential function of crumb density; thus, an increase in density or a decrease in specific volume can significantly enhance hardness. Additionally, in this case, the increased hardness resulted in reduced crumb resilience and cohesiveness, which are measures of the crumb ability to regain its original height after compression. These properties decreased markedly with the increase in particle size. As suggested by Verbauwhede et al. (2018) and Renzetti et al. (2021), crumb cohesiveness and resilience in wheat bread are mainly controlled by starch swelling and amylose leaching rather than by the gluten network. This is also confirmed by Bressiani et al. (2017), who reported an increase in viscosity for flour with finer particle size than the coarse one, due to the large contact surface and the consequent starch leaching and swelling. This could explain why SB, produced with fine flour was more resilient and cohesive than MB and LB which contain large clusters of encapsulated starch in intact cells. Therefore, the different textural parameters reported for the three bread samples mirrored the different disintegration behaviours observed during the *in vitro* digestion. Indeed, MB and LB, which were characterized by a lower cohesiveness and resilience compared to SB, presented more than 50% of particles smaller than 0.3 mm^2 after the first 20 min of simulated intestinal digestion. At the same time point, 70% of SB digesta particles were larger than 0.9 mm^2. The relatively bigger size of the compact digesta particles in SB could have limited the diffusivity of the enzymes inside the bread structure, slowing down starch digestibility, and therefore produced a higher amount of starch that escaped digestion, i.e., RS. Bread with the highest firmness and low moisture was the easiest to disintegrate during digestion and was not able to maintain its structure with the addition of digestive liquid (Bornhorst & Singh, 2012). The central role of disintegration rate in modulating starch digestibility was well explored *in vivo* by Vanhatalo et al. (2022). In the quoted manuscript, the disintegration rate of durum wheat food products with different textures, such as bread, cous cous and pasta, was evaluated and it was demonstrated that the food products with a more cohesive structure disintegrated less during the gastric phase, leading to bigger digesta particles, and therefore, to a reduced glycemic index. Hence, a highly packed brittle structure formed during bread processing is more easily weakened by intestinal movement during digestion. As shown in the manuscript, this facilitated the disintegration of the digesta into small particles and the consequent starch hydrolysis due to the greater contact surface between the enzyme and the substrate. The key role of crumb structure in modulating the starch digestibility of rye bread was also confirmed by Nordlund and coworkers (2016), who showed that, by increasing the porosity of the crumb structure through the addition of gluten in the bread recipe, the digestibility significantly increased. The formed porous aerated structure, indeed, was easier to disintegrate during digestion, and therefore, increased its insulin and glycemic response. From what was observed, it is possible to conclude that the driving mechanism in reducing starch digestibility in these rye model bread samples, where the structure is mainly formed by starch interaction and not gluten network, is the physical hindrance that enzymes face in reaching the starch. In support of this, also in starch gel, a simple food model, digestibility mainly

