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Antifungal peptides from faba bean flour fermented by *Levilactobacillus brevis* AM7 improve the shelf-life of composite faba-wheat bread

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ABSTRACT

Faba bean is an important crop from an ecological, nutritional, and economical perspective, hence its incorporation in plant-based products can play a key role in the sustainability of the whole food system. In this study, the antifungal properties of faba bean flour were investigated employing three bioprocessing treatments including hydrolysis with a commercial proteolytic enzyme preparation, and fermentation with selected or commercial mixed sourdough starters. Faba bean flour fermented by *Levilactobacillus brevis* AM7 showed the broadest inhibitory spectrum against the fungal species tested and antifungal activity ranging from 25 to 73 %. Bioactive proteins and peptides were found to be responsible for the antifungal activity. Polypeptides sequences corresponding to defensin-like and non-specific lipid-transfer proteins and seven antifungal peptides having 11–22 amino acid residues and mass from 1240.7 to 2624.2 Da, were identified via Liquid Chromatography-Electrospray Ionisation-Mass Spectra/Mass Spectra (nano-LC-ESI-MS/MS). To assess the antifungal effect in food processing conditions, fermented faba bean was used as ingredient in breadmaking at a substitution level of 30 % of the dough weight. Wheat composite-bread containing faba bean sourdough, which could be claimed as "source of proteins", according to the current EC Regulation, delayed the molds contamination, lasting up to 10 days before mold appearance, and behaving similarly to wheat bread containing calcium propionate.

1. Introduction

The use of nutrients-rich plant ingredients like faba bean protein preparations for the fortification of foods commonly included in the diet is among the proposed solutions for enabling the sustainability of the food systems (Augustin and Cole, 2022; Boukid and Castellari, 2022). Faba bean, belonging to the *Fabaceae* family, is cultivated all over the world and it is considered a staple dietary food in North Africa and Middle East. Its agronomic importance resides in large adaptability and productivity, but also in some ecosystem services, such as the nitrogen-fixation ability, which can significantly reduce synthetic fertilizers application, and the ameliorative effects on phosphorus solubilization and on the microbial activity in the soil, thus improving its fertility (Sharan et al., 2021). Faba bean, like other legumes, is a major source of proteins, fibers, vitamins (ascorbic acid, niacin, folate), minerals, and phytochemicals with antioxidant and anticarcinogenic properties; besides, compared to other legumes, it has a higher protein-carbohydrate ratio (Mudryj et al., 2014; Sharan et al., 2021) thus supporting its potential application as ingredient. For the reasons above, faba bean importance has significantly grown in the EU in these last years, where it is considered an important domestic protein source (Karkanis et al., 2018; Lybæk and Hauggaard-Nielsen, 2019). The interest of the research

Abbreviations: AM7, faba bean fermented with *Levilactobacillus brevis* AM7; cpWB, wheat bread added of calcium propionate; FAN, free amino nitrogen; FB, faba bean dough; fFWB, wheat bread made with faba bean slurry fermented with *Lv. brevis* AM7; FP, faba bean fermented with Florapan LA4; FWB, wheat bread made with faba bean flour; HPAEC-PAD, high performance anion exchange chromatography with pulse amperometric detection; HPP, faba bean treated with Veron HPP; LAB, lactic acid bacteria; MRS, de Man, Rogosa and Sharpe; Nano-LC-ESI-MS/MS, nano-Liquid Chromatography-Electrospray Ionisation-Mass Spectra/Mass Spectra; nsLTPs, non-specific lipid-transfer proteins; PDA, potato dextrose agar; RP-FPLC, reversed-phase fast performance liquid chromatography; TFAA, total free amino acids; TTA, total titratable acidity; WB, wheat bread.

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in the use of faba bean protein preparations (flour, protein concentrate and isolate) as food ingredients has had an exponential rise over the past few years. Indeed, roughly 700 documents, with the "faba bean" and "food" in either the title, abstract or keywords, have been published since the 80′, half of which in the past 5 years (Scopus, last access on January 30th, 2023). Nevertheless, the use of faba bean ingredients has its challenges. While its potential to increase protein content or allow amino acid complementation meeting the proper nutritional requirements are the main indisputable advantages of its use in cereal-based foods, the same cannot be said about its impact on other nutritional, rheological or sensory aspects. Fermentation inspired to sourdough biotechnology can positively affect baking performance and nutritional attributes of faba bean flour by reducing the content of anti-nutritional compounds, as well as producing or releasing a large range of compounds with biological activities and potentially beneficial to its techno-functional properties (Hoehnel et al., 2020; Sharan et al., 2021; Verni et al., 2019).

Among these, antibacterial and antifungal compounds symbolize a relevant feature in view of faba bean use as food ingredient, especially in bakery products. Indeed, microbial deterioration due to mold contamination is of serious concern and one of the leading causes of bread wastage, determining large economic losses for both the bakery industry and consumers. Fungi are also responsible for the production of mycotoxins and off-flavors, which might be generated even before fungal growth is visible (Hoehnel et al., 2020; Melikoglu and Webb, 2013). Thus, spoiled breads represent a hazardous risk to consumer's health, but chemical preservatives should not be the only solution. Antifungal proteins from legumes have been studied in previous works, especially in the form of water-extracts (for review see Mani-López et al., 2021). However, although they are effective, there is currently no information on their use in food industry. This might be due to practical reasons such as low yields, high costs of extraction, and poor safety regulatory status. Therefore, further research on their applicability is needed.

