Lactobacillus plantarum ITM21B fermentation product and chickpea flour enhance the nutritional profile of salt reduced bakery products

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ABSTRACT
The study aimed at improving the nutritional profile of yeast leavened salt reduced sliced bread and puccia type bread fortified with a wheat-based Lactobacillus plantarum ITM21B fermentation product (Bio21B). The protein content of bread made under laboratory conditions was increased by using: (i) chickpea flour (CF) (15% wt/wt flour) and Bio21B or (ii) the Bio21B containing a fungal protease to favour the gluten hydrolysis. Products showed increased protein and total amino acid content and improved protein digestibility. Moreover, the formula significantly affected the protein pattern of breads which, according to the results of the microfluidic two-dimensional electrophoresis (μ2DE) protein pattern, were discriminated as observed by the PCA plot. The use of CF was validated at industrial pilot plant producing salt reduced sliced bread and puccia type bread. The resulting products showed improved nutritional profile and a sensory quality comparable to the company’s products containing salt.

Introduction
The decrease of salt content in baked goods may represent a suitable tool to reduce sodium daily intake with positive effects on human health, as recommended by World Health Organization (WHO) (WHO 2006).

Amino acids and other molecules, including organic acids, contribute to improve the sensory quality of bread by compensating the negative perception of salt reduction, as recently demonstrated in salt reduced yeast leavened bread formulated with a Lactobacillus plantarum fermentation product (Valerio et al. 2017). Among the acidic molecules that may play a role in modulating the sensory quality, authors found phenyllactic (PLA), hydroxy-phenyllactic (OH-PLA) and indol-lactic acids, which derive from the amino acid catabolism and their content can be modulated by increasing the protein sources in bread formula. Among protein sources, legume flours, pseudo-cereals and hydrolysed proteins from wheat gluten can be used (Schlichtherle-Cerny and Amado 2002), the fortification of cereal-based products with proteins from legumes represents a good strategy to complement the nutritional profile of cereal based foods (Coda et al. 2017). Actually, legumes are an economical source of highly digestible, good quality proteins complementary to the cereal protein pattern (Mansoor and Yusuf 2002; Asif et al. 2013). Legumes have been incorporated in bread formula to improve the nutritional quality and increase the daily nutrient supply (Rachwa-Rosiak et al. 2015; Turfani et al. 2017; Shrivastava and Chakraborty 2018; Wanderslebe et al. 2018). In particular, chickpea flour (CF) (fermented or not) has been widely used in bread-making to improve the nutritional and technological quality (Coda et al. 2010; Mohammed et al. 2014; Rizzello et al. 2014a; Xiao et al. 2016; Melini et al. 2017; Shrivastava and Chakraborty 2018), due to the high amount of proteins (17–22%), available carbohydrates (50%), crude fibres (3.82%), vitamins (thiamine, niacin and ascorbic acid), minerals (Ca, Fe, K, Mg and P) and unsaturated fatty acids (linoleic, oleic) and low fat (6.48%) (Asif et al. 2013; Rachwa-Rosiak et al. 2015). The protein content
of CF is mainly represented by globulins which range between 53% and 60% (Asif et al. 2013). The use of wheat-chickpea composite flour in bread formula determines improved nutritional value by increasing the content of essential amino acids (including lysine, leucine, aspartic acid, glutamic acid and arginine) (Rachwa-Rosiak et al. 2015; Zafar et al. 2015). Generally, the substitution of the 10–20% (wt/wt) wheat flour with CF allows to obtain products acceptable to consumers and similar to wheat dough (Melini et al. 2017). However, the low digestibility of legume proteins due to the presence in seeds of antinutritional compounds such as trypsin inhibitors, lectins and polyphenols, limits their application as ingredients in food formula (Clemente et al. 2000; Rachwa-Rosiak et al. 2015). To face this drawback in bakery products, several researches have tried to increase the protein digestibility of legume flours by using sourdough technology (Coda et al. 2010; Rizzello, Calasso et al. 2014). The acidification operated by lactic acid bacteria (LAB) activates endogenous proteases that, flanked by LAB peptidase, favour the proteolysis with a consequent improvement of the protein digestibility (Coda et al. 2010; Rizzello, Curiel et al. 2014). The enhanced enzymatic activity of wheat proteases also increased the content of free amino acids thus improving bread flavour and nutritional quality (Thiele et al. 2002; Clarke et al. 2004; Rizzello et al. 2012). The acidification of dough can also be obtained in yeast leavened bread by including LAB fermentation products in bread formula: textural and physico-chemical properties of bread were improved by using wheat flour fermented with Lactobacillus brevis (Valerio et al. 2014). Moreover, acidic molecules contained in a wheat/gluten Lactobacillus plantarum fermentation product (Bio21B) included in salt reduced bread formula led to a taste improvement (Valerio et al. 2017).

Therefore, the aim of the current study was to increase the protein – and their digestibility – and total amino acid content of salt reduced bakery products by using either: (i) CF and the L. plantarum fermentation product Bio21B or (ii) the Bio21B containing a fungal protease (Bio21B_H). According to the results achieved at laboratory level, a formula of salt reduced sliced bread and puccia type bread with improved nutritional value was manufactured at industrial pilot plant.

**Materials and methods**

**Bacterial cultures**

*Lactobacillus plantarum* ITM21B was previously isolated from sourdough (Corsetti et al. 2001) and deposited in the Belgian Coordinated Collections of Microorganisms (BCCM/LGM, Gent, Belgium) as LMG P-22033. In this study, the strain is reported as *L. plantarum* ITM21B. For long-term storage, stock cultures were stored frozen (-80°C) in de Man Rogosa Sharpe medium broth (MRS, OXOID Ltd, Basingstoke, UK) supplemented with 20% Bacto glycerol (Difco, Becton Dickinson Co., Sparks, MD). To obtain a fresh culture, the strain was subcultured twice (37°C for 8 h and 37°C for 18 h).