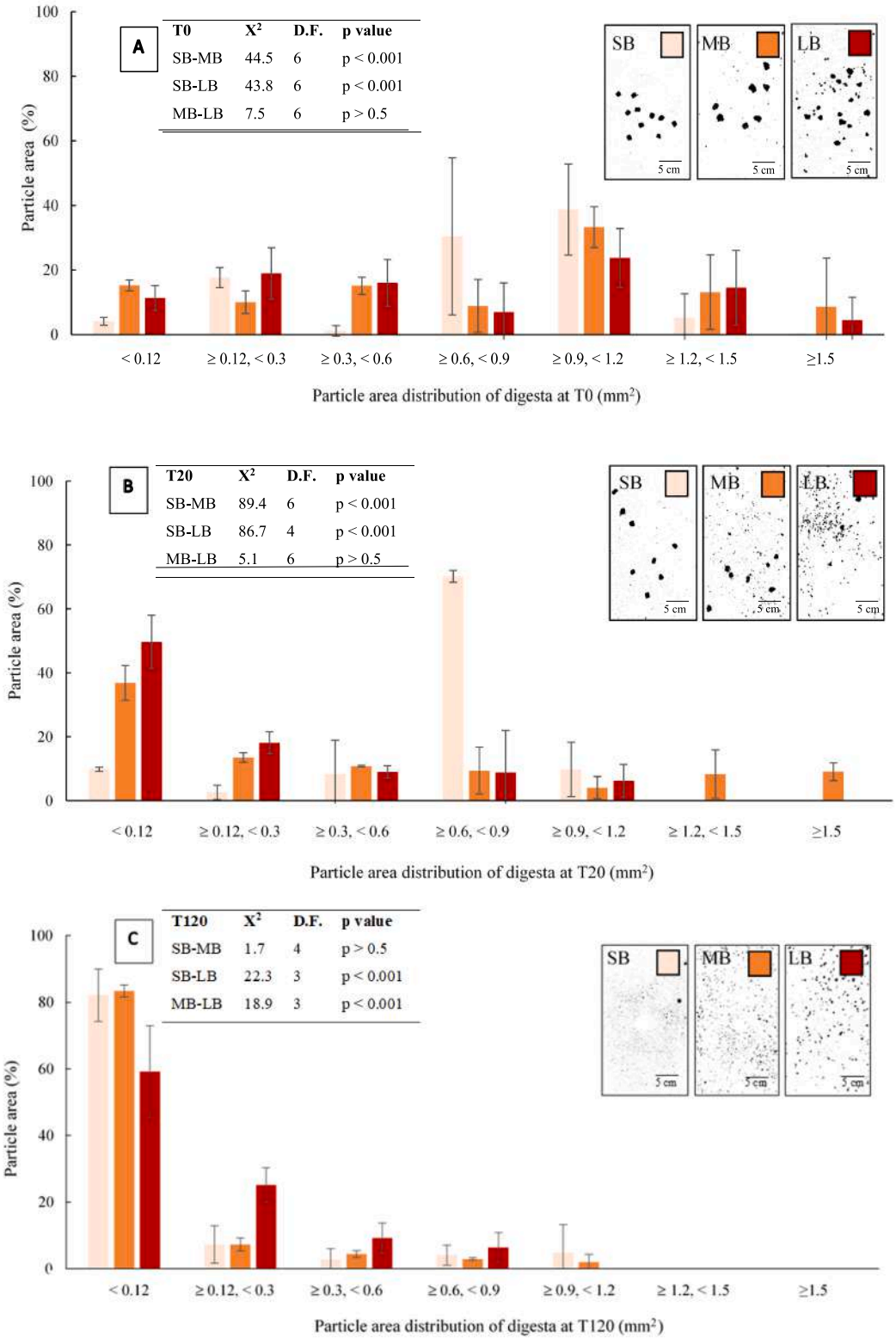

Fig. 4. Representative images (in the upper right corner) and particle size distribution of the entire digesta particles after the gastric phase (T0, panel a), after 20 min of intestinal phase (T20, panel b), and at the end of the intestinal phase (T120, panel c) of rye bread samples, captured through scanner and processed with Image J. SB = bread made with small particle size flour; MB = bread made with medium particle size flour; LB = bread made with large particle size flour. Particle size distribution of digesta divided into 7 intervals mm^2; **1**: < 0.12; **2**: ≥ 0.12, < 0.3; **3**: ≥ 0.3, < 0.6; **4**: ≥ 0.6, < 0.9; **5**: ≥ 0.9, < 1.2; **6**: ≥ 1.2, < 1.5; **7**: ≥ 1.5. P value ≤ 0.05 in chi-squared test showed significant differences between the digesta distribution: X2 = Chi squared value, D.F. = degree of freedom.