In this study, we investigated the antifungal properties of faba bean flour, employing three bioprocessing treatments. Faba bean was *i*) treated with a proteolytic enzyme preparation already studied for same scope (Rizzello et al., 2015); *ii*) fermented with a commercial mixed sourdough starter with anti-molding and anti-staling properties; and *iii*) fermented with *Levilactobacillus brevis* AM7, a strain which previously showed the release of antifungal peptides from wheat sourdough and bread hydrolysate (Coda et al., 2008, 2011; Nionelli et al., 2020). As consequence of the protein hydrolysis, induced by exogenous enzymes or by means of lactic acid bacteria (LAB) proteolytic system, bioactive peptides with antifungal activity might be released as previously observed. Alternatively, organic acids or other compounds deriving from the metabolism of LAB, known for their biopreservation potential (Sadiq et al., 2019) might be synthesized. The compounds responsible for the antifungal activity were selectively purified and characterized through liquid chromatography coupled with mass spectrometry and fermented faba bean was then used as ingredient in a composite bread to improve its shelf-life.

2. Materials and methods

2.1. Raw materials, enzymes, and microorganisms

Commercial faba bean (*Vicia faba* var. minor cv Kontu) flour, certified as organic and having the following composition: carbohydrates 60 %, proteins 30 %, lipids 3 %, and ash 3 %, was used in this study.

Veron HPP (AB enzymes) a commercial enzymatic preparation (1116 UHb/g, where 1 UHb/g corresponds to the release of 1 μmol/min of tyrosine from hemoglobin at 37 °C and pH 5.0) was used for faba bean flour treatment. Florapan LA4 (Lallemand Gmbh), a commercial mixed starter including selected strains of *Levilactobacillus brevis*, *Lactiplantibacillus plantarum* and *Saccharomyces cerevisiae* and *Lv. brevis* AM7, (formerly *Lactobacillus brevis*) belonging to the Culture Collection of the Department of Soil, Plant and Food Science (University of Bari, Bari, Italy) were used as starter for faba bean flour fermentation.

The strain AM7 was cultivated for 24 h at 30 °C in MRS (de Man, Rogosa and Sharpe) broth (Oxoid Laboratories, Hampshire, UK). For the inoculum, cells were harvested by centrifugation (10,000 ×*g* for 10 min), washed twice in phosphate-buffered saline, pH 7.0, and re-suspended in tap water before the inoculum in the faba bean dough.

Fungi used in antifungal assays as indicators were the following: *Penicillium commune* P3, *Penicillium paneum* CBS 101032, *Penicillium roqueforti* P1 and DPPMAF1, *Penicillium crustosum* P2, *Penicillium allocoremium* CBS 109582, *Eurotium herbarioum* CBS 117336, and *Aspergillus niger* P4. Fungi were cultivated in potato dextrose agar (PDA; Oxoid) for 4 days at 25 °C.

2.2. Bioprocessing treatments and chemical analyses

Faba bean flour was mixed with water (ratio 1:2, w/v) and subjected to the following bioprocesses: *i*) treatment with the proteolytic enzyme preparation Veron HPP (HPP); *ii*) fermentation with Florapan LA4 (FP); and *iii*) fermentation with *Lv. brevis* AM7 (AM7). In details: *i*) the enzyme Veron HPP was suspended in water before addition, at a concentration of 29.6 mg/100 g flour, and the mixture was incubated for 5 h at 50 °C; *ii*) the starter preparation Florapan LA4 (in dehydrated form) was inoculated at 0.1 % (w/w), according to manufacturer recommendations, whereas *iii*) *Lv. brevis* AM7 was inoculated at a final cell density of ca. 10^7 cfu/g. Both fermentations were carried out for 8 h at 30 °C. Afterwards, the supernatant, corresponding to the water soluble extract (WSE) was recovered by centrifugation at 12,000 ×*g* for 15 min at 4 °C. Enzymes inactivation was performed for 5–7 min at 90 °C and the extract was centrifuged again in the same conditions. The WSEs were then utilized for the characterization of the protein content, using Bio-Rad kit for protein assay based on the Bradford dye-binding method (Bradford, 1976), and the peptide content which was determined by the *o*-phtaldialdehyde method (Church et al., 1983). Free amino nitrogen (FAN) content was determined using the European Brewery Convention ninhydrin method 4.10 (EBC, 1998) and expressed as mg/ml of supernatant. The acidity of the samples was determined at the end of bioprocessing by measuring pH and Total Titratable Acidity (TTA). Ten grams of sample were homogenized with 90 ml of distilled water in a Stomacher for 2 min. Then, 25 ml of the mixture were used for pH and TTA determination with an automatic titrator, EasyPlus™ (Mettler Toledo International Inc., Switzerland). TTA was expressed as ml NaOH 0.1 M necessary to bring 10 g of sample to pH 8.5.

2.3. Antifungal assays

Antifungal activity was determined by hyphal radial growth rate assay as described by Quiroga et al. (2001) and Rizzello et al. (2011) with some modifications. The WSE to test was added to Petri dish containing PDA medium at 25 % of the total volume. Before antifungal assays, conidia suspensions were prepared from fungal cultures after 7 days of growth at 25 °C on PDA plates. Conidia were harvested in sterile saline solution containing 0.05 % (vol/vol) Tween 80. The suspension (25 μl) was then used to inoculate the Petri dish. The mycelia colony diameter was measured after 7 days of incubation and the inhibition percentage was calculated after 7 days. The percentage of growth inhibition was calculated from mean values as follows: [(mycelial growth under control conditions − mycelial growth in the presence of WSE) / mycelial growth under control conditions] * 100. The control conditions were represented by the WSE from non-fermented faba bean flour.

For the fractionated extracts, the antifungal activity was determined against *Penicillium roqueforti* DPPMAF1, with agar diffusion assays, on PDA agar, according to Coda et al., 2008. Sterile blank paper disks (0.5-cm diameter) were placed at ca. 0.5 cm away from the rim of the grown mycelial colony. Ten microliters of the WSE to be tested were added to the disks. Plates were incubated at 25 °C for 72 h until the mycelial

growth overlaid the paper disk containing the control (without WSE addition).