**Flours and enzyme**

Wheat (*Triticum aestivum*), chickpea (*Cicer arietinum*), wheat gluten flours and the protease enzyme VERON® “PS” were supplied by the Company Valle Fiorita Catering S.r.l. (Ostuni, Italy). The protein content was 14.5% of dry matter (d.m.), 21% of d.m. and 83% of d.m. for wheat flour type 0, CF and vital wheat gluten, respectively.

**Preparation of the fermentation product Bio21B and Bio21B_H**

The fermentation product Bio21B was prepared as reported in Valerio et al. (2017) with slight modifications. Briefly, cell suspension of *L. plantarum* ITM21B was prepared by inoculating (2%, vol/vol) the MRS medium and after overnight incubation at 37°C, the culture was centrifuged and bacterial cells were washed and re-suspended in sterile water at a final cell density of about 9.0 log CFU/mL. The fermentation product Bio21B was prepared by mixing 80 mL of the suspension of *L. plantarum* ITM21B, 240 mL of sterile tap water, 40 g of wheat flour and 40 g of wheat gluten and incubated at 30°C for 14 h. The resulting product Bio21B, having d.m. content of 20% and dough yield (DY, dough weight × 100/flour weight) of 500, was included in formula of breads made under laboratory conditions and containing (Bread CF_Bio21B) or not (Bread Bio21B) CF (Table 1). To produce Bio21B at industrial pilot plant, an amount of 10 L of fermentation product was incubated in a fermentation system (Novasilos SNL100, Forli, Italy).

The fermentation product Bio21B_H was prepared by adding the fungal protease VERON® PS (*Aspergillus oryzae*) to the Bio21B. In detail, an amount of 0.48 g of enzyme was solubilised in pre-warmed (45°C) sterile tap water (120 mL) and mixed with wheat gluten (20 g) and the *L. plantarum* culture (40 mL).
Assessment of the presence of *L. plantarum* ITM21B in Bio21B and Bio21B_H

To ensure the presence of *L. plantarum* ITM21B in the fermentation products, presumptive LAB of fermented samples was enumerated on MRS agar medium (Oxoid, Basingstoke, UK) supplemented with cycloheximide (0.1 g/L). Twenty percent of total colonies, randomly picked from MRS agar plates containing the two highest dilutions, were isolated and checked for purity. Bacterial DNA was extracted from each colony from overnight cultures grown in MRS broth at 37°C as previously described (De Bellis et al. 2010). Genetic identification of *L. plantarum* ITM21B was based on the comparison of the REP-PCR profile of each isolate with the specific pattern obtained from the pure culture of *L. plantarum* ITM21B strain. The amplification products were separated by microfluidic electrophoresis using the DNA7500 LabChip kit with the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Chips were prepared according to the manufacturer’s instructions and profiles were automatically generated. Data were analysed using the 2100Expert software provided by the same company.

**Bread-making**

The fermentation product was used to prepare bread samples either at laboratory level or by industrial pilot plant (Valle Fiorita Catering, Srl, Ostuni, Italy). As regard to the former, three different types of salt-reduced breads were prepared and compared to bread sample containing salt and not containing fermentation products (CTRL) (Table 1). Bread samples were manufactured using Princess®Home Breadmaker, type 1936 bread machine (Princess Household appliance BV, Breda, Netherland). All ingredients were mixed and the dough proofed and baked using the “Basic” programme. The CF was used (Bio21B_CF) at 15% (wt/wt) of the flour content. All dough samples had a DY of 160. Bread samples were cooled at room temperature for about two hours, sliced and frozen at –20°C until chemical analyses.

The formulation including the fermentation product Bio21B and CF was also used to produce sliced bread and puccia type bread at industrial pilot plant following the company recipes. Ingredients used are reported in Table 2. The CTRL doughs had DY of 170, while Bio21B_doughs showed a DY of 180.

| Table 1. Ingredients of salt reduced breads containing or not the fermentation product Bio21B prepared in laboratory in comparison to a standard recipe bread (CTRL) containing salt. |
|----------------|-----------------|-----------------|-----------------|-----------------|
| Ingredients    | CTRL            | Bio21B          | CF_Bio21B       | Bio21B_H        |
| Wheat flour (type 0) | 60.60           | 55.16           | 46.89           | 55.16           |
| Chickpea flour (CF) | –               | –               | 8.28            | –               |
| Water           | 36.36           | 13.25           | 13.25           | 13.25           |
| Baker’s yeast   | 1.51            | 1.52            | 1.52            | 1.52            |
| NaCl            | 0.76            | 0.76            | 0.76            | 0.76            |
| Fermentation product Bio 21B or Bio21B_Ha | 29.29 | 29.29 | 29.29 | 29.29 |

**Table 2. Ingredients used to produce sliced bread and puccia type bread in pilot plant production.**

| Sliced bread type | Puccia type bread |
|-------------------|-------------------|
| Wheat flour (0 type) | Wheat flour (0 type) |
| Chickpea flour (CF)b | Durum wheat Semolina |
| Baker’s yeast | Baker’s yeast |
| Water | Water |
| Dextrose | – |
| Liquid sourdough (LS) or Bio21B | Liquid sourdough (LS) or Bio21B |
| Salt | Salt |
| Extra virgin oil | Sunflower oil |

**Assessment of the presence of *L. plantarum* ITM21B in Bio21B and Bio21B_H**

To ensure the presence of *L. plantarum* ITM21B in the fermentation products, presumptive LAB of fermented samples was enumerated on MRS agar medium (Oxoid, Basingstoke, UK) supplemented with cycloheximide (0.1 g/L). Twenty percent of total colonies, randomly picked from MRS agar plates containing the two highest dilutions, were isolated and checked for purity. Bacterial DNA was extracted from each colony from overnight cultures grown in MRS broth at 37°C as previously described (De Bellis et al. 2010). Genetic identification of *L. plantarum* ITM21B was based on the comparison of the REP-PCR profile of each isolate with the specific pattern obtained from the pure culture of *L. plantarum* ITM21B strain. The amplification products were separated by microfluidic electrophoresis using the DNA7500 LabChip kit with the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Chips were prepared according to the manufacturer’s instructions and profiles were automatically generated. Data were analysed using the 2100Expert software provided by the same company.

