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Development of a composite bread exploiting common bean (*Phaseolus vulgaris* L.) fermented flour with lactic acid bacteria

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ABSTRACT

Legumes are sustainable and nutritionally valuable crops, yet their consumption is limited by antinutritional factors including lectins, phytic acid, and raffinose-family oligosaccharides (RFOs) impairing nutrient absorption and causing gastrointestinal discomfort. The study investigates the development of bread enriched with 25% fermented common bean (*Phaseolus vulgaris* L.) flour by exploiting lactic acid bacteria (LAB) fermentation from three different strains: *Lentilactobacillus buchneri* LBC01, *Lacticaseibacillus rhamnosus* LRH01, and *Leuconostoc lactis* LN01. Antioxidant activity and digestibility, but also technological aspects, including dough rheology, loaf volume, volatile organic compound profile, and shelf-life, were evaluated. Notably, LAB fermentation improved dough leavening and reduced staling within 24 h, enabling a higher incorporation of legume flour. *Ln. lactis* increased bread volume by 15% compared to *Saccharomyces cerevisiae*. RFOs were not detected in either the fermented flours or the resulting breads. The inclusion of fermented bean flour significantly enhanced antioxidant capacity, with radical scavenging activity nearly tenfold higher than that of the wheat bread. Analysis of volatile compounds showed that enriched bread exhibited the highest overall volatile content. 2-pentylfuran—associated with "beany" aroma—was elevated in breads containing fermented bean flour (FBF), while diacetyl and acetoin (contributing buttery and caramel notes) were most abundant in breads fermented with *L. rhamnosus*. Microbial proteolysis contributed to improved protein digestibility, and FBF breads also showed increased starch hydrolysis. Overall, LAB fermentation offers a viable approach for producing legume-enriched breads with superior nutritional quality, improved technological performance, and extended shelf life, while effectively mitigating common challenges associated with the use of legume-based ingredients.

1. Introduction

Legumes have been identified as one of the best alternatives to animal-based food, a global dietary shift identified as a critical necessity in the fight against malnutrition and sustainability-related issues [1,2]. However, the utilization of legume seeds by consumers is limited, since a considerable proportion of people abstain from eating them due to poor taste and texture and thus legume consumption is often far away from the recommended dose intake [3]. The incorporation of legume flours in novel, convenient and healthy staple food such as bread is one of the possible approaches that may increase legume consumption [4]. The development of legume-supplemented bread with a better consumer acceptability while being highly recommended is also challenging as the partial replacement of wheat flour with alternative flours generally

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results in a poor bread texture and loaf volume when exceeding 15% [5–8]. Common bean (*Phaseolus vulgaris* L.) is the most grown food legume crop worldwide. Nutritionally, common beans are an important source of proteins (16-33%), starch (30-60%), fiber (14-19%), essential vitamins and minerals, while being low in lipid (1-3%) and offering high energetic value [9,10]. Additionally, common beans are rich in phytochemicals, including a diverse range of flavonoids [11]. Their composition contributes to numerous health benefits, aiding in the prevention and management of conditions like obesity, cardiovascular diseases, diabetes, and cancer diseases prevalent in industrialized societies [10]. Despite this, the nutritional value of common bean is often limited by the presence of antinutritional factors (ANFs), such as lectins, protease inhibitors, phytic acid, raffinose-family oligosaccharides (RFOs), and phenolic compounds, although over the past 20 years the scientific community has recognized that some antinutrients may impart health benefits [12,13].

Lactic acid bacteria (LAB) fermentation is a widely used food processing technique in the food industry, applied to enhance the quality of various products, including dairy [14,15], meat [16], cereals [17,18], and also legume-based foods [19–21]. LAB fermentation promotes acidification, which inhibits pathogen growth and may extend food shelf life. Moreover, LAB can convert food components into bioactive metabolites with significant roles in food preservation, technical functionality, health benefits, such as bacteriocins, B-group vitamins, and exopolysaccharides (EPSs) [22]. In addition, LAB proteolytic activity produces bioactive peptides and free amino acids (FAAs), enhancing both the nutritional value and sensory qualities of fermented foods [23–25]. From the metabolic point of view, LAB fermentation effectively reduces ANFs and sugar content, while increasing the bioavailability of essential nutrients such as minerals and γ-aminobutyric acid (GABA), endowed with physiological benefits [26], including antihypertensive and antidepressant effects [27].

Despite the well-known health benefits of LAB-fermented foods [28], few studies adopted an interdisciplinary approach for the development of bread enriched in fermented common bean flour encountering relevant limitations with pulse inclusion level higher than 15% [29] due to technological and nutritional challenges, including antinutritional factors and reduced leavening properties. In this context, the present study addresses these challenges by fermenting common bean flour with a combination of LAB and yeast, enabling the development of bread with 25% legume content.

2. Materials and methods

2.1. Microbial strains for bean flour fermentation

Lentilactobacillus buchneri LBC01, *Lacticaseibacillus rhamnosus* LRH01, *Leuconostoc lactis* LN01 were purchased from Sacco srl (Cadorago, CO, Italy). Each LAB strain was routinely propagated at 30 °C in de Man – Rogosa – Sharpe (MRS) broth (Biolife Italiana, Milan, Italy) for 18 h. Fermentation by LAB was compared with two yeasts previously isolated from sourdough, *Kazachstania humilis* ITEM 19255 and *Saccharomyces cerevisiae* 2B belonging to CNR ISPA collection Agri-Food Microbial Culture Collection (ITEM) Bari, Italy. Yeast strains were propagated at 25 °C in Yeast Malt broth (Biolife Italiana) for 24 h. LAB content was assessed using MRS Agar supplemented with cycloheximide (0.1 g/L) incubated anaerobically at 30 °C for 72 h (Anaerocult A, Merck-Millipore, Darmstadt, Germany), while yeasts were enumerated on Yeast-extract-Glucose Chloramphenicol agar (Biolife Italiana) after 5-day incubation at 25°C. All microbiological analyses were conducted in duplicate.

2.2. Bean flour fermentation

Fermentation of common bean flour, Taylor's Horticultural variety, was performed according to a previous study [26]. A lab-scale kneading (Artisan 5KSM150PS KitchenAid, St. Joseph, USA) equipped with a hook was used to knead 1 kg of common bean flour and 2.5 L of tap water previously inoculated at ca. 7.0 log10 CFU/mL with the test microorganism. The dough was incubated at 30 °C for 48 h for fermentation. The pH value of fermented flour was determined by a pH meter with a food penetration probe (Hanna Instruments Inc., Woonsocket, Rhode Island, 02895, USA) and final counting cells in the fresh fermented product were then determined. Five g of the fermented material were homogenized in 45 mL of sterile 2% (w/v) Buffered Peptone Water for 2 min using a Stomacher. The homogenate was serially diluted in quarter-strength Ringer's solution and plated on MRS agar supplemented with 0.1 g/L cycloheximide for LAB, or on YGC agar for yeasts. Plates were incubated as previously described in paragraph 2.1.

2.3. Dough preparation and characterization

Fermented bean flour was dried in a laboratory oven at 45 °C for 24 h, ground (M20 Universal Mill, IKA, Werke Staufen, Germany) and sieved (particle size <150 μm). Each fermented common bean flour (FBF) was incorporated into a commercial wheat flour (Molino Quaglia S.p.A., Vighizzolo d'Este, Italy; protein content: 14 g/100 g; W = 300 × 10^{-4} J) at a substitution level of 25%. Blend with unfermented common bean and wheat (25:75 ratio) was used as control (CTRLnf).

Mixing properties were performed in the Farinograph-E (Brabender GmbH & Co. KG, Duisburg, Germany) equipped with 50 g mixing bowl, following the ICC official method (ICC 115/1).

Leavening properties were assessed in duplicate with a Rheofermentometer® (Chopin, Villeneuve La Garenne Cedex, France). Blends were mixed with dry bakers' yeast (2.7 g/100 g flour; Cameo S.p.A; Desenzano del Garda, Italy) previously dissolved in distilled water, olive oil (3.3 g/100 g flour; Carrefour; Milan, Italy), sugar (2.7 g/100 g flour; Carrefour, Milan; Italy), and salt (1.5 g/100 g flour; Carrefour; Milan, Italy). The amount of water was determined by the farinograph test. The ingredients were mixed in the mixing bowl of the Alveograph® (Chopin, Villeneuve La Garenne Cedex, France) for 8 min at 25 °C. Then, 315 g of dough were placed in the Rheofermentometer® chamber, and the leavening was carried out at 30 °C for 3 h.

2.4. Bread preparation and characterization

Bread was prepared as described by Bresciani et al. [30] following the recipe given above. Two independent baking trials were conducted for each formulation, resulting in six loaves per trial and twelve for each formulation.

Bread was analyzed 2 h after baking for volume, color, moisture, water activity and firmness. Two loaves of each sample were wrapped with aluminum foil and stored at 25 °C for 24 h until texture analysis. The volume of 12 loaves (six from each baking trial) was determined using the same displacement method. The weight was recorded using a technical scale (Europe 1700, Gibertini, Novate, Italy). The specific volume of each loaf was calculated by dividing the measured loaf volume by its mass, with results expressed in mL/g. Crumb and crust color profile was determined using a reflectance colorimeter (CR 300 Minolta Co., Osaka, Japan, with results expressed in CIE $L^*a^*b^*$ color space. Crust color measurements were performed in triplicate on three loaves per trial (n = 18), while crumb color was measured on 2.5 cm slices from three loaves per trial (n = 18). Crumb firmness (n = 6 for t0 and n = 4 for t1) was measured using a Texture Analyzer TA-XT plus C (Stable Micro Systems, Godalming, UK) equipped with a 10 kg (100 N) load cell and a Plexiglas probe 25 mm (P/25P; 25 mm Dia Cylinder Lap Perspex). For each loave, two slices of 2.5 cm in height were obtained and analyzed after 2 h (t0) and 24 h (t1) after baking. The moisture content and water activity of the crumb was determined on two samples from each batch by using the MA 50.R Radwag thermobalance (Radom, Poland) and an electronic hygrometer (Acqua Lab, CX-2 – Decagon Devices, Pullman, WA), respectively.