2.4. Microbiological and chemical characterization of faba bean fermented by Lv. brevis AM7

Microbial growth was monitored on native faba bean flour and on faba bean dough before and after 8 h of fermentation. For microbial enumeration, 10 g of sample were homogenized with 90 ml of sterile 0.9 % (w/v) sodium chloride solution using a stomacher (Colworth, UK), and serially diluted suspensions were plated accordingly. LAB were cultivated in MRS Agar (Negoen) in micro aerophilic conditions at 30 °C for 48 h; total aerobic mesophilic bacteria in Plate Count Agar (Neogen) 30 °C for 72 h, and *Enterobacteriaceae* in Violet Red Bile Glucose Agar (Neogen), at 37 °C for 24 h. *Bacillus cereus* was cultivated in Polymyxin Pyruvate Egg-Yolk Mannitol Bromothymol Blue Agar (Neogen), at 30 °C for 24 h, yeasts and molds were cultivated in Yeast Extract Peptone Dextrose Agar (Neogen) and Malt Extract Agar (Neogen) both supplemented with 0.01 % chloramphenicol (Oxoid, UK) at 25 °C for 72 h and 25 °C for 120 h respectively. The pH of the fermented dough was measured using an online pH meter (Knick, Germany).

Organic acids were analyzed from water/salt-soluble extracts prepared according to Weiss et al. (1993) using 50 mM Tris–HCl (pH 8.8). Lactic and acetic acids were respectively analyzed with K-DLATE and K-ACET kits (Megazyme International Ireland Limited, Bray, Ireland).

Galactose, glucose, sucrose, fructose, melibiose, raffinose, stachyose and verbascose were quantified from fermented faba bean flour doughs using high performance anion exchange chromatography with pulse amperometric detection (HPAEC-PAD) system. Sugars were separated in a CarboPac PA1 column (250 × 4 mm i.d., Dionex, Sunnyvale, CA) and were detected by a Waters 2465 pulsed amperometric detector (Waters, USA). Gradient elution method was applied to separate different carbohydrates using mobile phase A (200 mM NaOH) and mobile phase B (MQ water) at flow rate 1 ml/min and at 10 °C column temperature. The applied gradient run was 60 min starting with 1 % of mobile phase A and 99 % of B for 4 min, 30 % A and 70 % B until 30 min, 100 % A until 38 min, remain at 100 % A until 48 min, 1 % A and 99 % B until 50 min and stabilize for the final 10 min at 1 % A and 99 % B. Galactose, glucose, sucrose and fructose (MERCK, Germany), melibiose, raffinose and verbascose (Megazyme, Ireland), and stachyose (Sigma, USA) were used as standards and 2-deoxy-D-galactose (Sigma-Aldrich, UK) was used as internal standard. For sample preparation, 200 mg of freeze-dried sourdough was homogenized in 5 ml milli-Q water in a 15 ml falcon tube. The tube was then kept in a boiling water bath for 5 min, and then centrifuged at full speed (at +4 °C) for 10 min. The supernatant was filtered through Amicon® Ultra Centrifugal Filters 0.5 ml 10 K (Merck Millipore Ltd., Ireland). Finally, the filtrate was transferred into the vials, internal standard was added and used for the quantification.

The peptides profile of raw and fermented faba bean extracts, pretreated with trifluoroacetic acid (final concentration 0.05 %) aiming at protein precipitation, was first characterized by reversed-phase fast performance liquid chromatography (RP-FPLC), using a Resource RPC column (C18, 3 ml volume, 6.4 × 100 mm, 15 μm particle size) and an ÄKTA FPLC, with the UV detector operating at 214 nm (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) as described by Verni et al., 2021. Free amino acids of the extracts were also determined, after precipitation with 5 % cold sulfosalicylic acid, by using a Biochrom 30^{+} series Automatic Amino Acid Analyzer (Biochrom Ltd., Cambridge Science Park, United Kingdom), equipped with a Li-cation-exchange column (4.6 × 200 mm, Ultropack Cation Exchange Resin) (Verni et al., 2021).

2.5. Proteolysis and heat stability of antifungal compounds

WSE were treated with trypsin (EC 3.4.21.4; Sigma Aldrich Co.) as described by Atanassova et al. (2003). Briefly, a trypsin solution 0.25 M in Tris HCl was mixed in a 1:1 ratio with fermented faba bean extract. After 5 h of incubation at 25 °C, the reaction was stopped by boiling the mixture for 3 min and the residual activity was determined by hyphal radial growth using *P. roqueforti* DPPMAF1 as indicator. Similarly, heat stability was assessed after heating for 5 min at 100 °C.

2.6. Purification and identification of antifungal peptides

The WSE of faba bean fermented with *Lv. brevis* AM7, which resulted the most effective, was first fractionated by ultrafiltration (Amicon Ultra-15 centrifugal filter units; Millipore) by using three different membrane sizes with 50, 30, and 10 kDa cutoffs. The recovered fractions were then used for antifungal assessment with agar diffusion assay as previously described, aiming at identifying the molecular mass of the antifungal compounds. Then, an aliquot of the 30 kDa partially purified fraction was further fractionated (35 fractions) by RP-FPLC as described above. Solvents were removed from collected fractions by freeze-drying and the fractions were redissolved in sterile water and tested for the inhibitory activity based on hyphal radial growth rate of *P. roqueforti* DPPMAF1.

The three purified fractions displaying the highest antifungal activity on the indicator mold were subjected to digestion with trypsin (17,511 U/mg) (Promega, Mannheim, Germany) and analyzed through nano-Liquid Chromatography-Electrospray Ionisation-Mass Spectra/Mass Spectra (nano-LC-ESI-MS/MS), aiming at identifying peptides responsible for the activity. Aiming at identifying active low molecular mass peptides, aliquots obtained by ultrafiltration (10 kDa cut-off) from the same three active fractions were directly analyzed through nano-LC-ESI-MS/MS, without performing the tryptic digestion.