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The formulation including the fermentation product Bio21B and CF was also used to produce sliced bread and puccia type bread at industrial pilot plant following the company recipes. Ingredients used are reported in Table 2. The CTRL sliced bread contained salt, at 1.36% wt/wt of dough, and a liquid sourdough (LS) (fermented by a company *L. plantarum* strain) added at 15% wt/wt of dough. In salt-reduced sliced bread, the LS was replaced by Bio21B and contained a salt amount reduced by 50% respect to the CTRL (0.68% wt/wt of dough). The CTRL puccia type bread contained salt (1.24% wt/wt of dough) and the LS (20% wt/wt of dough). In the salt-reduced puccia, LS was replaced by Bio21B and a salt amount reduced by 50% (0.62% wt/wt of dough). CTRL doughs had DY of 170, while Bio21B_doughs showed a DY of 180.
**Determination of pH, TTA, organic acids in fermentation products, dough and bakery product samples**

Total titratable acidity (TTA) of laboratory and pilot plant bakery products was measured according to AOAC Method No. 981.12 (AOAC 1990). The pH of fermentation products was recorded with a pH-metre (Beckman Coulter, Ω340 pH/Temp metre, Brea, CA) supplied with a glass electrode (Beckman Coulter, Brea, CA) while for dough and breads a portable 110 pH-metre (type110, Eutech Instruments, Singapore) supplied with Double Pore D electrode (Hamilton, Bonaduz, Switzerland) was used.

Sample preparation and analysis of lactic, acetic, PLA and OH-PLA acids was performed as reported in Valerio et al. (2017) with slight modifications. Briefly, the fermentation products were centrifuged (9072×g, 10 min, 4°C) and the supernatants were freeze-dried; dough and bread samples, 10-g portions, were suspended in sterile tap water (90 mL), homogenised in a stomacher for 2 min, then the suspensions were centrifuged (9072×g, 10 min, 4°C) and the supernatants freeze-dried. The freeze-dried samples were re-suspended in the HPLC mobile phase (0.007 mol/L H2SO4) (Fluka, Deisenhofen, Germany) and filtered by centrifugation (7000×g, 1 h, 2°C) through a 3000 Da cut-off micro-concentrator (Ultracel-3k, Amicon, Danvers, MA). The fraction containing molecules with molecular weight (MW) lower than 3000 Da was analysed by HPLC (AKTAbasic10, P-900 series pump, Amersham Biosciences AB, Uppsala, Sweden; degasser Gastorr BG-12, FLOM Corporation, Tokyo, Japan), using a Rezex ROA organic acid Hþ (8%) column (7.80×300 mm, Phenomenex, Torrance, CA), an injection volume of 10 µL, a 3-channel UV detector (Amersham Biosciences 900, Uppsala, Sweden) set at 210 (lactic, acetic, PLA acids) and 220 nm (OH-PLA). The mobile phase was pumped at a flow rate of 0.7 mL/min through the column heated to 65°C. Quantification of the organic acids was performed by integrating calibration curves obtained from the relevant standards in CTRL bread and dough matrix. Limit of detection (LOD) and limit of quantification (LOQ) were calculated considering a signal-to-noise (S/N) ratio of 3 and 6, respectively. LOD values were the following: lactic acid, 0.233 mmol/kg; acetic acid, 0.285 mmol/kg; PLA, 1.1 µmol/kg; OH-PLA, 0.77 µmol/kg. LOQ values corresponded to 2×LOD. Final concentration of each organic acid in fermentation product, dough and bread samples was calculated taking into account concentration and/or dilution factors and expressed as mmol/kg of product (dough or bread). The fermentation quotient (FQ) was determined as the molar ration between lactic and acetic acids.

**Characterisation of bread proteins**

Protein content was measured using the BioRad protein assay dye reagent (BioRad Laboratories, Hercules, CA) and expressed as mg of protein per gram of bread. Protein fractions were analysed by the Lab-on-a-Chip (LoaC) capillary electrophoresis in combination with the Protein230 LabChip kit (Agilent Technologies, Waldbronn, Germany). Evaluation of data was performed by the dedicated 2100Expert software and results were displayed for each sample as peaks in an electropherogram, as bands in a gel-like image and in a tabular format. For each protein peak, data obtained were: time-corrected peak area (TCA), relative concentration based on a one-point calibration to the upper marker (60 mg/mL) and protein percentage as a function of the total protein detected in the sample. After each run, peaks with fluorescence units (FUs)>1 were manually integrated and relative quantification was carried out considering TCA: results were expressed as percentages by taking the sum of all protein peaks present in the electropherogram as 100%. All experiments were performed twice (n=2).