2.5. NMR analysis

Fermented flour and fresh bread crust and crumb were stored at −20 °C until lyophilization. Samples were prepared according to a previous study [26].

NMR spectra were acquired at 300 K on Bruker Avance 600 Neo spectrometer (Bruker Biospin, GmbH Rheinstetten, Karlsruhe, Germania), equipped with a z-gradient 5 mm cryoprobe (Prodigy). ^{1}H monodimensional spectra were acquired by using a modified 1D NOESY pulse sequence in a quantitative mode. Each ^{1}H NMR spectrum was acquired with 7812 Hz of sweep width, over 64K data points and 256 scans. Mixing time was set to 10 msec. Total Correlation Spectroscopy (TOCSY), ^{1}H-^{13}C Heteronuclear Single Quantum Coherence (HSQC) and Heteronuclear Multiple Bond Correlation spectra (HMBC) were acquired with NUS acquisition mode. All spectra were referenced to TMS and MeOD signals for proton and carbon, respectively.

2.6. SPME-GC-MS analysis

Volatiles were determined by means of a Head-Space Solid Phase Micro Extraction module (Combi-Pal automated sampler CTC Analytics, Zwingen, Switzerland) equipped with DVB/CAR/PDMS 50/30 m fiber (Supelco, Bellefonte, USA) and coupled to a gas chromatograph-mass spectrometer (6890N/5973N Agilent Technologies, Inc., Wilmington, DE). Crust or crumb samples (2 g; $n = 3$) were placed in 20 mL headspace vials and submitted to the following conditions: equilibrium, 5 min at 50 °C during stirring at 250 rpm; extraction, at 50 °C for 40 min maintaining stirring; desorption at 260 °C for 10 min directly in the injection port of the GC. Separation was achieved on a polar column (Zebron ZB-WAX plus, 60 m × 0.25 mm × 0.25 μm, Phenomenex, Torrance, CA) with the following analytical conditions: carrier gas, helium (1.2 mL/min, constant flow mode); inlet, split mode (split ratio 7:1); inlet temperature 260 °C; oven, 105 °C (10min), 5 °C/min to 150 °C (0min), 12 °C/min to 222 °C (13min). The transfer line to the mass spectrometer was maintained at 280 °C, the ion source was at 230 °C, and the quadrupole at 150 °C. Acquisition was performed in electronic impact mode. The mass range used was 39–220 amu. Data are expressed as arbitrary units, as log10 of the peak area of the corresponding selected ion.

2.7. Proximate composition analyses

The chemical composition of each flour and bread was assayed according to AOAC standard methods (AOAC, 2003) for dry matter (DM), ash, crude protein (CP), raw fiber (RF) and ether extract (EE). The samples were oven dried at 105 °C until constant weight to determine dry matter (DM; 934.01) and then incinerated in a muffle furnace at 550 °C for 4 h to calculate ash content (942.05). Total lipids were determined after acid-hydrolysis (EE; 920.29) and the percentage of crude protein was performed using Dumas method with MAX N exceed (Elementar Analysensystem GmbH, Langenselbold, Germany). Crude fiber was determined following the Weende analytical method (978.10). Starch content was enzymatically measured using the commercial Total Starch Assay Kit (Megazyme Ireland International, Ltd., Bray, Ireland) according to the manufacturer's recommendations.

2.8. Antinutrients analysis

Bean flour, or lyophilized bread samples, were extracted with 20 vol of phosphate buffered saline buffer (PBS: 10 mM $KHPO_4$, 15 mM NaCl, pH 7.4) and then used for the different analyses. Hemagglutinating activity of extracts was determined by a serial dilution method, previously described [31], using erythrocytes separated from the serum of a type A donor's blood by washing with about 10 vol (1.5 mL) of PBS. This step was repeated three times and the erythrocytes were stored at 4 °C in 10 vol of PBS. At the time of use, erythrocytes were diluted 1:10 with PBS. For each sample, serial dilutions in PBS, ranging from 1:2 to 1:256, were assayed. Agglutination was visually determined after 2 h incubation at room temperature.

RFO and phytic acid content analyses were performed with the Raffinose/Sucrose/D-glucose (K-RAFGA) and the Phytic Acid Assay (K-PHYT) Megazyme assay kits (Megazyme Ireland International, Ltd., Bray, Ireland), respectively according to the manufacturer's recommendations. Each sample was measured in duplicate.

2.9. DPPH radical scavenging activity assay

The free radical scavenging activities of different samples were determined using DPPH free radical assay, as previously described [32], using 50 μL of each sample extract and 2950 μL of 80% aqueous-methanolic 0.102 mM DPPH. After 15 min the absorbance reduction was spectrophotometrically measured at 515 nm. The ability to scavenge DPPH was calculated as follows:

$$(\%)\ \text{DPPH radical scavenging activity} = [(\text{Act} - \text{Asa})/\text{Act}] \times 100$$

where Act is the absorbance of DPPH radical plus methanol and Asa is that of DPPH radical plus the sample extract. Radical scavenging activity is shown as the percentage of DPPH inhibition for mg of DW. Each sample was measured in duplicate.

2.10. Digestibility test and determination of starch hydrolysis and protein breakdown

Bread samples were submitted to gastro-intestinal digestion (GID) according to the *in vitro* static protocol reported by Brodkorb et al. [33]. Five grams of bread were mixed with 5 mL of simulated salivary fluid containing 75 U/mL of human salivary α-amylase, and subjected to 2 min of simulated mastication (using a mincer) at pH = 7.0. The resulting oral bolus was then added to 10 mL of simulated gastric fluid (pH = 3). The gastric phase was performed using pepsin (2000 U/mL) and gastric lipase (60 U/mL). The intestinal phase was performed by adding 20 mL of simulated intestinal fluid (pH = 7.0) containing 10 mM of bile salts. The enzymes used for intestinal digestion were: trypsin (100 U/mL); chymotrypsin (250 U/mL); pancreatic amylase (100 U/mL); intestinal lipase (2000 U/mL); and colipase (mass ratio colipase to lipase was 1:2). The gastric and duodenal phases were both performed at 37 °C for 2 h. The samples were immediately frozen at the end of the GID until analysis. The enzymes and bile salts were from Merck (Darmstadt, Germany). Each digestion was carried out in triplicate. The degree of starch hydrolysis, before and after *in vitro* digestion of bread samples, was assessed by HPLC determining the content of maltotriose, maltose and glucose [34a,34b]. Protein breakdown during GID was evaluated by submitting aliquots of each *in vitro* digestate of bread to ultrafiltration (UF, 3 kDa). The permeates were submitted to the determination of nitrogen (N) content adopting Standard ISO 8968:2014. Protein breakdown was calculated as follows:

$$\text{Protein breakdown}\ (\%) = [(\text{NGID} - \text{Nb}) - \text{NSB}]\ /\text{NT} \times 100,$$

with NGID is the N content of the UF permeates of the bread samples after gastrointestinal digestion, Nb is the N content of the UF permeate of the blank sample (enzymes and simulated digestive fluids) after digestion, NSB is the N content of UF permeates of the bread dissolved in 30 mL of water, and NT is the total N content of the bread samples.

2.11. Statistical analysis

All experiments were performed with independent biological replicates as specified in each subsection. Data are reported as mean ± standard deviation (SD). Normality and homogeneity of variance were preliminarily assessed using Shapiro–Wilk and Levene's tests, respectively. When assumptions were satisfied, differences among samples

Table 1
Relative quantification of metabolites of interest produced by microorganisms in dried and milled fermented common bean flours. Values are reported in arbitrary units. CTRLnf contains not fermented bean flour.

	CRTLnf	*L. rhamnosus* LRH01	*L. buchneri* LBC01	*Ln. lactis* LN01	*K. humilis ITEM 19255*	*S. cerevisiae 2B*
FAA						
Leucine	0.26	1.47	1.95	1.66	1.80	1.41
Isoleucine	0.13	0.45	0.64	0.56	0.92	0.85
Valine	0.18	0.61	1.28	0.83	1.56	1.51
Alanine	0.70	1.54	2.74	1.86	4.46	4.53
Asparagine	1.56	0.88	1.17	0.92	0.84	0.67
Methionine	3.60	1.81	1.98	2.19	1.79	1.23
Tyrosine	nd[a]	0.23	nd[a]	0.17	0.12	nd[a]
OTHERS						
GABA	1.40	2.34	2.78	2.24	3.25	4.09
RFOs[b]	7.78	0.27	0.31	0.18	nd[a]	nd[a]

[a] Nd: not detected.

[b] Raffinose-family oligosaccharides.