A Finnigan LCQ Deca XP Max ion trap mass spectrometer (ThermoElectron, Waltham, MA, USA) was used through the nano-ESI interface. According to the manufacturer's instrument settings for nano-LC-ESI-MSMS analyses, MS spectra were automatically recorded by Xcalibur software (ThermoElectron), in positive ion mode. MS/MS spectra were processed using the BioWorks 3.2 software (ThermoElectron), which generated peak lists suitable for database searches. Proteins and peptides were identified using the MS/MS ion search of the Mascot search engine (Matrix Science, London, England) and NCBInr protein database (National Centre for Biotechnology Information, Bethesda, MD, USA).

For the identification of proteins and peptides the following parameters were considered: enzyme: “none” (<10 kDa subfractions) or “trypsin” (>10 kDa subfractions); instrument type: “ESI-trap”; peptide mass tolerance: ±0.1 % and fragment mass tolerance: ± 0.5 Da. Results from peptide identification were subjected to a manual evaluation, as described by Chen et al. (2005), and the validated peptide sequences explained all the major peaks in the MS/MS spectrum.

The APD3 (Antimicrobial Peptide Database) tools (https://aps.unmc.edu) were used for the study of the structure of the identified peptide sequences (Wang et al., 2016).

2.7. Bread making, characterization and mold-free shelf-life

The effects of fermented faba bean on mold-free shelf life of bread was determined after baking trials. Wheat flour used had the following characteristics: moisture content 15 %; proteins, 13 %; lipids, 2 %; ash 0.64–0.71 %; and total carbohydrates, 69 %. Four types of breads were produced, in the bakery facility of the University of Helsinki, including a wheat control bread (WB), a positive control wheat bread added of 0.1 % calcium propionate (cpWB), and breads containing faba bean raw (FWB) or fermented with *Lv. brevis* AM7 (fFWB) at 30 % substitution level on dough weight. The substitution level (17 %) of faba bean flour was determined based on calculation (nutritional composition) to obtain that 12 % of the total energy value would be provided by the protein, according to the regulation of the European Parliament and Council (20/12/2006 for calculations, see Table 1S). Formulas for bread making are described in Table 1. The optimal water content for the breads was based on wheat flour as determined with a Brabender Farinograph (Brabender

Table 1
Test bread recipes presented in weights and percentages of flour and dough weight (f.w. and d.w., respectively).

	WB			cpWB			FWB			fFWB		
	% f.w.	d.w.	Weight (g)	% f.w.	d.w.	Weight (g)	% f.w.	d.w.	Weight (g)	% f.w.	d.w.	Weight (g)
Wheat flour	100	57.31	920	100	57.21	920	83	47.34	760	83	47.34	760
Water	60	34.38	552	60	34.38	552	60	34.38	552	25	14.45	232
Faba sourdough	–	–	–	–	–	–	–	–	–	52	30	480
Faba flour	–	–	–	–	–	–	17	9.97	160	–	–	–
Salt	1.5	0.86	13.8	1.5	0.86	13.8	1.5	0.86	13.8	1.5	0.86	13.8
Sugar	2	1.15	18.4	2	1.15	18.4	2	1.15	18.4	2	1.15	18.4
Yeast	5	2.87	46	5	2.87	46	5	2.87	46	5	2.87	46
Fat	6	3.44	55.2	6	3.44	55.2	6	3.44	55.2	6	3.44	55.2
Calcium propionate	–	–	–	0.17	0.1	1.56	–	–	–	–	–	–
Total	174.5	100	1605.4	174.67	100	1606.96	174.5	100	1605.4	174.5	100	1605.4

WB, wheat bread; cpWB, wheat bread added of calcium propionate; FWB, wheat bread made with faba bean flour; fFWB, wheat bread made with faba bean slurry fermented with *Lv. Brevis* AM7 at 30 °C for 8 h.

GmbH & Co. KG, Germany), according to AACC method 54–21 (AACC 2000). Breads were prepared by mixing all the ingredients in a DIOSNA mixer bowl (Dierks & Söhne GmbH, Germany) for 3 min at low speed and 4 min at fast speed. After 15 min proofing in a fermentation cabinet (Lillnord, Odder, Denmark) at 35 °C and relative humidity (RH) of 75 %, the dough was divided into pieces of 250 g. The doughs were molded manually and rested in pans for 45 min (35 °C, RH 75 %). The breads were baked in a rotating rack oven (Sveba Dahlen, Fristad, Sweden) at 220 °C for 15 min with 15 s steaming at the beginning. After baking, the loaves were cooled for 1 h at room temperature before further analysis. Texture Profile Analysis (TPA) of breads was done on day 1 with a texture analyzer (TA, TA-XT2i, Stable Micro Systems Ltd., UK) using a 25-mm diameter aluminum probe with a 5 kg load cell as previously described by Wang et al. (2018). Samples for testing were prepared by cutting into 50 mm × 50 mm × 25 mm slices and the edges were removed.

For the observations of the mold appearance on the breads, bread loaves were cooled for 1 h and then loaves (two per type) and slices (three per each loaf) were packed in polyethylene bags and kept at room temperature. Mold growth was estimated by visual observation for a period of 15 days after baking and approximately quantified as % of spoiled surface on the total surface (whole loaf or slice) as described earlier (Nionelli et al., 2020).

2.8. 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.).

3. Results

3.1. Effect of bioprocessing treatments on faba bean antifungal properties

Faba bean flour was subjected to enzymatic treatment with proteases, fermented with a selected lactic acid bacteria strain previously known for its antifungal potential or with a commercial preparation of yeasts and lactic acid bacteria. Before treatment, pH and TTA of faba bean were 6.65 ± 0.07 and 5.09 ± 0.1 ml, respectively and bioprocessing did not cause significant changes except for TTA of HPP, which increased of 3.6 ml and pH of AM7 which was 5.82 ± 0.09 after 8 h of incubation.