Total proteins were characterised according to their isoelectric point (Ip) and MW using a micro two-dimensional electrophoresis (µ2DE) system by the means of the 3100OFFGEL fractionator (Agilent Technologies, Waldbronn, Germany) and the 2100Bioanalyzer. Total proteins were extracted from each sample as reported in Hurkman and Tanaka (2007) with minor modifications. Briefly, 80 mg of dried ground bread were extracted with 800 µL of SDS buffer for 1 h at room temperature afterward insoluble material was removed by centrifugation (16,000×g for 10 minutes). For Ip-based separations, isoelectrofocusing (IEF) was performed using the OFFGEL kit pH 3–10 with a 12-well setup. Before fractionation, SDS was removed from extracts by precipitating proteins with cold acetone prior to solubilisation in 1.8 mL of focussing buffer. According to the supplier’s protocol, gel strips were rehydrated with 40 µL focussing buffer per well and 150-µL sample volume was loaded into each well. Samples, with a protein content of about 0.7 mg, were focussed with a maximum current of 50 mA for approximately 25–30 h. Liquid fractions from each compartment were carefully collected and proteins were precipitated overnight at –20°C with four volumes of cold...
acetone. Pellets were collected by centrifugation at 16,000 \( g \) for 10 min at 4 \( ^\circ \)C and dissolved in 50 \( \mu \)L of Tris buffer (10 mM, pH 8). Each fraction was analysed by Loac on Protein230 LabChip kit. In order to better visualise changes in protein distribution, the gel-like image of the 2DE pattern was divided into 12 sectors on the base of Ip and MW and proteins were enumerated from the corresponding electropherograms (Figure S1). Sectors were coded as follows: the number indicates the MW zone 1 (14–19 kDa), 2 (20–39 kDa), 3 (40–69 kDa) and 4 (above 70 kDa) while the Ip regions (acidic, neutral, alkaline) are indicated by the abbreviations Ac (Ip 3–5.9), N (Ip 5.9–7.7) and Al (Ip 7.7–10).

Different protein fractions (albumins + globulins AG, glutenins + gliadins GG and CM proteins) were extracted from 80 mg of each dried bread sample (CTRL, Bio21B, Bio21B_H and CF_Bio21B) as reported in Hurkman and Tanaka (2007). The glutelin and prolamine fractions of CF_Bio21B samples were extracted in the GG fraction (Chang et al. 2011).

### Amino acid analysis in dough and bread samples

Water/salt-soluble extracts of the dough and bakery products samples (from laboratory and pilot plant experiments) were prepared according to the method originally described by Osborne (1907) and modified by Weiss et al. (1993) and used to analyse total free amino acids (TFAAs). TFAAs were determined by Cd-ninhydrin method as reported by Doi et al. (1981).

### Sensory analysis

A trained panel group composed of eight assessors (four male and four female, range: 30–45 years, researchers of the Department of Agricultural Sciences, Food and Environment of the University of Foggia), was used (Saccotelli et al. 2018). Although the assessors were experienced in the evaluation of bakery products prior to this study, they were retrained in three sessions held over three days (1 session/day, 2 h/session). Retraining samples included laboratory scale bread samples. Appropriate sensory attributes were discussed during the retraining sessions. After retraining, the assessors were able to evaluate product colour, aroma, taste, large bubbles structure, crust and crumb firmness and overall quality. Each sample was coded arbitrarily with three-digit numbers. Samples were served in random order and evaluated in two replicates by all panellists. Bread samples were evaluated on a scale of 1–9 (where, 

\( 1 = \) extremely unpleasant, \( 9 = \) extremely pleasant, 

\( 5 = \) sensory acceptability threshold), for the acceptance of the seven attributes. Sensory analysis was performed in duplicate.

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**Figure 1.** Electrophoretic analysis (Loac) of total proteins extracted from bread samples shown as overlaid electropherograms (a) and gel-like image (b) on Protein230 LabChip. CTRL bread (red line); Bio21B bread (blue line); Bio21B_H bread (green line); CF_Bio21B bread (light blue line); sizing Ladder (lane L). Numbered brackets indicate molecular weight zones: 1 (14–19 kDa), 2 (20–39 kDa), 3 (40–69 kDa), 4 (>70 kDa).
Statistical analysis

Data are presented as mean values ± standard deviation and were subjected to one-way ANOVA followed by Fisher’s post hoc test. Statistical significance was assessed at a level of 5%. Data analysis was performed using the software STATISTICA 6.0 (StatSoft, Tulsa, OK). The principal component analysis (PCA) was used to characterise laboratory bread samples according to their protein μ2DE pattern and in particular the number of proteins and the relevant TCA for each sector identified in the pattern were considered (see Section ‘Characterisation of bread proteins’). The first principal component explains as much of the variability in the data as possible, and each succeeding component explains as much of the remaining variability as possible. Data were pre-treated (mean centred and scaled) to give all variables the same variance and the same chance to influence the estimation of components, prior to the PCA calculation. PCA was carried out with the Unscrambler X (CAMO, Oslo, Norway).

Results and discussion

Chemical characterisation of fermentation products and laboratory dough and bread samples

After 14 h fermentation, Bio21B and Bio21B_H showed values of pH of 3.61 and 3.33, respectively (Table 3). The cell density of L. plantarum ITM21B reached ca. 8.0 log CFU/mL. The quantification of organic acids in the fermentation product Bio21B in the presence or not of the fungal protease is reported in Table 3. The use of the enzyme led to a higher amount of acids, mainly lactate, PLA and OH-PLA. Protease activity increased the concentration of phenylalanine (Phe) and tyrosine (Tyr) which are precursor compounds of PLA and OH-PLA, respectively (Valerio et al. 2004). Lactic acid generally derives from carbohydrate metabolism through the formation of pyruvate; however, it can also be formed by some non-conventional substrates such as amino acids. In particular, serine can be deaminated to ammonia and pyruvate, with the latter reduced to lactate (Liu et al. 2003). Alternatively, pyruvate can be produced directly (e.g. alanine) or indirectly (aspartate) from amino acids by transamination (Liu et al. 2003). The FQ was always higher than 1 due to the very low concentration of the acetic acid.