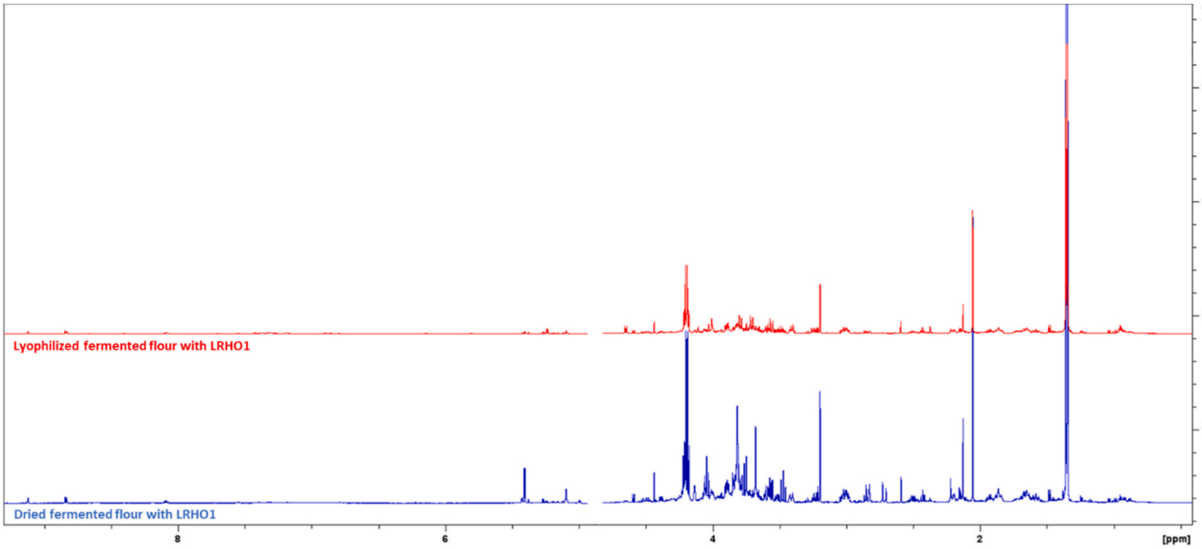

Fig. 1. Comparison between lyophilized (red) and dried at 105 °C (blue) fermented flour with *L. rhamnosus* LRH01. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

were evaluated by one-way ANOVA considering the type of fermented bean flour as the main factor. Post hoc comparisons were performed using Tukey's HSD test at a significance level of $p \leq 0.05$. For VOC analysis, statistical differences among formulations were assessed independently for crust and crumb. All statistical analyses were performed using Statgraphics Plus 5.1 (StatPoint Inc., Warrenton, USA).

3. Results and discussion

3.1. NMR-based metabolomics analysis of bean flour fermented with selected microorganisms

Semisolid fermentation was performed using three lactic acid bacteria (LAB) strains (*Lentilactobacillus buchneri* LBC01, *Lacticaseibacillus rhamnosus* LRH01, and *Leuconostoc lactis* LN01) as well as two yeast strains (*Kazachstania humilis* and *Saccharomyces cerevisiae*). After 48 h of incubation at 30 °C, all species exhibited a 2-log increase in cell count [26]. The fermented bean flours were analyzed using ^{1}H NMR spectroscopy to characterize their metabolite profiles and gain insights into the underlying metabolic pathways [34a,34b,35].

Among the detected metabolites, particular attention was given to the formation of valuable compounds such as FAAs and GABA, as well as the depletion of saccharides, including RFOs (Table 1). Additionally, the chemical composition was assessed in relation to the drying temperature (105 °C) to evaluate any potential alterations (Fig. 1).

Comparative analysis between fermented bean flours (FBFs) and non-fermented bean flours (NFBFs) revealed a marked increase in content of all FAAs (except asparagine and methionine) and GABA in FBFs, while RFOs were largely eliminated (Table 1), confirming previous findings [26].

3.2. Fermented common bean-enriched bread preparation and technological properties

Previous studies showed that enriching bread with 10–15% pulses enhances its nutritional profile, although higher inclusion levels typically require additives to offset negative effects on dough handling and sensory attributes [5]. In this study, a higher enrichment level (25%) was adopted, combined with a biotechnological approach.

The control dough was prepared with non-fermented bean flour (CTRnf), rather than wheat flour (CTRLwf), as the impact of bean flour addition to wheat has been previously documented [36]. Adding bean flour to wheat increased water absorption by approximately 6–7%, reduced dough development time by about 5 min, and decreased dough

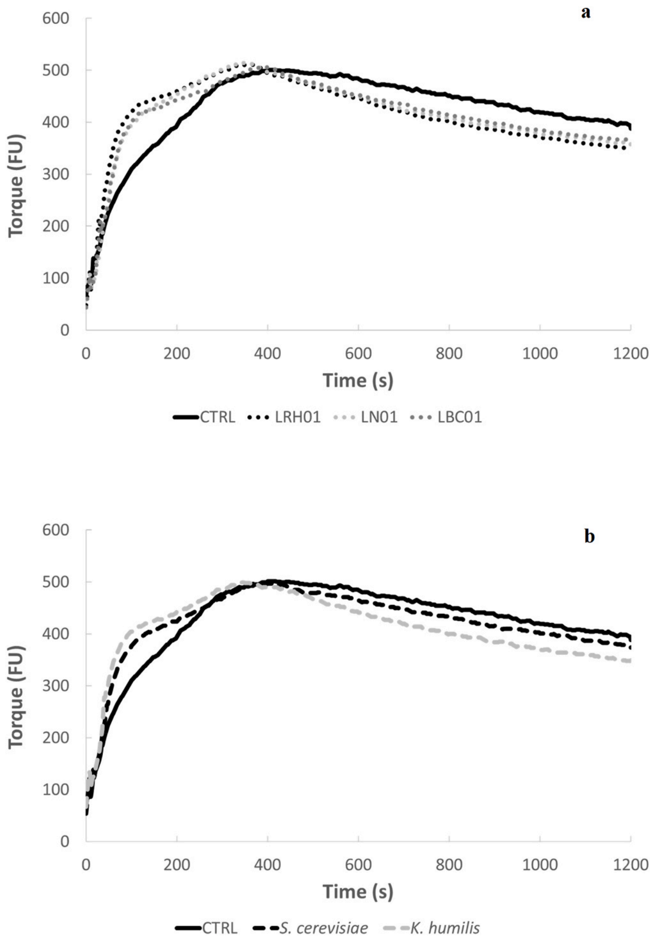

Fig. 2. Mixing properties of wheat flour containing 25% of NFBF (CTRL), and FBF by lactic acid bacteria (*L. rhamnosus* LRH01*, Ln. lactis* LN01*, L. casei* LBC01) (**a**) or yeasts (*S. cerevisiae*, *K. humilis*) (**b**). A representative curve for each sample is reported. FU, Farinograph Unit.

stability by a factor of four, resulting in a 20% reduction in bread volume (data not shown).

Mixing properties of doughs enriched with NFBF and FBFs were evaluated at a target consistency of 500 FU over a 20-min mixing period (Fig. 2). Water absorption remained consistent across samples (65.5–66.9%, Table S1), but fermentation slightly reduced dough development time: from 7 min in CTRnf to 6.2 min with LRH01 and 5.7 min with LN01 or *K. humilis* (Fig. 2a–Table S1). Dough stability was notably improved by LAB fermentation, decreasing from 5.7 min (CTRnf) to approximately 3.6 min with LABs and 4.7 min with yeasts (Fig. 2b–Table S1).

During leavening, CTRnf dough reached a maximum height of 37 mm after 92 min, followed by a 24% collapse after 3 h (Fig. 3). FBFs enhanced dough development, with LN01 achieving the highest dough height (52 mm after 82 min), while *S. cerevisiae* and *K. humilis* reached 44 mm and 47 mm after 62 and 83 min, respectively (Fig. 3a and b).

FBFs also reduced dough collapse during leavening: height loss decreased from 24% (CTRnf) to 19% (*K. humilis*), 17% (LN01), 13% (LRH01), and 8% (LBC01). In contrast, dough fermented with *S. cerevisiae* showed a 29% reduction in height. These results suggest that LAB-fermented common bean flours are most effective in minimizing dough collapse during leavening.

Regarding CO_2 production (Fig. 3c), all doughs initially retained the gas, which was later released. Doughs enriched with yeast-fermented FBFs produced similar total gas volumes, while LAB-fermented doughs showed slightly reduced gas production but maintained good retention capacity compared to CTRnf.

