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ORIGINAL ARTICLE

Biosynthesis of γ-aminobutyric acid by lactic acid bacteria in surplus bread and its use in bread making

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Abstract

Aims: The aim of this study was to investigate the effectiveness of bread as substrate for γ-aminobutyric acid (GABA) biosynthesis, establishing a valorization strategy for surplus bread, repurposing it within the food chain.

Methods and Results: Surplus bread was fermented by lactic acid bacteria (LAB) to produce GABA. *Pediococcus pentosaceus* F01, *Levilactobacillus brevis* MRS4, *Lactiplantibacillus plantarum* H64 and C48 were selected among 33 LAB strains for the ability to synthesize GABA. Four fermentation experiments were set up using surplus bread as such, added of amylolytic and proteolytic enzymes, modifying the pH or mixed with wheat bran. Enzyme-treated slurries led to the release of glucose (up to 20 mg g^{-1}) and free amino acid, whereas the addition of wheat bran (30% of bread weight) yielded the highest GABA content (*circa* 800 mg kg^{-1} of dry weight) and was the most suitable substrate for LAB growth. The selected slurry was ultimately used as an ingredient in bread making causing an increase in free amino acids.

Conclusions: Besides the high GABA concentration (148 mg kg^{-1} dough), the experimental bread developed in this study was characterized by good nutritional properties, highlighting the efficacy of tailored bioprocessing technologies as means to mitigate food wastage.

Significance and Impact of Study: Our results represent a proof of concept of effective strategies to repurpose food industry side streams.

KEYWORDS

bioprocessing, enzymes, fermentation, food, lactic acid bacteria

INTRODUCTION

Over the past decades, the rapid transformation of the food system determined significant challenges, among which high levels of food loss and waste (FAO, 2014). Bread, whose production volume is expected to reach 209,874.8 million kilograms in 2021 (Statista, 2021), represents a large portion of the global food waste, with economic and environmental repercussion (Brancoli et al., 2020). Most of the leftover bread is disposed as waste, and alternative

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pathways such as donation, ethanol production or re-use as feed are being considered in some European countries (Brancoli et al., 2020; Melikoglu & Webb, 2013). It is crucial, however, to build more sustainable food systems, that future utilization solutions focus on keeping the food waste still safe for human consumption within the food chain.

Currently, bakery waste and bread are mostly reutilized as food ingredients in the form of glucose syrup (Kwan et al., 2018; Riaukaite et al., 2019) or for brewing (Brancoli et al., 2020; Dlusskaya et al., 2008). Recently, bioprocessing treatments, for example enzymatic treatments and microbial fermentation, have been used for the production of a medium for the growth of food industry starters (Verni et al., 2020) or to convert bakery waste into products that fit human consumption. The use of fermented surplus bread slurry in bread baking had a positive impact on bread quality and conferred a longer mould-free shelf-life (Immonen et al., 2020; Nionelli et al., 2020).

Nowadays, the use of enzymes in the baking and food industry in general spans across different applications, from texturizing to flavouring. Enzymes are advantageous for many operations and have low environmental and physiological toxicity, which is why their global market is growing continually (Chapman et al., 2018; Kirk et al., 2002). At the same time, lactic acid bacteria fermentation, one of the most ancient biotechnologies, has been considered for decades a versatile tool to improve the nutritional and functional potential of baked goods, due to its ability to reduce undesired compounds and to the synthesis of bioactive compounds, such as γ-aminobutyric acid (for review see Gobbetti et al., 2019). γ-aminobutyric acid (GABA) is a four-carbon non-protein amino acid that works as a neurotransmitter in the mammalian central nervous system having different physiological effects, such as diuretic, anti-depressive, antioxidant and hypotensive (Sarasa et al., 2020). GABA is abundant in fermented foods such as kimchi, cheese and fermented milk products. Several micro-organisms (bacteria, fungi and yeasts) have shown GABA-producing properties (Dhakal et al., 2012). GABA enrichment of cereal-based foods through lactic acid bacteria (LAB) fermentation was proposed by many authors (Coda et al., 2010; Dhakal et al., 2012; Diana et al., 2011; Venturi et al., 2019). An important step toward the achievement of high GABA content is the LAB starter selection since certain metabolic features during fermentation are often species- or strain-dependent.

To the best of our knowledge, GABA biosynthesis in bread matrices has never been investigated. In this context, the aim of this study was to investigate the effectiveness of GABA production using surplus bread still fit for human consumption as the substrate for LAB fermentation. Thirty-three LAB strains, isolated from several plant matrices (wheat, quinoa, faba bean, hemp, hop, etc), were screened for the ability to synthesize GABA and the selected starters were used for bread fermentation. The fermented bread was used as an ingredient to obtain a GABA-enriched bread. The main microbiological and biochemical features of the bread slurries were evaluated as well as the nutritional and technological properties of the enriched bread.

MATERIALS AND METHODS

Raw materials, enzymes and micro-organisms

White wheat bread, having the following composition: moisture, 35.7%; proteins, 11.40% dry matter (d.m.); fats, 6.25% d.m.; carbohydrates, 77.44% d.m.; dietary fibres, 2.35% d.m., was used for the experiments. The bread was grinded into small crumbs (<1 mm), mixed with distilled water (1:3 w/w) and homogenized with a blender (Oster) to obtain slurries. Wheat bran was purchased from Oy Karl Fazer Ab, Fazer Mills, Lahti, Finland. Its composition, as reported by the manufacturer, was: 5 g fat, 36 g carbohydrates, 31 g fibre, 2.9 g fructan and 16 g protein, and 2.7 g ash/100 g.

Enzymes used to hydrolyse bread slurries were alpha-amylase from porcine pancreas purchased from Sigma-Aldrich, bakery enzyme Grindamyl A14000, an amyloglucosidase from *Aspergillus oryzae*, purchased from Danisco and Corolase 7089, an endopeptidase (metalloprotease and serine protease) enzyme preparation obtained from *Bacillus subtilis*, purchased from AB Enzymes.

Commercial amylolytic and proteolytic enzyme preparations were also used to produce the waste bread medium as described by Verni et al. (2020). Novamyl, a maltogenic amylase (1500 units g^{-1}) from *B. subtilis*, provided by Novozyme, was used to hydrolyse starch, whereas fungal proteases from *A. oryzae* (E1; 500,000 haemoglobin units on the tyrosine basis g^{-1}) and *Aspergillus niger* (E2; 3000 spectrophotometric acid protease units g^{-1}), purchased from BIOCAT Inc. were used to hydrolyse proteins.

Lactiplantibacillus plantarum C2, C48, LB1, H48, H64, T6B4, T0A10, T6C16, 18S9, MRS1, 1A7, PU1, PRO17, *Furfurilactobacillus rossiae* LB5, T0A16, *Levilactobacillus brevis* MRS4, AM7, *Pediococcus pentosaceus* H11, T1A13, I76, I214, I02, I014, F01, OA1, S3N3, BAR4, *Pediococcus acidilactici* 10MM0, *Pediococcus* sp. I56, *Leuconostoc mesenteroides* 12MM1, I57, *Weissella confusa* KAS3 and NEY6, all belonging to the Culture Collection of the Department of Soil, Plant and Food Sciences (University of Bari) were used in the study. The list of all the strains and their isolation source is reported in Table S1. LAB strains were routinely propagated on De Man, Rogosa and Sharpe (MRS)

(Oxoid,) at 30°C. When used for fermentation, cells grown till the late exponential phase of growth (*circa* 12 h) were harvested by centrifugation at 9000 × g for 10 min at 4°C, washed twice in sterile physiological solution (NaCl 0.9%, w/v) and resuspended in distilled water.