When the fermentation products Bio21B and Bio21B_H were used in the laboratory bread-making process, the resulting dough samples showed pH values significantly lower than those obtained in the CTRL dough (Table 4) and comparable to those obtained in sourdough breads (Rizzello, Curiel et al. 2014). Dough samples containing the Bio21B were characterised by the presence of PLA and OH-PLA not detected in the CTRL dough. In particular, bread manufactured with Bio21B and CF contained the higher amount of OH-PLA. Moreover, as a result of the low amount of acetic acid found in the fermentation products (Table 3), dough samples did not contain (or at low level) this acid and the resulting FQ was higher than 1. This condition can positively affect the sensory properties of sourdough bread and the textural properties of final products as reported by several authors (Hammes and Gänzle 1998; Gobbetti et al. 2005). As expected, the concentration of lactic acid in all dough samples containing the fermentation product was higher (p < .05) than the CTRL (<LOD) and comparable among samples. The organic acid content remained almost unvaried after the baking process (Table 4). The TTA values indicated the higher acidity of CF_Bio21B (6.5), Bio21B_H (5.5) and Bio21B (4.9) compared to the control (2.3).

Amino acid amounts in laboratory dough and bread samples

TFAA content in dough and bread samples was evaluated and as expected the baking process determined a significant decrease in TFAA content in all samples (Table 5). However, results confirmed the contribution of the fermentation product to the amino acid content. In fact, dough and bread samples containing the fermentation products

Table 3. Organic acid concentrations in the fermentation product Bio21B containing or not the A. oryzae protease used in bread formula.

| Fermentation product | Lactic acid (mmol/L ± SD) | Acetic acid (mmol/L ± SD) | PLA (mmol/L ± SD) | OH-PLA (mmol/L ± SD) | pH | FQ (mmol/L ± SD) |
|----------------------|---------------------------|--------------------------|------------------|---------------------|----|-----------------|
| Bio21B               | 20.99 ± 0.86             | 1.08 ± 0.32             | 0.025 ± 0.002    | 0.012 ± 0.0002      | 3.61 ± 0.19 | 19.43           |
| Bio21B_H             | 43.75 ± 11.08            | 0.96 ± 0.21             | 0.088 ± 0.03     | 0.024 ± 0.0002      | 3.33 ± 0.09 | 45.57           |

Mean ± SD in the same column followed by different letters differs significantly (p < .05) by the one-way ANOVA followed by Fisher’s test.
were characterised by higher (p < .05) TFAA concentrations compared to the CTRL. It is known that the combined effect of the flour endogenous proteases activated by the acidification and LAB peptidases leads to a TFAA concentration higher in fermented flours than in non-fermented samples (Gänzle 2014). In this study, sample coded as Bio21B, thus only containing the fermentation product, had a lower (p < .05) concentration of amino acids compared to samples CF_Bio21B and Bio21B_H. In particular, bread coded as CF_Bio21B, manufactured with the addition of CF as additional source of proteins, contained the highest (p < .05) amount of TFAA. This alternative flour contains additional proteins (mainly albumins and globulins) and a higher content of total amino acids (Rachwa-Rosiak et al. 2015). A lower value of TFAA was found in bread manufactured with the fermentation product containing the wheat gluten hydrolysate (Bio21B_H).

### Table 5. Content of TFAA in laboratory and pilot plant bakery products.

| Sample                  | TFAA (mg/kg)±SD |
|-------------------------|-----------------|
| **Laboratory dough**    |                 |
| CTRL                    | <LOD           |
| Bio21B                  | 867.2 ± 9.2a    |
| CF_Bio21B               | 1288.8 ± 13.6c  |
| Bio21B_H                | 1987.1 ± 15.3a  |
| **Laboratory bread**    |                 |
| CTRL                    | <LOD           |
| Bio21B                  | 958.6 ± 7.8a    |
| CF_Bio21B               | 1829.9 ± 6.4b   |
| Bio21B_H                | 1787.23 ± 9.3f  |
| **Pilot plant products**|                 |
| Sliced bread CTRL       | 1727.8 ± 11.4a  |
| Sliced bread CF_Bio21B  | 1969.7 ± 12.7g  |
| Puccia type bread CTRL  | 1688.2 ± 13.3c  |
| Puccia type bread CF_Bio21B | 2435.8 ± 12.8a |

Mean ± SD in the same column followed by different letters differs significantly (p < .05) by the one-way ANOVA followed by Fisher’s test.

Characterisation of the bread proteins

To characterise the bread proteins, two different approaches (based either on Ip and MW or on solubility) were used. According to their MW, total proteins from laboratory bread samples were analysed by Loac to reveal differences in their protein pattern. Electrophoretic profiles are shown in Figure 1 as overlaid electropherograms (Figure 1(a)) and gel-like images (Figure 1(b)). Total protein extracts from bread samples showed similar patterns characterised by about 20 protein bands/peaks ranging from 14 to 220 kDa on Protein230 LabChip. Electrophoretic profiles were separated in four zones according to protein MW: 1 (14–19 kDa), 2 (20–39 kDa), 3 (40–69 kDa) and 4 (above 70 kDa) (Figure 1). In all samples, MW zone 3 accounted for the major protein content (expressed as %) while some differences in the protein profile were observed mainly in zones 1 and 2. In CTRL bread, zone 3 accounted for the 77.7 ± 3.1% and was characterised by three main protein bands of 45, 48 and 62 kDa. Minor protein contents, i.e. 9.0 ± 0.8%, 0.9 ± 0.3% and 12.5 ± 2.1% were observed in zones 1, 2 and 4, respectively. A slight increase of low MW proteins was observed for all samples containing the fermentation products in zones 1 and 2 (9.1–13.6% and 2.2–3.1%, respectively) with a concomitant decrease of larger MW proteins (zone 3) to 72.2–74.9%, maybe due to the dough acidification determined by the fermentation products. Finally, in zone 4, the protein content was in the range 9.5–14.4%.