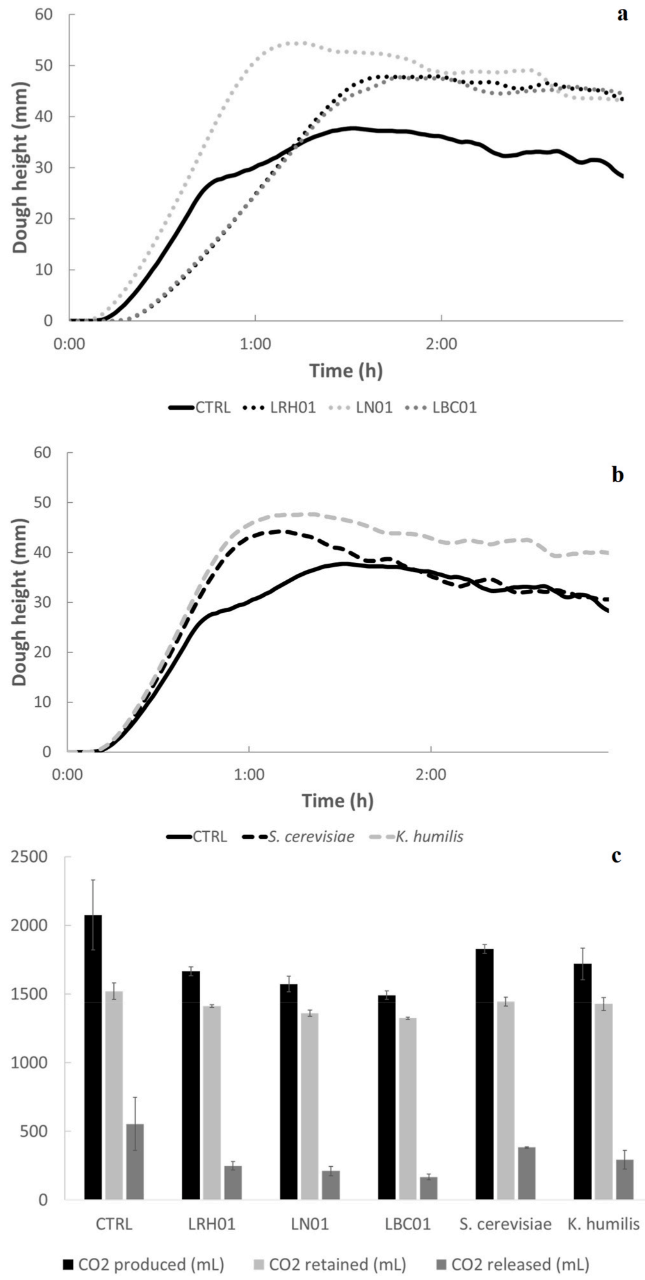

Fig. 3. Leavening properties (dough development in panels **a** and **b**; gas production in panel **c**) of wheat flour containing 25% of NFBF (CTRL), and FBF by lactic acid bacteria (*L. rhamnosus* LRH01*, Ln. lactis* LN01*, L. casei* LBC01) (a) and yeasts (*S. cerevisiae*, *K. humilis*) (b). A representative curve for each sample is reported.

FBFs did not affect crust luminosity but increased redness. Crust yellowness significantly increased only with yeast-fermented FBFs (Table 2). In the crumb, fermentation enhanced both redness and yellowness, desirable traits linked to Maillard reactions and caramelization, which contribute to appealing color, flavor, and aroma [37]. LN01 fermentation had the most pronounced effect, reducing crumb luminosity and increasing both redness and yellowness (Table 2). LN01 also improved bread height (Fig. 4) and specific volume by 15% (Table 2).

Although FBFs increased crumb firmness, the biotechnological approach effectively delayed staling after one day of storage. Firmness in CTRnf increased 2.3-fold, while bread made with FBFs showed lower increases: 1.2 (LBC01), 1.5 (LN01 and *S. cerevisiae*), 1.8 (*K. humilis*), and

Table 2
Effect of wheat flour 25% substitution by fermented bean flour on technological properties (specific volume, and firmness) and bread colour. CTRLnf contains not fermented bean flour.

	CTRLnf	*L. rhamnosus* LRH01	*Ln. lactis* LN01	*L. buchneri* LBC01	*S. cerevisiae 2B*	*K. humilis ITEM 19255*
Crust luminosity	47.82 ± 4.77^{ab}	45.78 ± 4.19^{a}	45.09 ± 2.63^{a}	45.37 ± 4.33^{a}	49.81 ± 2.57^{b}	51.11 ± 3.49^{b}
Crust redness	15.36 ± 1.16^{a}	16.88 ± 0.48^{b}	17.30 ± 0.77^{b}	17.25 ± 0.94^{b}	16.58 ± 0.68^{b}	17.33 ± 1.02^{b}
Crust yellowness	25.24 ± 2.20^{a}	24.31 ± 2.71^{a}	24.67 ± 2.36^{a}	24.35 ± 4.09^{a}	27.92 ± 1.52^{b}	29.84 ± 1.92^{b}
Crumb luminosity	58.96 ± 3.02^{bc}	57.53 ± 1.90^{ab}	55.82 ± 2.31^{a}	59.98 ± 1.70^{c}	58.97 ± 1.20^{bc}	59.36 ± 1.53^{bc}
Crumb redness	5.44 ± 0.33^{a}	7.93 ± 0.17^{c}	8.52 ± 0.24^{d}	7.70 ± 0.24^{c}	5.61 ± 0.18^{a}	6.26 ± 0.41^{b}
Crumb yellowness	10.92 ± 0.42^{a}	18.73 ± 0.33^{d}	23.62 ± 0.44^{e}	16.91 ± 0.52^{c}	14.86 ± 0.29^{b}	17.40 ± 0.83^{c}
Specific Volume (mL/g)	2.46 ± 0.12^{a}	2.42 ± 0.12^{a}	2.84 ± 0.26^{b}	2.57 ± 0.11^{a}	2.49 ± 0.01^{a}	2.40 ± 0.06^{a}
Firmness after baking (N)	7.43 ± 1.57^{a}	9.02 ± 0.50^{ab}	9.29 ± 0.49^{b}	9.70 ± 0.97^{b}	11.44 ± 1.50^{c}	11.52 ± 1.81^{c}
Firmness after 1 day (N)	17.20 ± 2.66^{bc}	16.97 ± 2.48^{b}	14.46 ± 0.98^{ab}	12.37 ± 2.66^{a}	17.25 ± 2.36^{bc}	21.09 ± 3.86^{c}

Different letters in the same row indicate a significant difference among samples (one-way ANOVA; Tukey HSD test; $p \leq 0.05$).

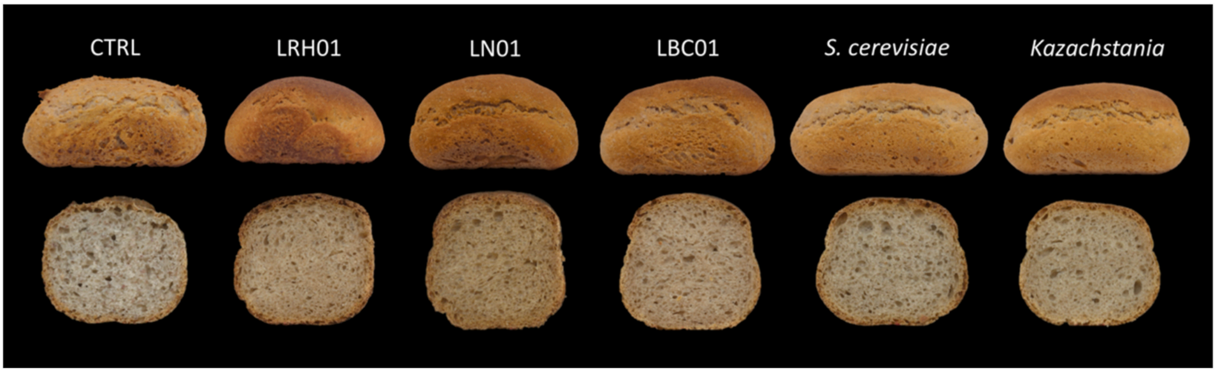

Fig. 4. Pictures of the bread prepared from wheat flour containing 25% of NFBF (CTRL), and FBFs by lactic acid bacteria (*L. rhamnosus* LRH01*, Ln. lactis* LN01*, L. buchneri* LBC01) and yeasts (*S. cerevisiae*, *K. humilis*).

1.9 (LRH01).

Overall, FBF-enriched breads exhibited lower specific volume (−20%) and higher crumb firmness (+40%) compared to wheat control (CTRLwf, data not shown). However, the impact of these technological changes on consumer acceptance warrants further investigation, particularly considering growing consumer interest in health and sustainability.

3.3. SPME-GC_MS analysis of VOCs in bread

Bread aroma consists of a wide combination of hundreds of molecules having different origins [38,40] and is crucial for consumer acceptance. The crust is mostly characterized by volatiles produced by thermal reactions in baking stage i.e. aldehydes, sulfuric compounds, and heterocyclic compounds (furans, pyrazines), whereas the crumb is mostly characterized by volatiles produced by enzymatic reactions and lipid oxidation i.e. alcohols, aldehydes and ketones. The final distribution of volatiles also takes account of the displacement of molecules between crust and crumb in both directions [39].

Crumb and crust samples were analyzed separately, with a focus on volatile compounds known to have high flavor dilution factors [38], positively or negatively correlated with bread aroma [39,40], and compounds that could potentially enable discrimination based on the microorganism used for bean flour fermentation [41,42].

Compared to bread obtained with fermented bean flour, wheat (CTRLwf) and non-fermented bean flour (CTRLnf) controls exhibited a higher content of alcohols and a lower content of other reported volatiles (Table 3).

Volatiles derived from sugar fermentation—such as ethanol, diacetyl, and acetoin—were present at similar levels in both wheat (CTRLwf) and non-fermented bean flour (CTRLnf) controls. However, CTRLnf showed slightly elevated levels of sulfur compounds (e.g., methional, dimethyltrisulfide), aldehydes (e.g., nonanal, nonadienal), and heterocyclic compounds (e.g., 2-pentylfuran, maltol, 2-methylpyrazine), reflecting the distinct composition of bean flour. Notably, 2-pentylfuran, a key contributor to “beany” aroma [45], was consistently higher in all FBF-enriched breads, with microbial species not affecting its final content.

CTRLnf and *S. cerevisiae* samples differ in number of yeast fermentations; the latter being twice fermented by *S. cerevisae*. The volatile profile originating from both yeast activity and baking reflects a balance between losses occurring in flour during drying, residual substrate and newly developed precursors available for the further leavening step. This may explain a lower content of 3 methyl 1 butanol and a higher content of diacetyl in *S. cerevisiae* samples compared to CTRLnf samples, given also their different origin (fermentation *vs* fermentation and Maillard reaction).