Starter selection

To select the LAB strains showing high GABA production in bread slurries, a medium obtained from wasted bread (Wasted Bread Medium, WBM) mimicking such conditions, and prepared as described in Verni et al. (2020), was used. The 34 strains were cultivated in MRS until the late exponential phase of growth was reached, harvested by centrifugation at 9000 × g for 10 min at 4°C, washed twice in 50 mM sterile phosphate buffer (4°C, pH 7.0), resuspended in sterile distilled water and used to inoculate WBM (4% [v/v], corresponding to initial cell density of *circa* 7.0 log cfu g^{-1}).

To compare the growth in WBM against MRS, microbial kinetics of growth were determined and modelled in agreement with the Gompertz equation, as modified by Zwietering et al. (1990):

$$y = k + A\exp\left\{-\exp[(\mu max\, e/A)(\lambda - t) + 1]\right\}.$$

where y is the OD_{620}; k is the initial level of the dependent variable to be modelled (OD_{620}); A is the cell density variation (between inoculation and the stationary phase); μmax is the maximum growth rate expressed as OD_{620} units/h; λ is the length of the lag phase measured in hours. The experimental data were modelled by the nonlinear regression procedure of the Statistica 12.5 software (Statsoft).

GABA produced in WBM was analysed by a Biochrom 30^{+} series Automatic Amino Acid Analyzer (Biochrom Ltd.,), equipped with a Li-cation-exchange column (4.6 × 200 mm internal diameter), as described by De Pasquale et al. (2021). To exclude the possibility of lacking GABA production, due to the absence of substrate and cofactor necessary for the reaction, WBM was also supplemented with 18.4 mmol L^{-1} L-glutamate (Sigma-Aldrich) and 0.1 mmol L^{-1} pyridoxal phosphate (Sigma-Aldrich) as previously described by Di Cagno et al. (2010). GABA production on WBM with and without the supplements was determined after incubation at 30°C for 24 h.

Set up of the fermentation process

Four different laboratory-scale fermentations were set up to investigate optimal parameters for the *in situ* production of GABA by selected LAB starters. Bread slurries were prepared according to the ingredients listed in Table 1 and

TABLE 1 Ingredient composition of surplus bread slurries singly fermented with the selected LAB strains for 24 h at 30°C

	Experiment I	Experiment II		Experiment III [a]		Experiment IV	
		α	αp	α6.5	αp6.5	b15	b30
Surplus bread (g)	50	50	50	50	50	42.5	35
Water (g)	150	150	150	150	150	150	150
α-amylase (g)	—	0.0025	0.0025	0.0025	0.0025	—	—
Grindamyl A14000 (g)	—	0.125	0.125	0.125	0.125	—	—
Corolase® 7089 (ml)	—	—	0.245	—	0.245	—	—
Wheat bran (g)	—	—	—	—	—	7.5	15
Starter LAB strains	*L. plantarum* H64, C48 *P. pentosaceus* F01 *Lv. brevis* MRS4	*L. plantarum* H64, C48	*L. plantarum* H64, C48	*L. plantarum* H64, C48	*L. plantarum* H64, C48	*L. plantarum* H64, C48	*L. plantarum* H64, C48

[a]pH was adjusted to 6.5 with 1 mol l^{-1} NaOH at the beginning of fermentation and again after 6 h of incubation.

incubated at 30°C for 24 h. The plain slurry was prepared by mixing one part of grinded bread and three parts of distilled water.

Effect of the selected starters

In the first fermentation experiment (I), the plain slurry was singly inoculated with *P. pentosaceus* F01, *Lv. brevis* MRS4, *L. plantarum* H64 and C48.

Enzymatic treatments

In the second experiment (II), plain slurries were treated with α-amylase (0.05 g kg^{-1}) and amyloglucosidase (2.5 g kg^{-1}) without (slurry α) and with the addition of Corolase (4.9 ml kg^{-1}) (slurry αp) and inoculated with *L. plantarum* H64 and C48.

Effect of the pH

In the third experiment (III), the pH of the slurries was adjusted to 6.5 with 1 mol L^{-1} NaOH at the beginning of fermentation and again after 6 h of incubation. Similar to the II experiment, slurries were treated with α-amylase and amyloglucosidase without (slurry α6.5) and with the addition of Corolase (αp6.5).

Wheat bran supplementation

In the fourth fermentation experiment, 15% and 30% of the weight of the grinded bread was replaced with commercial wheat bran (IV, b15 and b30) without enzyme addition nor pH adjustment.

Acidification and microbiological characterization of the slurries

The pH of the slurries was determined by a pH-meter (Mettler Toledo Model MP220) with a food penetration probe and total titratable acidity (TTA) was determined with a Mettler Toledo Titrator (model GL53). Ten grams of sample in 100 ml of distilled water were titrated with 0.1 mol L^{-1} NaOH to a final pH of 8.5. TTA was expressed as the volume of NaOH used (ml).

LAB cell density in the slurries, prior and after fermentation, was also evaluated. For microbiological analysis, 10 g of sample was mixed with 90 ml of sterile physiological solution (NaCl 0.9%, w/v) and homogenized with a Stomacher 400 lab blender (Seward Medical) followed by serial dilution. Presumptive LAB were enumerated using MRS (Lab M) agar, supplemented with cycloheximide (0.1 g L^{-1}), plates were incubated for 48 h at 30°C in microaerophilic conditions.

Biochemical characterization of the slurries

Organic acids and mono- and disaccharides were extracted from 0.5 g of sample weighed into 50 ml Falcon-tube. MilliQ-water was added to the tube so that the weight, together with the sample, was 5 g. The sample was held at 4°C for 1 h and vortexed at 15-min intervals and centrifuged at 10,000 × *g* for 15 min at 18–24°C. After centrifuging, 0.8 ml of supernatant and 0.8 ml of 5% perchloric acid were transferred into an Eppendorf tube and vortexed well. The sample was stored at 4°C overnight and centrifuged at 10,000× *g* for 15 min at 18–24°C.

For organic acid quantification, 1 ml of supernatant was syringe-filtered (Acrodisc GHP Minispike 45 μm, Pall Corporation) into HPLC vials, ready to be analysed. The analysis was performed with HPLC system that consisted of a Waters 515 pump (Waters Corp.), an autosampler (Waters Corp.), an ultraviolet (UV) detector (Waters 717), a refractive index detector (HP 1047A, HP), a Hi-Plex H column (Agilent Technologies, Inc.; 300 × 6.5 mm) and a Hi-Plex H guard column (Agilent; 50 × 7.7 mm). 10 mmol L^{-1} H_2SO_4 was used as a mobile phase (0.5 ml/min). The temperature of the column was 40°C, injection volume of 20 μl and runtime per sample 35 min. Software used for the data collection was Empower2 (Waters Corp.). Organic acids were quantified against external organic acid standard curves with UV-chromatogram from duplicates of two biological replicate samples.

For mono- and disaccharide quantification, 0.5 ml of extracted supernatant was transferred to a centrifuge filter tube (Amicon Ultra – 0.5 Centrifugal Filter Unit). The sample was centrifuged at 13,000 × *g* for 10 min at 18–24°C and transferred into HPLC vials for analysis. The total dilution for the samples was 1:100 of the original. Glucose, fructose and maltose were analysed by HPAEC-PAD according to Xu et al. (2017). The system consisted of a Waters 2707 autosampler (Waters Corp.), a Waters 515 HPLC pump (Waters Corp.), an SSI pulse dampener model LP-21 (Scientific Systems Inc.), a CarboPac PA-1 Guard columns (4 × 50 mm) (Dionex Corporation), a CarboPac PA-1 anion exchange columns (4 × 250 mm) (Dionex Corporation) and a Waters 2465 pulsed amperometric detector (Waters Corp.).