The analysis of proteolysis showed that, to a different extent, all treatments determined a modification of peptides and free amino nitrogen profiles, although the total protein content resulted similar (Fig. 1). The enzymatic treatment led to the highest increases, up to 3 times compared to untreated faba bean. Fermentation with Florapan did

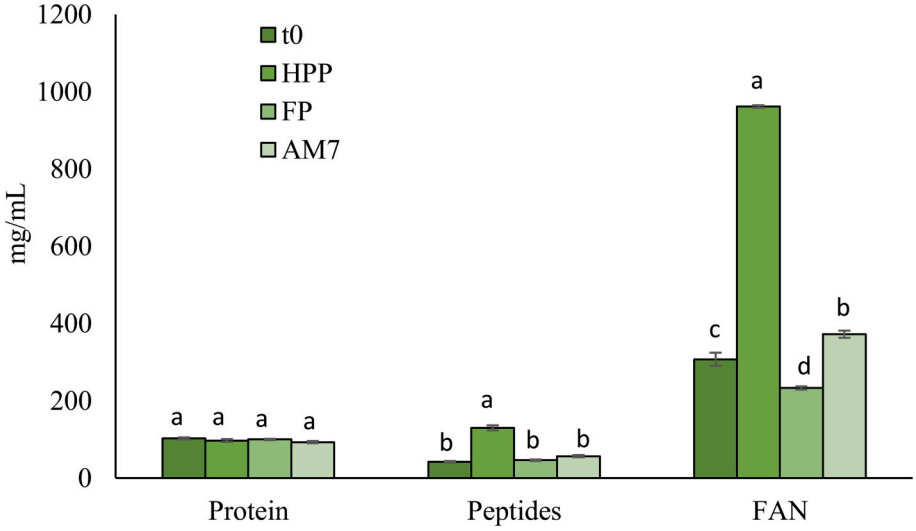

Fig. 1. Concentration of proteins, peptides and free amino nitrogen (FAN, mg/ml of water soluble extract) of faba bean before treatments (t0), enzymatically treated with commercial protease Veron HPP (HPP), and fermented with Florapan LA4 (FP), or *Lv. brevis* AM7 (AM7). [a–d]Values obtained in the same analysis (peptides/FAN) with different superscript letters differ significantly ($P < 0.05$).

not vary peptides concentration and decreased free amino nitrogen of roughly 32 %, whereas in faba bean fermented with *Lv. brevis* AM7 slight but significant ($P < 0.05$) increases of peptides and FAN (34 and 21 %, respectively) were observed.

The antifungal effect of bioprocessed faba bean extract was determined on several fungi commonly contaminating bakery products (Fig. 2). All of them, including the WSE from untreated faba bean, appeared to be particularly efficient at inhibiting several mold species, especially *P. commune*, *P. paneum* and *P. roqueforti*, exceeding 60 % of growth inhibition rate. To a lesser extent, *A. niger* was also inhibited by all extract. The extract obtained from faba bean fermented with *Lv. brevis* AM7 was characterized by a slightly lower activity towards *P. paneum*, with a 47 % inhibition rate, but on the other hand, it was the only extract significantly inhibiting all the tested mold species. Indeed, HPP and FP did not show antifungal activity against *P. roqueforti* P1, *P. crustosum* and *E. herbariorum*. Hence, fermentation with *Lv. brevis* AM7 was selected as the most effective treatment for faba bean flour, to be further characterized and used as bread ingredient.

3.2. Characterization of faba bean fermented with Lv. brevis AM7: organic acids, sugar and proteolysis

In the native flour, the total presumptive LAB cell density was 3.3 ± 0.05 Log cfu/g, and the total aerobic mesophilic bacteria density was 5.3 ± 0.09 Log cfu/g. During controlled fermentation with *Lv. brevis* AM7, the cell density of presumptive LAB increased from the initial value 7.1 ± 0.02 to 8.5 ± 0.03 Log cfu/g after 8 h of fermentation, while total aerobic mesophilic bacteria increased and from 7.1 ± 0.07 to 8.6 ± 0.10 Log cfu/g. *Bacillus cereus*, *Enterobacteriaceae*, yeasts, and molds were not detected in either the native faba bean flour or the fermented faba bean flour doughs.

Before fermentation, no organic acids were found. According to the acidification observed, a relevant amount of lactic and small amount of acetic acid were synthesized during fermentation (34.4 ± 1.2 mmol/kg and 0.8 ± 0.1 mmol/kg, respectively).

Galactose, glucose, sucrose, fructose, melibiose, raffinose, stachyose and verbascose were detected at T0. A significant increase in the amount of galactose (309.45 ± 27.48 to 522.56 ± 23.46 mg/100 g of dough dry weight) and melibiose (from 25.61 ± 7.01 to 108.37 ± 11.93 mg/100 g) was observed after 8 h of fermentation, while there was a significant decrease in the amount of glucose (501.41 ± 31.04 to 195.88 ± 16.18 mg/100 g of dough), raffinose (from 63.23 ± 1.1 to 32.09 ± 5.9 mg/100 g) and verbascose (from 40.03 ± 1.67 to undetectable levels). Sucrose, fructose, and stachyose remained at a similar level before (46.06 ± 9.34, 495.31 ± 61.27, and 23.28 ± 0.69 mg/100 g of dough, respectively) and after 8 h of fermentation (47.1 ± 6.91, 435.06 ± 16.47, and 22.02 ± 0.61 mg/100 g of dough, respectively).