Bread samples from yeasts-FBFs showed in-between amounts of volatiles; some compounds (e.g., acetoin, diacetyl, acetate, 2-methylpropanal) were found at levels comparable to LAB-FBF samples, while others (e.g., 3-methylbutanoate, acetylfuran, furfural) at levels similar to control samples. Butyrate (a product of fatty acids synthesis pathway) and methional (a sulfur containing compound originating from yeast fermentation and Maillard reactions) content appeared to be distinctive for *S. cerevisiae* and *K. humilis* samples.

Volatile profiles of LAB-FBF breads were strongly influenced by the LAB species used, their enzymatic activities, fermentation pathways (homo-vs. heterolactic), precursor availability, and the lower pH of FBFs. LAB-FBF breads exhibited the highest levels of most of the volatiles, with some exceptions—particularly in LN01 samples. The lower enzymatic activity of *Leuconostoc* [46] may account for reduced precursor availability and lower levels of certain volatiles, such as pyrazines, which contribute roasted and burnt notes.

FAA in FBFs may influence the content of volatiles originating from Ehrlich pathways (a distinguishing trait of yeast metabolism) in the dough and from Maillard reactions during baking. LAB-FBF breads were

Table 3
Volatiles content in crust and crumb of bread samples (data expressed as arbitrary units, as log10 of the peak area of the corresponding selected ion). CTRLwf (bread from wheat flour), CTRLnf (bread from wheat flour containing 25% NFBF), LRH01, LN01, LBC01 (bread from wheat flour containing 25% of bean flour fermented by lactic acid bacteria *L. rhamnosus, Ln. lactis, L. buchneri*), *S. cerevisiae*, *K. humilis* (bread from wheat flour containing 25% of bean flour fermented by yeasts).

origin [a]	**cas**	**volatile compounds**		**CTRL wheat**	**CRTLnf**	***S. cerevisiae* *2B***	***K. humilis* *ITEM 19255***	***L. rhamnosus* LRH01**	***Ln. lactis* LN01**	***L. buchneri* LBC01**	*p*	**odorant correlation with bread aroma** [b]	**odor** [c,d]
Alcohols													
F	64-17-5	ethanol	crumb	6.99 ^{a}	6.99 ^{a}	691 ^{a}	6.91 ^{a}	6.76 ^{b}	6.99 ^{a}	6.97 ^{a}	*0.002*		ethanolic*
			crust	6.90 ^{b}	6.90 ^{b}	7.04 ^{a}	7.04 ^{a}	6.68 ^{c}	6.97 ^{a,b}	6.93 ^{a,b}	*<0.001*		*alcoholic*
F	123-51-3	3 methyl 1 butanol (isoamyl alcohol)	crumb	6.07 ^{a}	606 ^{a}	565 ^{d}	576 ^{c}	5.84 ^{b,c}	5.89 ^{b}	5.68 ^{c,d}	*<0.001*	POS	fusel-like, malty
			crust	6.06 ^{a}	6.02 ^{a}	5.72 ^{b}	5.83 ^{b}	5.73 ^{b}	5.84 ^{b}	5.56 ^{c}	*<0.001*		*balsamic, alcoholic, malty*
F.M	60-12-8	2 phenylethanol	crumb	5.92 ^{a}	5.91 ^{a}	5.64 ^{a,b}	5.78 ^{a}	5.53 ^{a,b}	5.27 ^{b}	5.27 ^{b}	*0.001*	POS	flowery, honey-like, yeast-like
			crust	5.59 ^{a,b}	5.76 ^{a}	5.45 ^{b}	5.63 ^{a,b}	5.42 ^{b}	5.13 ^{c}	5.14 ^{c}	*<0.001*		*flowery, honey-like, yeast-like*
ketones													
F,M	513-86-0	3 hydroxy 2 butanone (acetoin)	crumb	5.17 ^{c}	5.29 ^{c}	5.81 ^{b}	5.66 ^{b}	6.30 ^{a}	5.21 ^{c}	5.86 ^{b}	*<0.001*	POS	butter-like, carrot-like
			crust	5.08 ^{c}	5.27 ^{c}	6.08 ^{a}	6.00 ^{a}	6.17 ^{a}	5.56 ^{b}	6.04 ^{a}	*<0.001*		*butterscotch, butter, yogurt, cream*
F,M	431-03-8	2,3 butanedione (diacetyl)	crumb	5.16 ^{c,d}	5.16 ^{c,d}	5,49 ^{b}	5.39 ^{b}	5.69 ^{a}	5.08 ^{d}	5.34 ^{b,c}	*<0.001*	POS	butter-like
			crust	5.22 $^{c.d}$	5.24 ^{c,d}	5.52 ^{a,b}	5.41 ^{b,c}	5.57 ^{a}	5.18 ^{d}	5.35 ^{c}	*<0.001*		*buttery, caramel*
acids													
F,M	64-19-7	acetic acid	crumb	5.63 ^{c}	5.57 ^{c}	6.16 ^{b}	6.19 ^{b}	6.52 ^{a,b}	6.41 ^{a,b}	6.60 ^{a}	*<0.001*		sour, pungent, vinegar-like
			crust	5.77 ^{d}	5.78 ^{d}	6.42 ^{b,c}	6.41 ^{c}	6.58 ^{a,b}	6.53 ^{b,c}	6.74 ^{a}	*<0.001*		*sour, acid, pungent*
F	107-92-6	butyric acid	crumb	4.41 ^{c}	4.36 ^{c}	4.74 ^{b}	5.23 ^{a}	4.73 ^{b}	4.81 ^{b}	4.89 ^{b}	*<0.001*	NEG	sweaty, rancid, butter-like
			crust	4.29 ^{d}	4.36 ^{d}	4.88 ^{b}	5.32 ^{a}	4.61 ^{c}	4.88 ^{b}	4.88 ^{b}	*<0.001*		*sweaty, rancid*
F	79-31-2	2 methylpropanoic acid (isobutyric acid)	crumb	5.43 ^{a,b}	5.37 ^{b,c}	5.28 ^{c}	5.41 ^{a,b,c}	5,53 ^{a}	5.54 ^{a}	5.54 ^{a}	*0.001*	POS	sweaty, rancid, cheesy, sour
			crust	5.34 ^{b}	5.41 ^{b}	5.41 ^{b}	5.54 ^{a}	5.37 ^{b}	5.52 ^{a}	5.52 ^{a}	*<0.001*		*sweaty, butter, fatty, sour, rancid*
F,L,M	503-74-2	3 methylbutanoic acid (isovaleric acid)	crumb	5.12 ^{c}	5.04 ^{c}	5.12 ^{c}	5.18 ^{b,c}	5.43 ^{a}	5.36 ^{a,b}	5.41 ^{a}	*0.001*	POS and NEG	sweaty, cheesy, rancid, yeast-like
			crust	4.89 ^{c}	4.98 ^{c}	5.19 ^{b}	5.23 ^{a,b}	5.28 ^{a,b}	5.27 ^{a,b}	5.31 ^{a}	*<0.001*		*sweaty*
aldehydes													
F,M	122-78-1	phenylacetaldehyde	crumb	4.39 ^{c}	4.35 ^{c}	4.70 ^{b}	4.84 ^{b}	5.14 ^{a}	5.13 ^{a}	5.24 ^{a}	*<0.001*	POS	sweet, honey-like, floral*
			crust	4.49 ^{e}	4.37 ^{e}	4.99 ^{d}	5.24 ^{c}	5.39 ^{b,c}	5.51 ^{a,b}	5.68 ^{a}	*<0.001*		*honey-like, sweet*
F,L,M	100-52-7	benzaldehyde	crumb	4.61 ^{b,c}	4.40 ^{c}	4.75 ^{b}	4.73 ^{b}	5.08 ^{a}	5.09 ^{a}	4.82 ^{b}	*<0.001*	NEG	bitter almond-like, marzipan-like*
			crust	4.42 ^{d}	4.34 ^{c,d}	4.60 ^{b,c}	4.74 ^{b}	5.17 ^{a}	5.03 ^{a}	5.01 ^{a}	*<0.001*		*almond, caramel*
F,L	66-25-1	hexanal	crumb	4.90	5.04	5.07	5.06	4.82	5.04	4.96	*0.091*	NEG	green, grassy
			crust	5.06	5.12	4.91	5.06	5.03	5.07	4.96	*0.055*		*green, grassy, tallow*
F,M	78-84-2	2 methylpropanal	crumb	4.46 ^{b}	4.73 ^{a,b}	4.90 ^{a,b}	5.00 ^{a}	4.64 ^{a,b}	4.96 ^{a}	4.86 ^{a,b}	*0.017*	POS	malty
			crust	4.51 ^{c}	4.68 ^{b,c}	5.09 ^{a,b}	5.18 ^{a,b}	4.99 ^{a,b,c}	5.07 ^{a,b,c}	5.26 ^{a}	*0.010*		*malty*

(*continued on next page*)