Empower2 Software (Waters Corp.) was used for the data collection. All the solvents were at least HPLC grade.

Free amino acids and GABA content were measured from freeze-dried fermented slurry samples. Briefly, water/salt-soluble extracts were prepared from lyophilized bread following the method modified by Weiss et al. (1993). Aliquots of each sample were diluted with 50 mmol L^{-1} Tris–HCl (pH 8.8), held at 4°C for 1 h, vortexing at 15-min intervals and centrifuged at 20,000 × *g* for 20 min. The supernatant containing the water/salt-soluble fraction was filtered through a Millex-HA 0.22-μm pore size filter (Millipore Co.) and used for the analysis of GABA and free amino acids (FAA) as reported above.

Bread making

Based on the screening performed above, the process corresponding to the highest GABA biosynthesis (H64-b30) was selected for the manufacture of a fortified bread, that was characterized and compared to a wheat flour bread (wfB).

Before bread making, the slurry was also characterized for the presence of yeasts, moulds and *Enterobacteriaceae*. Yeasts and moulds were cultivated on Yeast Peptone Dextrose Agar medium (YPDA) (Sigma-Merck), supplemented with 0.01% chloramphenicol, through pour and spread plate enumeration, respectively, and incubated at 25°C. *Enterobacteriaceae* were determined on Violet Red Bile Glucose Agar (VRBGA, Oxoid) at 37°C for 24 h.

Bread doughs, manufactured at the pilot plant of the Department of Soil, Plant and Food Science (University of Bari), having dough yield of 180 (corresponding to 55% and 45% of flour and water, respectively), were mixed at 60 × *g* for 5 min with an IM 5–8 high-speed mixer.

For the experimental bread making (slB, surplus bread slurry bread), fermented slurry addition was adjusted to match 10% surplus bread per dough. More specifically, the dough had the following formula: 40.7 g of white flour, 57.2 g of fermented slurry and 2.1 g of water. Compressed baker's yeast was added (2% w/w, corresponding to a final cell density of yeasts of *circa* 7 log cfu g^{-1}) to both breads and fermentation was performed at 30°C for 1.5 h. All breads were baked at 220°C for 20 min using a Combo 3 oven (Zucchelli). Wheat flour (type '0') used for bread making had the following chemical composition: moisture, 14.1%; protein, 11.2% of d.m.; fat, 1.2% of d.m.; carbohydrates, 86.9% of d.m.; fibre, 3.1% of d.m.; and ash, 0.6% of d.m.

Bread characterization

The pH, TTA, organic acids and FAA of bread doughs were determined as described above. FAA were also determined after baking. Acrylamide content in control and experimental bread, as well as in the selected slurry, was determined by liquid chromatography-tandem mass spectrometry according to the UNI EN 16618:2015 regulation.

The *in vitro* protein digestibility (IVPD) was determined by the method of Akeson and Stahmann (1964). Samples were subjected to a sequential enzyme treatment mimicking digestion in the gastro-intestinal tract using pepsin and pancreatin. Enzymes were inactivated by the addition of trichloroacetic acid, and the undigested protein was precipitated. The concentration of protein of the supernatant was determined by the Bradford method (Bradford, 1976), whereas the precipitate was subjected to protein extraction, according to Weiss et al. (1993), and the concentration of protein was determined. The IVPD was expressed as the percentage of the total protein, which was solubilized after enzyme hydrolysis.

The analysis of starch hydrolysis was carried out with a procedure mimicking the *in vivo* digestion of starch previously proposed by De Angelis et al. (2009). The degree of starch digestion was expressed as a percentage of potentially available starch hydrolysed at different times (30, 60, 90, 120 and 180 min). Wheat flour bread was used as a control to estimate the hydrolysis index (HI = 100). The predicted glycaemic index (pGI) was calculated using the equation: Pgi = 0.549 × HI + 39.71 (Capriles & Arêas, 2013).

Instrumental Texture Profile Analysis (TPA) was performed with an FRTS-100N Texture Analyzer (Imada) using a cylinder probe FR-HA-30J (30 mm in diameter). For the analysis, breads of approximately 200 g were used in full and in triplicate. The determination was carried out in two compression cycles by using the following parameters: test speed 1 mm/s, 30% deformation of the sample. The chromaticity coordinates of the samples (obtained by a Minolta CR-10 camera) were reported in the form of a colour difference, ΔE^*_{ab}, calculated by the following equation, where ΔL, Δa and Δb are the differences for L, a and b values between sample and reference (a white ceramic plate having $L = 93.4$, $a = -1.8$ and $b = 4.4$).

$$\Delta E*_{ab} = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2}.$$

Statistical analysis

The chemical and physical analyses were carried out in duplicate or triplicate for each batch of slurry and bread

samples. Data were subjected to one-way ANOVA; paired comparison of treatment means was achieved by Tukey's procedure at $p < 0.05$, using the statistical software Statistica 12.5 (StatSoft Inc.,). Data sets related to the main microbiological and biochemical features of bread slurries were analysed through Principal Component Analysis (PCA), using the software Statistica 12.5.

RESULTS

Screening of GABA-producing strains

To select LAB strains with the best adaptation to bread substrate, the growth of 33 different LAB starters was monitored for 24 h of incubation in WBM and compared to that in MRS. After 24 h of incubation, the pH of WBM ranged from 3.50 to 4.01, while MRS final pH values were slightly higher (3.97–4.53). The cell density variation between the inoculum and the stationary phase was significantly higher in WBM only for *L. plantarum* 18S9, C2 and H64, remaining similar to that in MRS for most of the other strains (Figure S1). Whereas, except for *Pediococcus* spp. I56, *P. pentosaceus* T1A13, BAR4, S3N3, *L. plantarum* C48, T6C16, T0A10, PU1, PRO17, *F. rossiae* LB5 and *W. confusa* KAS 3, the latency phase was similar or slightly, but significantly, lower in WBM than that of the respective strain grown in MRS. Overall, compared to MRS, the maximum velocity of growth was significantly lower in WBM for 25 out of 33 strains (Figure S1).

The ability to produce GABA, was tested on WBM as such or supplemented with L-glutamate and pyridoxal phosphate and represented by the boxplots in Figure 1. Except for *P. pentosaceus* BAR4, I214, I02 and *W. confusa* NEY6, which did not cause significant changes, all the strains determined increments of GABA, ranging from 20 to 100%, compared to not inoculated WBM (*circa* 35 g L^{-1}). *P. pentosaceus* F01, *Lv. brevis* MRS4 and *L. plantarum* C48, determined considerable increases of GABA (up to 3-fold the initial content), while the highest value was found for *L. plantarum* H64 with 328 mg L^{-1}, corresponding to the extreme in Figure 1. Glutamate content in the medium, before fermentation, was 550.9 mg/L, thus resulting in an almost complete conversion of glutamate into GABA for *L. plantarum* H64. For this reason, the medium was supplemented with glutamate and pyridoxal phosphate. Indeed, higher concentrations of GABA were obtained when substrate and cofactor were added to the medium, even for the strains that synthesized an extremely low concentration in plain WBM, except for *P. pentosaceus* T1A13. However, the trend remained similar, showing *P. pentosaceus* F01, *Lv. brevis* MRS4, *L. plantarum* C48 and H64 as extremes and outliers, with 169 ± 6, 193 ± 8, 145 ± 3 and 521 ± 10 mg L^{-1} of GABA respectively (Figure 1). Hence, these strains were selected for further experiments.