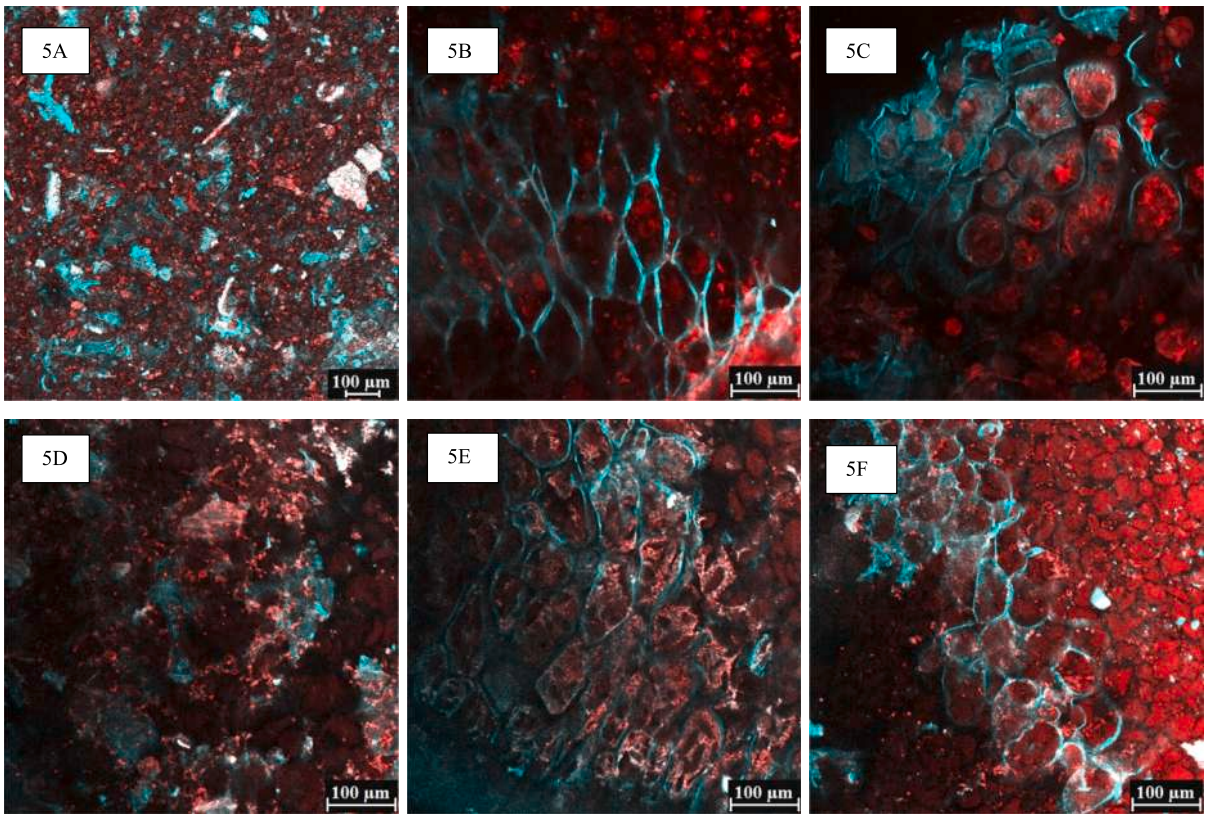

Fig. 5. Representative confocal laser scanning microscopy images of SF = small particle size flour (5A); MF = medium particle size flour (5B); LF = large particle size flour (5C); SB = bread made with small particle size flour (5D); MB = bread made with medium particle size flour (5E); LB = bread made with large particle size flour (5F). Calcofluor White (0.01%) and Rhodamine B (0.005%) were used as dyes to stain simultaneously the cell wall (light blue) and the protein (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

depends on the texture and the mechanical properties of the matrix and, therefore, on the capacity of the enzyme to move into the gel. As Santamaria et al. (2021, 2022) reported, gels characterized by strong and highly structured architecture, including high firmness and a high elastic modulus (G'), show a lower starch digestibility than gel characterized by a porous and less organized structure with more viscous behavior (higher tan δ (G″/G′)). This loose structure may facilitate easier enzyme penetration, thereby promoting the initial hydrolysis of starch.

In conclusion, producing rye bread with flour of different particle sizes allowed to better understand the effect of intact cell walls on *in vitro* starch digestion in rye flour and bread. The intactness of cell walls is a limiting factor that controls the extent of hydrolysis of starch only in rye flour but not in a bread matrix. Instead, bread made with fine flour that disintegrated less during digestion was the one with the lower starch accessibility, confirming the central role of bread texture in modulating starch digestibility. The findings of the present study can guide future experiments in enhancing crumb cohesiveness to reduce the disintegration during *in vitro* digestion and consequently lower starch digestibility. For example, the addition of gluten to increase the cohesiveness and reduce the hardness of bread made with coarse rye might be an effective strategy to improve the textural quality of the bread. This approach can also reduce mixing time, thereby decreasing cell wall porosity.

Moreover, in the future, it is advised to incorporate real mastication as a first step in the *in vitro* model of intestinal digestion. In such a way, a more realistic bolus disintegration can be achieved compared to the simplified sample preparation of the existing *in vitro* models of digestion. Even better, an acute intervention with bread with different textural properties where bolus properties are also characterized, should be carried out to confirm our hypothesis.

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CRediT authorship contribution statement

Marianna Tagliasco: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Guillem Font:** Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Stefano Renzetti:** Writing – review & editing, Visualization, Supervision, Project administration, Methodology, Data curation, Conceptualization. **Edoardo Capuano:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Methodology, Data curation. **Nicoletta Pellegrini:** Writing – review & editing, Visualization, Supervision, Project administration, Methodology, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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