Table 3 (*continued*)

origin [a]	cas	volatile compounds		CTRL wheat	CRTLnf	*S. cerevisiae* 2B	*K. humilis* ITEM 19255	*L. rhamnosus* LRH01	*Ln. lactis* LN01	*L. buchneri* LBC01	*p*	odorant correlation with bread aroma [b]	odor [c,d]
F,L	124-19-6	nonanal	crumb	4.19	4.37	4.05	3.98	4.00	4.37	4.09	*0.448*	POS	soapy, fruity, citrus-like*
			crust	2.01^{c}	4.36^{a,b}	4.09^{a}	3.96^{b}	4.40^{a}	4.09^{a,b}	4.03^{a,b}	*<0.001*		*citrus, soapy*
L	5910-87-2	2,4 nonadienal	crumb	4.47^{b}	5.23^{a}	4.84^{a,b}	4.74^{a,b}	5.10^{a}	4.58^{b}	4.53^{b}	*0.003*		fatty, green, wax-like *deep fat fried*
			crust	4.29	4.71	4.72	4.64	4.97	4.54	4.68	*0.115*		
sulfur compounds													
F,M	3268-49-3	3 methylthio propanal (methional)	crumb	1.00^{c}	4.76^{b}	5.18^{a}	5.12^{a}	4.54^{b}	4.61^{b}	4.65^{b}	*<0.001*	NEG	cooked potato-like, sweet
			crust	1.00^{d}	4.65^{b}	5.18^{a}	5.21^{a}	4.33^{c}	4.67^{b}	4.65^{b}	*<0.001*		*boiled-potato, cooked-potato, malty, waxy*
	3658-80-8	dimethyltrisulfde	crumb	1.00^{e}	2.01^{e}	3.35^{b,c}	3.26^{c,d}	4.01^{a}	3.77^{a,b}	3.58^{b}	*<0.001*	POS	cabbage-like, sulfuric like*
			crust	1.00^{d}	3.65^{b}	4.65^{a}	4.56^{a,b}	4.99^{a}	4.47^{a,b}	4.87^{a}	*<0.001*		*cabbage-like*
heterocyclic compounds													
F,L,M	3777-69-3	2 pentylfuran	crumb	4.55	4.57	4.72	5.14	4.69	5.15	5.14	*0.060*		vegetable-like * *butter, green bean, floral, fruity, mushroom, raw nuts*
			crust	4.19^{b}	4.96^{a}	5.08^{a}	5.13^{a}	5.04^{a}	4.99^{a}	5.16^{a}	*<0.001*		
L,M	1192-62-7	2 acetylfuran	crumb	4.11^{c}	4.47^{b,c}	4.24^{c}	4.31^{c}	5.30^{a}	4.32^{c}	4.83^{b}	*<0.001*		smoky *smoky, roasty*
			crust	4.19^{d}	4.51^{c,d}	4.49^{c,d}	4.64^{c}	5.69^{a}	4.66^{c}	5.13^{b}	*<0.001*		
F,M	98-01-1	furfural (2 furaldehyde)	crumb	4.46^{b,c}	4.41^{b,c}	4.18^{c}	4.27^{b,c}	5.43^{a}	5.06^{a,b}	4.78^{a,b,c}	*0.004*	POS	sweet, cereal-like, bakery-like, bread-like
			crust	4.25^{d}	4.59^{c,d}	4.40^{d}	4.62^{c,d}	5.87^{a}	5.17^{b}	5.07^{b,c}	*<0.001*		*almond, bread-like, soil, burnt, roasted, sweet, toasted*
M	98-00-0	furfuryl alcohol	crumb	4.21^{a,b}	4.52^{a}	4.46^{a,b}	4.40^{a,b}	4.52^{a}	4.19^{b}	4.38^{a,b}	*0.020*		cooked ham-like, sweaty*
			crust	4.90	5.10	5.23	5.20	5.50	4.92	5.19	*0.066*		*burnt, warmy oil, mild*
M	118-71-8	3 hydroxy 2 methyl 4 pyrone (maltol)	crumb	1.47^{b}	4.05^{a}	4.55^{a}	4.48^{a}	4.50^{a}	4.11^{a}	4.52^{a}	*<0.001*		caramel-like, sweet
			crust	4.93	5.29	5.65	5.65	5.68	5.20	5.71	*0.052*		*warmy-fruity, caramellic-sweet*
M	5910-89-4	2,3 dimethyl pyrazine	crumb	3.97^{e}	4.32^{c,d}	4.85^{b}	4.49^{c}	5.36^{a}	4.14^{d,e}	4.82^{b}	*<0.001*		leather-like, linseed oil-like, earthy
			crust	4.29^{d}	4.49^{d}	5.36^{b}	5.08^{b,c}	5.88^{a}	4.69^{c,d}	5.45^{a,b}	*<0.001*		*popcorn, roasted*
M	109-08-0	2 methylpyrazine	crumb	4.13^{d}	4.57^{b}	4.60^{b}	4.45^{b,c}	5.45^{a}	4.21^{c,d}	4.70^{b}	*<0.001*		burned, burnt, roasty *roasted, burnt, sweet*
			crust	4.07^{d}	4.43^{c,d}	4.89^{b}	4.79^{b,c}	5.65^{a}	4.48^{c}	5.04^{b}	*<0.001*		
M	18138-05-1	3,5 diethyl 2 methyl pyrazine	crumb	1.00^{c}	1.00^{c}	2.59^{a,b}	2.34^{a,b}	3.21^{a,b}	2.00^{b,c}	3.63^{a}	*0.001*		bell pepper-like* *roasted, peanut butter*
			crust	1.00^{c}	1.00^{c}	4.10^{a,b}	4.02^{a,b}	4.51^{a,b}	3.85^{b}	4.63^{a}	*<0.001*		

a-d Statistically significant differences in rows ($p \leq 0.05$) are indicated by different superscripts.

[a] F (fermentation); L (lipid oxidation); M (Maillard reactions) [39,43].

[b] POS (compounds that correlate positively with bread aroma - pleasant aroma when concentration increases); NEG (compounds that correlate negatively with bread aroma - unpleasant aroma when concentration increases) according to [39].

[c] Description obtained from Leibniz-LSB@TUM Odorant Database by CAS RN entry followed by bread occurrence entry (* occurrence general), according to [44].

[d] Description according to [39].

characterized by a higher amount of phenylacetaldehyde (positively associated to bread aroma) and benzaldehyde (negatively associated to bread aroma). Both aldehydes derive from phenylalanine in the yeast cell and from Maillard reactions.

The lower pH of FBFs [26] likely contributes to the enhanced formation of Maillard-derived volatiles in LAB breads, including diacetyl, acetoin, acetic acid, 3-methylbutanoic acid, phenylacetaldehyde, benzaldehyde, furaldehyde, 2 acetylfuran and pyrazines.

Diacetyl and acetoin—key aroma compounds imparting buttery and caramel notes [47]—have dual origins: they are formed during fermentation in the crumb from α-acetolactate and during baking in the crust via Maillard reactions [48]. Diacetyl is generated by both, LAB and yeasts, with homofermentative LAB strains generating higher levels than heterofermentative ones [39,41]. The highest concentrations of diacetyl and acetoin were found in LRH01 breads, likely due to their elevated precursor content and lower pH in FBF, which further promotes Maillard reactions. In contrast, LN01-FBF may contain less glucose due to reduced α-glucosidase activity in *Leuconostoc* [46], slowing down the yeast metabolism to produce acetolactate during dough fermentation resulting in a lower content of diacetyl and acetoin.

Furfural, associated with sweet, cereal-like aromas, is primarily formed through thermal reactions, with lower pH favoring its production [39]. A positive correlation between furfural and *Lactobacillus* species and bread aroma has been reported [42], while *S. cerevisiae* appears to play a negligible support in its formation [39]. Consistent with these findings, the highest furfural levels in this study were observed in LRH01-FBF breads, likely due to the availability of precursors and the lower flour pH.

3.4. NMR-based metabolomic analysis of fermented bean-enriched bread

Bread crumb and crust were independently analyzed using NMR spectroscopy to assess the relative abundance of selected metabolites in breads enriched with 25% fermented bean flour (FBF), compared to two controls: a standard wheat bread (100% wheat flour) and a bread containing 25% non-fermented bean flour (NFBF). The results are summarized in Table 4.

Overall, the metabolite profiles of crust and crumb did not exhibit substantial variation across samples. However, all FBF-enriched breads consistently showed elevated levels of FAAs, including essential amino acids, and GABA, relative to both control breads. Additionally, RFOs were undetectable in the FBF breads.

These findings suggest that the incorporation of FBF significantly enhances the nutritional value of bread, particularly with respect to FAA content and GABA levels, while effectively eliminating RFOs that are typically associated with digestive discomfort.

3.5. Proximate composition, antinutrient content and radical scavenging activity of fermented common bean-enriched bread

As expected, replacing 25% of wheat flour with NFBF led to an increase in protein and dietary fiber content. Specifically, protein levels rose from an average of 13% in the wheat control bread (CTRLwf) to 14.8% in bread containing FBF, while fiber content increased from 2.3% to 3.1%. Conversely, starch content decreased from 71.4% in CTRLwf to 61.6% in CTRLnf, with a further reduction observed in FBF breads, reaching an average of 57.8% in the bread and 38.5% in the FBFs themselves (Table 5).

Regarding ANFs, RFOs were undetectable in all FBFs and their corresponding bread samples (Table 6), with the sole exception of CTRLnf and its related bread. Even in this case, RFO levels were minimal, likely due to the presence of 75% wheat flour, which does not contain RFOs.

Phytic acid levels were reduced across all bread formulations, compared to its content in NFBF, regardless of whether the bean flour was fermented. In CTRLwf bread, phytic acid was already low in the flour (0.322 g/100 g), resulting in similarly low levels in the final product. Given that all bean-enriched bread contained 75% wheat flour, the dilution effect likely contributed to the overall reduction in phytic acid content.