As shown by the spectrophotometric assays, fermentation with *Lv. brevis* AM7 led to a moderate proteolysis involving the release of peptides and free amino acids. Indeed, although the number of peaks detected by FPLC was the same before and after incubation, total peak area was >22 % higher after fermentation (Table 2S). The distribution of peptides within the chromatogram also changed with fermentation, showing a decrease of less hydrophilic peptides (46–100 % of acetonitrile) and an increase of more hydrophilic peptides. Before fermentation the most abundant amino acid in faba bean was Pro (200 mg/kg) followed by Ala and Cys (ca. 100 mg/kg). Total free amino acids content more than doubled during the incubation reaching 2 g/kg, with increases up to 18-folds except for Ser and Ala which rose of roughly 24 % (Fig. 3). With fermentation, the overall FAA profile changed and although Pro, was still the most abundant amino acid, Glu, Ala, Cys, and Arg followed ranging from 150 to 200 mg/kg.

3.3. Purification and identification of antifungal peptides

Before purification, to verify the nature of the compounds involved in the antifungal activity, the WSE of fermented faba bean was subjected to trypsin digestion. The inhibitory activity of the supernatant was almost completely lost after the enzymatic treatment (−80 %) supporting the hypothesis of the protein-nature of the antifungal compounds, whole whereas after the heat treatment roughly 50 % of the activity was retained.

The extract was then fractionated by ultrafiltration and tested against *P. roqueforti* DPPMAF1, showing that the antifungal activity was

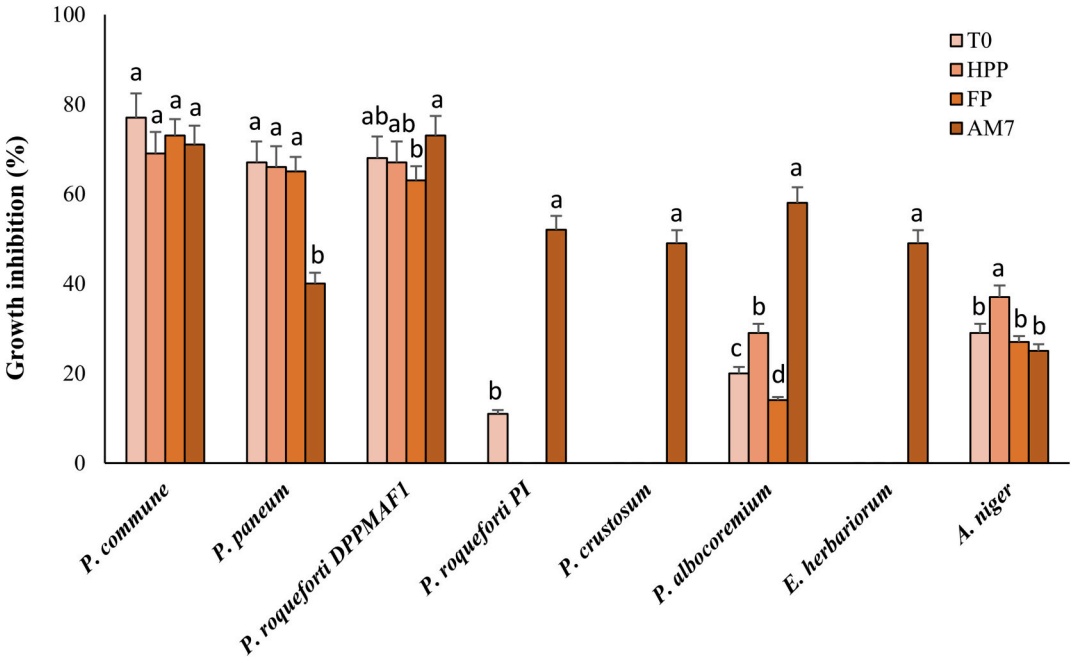

Fig. 2. Mold growth inhibition, after 7 days of incubation, of water-soluble extracts of faba bean without treatment (t0), enzymatically treated with commercial protease Veron HPP (HPP), and fermented with Florapan LA4 (FP), or *Lv. brevis* AM7 (AM7). [a–d]Values obtained for the same fungal strain with different superscript letters differ significantly ($P < 0.05$).