In order to deeply investigate the differences in the protein profile among samples, the total protein extract from each laboratory bread was also analysed by μ2DE and proteins were enumerated and quantified in each of the 12 sectors shown in the gel-like image (Figure S1). Bread samples CTRL, Bio21B, CF_Bio21B and Bio21B_H were

### Table 4. Organic acids (lactic, acetic, PLA and OH-PLA), pH and fermentation quotients (FQ) values in dough samples produced under laboratory conditions.

| Sample     | Lactic Acid | Acetic Acid | PLA         | OH-PLA      | pH    | FQ     |
|------------|-------------|-------------|-------------|-------------|-------|--------|
| **Dough**  |             |             |             |             |       |        |
| CTRL       | <LOD        | n.d.        | n.d.        | n.d.        | 5.49  | n.d.   |
| Bio21B     | 18.30 ± 2.52 | n.d.        | 30.49 ± 0.85 | 21.47 ± 0.53 | 4.45  | 1.03   |
| CF_Bio21B  | 14.01 ± 0.50 | n.d.        | 41.75 ± 0.49 | 40.86 ± 0.41 | 4.36  | 1.03   |
| Bio21B_H   | 16.17 ± 0.99 | 0.62 ± 0.11 | 74.14 ± 5.35 | 17.58 ± 0.25 | 4.32  | 2.06   |
| **Bread**  |             |             |             |             |       |        |
| CTRL       | <LOD        | n.d.        | n.d.        | n.d.        | 5.52  | 10.04  |
| Bio21B     | 22.21 ± 0.70 | n.d.        | 46.22 ± 0.89 | 34.45 ± 0.82 | 4.99  | 0.01   |
| CF_Bio21B  | 10.52 ± 0.43 | n.d.        | 40.81 ± 0.71 | 42.00 ± 0.71 | 5.13  | 0.04   |
| Bio21B_H   | 17.16 ± 0.50 | 0.97 ± 0.14 | 62.98 ± 1.59 | 23.72 ± 0.39 | 5.04  | 0.07   |

n.d.: not detected; FQ: fermentation quotient (ratio between molar lactic and acetic acids concentration).

Mean ± SD in the same column followed by different letters differs significantly (p < .05) by the one-way ANOVA followed by Fisher’s test.

Lower than the limit of detection (<LOD).
characterised by 71, 101, 96 and 98 proteins (Table S1). Data from the μ2DE pattern were used in the PCA to discriminate samples (Table S1; Figure 2). PC1 and PC2 in the resulting model described 79% of the total variance of the data. An effect of bread formula on protein distribution was observed since samples were clearly separated on the plane. Overall, acidic proteins characterised CF_Bio21B bread, the neutral proteins were mainly represented in Bio21B and Bio21B_H samples while alkaline proteins distinguished the CTRL bread. In particular, CF_Bio21B bread was characterised by a high number of acidic proteins – sectors S1_Ac (14–19 kDa), S3_Ac (40–69 kDa) and S4_Ac (>70 kDa) – and alkaline proteins in sector S2_Al (20–39 kDa) and mainly by a high protein content in sector S2_Ac in which only three proteins accounted for the 28.3% (Table S1; Figure 2). A high acidic protein content has been already reported by Arcan and Yemenicioğlu (2010) in the albumin and globulin fraction of CF. Authors correlated these proteins to the improved free radical scavenging activity. Breads Bio21B and Bio21B_H located in the lower left part of the plane and they were mainly characterised by the protein content relevant to the neutral zone of all sectors and by low MW acidic proteins (S1_Ac). As a result of the different formula, CTRL bread was separated from the other samples and located in the lower right part of the plane. It was mainly characterised by high contents of proteins relevant to alkaline zones S1_Al, S2_Al, S4_Al. Additional information on the protein content of bread samples was obtained when proteins were fractionated according to their solubility in gliadins and glutenins (GG), albumins and globulins (AG) and CM proteins (CM) fractions (Table 6). The addition of CF (CF_Bio21B) or wheat gluten hydrolysate (Bio21B_H) to the bread formula significantly increased (p < .05) the total protein content of the relevant bread compared to the CTRL and Bio21B bread samples (Table 6). Among samples, those enriched with the fermentation product and CF showed the highest (p < .05) content of GG and AG fractions, while the remaining protein fractions resulted to be comparable.

Table 6. Content and digestibility (IVPD) of proteins of laboratory breads.

| Sample     | GG           | AG           | CM           | Total proteins | IVPD         |
|------------|--------------|--------------|--------------|----------------|--------------|
| CTRL       | 7.42 ± 0.33a | 0.38 ± 0.10a | 10.84 ± 1.27a| 18.63 ± 1.05a  | 57.89 ± 0.49a|
| Bio 21B    | 6.51 ± 0.16a | 0.44 ± 0.02a | 11.23 ± 0.69a| 18.18 ± 0.86a  | 56.20 ± 0.25a|
| CF Bio21B  | 9.48 ± 0.61b | 1.40 ± 0.09b | 11.51 ± 0.69b| 22.38 ± 0.22b  | 63.45 ± 1.29b|
| Bio 21B_H  | 8.00 ± 0.05a | 0.34 ± 0.07a | 12.95 ± 0.08a| 21.30 ± 0.11b  | 62.68 ± 1.03b|

Mean ± SD in the same column followed by different letters differs significantly (p < .05) by one-way ANOVA followed by the Fisher’s test.
Figure 3. Electrophoretic analysis (Loac) of the GG fraction extracted from bread samples (on the left) and of the relevant not digested GG fraction recovered after in vitro digestion (on the right) shown as electropherograms on Protein230 LabChip kit. CTRL bread (a, e); Bio21B bread (b, f); CF_Bio21B bread (c, g); Bio21B_H bread (d, h). Numbered brackets indicate molecular weight zones: 1 (14–19 kDa), 2 (20–39 kDa), 3 (40–69 kDa), 4 (>70 kDa).
Digestion of bread proteins