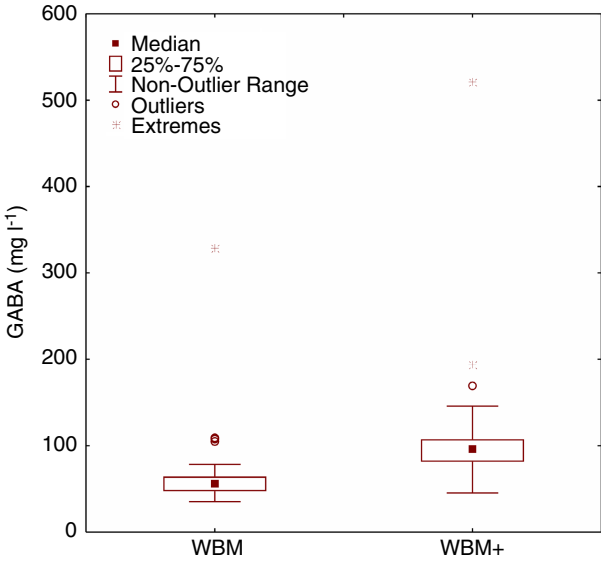

FIGURE 1 Boxplot showing the distribution of 33 LAB strains based on the ability to produce GABA in WBM (wasted bread medium) as such or supplemented with 18.4 mmol L^{-1} L-glutamate and 0.1 mmol L^{-1} pyridoxal phosphate (WMB+)

Optimization of GABA production in bread slurry

In situ GABA production

The microbiological and biochemical characterization of all the slurries is reported in Table 2. First, the four strains selected based on the ability to produce GABA on WBM, were used to ferment plain bread slurries (Experiment I). The initial cell density corresponded to the targeted inoculum and, after 24 h of fermentation, presumptive LAB cell density increased of approximately 1 log cycle for the slurries fermented by *L. plantarum* H64 and C48 and *circa* 20% less for *Lv. brevis* MRS4 and *P. pentosaceus* F01, which barely reached 8.4 log cfu g^{-1}. Nevertheless, a relevant acidification was obtained in all samples, with an average production of 35 mmol kg^{-1} of lactic acid. Consequentially, values of TTA increased, with H64 and F01 showing the highest and lowest respectively (Table 2). Acetic acid was not detected in any of the slurries.

Plain slurry contained traces of glucose and fructose and less than 4 mg g^{-1} of maltose. During fermentation, the first two were consumed while, for the latter, increases up to 3-fold, were found (Table 2). Compared to a plain slurry (*circa* 97 mg kg^{-1}), no statistical differences ($p > 0.05$) were observed in GABA content after

TABLE 2 Main microbiological and biochemical features of surplus bread slurries singly fermented with *Lactiplantibacillus plantarum* H64 (H64), and C48 (C48), *Pediococcus pentosaceus* F01 (F01) and *Levilactobacillus brevis* MRS4 (MRS4) for 24 h at 30°C (Experiment I). As reported in Table 1, amylolytic enzymes were used alone (**α**) or in combination with proteolytic enzymes (**α**p) with (Experiment III) or without (Experiment II) the modification of pH (6.5). Slurries were also fermented after the addition of wheat bran substituting surplus bread at 15 (b15) or 30% (b30) (Experiment IV). Not fermented slurries composed of surplus bread alone (SB) or supplemented with wheat bran at 15% (SB-b15) or 30% (SB-b30) were used as controls

	LAB (log cfu/g)								
	0	24	pH	TTA (ml)	Lactic acid (mmol kg^{-1})	Glucose (mg g^{-1})	Fructose (mg g^{-1})	Maltose (mg g^{-1})	GABA (mg kg^{-1} d.m.)
I experiment									
SB			5.31 ± 0.02^{c}	1.60 ± 0.01^{i}	15.01 ± 1.31^{i}	0.29 ± 0.02^{f}	0.76 ± 0.16^{a}	3.71 ± 0.34^{d}	96.84 ± 1.57^{g}
H64	7.78 ± 0.06^{a}	8.97 ± 0.11^{cd}	3.88 ± 0.01^{e}	4.35 ± 0.02^{e}	40.17 ± 2.21^{g}	n.d.	n.d.	5.46 ± 0.28^{d}	90.79 ± 1.32^{g}
C48	7.66 ± 0.01^{a}	8.72 ± 0.01^{d}	3.84 ± 0.01^{e}	3.89 ± 0.08^{f}	35.28 ± 2.89^{g}	0.05 ± 0.3^{f}	n.d.	5.69 ± 0.44^{d}	97.22 ± 1.97^{g}
MRS4	7.70 ± 0.12^{a}	8.45 ± 0.03^{e}	3.87 ± 0.04^{e}	3.76 ± 0.12^{f}	36.89 ± 0.74^{g}	n.d.	n.d.	7.83 ± 0.27^{c}	94.87 ± 1.23^{g}
F01	7.42 ± 0.03^{b}	8.22 ± 0.32^{e}	4.26 ± 0.00^{d}	2.76 ± 0.06^{g}	26.44 ± 0.41^{h}	0.05 ± 0.04^{f}	0.12 ± 0.06^{b}	7.95 ± 0.58^{c}	85.58 ± 1.97^{gh}
II experiment									
H64-α	7.80 ± 0.09^{a}	9.05 ± 0.02^{c}	3.68 ± 0.01^{f}	5.40 ± 0.16^{d}	77.34 ± 0.92^{e}	18.63 ± 0.93^{a}	n.d.	n.d.	111.76 ± 2.29^{f}
H64-αp	7.71 ± 0.03^{a}	9.30 ± 0.01^{b}	3.50 ± 0.01^{g}	9.65 ± 0.03^{c}	143.05 ± 1.53^{a}	17.00 ± 0.76^{a}	n.d.	n.d.	268.40 ± 3.23^{d}
C48-α	7.64 ± 0.06^{a}	8.77 ± 0.01^{d}	3.75 ± 0.01^{e}	4.67 ± 0.10^{e}	65.49 ± 1.85^{f}	18.50 ± 0.25^{a}	n.d.	n.d.	110.71 ± 4.16^{f}
C48-αp	7.71 ± 0.06^{a}	9.19 ± 0.05^{c}	3.52 ± 0.00^{g}	9.17 ± 0.07^{c}	132.10 ± 5.4^{b}	19.43 ± 0.45^{a}	n.d.	n.d.	121.22 ± 2.03^{f}
III experiment									
H64-α6.5	7.76 ± 0.06^{a}	8.84 ± 0.34	4.19 ± 0.02^{d}	3.74 ± 0.24^{f}	31.66 ± 0.85	8.62 ± 0.57^{b}	n.d.	n.d.	113.56 ± 1.06^{f}
H64-αp6.5	7.76 ± 0.02^{a}	9.58 ± 0.02^{a}	3.77 ± 0.01^{e}	9.99 ± 0.01^{c}	71.03 ± 1.69^{e}	7.97 ± 0.50^{b}	n.d.	n.d.	156.11 ± 2.93^{e}
C48-α6.5	7.60 ± 0.06^{a}	9.02 ± 0.25^{bc}	4.25 ± 0.10^{d}	3.37 ± 0.26^{f}	29.20 ± 1.46^{gh}	8.81 ± 0.41^{b}	n.d.	n.d.	109.93 ± 2.27^{f}
C48-αp6.5	7.59 ± 0.02^{ab}	9.55 ± 0.10^{a}	3.76 ± 0.01^{e}	9.70 ± 0.03^{c}	68.67 ± 0.93^{ef}	8.15 ± 0.84^{b}	n.d.	n.d.	148.90 ± 1.56^{e}
IV experiment									
SB-b15			6.07 ± 0.01^{b}	2.41 ± 0.04^{h}	n.d.	n.d.	n.d.	9.51 ± 0.24^{b}	83.21 ± 1.63^{h}
SB-b30			6.29 ± 0.02^{a}	3.53 ± 0.05^{f}	n.d.	1.50 ± 0.14^{e}	n.d.	12.67 ± 5.22^{b}	78.94 ± 1.55^{i}
H64-b15	7.83 ± 0.01^{a}	9.59 ± 0.01^{a}	3.39 ± 0.01^{g}	14.25 ± 0.21^{b}	123.86 ± 0.38^{c}	3.52 ± 0.24^{d}	n.d.	17.58 ± 2.01^{a}	543.08 ± 35.02^{b}
H64-b30	7.73 ± 0.07^{a}	9.48 ± 0.20^{a}	3.47 ± 0.01^{g}	17.49 ± 0.08^{a}	141.42 ± 1.53^{a}	10.94 ± 2.04^{b}	n.d.	19.24 ± 5.84^{a}	798.77 ± 14.47^{a}
C48-b15	7.66 ± 0.03^{a}	9.50 ± 0.18^{a}	3.41 ± 0.01^{g}	13.67 ± 0.21^{b}	111.83 ± 1.42^{d}	5.17 ± 0.05^{c}	n.d.	19.58 ± 4.53^{a}	258.83 ± 8.07^{d}
C48-b30	7.71 ± 0.06^{a}	9.62 ± 0.07^{a}	3.45 ± 0.01^{g}	17.13 ± 0.36^{a}	137.75 ± 1.06^{b}	13.28 ± 2.26^{b}	n.d.	20.10 ± 6.53^{a}	396.15 ± 2.34^{c}