Legumes and fermentation are known to enhance the antioxidant properties of food matrices [49,50]. Therefore, the radical scavenging activity of extracts from wheat flour, NFBF, FBFs, and their corresponding breads (Table 6) were assessed. Bean-containing flours—both fermented and non-fermented—exhibited the highest antioxidant activity, with DPPH inhibition values ranging from 8.0 to 9.0% per mg dry weight, nearly tenfold higher than the wheat control (0.829% inhibition/mg DW).

Elevated antioxidant activity was also observed in both crust and crumb extracts of NFBF- and FBF-enriched breads. Interestingly, DPPH inhibition values were consistently lower in crumb samples, suggesting that antioxidant compounds, including phytic acid, may be more unstable in the inner part of the bread. This could be attributed to the higher and prolonged heat exposure of the crust during baking, which may promote greater degradation of phytic acid and other antioxidant compounds (Table 6).

3.6. Protein and starch in vitro digestibility

Protein breakdown in control and experimental bread samples, both before and after *in vitro* digestion, is presented in Table 7. Proteolysis observed in undigested samples is primarily attributed to endogenous proteases. However, the higher levels of protein degradation in undigested FBF breads suggest additional proteolytic activity contributed by microbial fermentation starters.

Following the *in vitro* digestion, CTRLnf bread exhibited greater protein breakdown compared to the wheat control (CTRLwf). This difference can be explained by the partial substitution of wheat flour with bean flour (25%), which reduces gluten content. Previous studies have demonstrated that gluten can hinder protein digestibility. For instance, Montemurro et al. [51] reported that proteins from fermented wheat and barley flours were less digestible *in vitro* than those from chickpea and lentil flours. Additionally, the lower protein digestibility observed in CTRLwf may be linked to its higher starch content (Table 5), as starch has been shown to interfere with protein degradation during digestion [52].

FBF-enriched breads showed the highest levels of protein breakdown after *in vitro* digestion, consistent with findings by Sozer et al. [53]. Enhanced protein digestibility in fermented pulse-based products has also been reported by different authors [51,54–56], our findings together with these studies collectively suggest that fermentation improves protein accessibility to digestive enzymes, primarily through microbial proteolysis during flour fermentation.

Starch hydrolysis data before and after *in vitro* digestion are shown in Table 8. CTRLwf bread was the sample that showed the lowest starch digestion. The gluten matrix in wheat bread may inhibit starch digestion, which could explain the higher hydrolysis rates observed in breads containing common bean flour. The partial replacement of wheat flour reduces gluten content, thereby diminishing its restrictive effect on starch accessibility.

The interaction between proteins and starch within the food matrix plays a critical role in starch digestibility. Singh et al. [57] emphasized that both the presence and degradation of proteins significantly influence starch hydrolysis. Hydrolysis of the protein barrier surrounding starch granules has been shown to markedly enhance digestibility [58]. Supporting this, Jenkins et al. [59] found that starch digestion products were lower in white bread compared to gluten-free bread. Similarly, De Angelis et al. [60] reported a reduced starch hydrolysis index in wheat sourdough bread fermented with *Lactobacillus* strains compared to yeast-fermented wheat bread, attributing this to enhanced gluten-starch interactions under low pH conditions. Curiel et al. [61] observed comparable trends in durum wheat versus gluten-free pasta.

Starch hydrolysis levels in FBF breads were comparable or only

Table 4
NMR relative metabolites quantification of fermented bean-enriched breads at 25% levels, not fermented bean-enriched bread at 25% levels control, and 100% wheat bread control, crust and crumb and their respective flours. CTRLnf contains not fermented bean flour.

	CTRL wheat		CRTLnf		*L. rhamnosus* LRH01		*L. buchneri* LBC01		Ln. lactis LN01		*K. humilis ITEM 19255*		*S. cerevisiae 2B*	
	crust	crumb	crust	crumb	crust	crumb	crust	crumb	crust	crumb	crust	crumb	crust	crumb
FAA														
Leucine	nd[a]	0.13	0.11	0.18	0.60	0.84	1.18	1.22	1,00	0.96	0.85	0.82	0.69	0.61
Isoleucine	0.053	0.80	0.05	0.07	0.22	0.28	0.44	0.39	0.3	0.36	0.49	0.41	0.41	0.35
Valine	0.09	0.10	0.09	0.12	0.42	0.51	0.82	0.83	0.67	0.75	0.91	0.9	0.84	0.78
Alanine	0.62	0.6	0.64	0.72	1.22	1.46	2.51	2.4	2.1	2.03	3.4	3.19	3.7	3.63
Asparagine	nd	nd	0.37	0.48	0.85	0.97	0.96	0.98	0.65	1.03	nd	1.05	0.95	0.95
Methionine	nd	nd	1.19	1.61	1.14	1.56	1.03	1.19	1.66	1.7	0.81	0.91	0.69	0.75
Tyrosine	nd	nd	nd	nd	0.12	0.11	nd	nd	nd	nd	nd	nd	nd	nd
OTHERS														
GABA[c]	1.03	1.34	nd	1.88	1.51	1.73	2.48	2.59	2.54	2.25	2.92	2.62	3.26	2.55
RFOs[b]	nd	nd	1.1	1.2	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

[a] Nd: not detected.
[b] Raffinose-family oligosaccharides.

Table 5
Proximate composition of crust and crumb of 100% wheat bread control, not fermented bean-enriched bread at 25% levels control (CTRLnf), fermented bean-enriched bread at 25% levels, and their respective flours. DM: dry matter, CP: crude protein, RF: raw fiber, EE: ether extract.

	sample	DM	Ashes	CP	RF	Starch	EE
100% wheat	**CTRL crust**	92.9 ± 0.1	2.24 ± 0.08	12.9 ± 0.0	2.07 ± 0.67	73.0 ± 0.4	2.15 ± 0.26
	CTRL crumb	93.8 ± 0.1	2.13 ± 0.02	13.0 ± 0.0	2.61 ± 0.75	69.7 ± 0.6	1.79 ± 0.13
	CTRL flour	92.5 ± 0.9	0.46 ± 0.10	13.5 ± 0.0	2.87 ± 0.30	74.7 ± 3.3	1.88 ± 0.33
25% bean	**CTRLnf[a] crust**	94.7 ± 0.1	3.07 ± 0.03	14.6 ± 0.0	3.15 ± 0.74	61.8 ± 0.3	2.05 ± 0.17
	CTRLnf crumb	93.9 ± 0.1	3.03 ± 0.05	14.9 ± 0.0	3.06 ± 0.78	61.4 ± 0.6	2.39 ± 0.58
	CTRLnf flour	89.9 ± 0.2	5.23 ± 0.00	20.5 ± 0.2	3.44 ± 0.65	37.2 ± 0.1	1.99 ± 0.09
25% common bean	***S. cerevisiae* 2B crust**	94.9 ± 0.3	3.14 ± 0.08	14.7 ± 0.0	3.13 ± 0.79	57.0 ± 0.2	2.25 ± 0.77
	***S. cerevisiae* 2B crumb**	94.8 ± 0.1	3.05 ± 0.10	14.7 ± 0.0	3.02 ± 0.65	56.9 ± 0.6	1.83 ± 0.58
	***S. cerevisiae* 2B flour**	91.33[b]	na[a]	22.1 ± 0.0	3.38 ± 0.39	33.1 ± 0.9	2.19 ± 0.70
	***K. humilis* ITEM 19255 crust**	93.7 ± 0.0	3 .00 ± 0.04	14.6 ± 0.0	2.76 ± 0.21	60.7 ± 0.4	2.28 ± 0.74
	***K. humilis* ITEM 19255 crumb**	94.8 ± 0.2	3.13 ± 0.04	14.8 ± 0.0	3.20 ± 0.04	60.8 ± 0.1	2.26 ± 0.70
	***K. humilis* ITEM 19255 flour**	91.33[b]	na[a]	21.8 ± 0.1	2.84 ± 0.61	33.8 ± 0.7	2.61 ± 0.09
	***L. rhamnosus* LRH01 crust**	94.0 ± 0.0	0.00 ± 0.00	14.8 ± 0.0	3.19 ± 0.35	57.2 ± 0.6	2.12 ± 0.41
	***L. rhamnosus* LRH01 crumb**	95.2 ± 0.3	3.03 ± 0.05	14.8 ± 0.0	2.66 ± 0.81	57.0 ± 0.2	2.13 ± 0.66
	***L. rhamnosus* LRH01 flour**	91.6 ± 0.1	4.75 ± 0.56	21.9 ± 0.0	2.16 ± 0.03	42.9 ± 0.0	2.46 ± 0.27
	***Ln. lactis* LN01 crust**	94.9 ± 0.0	2.89 ± 0.11	15.2 ± 0.1	3.42 ± 0.20	57.4 ± 0.2	2.56 ± 0.34
	***Ln. lactis* LN01 crumb**	95.3 ± 0.1	3.03 ± 0.01	14.9 ± 0.0	3.43 ± 0.54	57.4 ± 0.6	1.98 ± 0.78
	***Ln. lactis* LN01 flour**	90.5 ± 0.0	4.42 ± 0.02	22.0 ± 0.0	3.24 ± 0.17	41.9 ± 0.5	2.09 ± 0.25
	***L. buchneri* LBC01 crust**	95.0 ± 0.2	2.76 ± 0.26	15.0 ± 0.0	3.67 ± 0.74	56.7 ± 0.9	2.45 ± 0.23
	***L. buchneri* LBC01 crumb**	94.6 ± 0.0	3.04 ± 0.04	15.0 ± 0.1	2.76 ± 0.56	56.6 ± 0.2	2.56 ± 0.14
	***L. buchneri* LBC01 flour**	91.9 ± 0.0	4.99 ± 0.60	22.5 ± 0.0	2.41 ± 0.39	41.9 ± 1.4	2.02 ± 0.37

[a] Na: not analyzed.
[b] Assumed value, as the amount of the sample was too low for the analysis.

slightly different from those in NFBF breads. Literature on the impact of FBF inclusion on starch digestibility in bread is limited, and findings vary depending on the food matrix. Sozer et al. [53] reported no significant difference in starch hydrolysis between breads made with LAB-fermented and unfermented faba bean flours. In contrast, increased starch digestibility has been observed in quinoa and buckwheat sourdough breads, while inhibitory effects were noted in sorghum and teff breads [19]. De Angelis et al. [60] attributed reduced starch hydrolysis in wheat sourdough to stronger gluten-starch interactions facilitated by fermentation-induced acidification.