The addition of Bio21B and CF (CF_Bio21B) or wheat gluten hydrolysate (Bio21B_H) to the bread formula significantly increased \( (p < 0.05) \) the protein digestibility respect to CTRL and Bio21B bread samples (Table 6). Interestingly, the AG fractions were completely digested in all samples, including the CF_Bio21B bread, which contained the highest amount of these proteins known to be poorly digested in the case of legume flours (Singh 2017). In all samples, a comparable digestibility of CM enriched fractions was observed (data not shown) while differences in digestibility of the GG fractions were registered among samples. Therefore, to visualise changes in protein patterns, the GG fraction extracted from bread samples and the non-digested GG fraction recovered after in vitro digestion, were subjected to Loac analysis. In all bread samples, the protein fraction showed about 20 protein bands/peaks ranging from 14 to 220 kDa on Protein230 LabChip (Figure 3(a–d)). Patterns showed the most abundant percentage of proteins \( (77.7–79.5\%) \) in the 44–60 kDa range. After digestion (Figure 3(e–h)), the high MW GG proteins (>70 kDa) disappeared in bread samples, except for bread Bio21B that showed a protein peak at 94 kDa, confirming the hydrolysis of these proteins by digestive enzymes as previously reported by Pasini et al. (2001) and Świeca et al. (2017). TCA of protein peaks relevant to the whole electropherogram of bread samples after digestion was compared to the relevant values before digestion and expressed as percentages. As a result of the digestion process, the areas decreased to 4.41\% in CF_Bio21B, 1.33\% in Bio21B_H, 23.2\% in Bio21B and 75.7\% in CTRL breads thus indicating a different digestion of the GG fraction among samples (Figure 3(e–h)).

It is known that CF contains globulins, classified as storage proteins, mainly represented by legumin and vicilin, which are poorly hydrolysed by digestive proteases due to their structure and the presence of anti-nutritional compounds (Clemente et al. 2000). In this study, the albumin and globulin fractions (higher in CF_Bio21B bread than in the other samples) resulted to be completely digested also in bread containing CF. This is maybe due to the contribution of dough acidification operated by the \textit{L. plantarum} fermentation product. Similarly, the addition to the wheat bread of CF fermented by LAB led to an improved protein digestibility and a higher content of TFAA compared to baker’s yeast bread (Rizzello, Calasso et al. 2014). Moreover, the increase of GABA and soluble fibre concentrations, antioxidant and phytase activities and the decrease of starch hydrolysis index, were observed. The use of fungal protease in bread-making process has also been proposed as tool to increase the protein content in bread (Rizzello, Curiel et al. 2014). In this study, bread prepared with the Bio21B_H showed a protein content and protein digestibility value comparable to CF_Bio21B bread and higher than CTRL and Bio21B samples. The presence of the protease allowed the hydrolysis of proteins during fermentation of Bio21B thus increasing the amino acid content of the corresponding bread. The addition of a wheat/gluten hydrolysate in bread formula can contribute to enrich the final product with savoury molecules that can compensate the reduction of salt content. As demonstrated by Aaslyng et al. (1998), the savoury flavour of protein hydrolysates is mainly related to the high content of free amino acids, especially glutamic acid, low MW peptides and organic acids. Actually, Bio21B, applied in salt reduced bread formula, containing high amount of TFAAs and organic acids, compensated the negative perception of salt reduction (Valerio et al. 2017).

Pilot plant production

The improved IVPD and increased TFAA content suggested the use of Bio21B and CF as an optimal strategy to produce, at industrial level, reduced salt bakery products with improved nutritional profile. A pilot plant production in a bakery industry was carried out to produce sliced bread and puccia type bread (Figure S2). The resulting products were characterised for protein content, TFAA, IVPD and sensory

| Table 7. Content and digestibility (IVPD) of proteins of industrial pilot plant sliced and puccia type bread. |
| Sample | GG | AG | CM | Total proteins | %±SD | IVPD |
|-----------------|---------|---------|---------|----------------|---------|---------|
| Sliced bread CTRL | 6.68 ± 0.58a | 0.68 ± 0.13a | 6.53 ± 1.18a | 13.88 ± 1.63a | 56.02 ± 1.70a | 0.42a |
| Sliced bread Bio21B | 6.80 ± 0.74a | 0.69 ± 0.01a | 6.04 ± 1.04a | 13.53 ± 1.80a | 55.32 ± 2.22a | 9.84a |
| Puccia CTRL | 6.47 ± 0.28a | 0.26 ± 0.07b | 8.12 ± 1.76a | 14.83 ± 1.97a | 59.49 ± 2.02a | 1.75b |
| Puccia Bio21B | 6.68 ± 0.58a | 0.68 ± 0.13a | 6.53 ± 1.18a | 13.88 ± 1.63a | 56.02 ± 1.70a | 0.42a |

Mean ± SD in the same column followed by different letters differs significantly \( (p < .05) \) by one-way ANOVA followed by the Fisher’s test.
profile. Salt reduced (50%) products, containing the Bio21B and CF, were compared to the reference commercial products prepared following the industrial recipe including salt (1.36% and 1.24% for sliced bread and puccia bread, respectively, respect to the dough weight) and a L. plantarum LS produced by the company.

Total protein extracts from sliced bread and puccia type bread samples (CTRL and CF_Bio21B) showed similar patterns when analysed by Loac electrophoresis (Figure S3) and their profiles were comparable with the laboratory scale products: about 20 protein peaks in the 14–220 kDa range were visualised on Protein230 LabChip kit. In particular, in sliced bread containing Bio21B and CF a twofold increase of protein peak areas in the 20–39 kDa range, was observed. In puccia type bread CF_Bio21B, a sixfold increase of low MW proteins (zones 1 and 2) in total protein extracts was found.

When proteins were extracted on the base of their solubility, the total protein content did not differ among samples (p > .05) while a significant improvement of protein digestibility was observed for CF_Bio21B sliced bread respect to the commercial reference product (Table 7).