Note: Data are the means of at least three independent analyses.

Abbreviation: n.d., not detected.

$^{a-i}$Values in the same column with different superscript letters differ significantly ($p < 0.05$).

incubation with the selected strains. On the contrary, a 50% decrease in total free amino acids (TFAA) was found in all fermented slurries (Table S2) compared to the plain slurry (409 ± 19 mg kg^{-1}).

Enzyme supplementation

For the following experiments, only *L. plantarum* H64 and C48 were used as starters and bread was added of amylolytic enzymes alone (α) or combined with proteolytic enzymes (αp) (Experiment II). Compared to the respective fermented slurries (Experiment I), higher cell densities (up to 9.30 log cfu g^{-1}) and lower pH (up to 3.50) were reached, especially when both hydrolytic enzymes were used. Accordingly, greater increases of lactic acid and, therefore TTA, were observed, almost double in αp, compared to α in slurries fermented with both *L. plantarum* H64 and C48 (Table 2).

As for the sugar content, fructose and maltose, were not detected in any of the samples, whereas substantial increases in glucose concentration (on average 18 mg g^{-1}) were detected. Overall, the enzyme treatment slightly affected GABA content. Except for H64-αp, which contained up to 270 mg kg^{-1} of GABA, in all the other slurries GABA concentration increased roughly of 20%, compared to a plain slurry (Table 2). Increases in the TFAA, were observed only in samples treated with proteases, reaching up to 2.3 g kg^{-1} (Table S3).

Effect of the pH

In the third experiment, the pH was adjusted to 6.5 at the beginning and after 6h of fermentation of the slurries subjected to hydrolytic enzymes (as described in the II fermentation batch). After 24 h of incubation, an increase in presumptive LAB cell density, approximately 1.4 and 2 log cycle, was found in α and αp samples, respectively (Table 2). Similar to the previous experiment, slurries treated with amylases alone had higher pH and lower presumptive LAB cell density, lactic acid and TTA compared to those treated with amylases and proteases. However, of all slurries fermented with *L. plantarum* H64 and C48, the pH-controlled and amylases hydrolysed slurries were those having the lowest (i) concentration of lactic acid, (ii) increases of TTA and (iii) decreases of pH (Table 2).

The pH adjustment of hydrolysed slurries resulted in approximately 50% lower glucose content than those without the pH adjustment (α and αp), while fructose and maltose were not detected. Despite increments being observed, pH-controlled fermentation did not seem to positively affect GABA concentration, which was almost halved in H64-αp6.5, compared to H64-αp (Table 2). Like in experiment II, significant increments of TFAA (up to 3.7 mg kg^{-1}, Table S4) were found only in αp samples, with C48-αp6.5 showing the highest value.

Wheat bran supplementation

In the fourth fermentation experiment, 15 (SB-b15) or 30% (SB-b30) of bread was replaced by wheat bran, thus leading to relevant changes in the fermentation performances. LAB cell density increased by approximately 2 log cycles in both the fermented slurries, with no differences between the two substitution levels. Overall a better acidification was obtained, with lactic acid values up to 140 mmol kg^{-1} in H64-b30 (Table 2).

Substitution of surplus bread with 15% of wheat bran resulted in glucose content of 3.5 and 5.2 mg g^{-1} in slurries fermented by *L. plantarum* H64 and C48 respectively. Whereas the addition of 30% resulted in approximately three times higher glucose content compared to their respective with 15%. The initial maltose content of non-fermented SB-b15 and SB-b30 slurries was 9.5 and 12.7 mg g^{-1} respectively. After 24 h fermentation, it ranged from 17.6 to 20.1 mg g^{-1}, not showing statistical differences between wheat bran content nor the strain used.

The highest levels of GABA production were achieved during experiment IV for both *L. plantarum* H64 and C48, even though the concentration was 2-fold higher for the former, compared to the latter, regardless of the amount of bran added (Table 2). Additionally, values of TFAA were 2.3 ± 0.09, 7.6 ± 0.09, 2.2 ± 0.04 and 4.9 ± 0.05 g kg^{-1} for H64-b15, H64-b30, C48-b15 and C48-b30 respectively (Table S5).

Principal component analysis

Data collected from the microbiological and biochemical characterization of bread slurries were subjected to principal component analysis as shown in Figure 2. The first and second factors explained, respectively, 58.81 and 17.16% of the total variance. Slurries were clearly defined by each fermentation experiment, being tidily separated within the plane. Slurries from the first fermentation experiment (top right frame) were characterized by the lowest increase in cell density, and lower acidification. The PCA shows F01 isolated from the other three slurries mostly because of the lowest utilization of fructose as well as the lowest acidification.

The second factor separated enzyme-treated slurries from those that were not subjected to hydrolysis. Enzyme-treated slurries from the second and third fermentation

experiments had the highest content of glucose, released by amyloglucosidases. Nevertheless, fermented slurries hydrolysed with amylolytic enzymes alone (bottom right frame of Figure 2) were characterized by lower LAB growth and lactic acid production, compared to those hydrolysed with amylases and proteases (bottom left frame of the PCA). Slurries of the fourth experiment (top left frame of the PCA) presented a very different profile, having the highest levels of organic acids, maltose, TFAA and GABA, as well as providing the most suitable substrate for LAB growth.

Bread characterization

The slurry obtained fermenting surplus bread added of wheat bran (substitution level of 30% on bread basis) with *L. plantarum* H64 was used as ingredient in the production of a fortified bread and compared to a control white bread. Before bread making, the slurry was characterized for the presence of yeasts, moulds and *Enterobacteriaceae*. All microbial species investigated were in notably low range, yeasts and moulds remained below 3 log cfu g^{-1}, whereas *Enterobacteriaceae* were not detected.