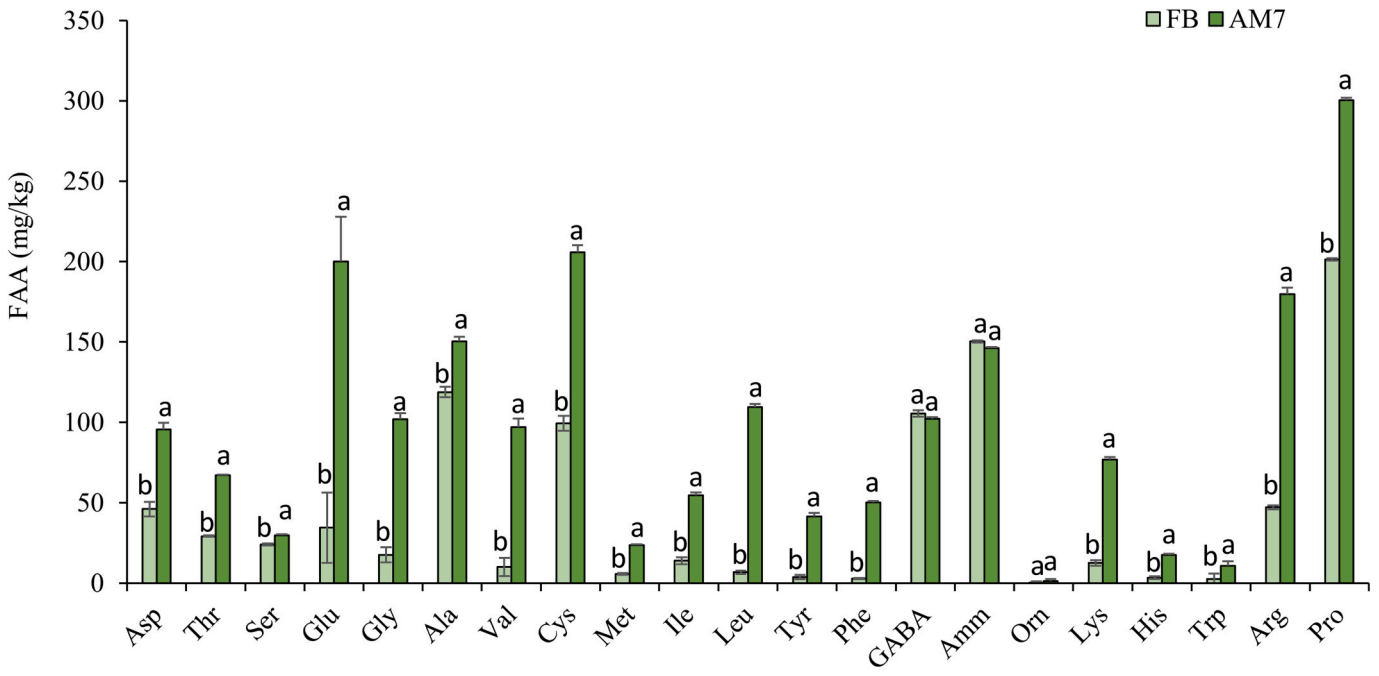

Fig. 3. Free amino acids (FAA) composition of faba bean flour before (FB) and after fermentation with *Lv. brevis* AM7 (AM7). [a–b]Values obtained for the same FAA with different superscript letters differ significantly ($P < 0.05$).

mostly retained in the fractions under 30 and 10 kDa, indicating that both proteins between 30 and 10 kDa and peptides (<10 kDa) are involved in the antifungal effect. The partially purified and active fraction obtained by ultrafiltration at the 30 KDa cut-off was further fractionated by reversed-phase liquid chromatography in thirty-five fractions, three of which (16–18) exhibited inhibitory activity on *P. roqueforti* DPPMAF1, used as indicator (Fig. 1S). The defensin-like protein ACI02059.1 (8792 Da) and a non-specific lipid-transfer protein NLTP4_LENCU (11,588 Da) were identified in the active fractions (Table 3S). Moreover, 7 peptides, having 11–22 amino acid residues, mass from 1240.7 to 2624.2 Da were identified in the <10 kDa permeate of the fraction 18 (Table 2). Except for the peptide IINPEGQQEEEEQEEEEKQR, all the others were characterized by hydrophobic ratio between 23 and 55 %. Peptides identified were reported in the NCBI database as encrypted into sequences of vicilin and legumin type B and were rich in valine (V) and glutamic acid (E). The peaks corresponding to the active peptides were characterized by a markedly lower area in the WSE of untreated faba bean flour (Fig. 2S).

3.4. Baking trials: assessment of bread quality and mold-free shelf-life

The addition of 30 % faba bean caused significant modifications of volume and hardness of the composite faba-wheat breads (Table 3); however, fermentation with *Lv. brevis* AM7 was less detrimental to bread properties, especially concerning hardness, which was not significantly different from control wheat bread (141.4 ± 11.3 g and 129.0 ± 13.0 g, respectively). Based on informal preliminary sensory analysis, fFWB had more elastic texture and better mouthfeel compared to FWB (Fig. 3S).

Table 3
Technological quality of experimental breads.

Breads	Specific Volume (ml/g)	Crumb hardness (g)
WB	4.50 ± 0.08^{c}	129.0 ± 13.0^{a}
cpWB	4.41 ± 0.09^{c}	124.4 ± 14.6^{a}
FWB	3.58 ± 0.05^{a}	176.3 ± 24.5^{b}
fFWB	3.74 ± 0.03^{b}	141.4 ± 11.3^{a}

[a-c] Values in the same column with different superscript letters differ significantly.
WB, wheat bread; cpWB, wheat bread added of calcium propionate; FWB, wheat bread made with faba bean flour; fFWB, wheat bread made with faba bean slurry fermented with *Lv. brevis* AM7 at 30 °C for 8 h.

Regarding the mold-free shelf life, after 5 days, between 50 % and 90 % of WB and FWB slices started to show signs of contamination (ca. 10–25 % of the surface) and, after 6 days, both the loaves too (ca. 2–3 % of the loaf surface). Loaves of cpWB and fFWB started to spoil after 8 days on average (ca. 5 % of the surface) lasting up to 12 days, when several fungal spots of ca. 2–6 mm diameter appeared on the surface of both the types of breads, on all the loaves. As for slices, after 8–10 days, on average, only 50 % of fFWB slices showed 1 fungal spot, similarly to cpWB slices. Overall, bread containing fermented faba bean resisted 2–5 days longer than control breads, showing similar trend to bread with calcium propionate.

Table 2
Sequences of peptides identified in the purified FPLC fraction 18 of the extract of faba bean slurry fermented with *Lv. brevis* AM7.