A nutritional improvement in the two bakery products was achieved thanks to the increased amount of TFAA: indeed, in both sliced and puccia type breads, an increase of the concentration due to the addition of the fermentation product and the CF, was observed (Table 5). In particular, compared to controls TFAA increased up (p < .05) in sliced and puccia type breads, respectively. The pH values of the products produced at industrial pilot plant were slightly higher than the experimental breads (Table 4). In detail, pH values were 5.23 ± 0.03 and 5.39 ± 0.08 (sliced bread), 5.54 ± 0.01 and 5.76 ± 0.01 (puccia type bread), for CTRL and CF_Bio21B products, respectively. As expected, the TTA values were lower respect to laboratory breads (3.05 ± 0.05 and 3.20 ± 0.2, sliced bread; 3.10 ± 0.10 and 2.62 ± 0.15, puccia type bread). In fact, the industrial products contained a lower content of organic acids, mainly lactic acid, respect to the laboratory bread samples while the presence of PLA and OH-PLA was ascertained only in Bio21B samples (data not shown). As previously reported, the two metabolites produced in particular by the strain L. plantarum ITM21B, were supposed to balance the negative perception of salt reduction together with other molecules. Differences of the physico-chemical parameters between laboratory and pilot plant products may be related to the lower amount of the fermentation product Bio21B used in the company recipes (15% for sliced bread and 20% for puccia type bread, and 30% in laboratory products, based on dough weight).

In addition to the nutritional features, the most relevant aspect to consider for the commercial success of a new food formula is the sensory acceptance by consumers. In the current study, the sensory analysis indicated that the reduced salt products received a good acceptance by the panellists comparable to that of the reference products (Table 8). In particular, the Bio21B products resulted similar (p > .05) to the controls for almost all properties. Panelists did not perceive the reduction of salt, as indicated by the taste scores. In the case of puccia type bread, the appearance of the two products was considered different (p < .05) since breads containing the Bio21B showed a lighter colour respect to the CTRL, even though this aspect did not compromise the global acceptability. Regarding the sliced bread, samples resulted to be fragrant and crisp externally and soft inside, with uniform bubbles. Regarding the olfactory and gustatory characteristics, the samples recorded excellent judgments: the smell was typical of a fresh product and delicate and pleasant taste, regard to the amount of salt, was perceived during chewing.

**Table 8. Sensory analysis of bread manufactured at industrial pilot plant.**

| Sample            | Colour   | Appearance | Crust firmness | Crumb firmness | Large bubbles | Aroma | Taste | Overall quality |
|-------------------|----------|------------|----------------|----------------|---------------|-------|-------|-----------------|
| Sliced bread CTRL | 7.84 ± 0.25<sub>a</sub> | 7.77 ± 0.19<sub>a</sub> | 7.75 ± 0.28<sub>a</sub> | 7.66 ± 0.25<sub>a</sub> | 7.39 ± 0.39<sub>a</sub> | 7.30 ± 0.50<sub>a</sub> | 7.82 ± 0.36<sub>a</sub> | 7.58 ± 0.33<sub>a</sub> |
| Sliced bread Bio21B | 7.78 ± 0.32<sub>b</sub> | 7.98 ± 0.02<sub>b</sub> | 7.81 ± 0.25<sub>b</sub> | 7.57 ± 0.31<sub>b</sub> | 7.88 ± 0.23<sub>b</sub> | 7.80 ± 0.33<sub>b</sub> | 7.78 ± 0.39<sub>b</sub> | 7.70 ± 0.27<sub>b</sub> |
| Puccia bread CTRL | 7.73 ± 0.47<sub>a</sub> | 8.10 ± 0.08<sub>a</sub> | 7.59 ± 0.29<sub>a</sub> | 7.56 ± 0.31<sub>a</sub> | 7.40 ± 0.43<sub>a</sub> | 7.63 ± 0.29<sub>a</sub> | 7.76 ± 0.33<sub>a</sub> | 7.78 ± 0.17<sub>a</sub> |
| Puccia Bio21B     | 7.09 ± 0.31<sub>b</sub> | 7.33 ± 0.13<sub>b</sub> | 7.69 ± 0.22<sub>b</sub> | 7.56 ± 0.52<sub>b</sub> | 7.69 ± 0.33<sub>b</sub> | 7.33 ± 0.14<sub>b</sub> | 7.50 ± 0.47<sub>b</sub> | 7.49 ± 0.15<sub>b</sub> |

Mean ± SD in the same column for each bakery product type followed by different letters differs significantly (p < .05) by one-way ANOVA followed by the Fisher’s test.

Conclusions

This study highlights that the combination of L. plantarum ITM21B fermentation product and CF can be considered as valuable approach to produce salt reduced bakery products with improved nutritional profile. Indeed, increased amino acid and protein content and digestibility were achieved and breads with peculiar sensory characteristics were manufactured at laboratory and industrial levels. In particular, the laboratory bread prepared with Bio21B and CF...
showed higher amounts of TF AA and acidic metabolites, mainly PLA and OH-PLA, with respect to the reference bread. The addition of CF to the bread formula significantly increased the protein content, mainly acidic GG and AG fractions. Interestingly, the acidification operated by the fermentation product allowed the complete digestion of AG fractions, which include the storage proteins of the legume, generally poorly hydrolysed by digestive enzymes. Therefore, the protocol developed in this study represents a strategy to overcome the downsides of the legume flour application in bakery products related to the limited digestibility of proteins due to their structure and the presence of antinutritional compounds. The suitability of the protocol in improving the nutritional features of bakery products was validated mainly for the sliced bread, at pilot plant level: an increase of TF AA and protein digestibility was observed and the salt reduced product was perceived as comparable to the reference product during the sensory test. In addition, the economic sustainability of the process valorises the results of the study. Actually, the fermentation product requires only an overnight incubation in fermentation systems generally available in SMEs producing yeast-leavened products, as the company involved in this study. Moreover, its use as an ingredient added to the dough in the mixing step, does not modify the usual industrial process but allows to obtain the same advantages offered by the traditional sourdough fermentation with shorter leavening time and easier management and reproducibility.

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