4. Conclusions

Bread was selected as a model product because it represents one of the most widely consumed staple foods worldwide. Therefore, its reformulation or fortification with bean flour could have a significant nutritional impact at the population level, particularly in terms of improving protein and fiber intake and overall dietary quality. Our study offers significant insights into the feasibility of using common bean flour as a substrate for fermentation. The findings provide compelling evidence that fermentation with lactic acid bacteria (LAB) and yeasts effectively reduces specific ANFs—such as RFOs—while simultaneously enhancing the nutritional profile by increasing bioactive compounds, including GABA, FAAs, and antioxidant activity. Moreover, composite flours incorporating FBFs demonstrated superior suitability for breadmaking compared to those with NFBFs, particularly due to improved leavening performance and reduced staling.

Nonetheless, certain limitations persist with FBFs, as substantial residual levels of phytic acid and lectins were still detectable post-fermentation as it has been already shown in previous studies [26]. To mitigate these drawbacks, the use of flours derived from genetically tailored bean genotypes lacking lectins and phytic acid (*lec*-, *lpa*), previously validated as health-promoting and effective ingredients for the formulation of various bean-based products, including biscuits,

Table 6
Antinutrients content (raffinose-family oligosaccharides (RFOs) and Phytic acid) and radical scavenging activity (DPPH method) of crust and crumb of 100% wheat bread control, not fermented bean-enriched bread at 25% levels control (CTRLnf), fermented bean-enriched breads at 25% levels, and their respective flours.

Sample	RFOs g/100g	Phytic acid g/100g	DPPH mean % inib/mg DW
CTRL – Wheat flour	nd[b]	0.322	0.829
CTRL – Wheat crust	nd[b]	0.329	1.785
CTRL – Wheat crumb	nd[b]	0.241	1.162
CTRLnf – Bean flour	4.330	1.850	8.023
CTRLnf – Crust	0.174	0.455	5.140
CTRLnf – Crumb	0.157	0.405	2.964
***L. rhamnosus* LRH01 – Fermented bean flour**	nd[b]	1.380	7.434
***L. rhamnosus* LRH01 – Crust**	nd[b]	0.471	4.743
***L. rhamnosus* LRH01 – Crumb**	nd[b]	0.444	3.729
***L. buchneri* LBC01 – Fermented bean flour**	nd[b]	1.819	8.307
***L. buchneri* LBC01 – Crust**	nd[b]	0.389	5.110
***L. buchneri* LBC01 – Crumb**	nd[b]	0.391	2.115
***Ln. lactis* LN01 – Fermented bean flour**	nd[b]	1.954	7.520
***Ln. lactis* LN01 – Crust**	nd[b]	0.503	5.893
***Ln. lactis* LN01 – Crumb**	nd[b]	0.424	4.440
***K. humilis* – Fermented bean flour**	nd[b]	0.935	7.862
***K. humilis* – Crust**	nd[b]	0.327	4.113
***K. humilis* – Crumb**	nd[b]	0.372	2.674
***S. cerevisiae* – Fermented bean flour**	nd[b]	1.053	9.077
***S. cerevisiae* – Crust**	nd[b]	0.283	4.550
***S. cerevisiae* – Crumb**	nd[b]	0.377	3.000

[b] Nd: not detected.

Table 7
Degree of protein breakdown (%) before and after *in vitro* digestion of bread from wheat flour (CTRLwf), wheat flour containing 25% of unfermented beans (CTRLnf) and fermented beans by lactic acid bacteria (LRH01, LN01, LBC01) and yeasts (*S. cerevisiae*, *K. humilis*).

	protein breakdown (%)	
	ante digestion	post digestion
CTRL	1.6 ± 0.4a	19.6 ± 1.5d
CTRLnf	3.4 ± 0.6b	41.0 ± 2.1e
***L. buchneri* LBC01**	5.9 ± 0.5c	71.8 ± 0.9f
***Ln. lactis* LN01**	5.7 ± 0.6c	69.1 ± 1.1 fg
***L. rhamnosus* LRH01**	5.4 ± 0.6c	64.8 ± 0.7g
***S. cerevisiae* 2B**	5.1 ± 0.9c	61.6 ± 1.8g
***K. humilis* ITEM 19255**	5.3 ± 0.3c	63.7 ± 0.9 g

[a-g] Statistically significant differences in rows ($p \leq 0.05$) are indicated by different superscripts.

crackers, and sweet spreads were proposed [31,62].

Finally, although the volatile organic compound (VOC) profiles of bread varied among control, LAB-fermented, and yeast-fermented samples, further sensory evaluation is warranted to determine consumer acceptability and preference.

The proposed approach enabled the development of bread with 25% legume content not only enhancing nutritional and functional properties, such as antioxidant activity and digestibility, but also improving technological aspects, including dough rheology, loaf volume, and shelf-life, thus representing a novel contribution to the field of legume-enriched bakery products.

CRediT authorship contribution statement

Giuseppina Tatulli: Writing – original draft, Investigation, Formal analysis. **Alessandra Marti:** Writing – review & editing, Resources, Methodology, Formal analysis. **Roberto Consonni:** Writing – review & editing, Resources, Methodology. **Tiziana Silvetti:** Formal analysis. **Eleonora Cominelli:** Writing – review & editing. **Claudia Liberatore:** Formal analysis. **Ivan Toschi:** Resources, Methodology. **Valentina Cesari:** Formal analysis. **Sara Pozzo:** Writing – original draft, Methodology, Formal analysis. **Stefano Cattaneo:** Writing – original draft, Methodology, Formal analysis. **Milena Brasca:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Francesca Sparvoli:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT based on the GPT-4 architecture in order to improve readability of the text and to assist with the drafting and conceptualization of the graphical abstract. After using this tool, the authors reviewed and edited the content as needed and took full responsibility for the content of the published article.

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Table 8
Levels (g 100 g^{-1} starch) of maltotriose, maltose and glucose before and after *in vitro* digestion of bread from wheat flour (CTRLwf), wheat flour containing 25% of unfermented beans (CTRLnf) and fermented beans by LAB (LRH01, LN01, LBC01) and yeasts (*S. cerevisiae 2B*, *K. humilis ITEM 19255*).

		CTRLwf	CTRLnf	*L. buchneri* LBC01	*Ln. lactis* LN01	*L. rhamnosus* LRH01	*S. cerevisiae 2B*	*K. humilsi ITEM 19255s*
maltotriose	*ante digestion*	0.35 ± 0.06a	0.90 ± 0.08b	0.35 ± 0.04a	0.31 ± 0.07a	0.55 ± 0.07c	0.32 ± 0.02a	0.27 ± 0.04a
	post digestion	8.03 ± 0.21d	7.57 ± 0.33d	7.71 ± 0.24d	7.93 ± 0.61d	5.71 ± 0.44e	8.14 ± 0.38d	7.24 ± 0.57d
maltose	*ante digestion*	1.82 ± 0.08a	2.43 ± 0.07b	1.08 ± 0.03c	1.06 ± 0.07c	1.15 ± 0.04c	1.53 ± 0.03d	1.04 ± 0.05c
	post digestion	26.22 ± 0.47e	31.54 ± 0.18f	32.79 ± 0.39f	32.60 ± 0.69f	30.34 ± 0.17f	35.52 ± 0.68g	32.98 ± 0.39 fg
glucose	*ante digestion*	0.06 ± 0.01a	0.06 ± 0.01a	0.07 ± 0.00a	0.07 ± 0.01a	0.25 ± 0.00b	0.07 ± 0.00a	0.09 ± 0.01a
	post digestion	3.76 ± 0.19c	5.91 ± 0.14d	5.71 ± 0.33d	5.49 ± 0.27d	5.06 ± 0.11d	5.98 ± 0.51d	5.80 ± 0.21d
total	*ante digestion*	2.23 ± 0.15a	3.39 ± 0.16b	1.50 ± 0.07c	1.44 ± 0.15c	1.95 ± 0.11a	1.92 ± 0.05a	1.40 ± 0.10c
	post digestion	38.01 ± 0.87d	45.02 ± 0.65e	46.20 ± 0.96e	46.02 ± 1.57e	43.31 ± 0.72e	49.63 ± 1.57f	46.02 ± 1.17ef

Different letters indicate a significant difference among samples (one-way ANOVA; Tukey HSD test; $p \leq 0.05$).

AD005.225.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jafr.2026.102919.

Data availability

Data will be made available on request.

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