The physical-chemical, biochemical, nutritional and technological features of the breads are summarized in Table 3. Before baking, the pH of the dough fermented with baker's yeast alone was significantly ($p < 0.05$) higher than that of the dough containing bread slurry, whereas TTA was more than 3-fold higher than the control bread. The use of H64-b30 in the bread formula, also led to higher concentrations of lactic acid, which was not detected in wfB. TFAA also increased approximately three times, compared to control bread dough (Table 3). Except for Gly, Cys and Tyr, all free amino acids increased from 1- to 20-fold in slB compared to wfB. The highest increment was found for Arg, 82.9 ± 0.38 against 1.9 ± 0.03 mg kg^{-1} of the control (Figure 3). Higher concentration of GABA was also detected, with values up to 148 mg kg^{-1} dough. After baking, TFFA decreased in both breads with 378 ± 13 and 783 ± 29 mg kg^{-1} for wfB and slB respectively.

The acrylamide content resulted in lower than 10 μg kg^{-1} for the slurry used for bread making as well as both the breads.

The main nutritional properties of the experimental bread, *in vitro* protein digestibility and starch hydrolysis index were determined. Experimental bread had IVPD roughly 10% higher than the control and significantly lower HI (Table 3). Consequentially, the predicted glycaemix index resulted 73.9 against 94.6 of the wfB.

The textural properties of breads were also determined after baking. Compared to wfB, the experimental bread was characterized by higher hardness, whereas no significant differences were observed for the other

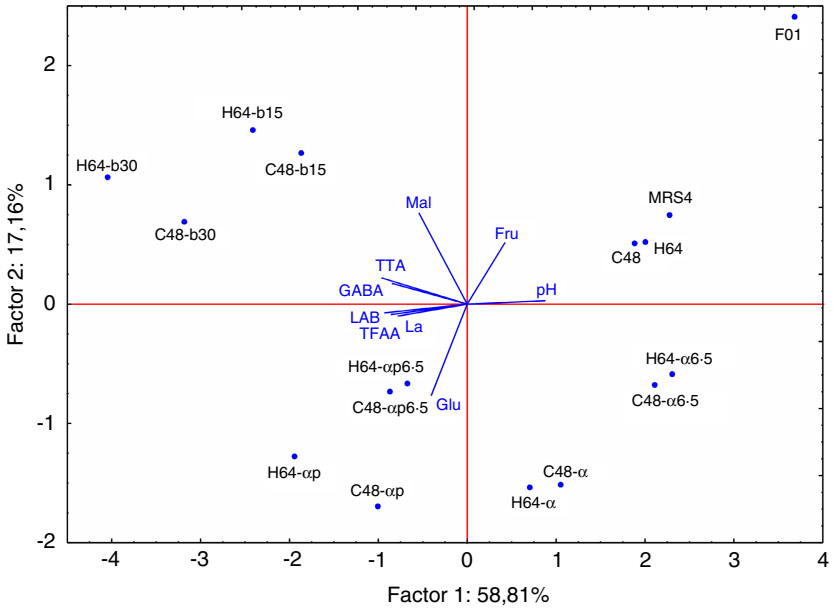

FIGURE 2 Principal component analysis of the main microbiological and biochemical features (pH; TTA, total tritatable acidity; LAB, lactic acid bacteria cell density; Glu, glucose concentration; Fru, fructose concentration; Mal, maltose concentration; TFAA, total free amino acids; GABA, γ-aminobutyric acid concentration) of surplus bread slurries fermented with *Lactiplantibacillus plantarum* H64 (H64), and C48 (C48), *Pediococcus pentosaceus* F01 (F01) and *Levilactobacillus brevis* MRS4 (MRS4) for 24 h at 30°C (I batch). As reported in Table 1, amylolytic enzymes were used alone (α) or in combination with proteolytic enzymes (αp) with (III batch) or without (II batch) the modification of pH (6.5). Slurries were also fermented after the addition of wheat bran substituting surplus bread at 15 (b15) or 30% (b30)

TABLE 3 Biochemical, nutritional and technological characterization of common wheat flour bread (wfB) and bread produced with surplus bread slurry (slB)

	wfB	slB
Biochemical and nutritional characteristics		
pH	5.4 ± 0.1[a]	4.2 ± 0.2[b]
TTA (ml)	5.7 ± 0.3[b]	17.4 ± 0.5[a]
Lactic acid (mmol kg^{-1})	n.d.	13.31 ± 0.42
TFAA (mg kg^{-1})	453 ± 12[b]	1329 ± 46[a]
Acrylamide (μg kg^{-1})	<10	<10
In Vitro Protein Digestibility (%)	64.2 ± 3.8[b]	75.8 ± 2.9[a]
Hidrolysis Index (%)	100[a]	62.4 ± 1.5[b]
Texture profile analysis		
Hardness (N)	2258 ± 31[b]	2365 ± 23[a]
Fracturability (N)	652 ± 10[a]	678 ± 9[a]
Cohesiveness	0.409 ± 0.2[a]	0.398 ± 0.3[a]
Springiness	4.74 ± 0.3[a]	4.69 ± 0.1[a]
Gumminess (N)	4438 ± 41[a]	4462 ± 39[a]
Colour analysis		
L	68.1 ± 0.7[a]	58.7 ± 0.8[b]
a	2.5 ± 0.1[b]	3.4 ± 0.3[a]
b	23.4 ± 0.3[a]	23.7 ± 0.4[a]
ΔE	33.1 ± 0.5[b]	39.7 ± 0.9[a]

Note: Data are the means of at least three independent analyses.

Abbreviation: n.d., not detected.

[a,b]Values in the same raw with different superscript letters differ significantly ($p < 0.05$).

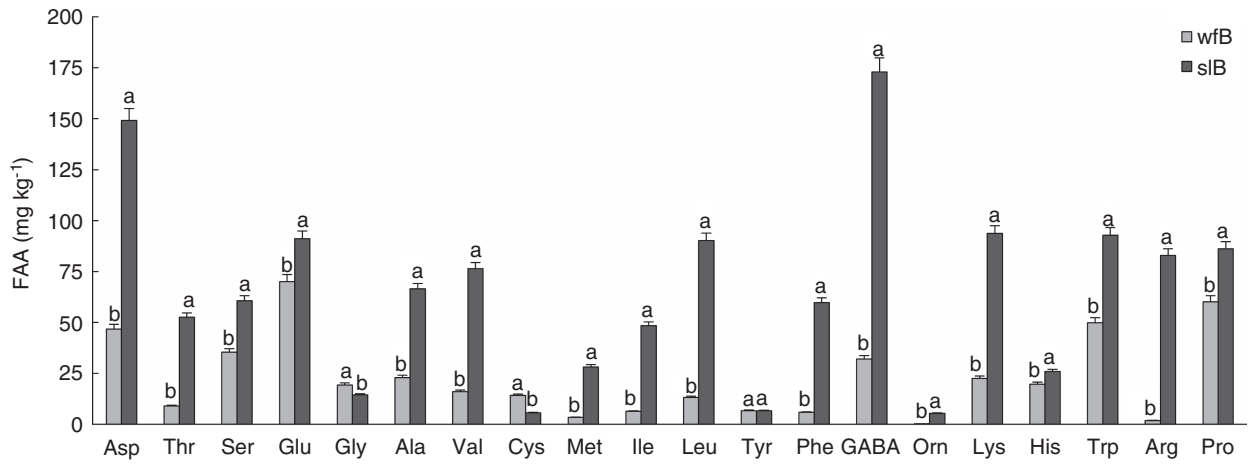

FIGURE 3 Free amino acids concentration (mg kg^{-1} of dough) of common wheat flour bread (wfB) and bread produced with surplus bread slurry (slB)

parameters (Table 3). Moreover, the addition of bread slurry, significantly ($p < 0.05$) influenced the colour of the crust, leading to a decrease in lightness (*L*) and to an increase in the *a* value (red/green colorimetric coordinate), while the *b* value (yellow/blue colorimetric coordinate) did not differ significantly ($p > 0.05$) between breads (Table 3).

DISCUSSION

Bread covers a big portion of the food wasted daily on a global scale, representing one of the burdens of modern society. In most European countries, bread distribution is conducted within a full-service strategy that involves a take-back agreement between retailer and supplier,

meaning that the bakeries are not only responsible for providing the product, but also for removing the one unsold (Brancoli et al., 2020). Of course, part of the waste is also produced in the bakery during the manufacturing process, due to substandard products, processing factors and sometimes consumers' requests. These factors enable a clean flow of bread, not mixed with other food fractions, and provide opportunities for a treatment alternative to incineration or anaerobic digestion (Brancoli et al., 2020) such as, for example, processing as a food ingredient.

In this framework, this study aimed at repurposing surplus bread within the food chain, increasing its nutritional value while responding to the modern vision of a circular economy. Thus, 33 LAB strains were screened for the ability to produce GABA in WBM, a medium obtained from the hydrolysis of bread, which proved to be suitable for the growth of lactic acid bacteria but also yeasts and fungi (Verni et al., 2020). The ability to synthesize GABA varies greatly between LAB strains, among which *Lactobacillus paracasei* (*Lacticaseibacillus paracasei*), *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactococcus lactis*, *L. plantarum* and *Lv. brevis* are among the most studied (Cui et al., 2020). This ability relies on the glutamate decarboxylase (GAD), a pyridoxal 5'-phosphate-dependent enzyme, which catalyses the irreversible decarboxylation of L-glutamate to GABA (Sarasa et al., 2020). GAD genes are widely distributed among LAB. However, the amino acid sequence of GAD from different LAB strains can differ significantly, especially in the N-terminal and C-terminal regions (Yogeswara et al., 2020). In this study, the 4 strains that distinguished for the production of GABA *P. pentosaceus* F01, *Lv. brevis* MRS4, *L. plantarum* C48 and H64, were among those with the shortest latency phase of growth, reaching quite low pH values (below 3.8). Indeed, GABA synthesis is considered a resistance mechanism against acidic conditions, since the decarboxylation of glutamate requires protons, which are taken from the intracellular environment under acidic conditions (Sarasa et al., 2020). pH, temperature and glutamate concentration are the main factors influencing GABA synthesis; however, the optimal conditions vary greatly among the strains (Li & Cao, 2010). For this reason, the supplementation of WBM with glutamate and pyridoxal phosphate, did not improve GABA biosynthesis for a few strains, while it was effective for others. It should also be considered that WBM already contained a fair amount of glutamic acid which was consumed in its entirety only by few strains. Hence the addition of more glutamate, for low GABA-producing strains, was not as essential as the addition of pyridoxal phosphate. It was also previously reported that increasing glutamate supplementation is not useful for all LAB strains since they are not able to tolerate high glutamate concentrations because of the osmotic pressure generated in the cells (Seo et al., 2013; Yang et al., 2008).

Consequentially, the 4 LAB strains with the highest GABA synthesis in plain and supplemented WBM were selected for the optimization experiments using bread as substrate. Four experimental fermentation processes were set up; plain bread was used as such, added of amylolytic and proteolytic enzymes, modifying the pH or supplementing it with wheat bran as a source of glutamate and free amino acids (Table 1).

When used for the *in situ* GABA production, although all the strains acidified the substrate, except for *P. pentosaceus* F01, the acidification remained relatively moderate compared, for instance to wheat flour. In fact, it was previously noted that, in fermented bread slurry a quick pH drop did not correspond to a high TTA value (Immonen et al., 2020). Compared to wheat flour, bread slurry is a more challenging fermentation substrate. In fact, starch is gelatinized and retrograded, while amino groups of proteins and amino acids and reducing sugars initially present in bread dough, during baking react to form the products of Maillard reaction, thus are not available for LAB growth (Zhang & Zhang, 2007). *Lv. brevis* MRS4 and *P. pentosaceus* F01 were the strains that worse adapted to the matrix, in terms of growth (with less than 0.8 log cycle between the inoculum and the end of fermentation), thus were not used further. Hence, for the second fermentation experiment, amylolytic and proteolytic enzymes were used to hydrolyse starch and proteins to provide more available carbon and nitrogen sources, possibly increasing GABA production.

The addition of starch hydrolysing enzymes alone did not enhance GABA synthesis significantly, while the use of the above enzymes and protease together in bread slurry fermented with *L. plantarum* H64 yielded almost 3-fold higher GABA production compared to the fermentation without enzyme hydrolysis. This was probably due to the release of high amounts of glutamate (up to 600 mg kg^{-1}) and other amino acids in the slurry (Table S3). Glutamate content is one of the most critical factors affecting GABA production, and its addition has been shown to increase its production during fermentations (Diana et al., 2014; Li & Cao, 2010). Besides enhancing GABA production, enzymatic hydrolysis also provides other potential advantages in waste bread baking. A previous study showed that the use of Corolase® increased peptide concentration *circa* three times in bread hydrolysate fermented by LAB. Some of the peptides released during bioprocessing showed antifungal properties and contributed to extend the mould-free shelf-life of bread (Nionelli et al., 2020).

Since pH is another important factor in GABA production, the pH of the slurries treated with hydrolytic

enzymes was raised to 6.5. In previous studies, the highest GABA production by LAB was achieved when the initial pH of culture medium fermentation was adjusted to 4.6 (Zhang et al., 2012), or to 6.0 when *L. plantarum* was used to ferment a grape must beverage (Di Cagno et al., 2010). In this study, however, the pH adjustment of hydrolysed slurries did not result in higher GABA production or acidification compared to the trial without pH control (Experiment II) especially in α6.5 samples. A significant increase in GABA production was observed only in C48-αp6.5 compared to C48-αp. It was previously shown that the optimal pH for GABA production depends on GAD characteristics and is strain-dependent (Dhakal et al., 2012), which might explain why an improvement was obtained in slurries fermented by *L. plantarum C48* but not *L. plantarum* H64. Moreover, the glutamate content in pH-controlled hydrolysed slurries was two times higher than in non-pH-controlled trials (Table S4), which indicates the low conversion of glutamate to GABA. Since GABA biosynthesis is also a response to acidic environment, the overall balance between optimal GAD activity and environmental favourable conditions should be addressed based on the specific strain.

In the fourth fermentation experiment, instead of using enzymes, surplus bread was supplemented with 15% or 30% (of bread weight) of wheat bran, leading to the release of sugars as a consequence of fibre and starch degradation carried out by bran endogenous enzymes or LAB activity. Wheat bran, an underutilized milling by-product, is an excellent nutrient source, rich in fibres and proteins with high biological value (Prueckler et al., 2014). The addition of wheat bran, especially at 30%, resulted in a significantly lower pH level and higher TTA than the other fermentations (Table 2). As the glutamate content in wheat bran was high (almost 800 mg kg^{-1}), it increased the initial glutamate content of the slurries. Furthermore, the amount of glutamate increased during fermentation, even if high amounts of GABA were produced simultaneously. Indeed, wheat bran endogenous proteases can be activated during bioprocessing, liberating glutamate (Arte et al., 2015), also providing endogenous GAD that converts glutamate to GABA (Jin et al., 2013).

Due to the significantly higher GABA production compared to the other fermentation trials, the slurry containing 30% wheat bran fermented by *L. plantarum* H64 was used in bread making. Since the worldwide bread consumption is estimated to be 26.6 kg per capita in 2021, with a peak of over 56 kg per capita in Europe (Statista, 2021), it is safe to assume that a staple food like bread, can be the carrier of compounds beneficial to the human body. Compared to the control, the experimental bread showed a better nutritional profile, with more FFA, higher protein digestibility and lower starch hydrolysis index (Table 3). These benefits, as well as the mechanisms behind them, derive from the addition of plant matrices fermented with selected lactic acid bacteria, and have been abundantly reviewed (Gobbetti et al. 2020; Petrova & Petrov, 2020).

Despite all the advantages generated by the addition of a fermented slurry constituted by surplus bread, some anti-nutritional features might have occurred. Among the modifications determined by the baking process, Maillard reactions and the formation of the compounds responsible for bread aroma and crust colour, are the most concerning. Among these compounds, acrylamide, which is formed during processing of carbohydrate-rich foods at elevated temperatures, is classified as a potential carcinogen to humans by European Food Safety Authority (EFSA) (Lineback et al., 2012). For this reason, a couple of years ago, the EU Regulation 2017/2158 established the mitigation procedures for the reduction of acrylamide in food as well as the benchmark levels, which for bread, depending on the type, range from 50 to 100 μg kg^{-1}. However, in our study, both the slurry and the bread had an acrylamide content below 10 μg kg^{-1}.

Although in Europe, GABA does not have any authorized nutrition or health claims by EFSA, evidence of beneficial physiological effects of GABA, are present in the literature (Inoue et al., 2003; Matsubara et al., 2002). Its oral administration elevates GABA levels in blood but is believed to metabolize quickly and it is still unclear if it can cross the blood–brain barrier (Boonstra et al., 2015). However, several benefits have been reported in mice after oral administration of GABA, with increases in the brain up to 33% (Shyamaladevi et al., 2002).

The amount of GABA in experimental bread dough was approximately 150 mg kg^{-1}, observing after baking a 10% loss (136 mg kg^{-1}). GABA itself is a stable molecule, however, during bread baking, may be lost in the Maillard reaction (Lamberts et al., 2012; Le et al., 2020), although, in a recent study, it was found to survive the baking process, resulting in bread with 39 mg kg^{-1} of GABA (Venturi et al., 2019). The effect of oral GABA intake in mildly hypertensive patients showed that the daily administration of 10–12 mg of GABA in a fermented beverage for 12 weeks was able to lower significantly blood pressure, both diastolic and systolic (Inoue et al., 2003). Hypothetically, this means that 100 g of the GABA-enriched bread obtained in this study, equaling *circa* 14 mg of GABA intake, could have similar effects. Analogous results to those obtained by Inoue et al. (2003) were also shown by Matsubara et al. (2002) but with higher GABA doses (80 mg). In our study, although even higher GABA content in bread could have been obtained by increasing the percentage of slurry added, the supplementation was kept relatively low at 10% to avoid strong repercussions on bread quality. Indeed, the use of

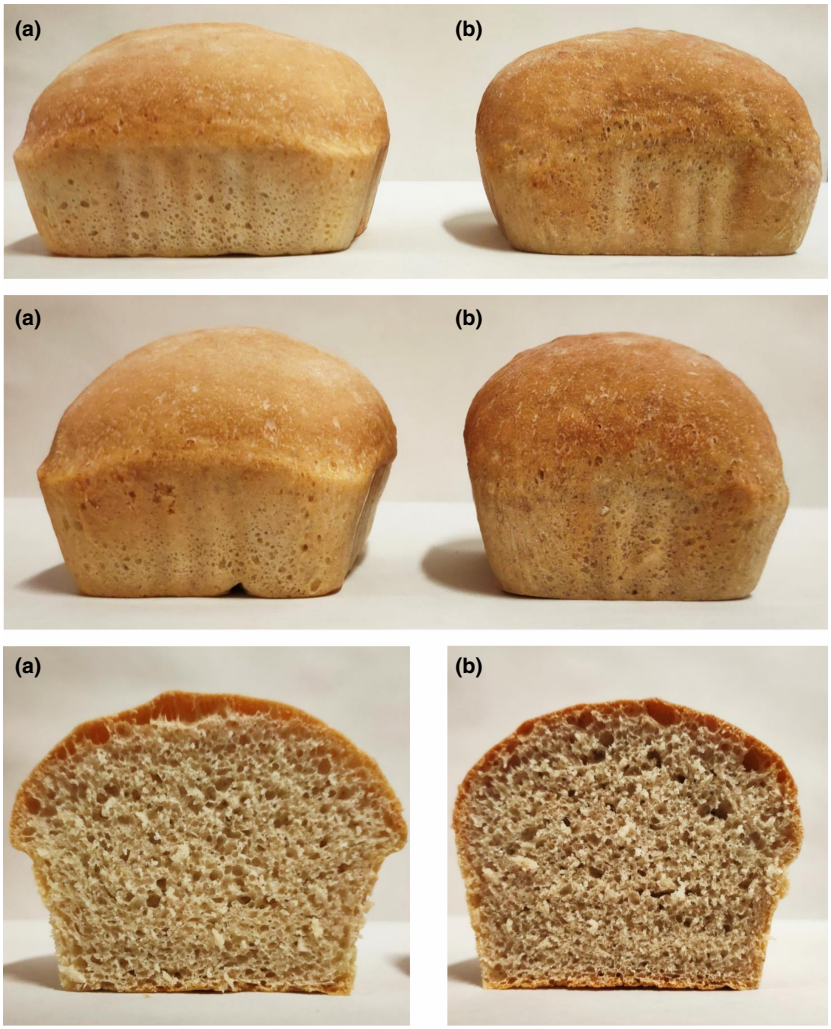

FIGURE 4 Image and cross section of common wheat flour bread (a) and bread produced with surplus bread slurry (b)

fermented surplus bread in bread making caused a moderate quality issue (*circa* 5% higher hardness compared to control bread) yet all the other textural properties remained unaffected, leading to a bread loaf as appealing as the control (Figure 4). During baking, starch gelatinization and gluten polymerization affect bread technological functions (Ortolan et al., 2015; Van Steertegem et al., 2013), hence, they do not contribute again to the structural properties of the new dough. The use of wheat bran also causes detrimental effects on bread volume and hardness, by weakening the gluten network (Boita et al., 2016; Hartikainen et al., 2014). However, the addition of wheat bran enhanced GABA biosynthesis and potentially also the nutritional value of the experimental bread, increasing its amount of fibre. In fact, with a calculated amount of fibres corresponding to 2.5%, the consumption of 100 g of GABA-enriched bread makes up for one-tenth of the daily fibre intake recommended by EFSA (2010).

Maintaining good technological properties when re-utilizing bread as a baking ingredient requires tailored processing. The use of selected starter cultures and conditions for surplus bread fermentation can produce functional molecules such as exopolysaccharides, and others that can add value to the bread recycling process (Immonen et al., 2020; Nionelli et al., 2020).

In this study, two major side streams from the cereal processing industry were used as a substrate for tailored LAB fermentation generating new added-value bakery products with good technological and nutritional properties. Hence, our results represent a proof-of concept of the effectiveness of tailored fermentation strategies to repurpose food industry side streams.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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