Sequence	Score	Net charge	Hydrophobic ratio (%)	Calculated mass (Da)	Expected mass (Da)	Source protein (NCBI accession no.)
ELAFPGSAQEVDTLLENQK	112	−3	37	2088.0375	2088.0384	Vicilin VCL_VICFA
LSPGDVLVIPAGYPVAIK	80	0	50	1808.0448	1808.0442	Vicilin VCL_VICFA
VEINEGSLLLPHYNSR	85	−0.75	31	1839.9479	1839.9491	Vicilin VCL_VICFA
SDQDNPFVFVFESNR	76	−2	33	1553.6747	1553.6731	Vicilin VCL_VICFA
VFYLGGNPEVEFPETQEEQQER	70	−5	23	2624.2031	2624.2014	Legumin type B LEGB4_VICFA
IINPEGQQEEEEQEEEEKQR	107	−7	10	2186.9338	2186.9379	Legumin type B LEGB4_VICFA
AAVSHVQQVFR	54	+1.25	55	1240.6677	1240.6691	Legumin type B LEGB4_VICFA

4. Discussion

The implementation of a more sustainable food system can be achieved integrating different strategies, including the improvement of dietary habits, by providing more nutritionally balanced foods, and fostering the use of plant-based raw materials that increase the sustainability of the food chain while reducing waste (Perez-Cueto, 2020). In this context, faba bean can play a key role, since it is an important crop from an ecological, nutritional, and economical perspective (Dhull et al., 2022). Yet, to utilize fully its potential as a food ingredient, several aspects must be considered, including the presence of antinutritional factors or the negative influence on sensory and technological properties of food. Bioprocessing treatments such as sourdough fermentation of cereal, pseudo-cereal, and legume flours have been found to be a unique tool for improving several attributes of baked goods made thereof (Arora et al., 2021).

In this study, fermented faba bean was employed as antifungal ingredient for wheat-faba composite bread. Bread has been successfully fortified with fermented legume flour or other fiber-rich grains in several previous studies (reviewed by Arora et al., 2021) achieving enhanced nutritional quality. Bread, a staple food worldwide, is also one of the most discarded foods at household and industry level, due to its short physical shelf-life (i.e. staling) and often also microbiological shelf-life, typically due to mold contamination (Melikoglu and Webb, 2013). Here, three bioprocesses including use of proteases and fermentation have been assessed as antifungal treatments against mold commonly contaminating bakery products. All the bioprocessed samples were able to inhibit mold growth to some extent, showing higher inhibitory activity on *Penicillium* spp. rather than *A. niger*. The faba bean flour used in this study already possessed a certain inhibitory activity on all the molds tested (except for *P. crustosum* and *E. herbariorum*) before bioprocessing, most likely due to proteins involved in the plant defense against fungal attack (Mani-López et al., 2021). Some contribution to this activity could be due to tannins, which have been shown to possess antimicrobial activity (Latté and Kolodziej, 2000). However, some of the tannins are typically removed through dehulling (to an extent of 83–97 %, Rao and Deosthale, 1982), a technological process needed to obtain faba bean flour suitable for human consumption (Saha et al., 2022). When compared to untreated faba bean flour, the only treatment that generated a significant further growth inhibition, especially of *E. herbariorium* and *P. crustosum*, was faba bean fermented by *Lv. brevis* AM7. This result is comparable to previous findings, when the same starter was used for wheat sourdough and bread slurry fermentation showing the strongest antifungal effect among several LAB (Coda et al., 2008; Nionelli et al., 2020). Due to the broadest inhibitory spectrum among all bioprocessing treatments, faba bean fermented with *Lv. brevis* AM7 was chosen for further characterization.

The native faba bean flour had a very low content of indigenous microbes. During fermentation, *Lv. brevis* AM7 grew approximately 1 log cycle, thus reaching cell densities below those previously reported in faba bean sourdough propagated for longer time (Coda et al., 2017a). This also reflected on the mild acidity, including lactic and acetic acid production which were lower than those typically observed (Coda et al., 2017a). During fermentation, *Lv. brevis* AM7 metabolized especially glucose while sucrose, fructose and stachyose did not change significantly. Melibiose and galactose increase was likely due to the degradation of verbascose and raffinose as consequence of alfa-galactosidase activity of the starter (data not shown) and the flour. The short fermentation time was able to avoid intense proteolysis, which occurred only to a moderate extent. A moderate degree of proteolysis has been recognized as optimal for taking advantage of peptides bioactivity. This avoids further degradation of the sequences released in the first steps of protein hydrolysis which can potentially lead to activity loss (Rizzello et al., 2017). On the contrary, the selected fermentation conditions determined a consistent increase in free amino acids (especially Val, Leu, Tyr, and Phe) probably due to a combined action of flour endogenous and starter enzymes.

Since organic acids content was too low to justify the overall antifungal effect (Lind et al., 2005), the presence of antifungal compounds of protein origin was hypothesized. Thus, purification of peptides from fermented faba bean extract was performed, followed by an identification through mass spectrometry. The sequences potentially responsible for the antifungal activity found in fermented faba bean extract originated from defensin-like and non-specific lipid-transfer proteins (nsLTPs, Table 3S). Defensins, along with lectins and chitinases, are the main antifungal proteins in legumes, protecting the plant from fungal attack. Defensins can permeate microbial cell membrane and interact with different organelles or cell components, including the nucleus, resulting in growth inhibition or cell death (Mani-López et al., 2021). Non-specific lipid-transfer protein NLTP4_LENCU belongs to a group of legume-nsLTP showing high sequence similarity. Indeed, NLTP2_PEA and NLTP3_PEA were also reported as MS/MS match by the Mascot search engine research (at lower score). Similarly to defensins, nsLTPs which can bind and transfer a variety of very different lipids between membranes *in vitro*, have been reported to have antifungal activity against plant pathogens (Missaoui et al., 2022). Moreover, it was shown that nsLTPs are capable of synergistically act with other antimicrobial peptides from defensins and thionines (Safi et al., 2015; Missaoui et al., 2022) which correspond to the conditions of this study, reporting the identification of seven potentially antifungal peptides. In fermented faba bean extract, most of the peptides identified had a net negative charge. Antimicrobial peptides are generally composed of 12 to 54 amino acids, with an overall net positive charge, although also negatively charged peptides exist (Struyfs et al., 2021). Hydrophobicity, another trait of antimicrobial peptides, is based on the proportion of hydrophobic residues (Val, Ile, Trp, Phe, Leu, Cys, Met, and Ala) (Thery et al., 2019) and AAVSHVQQVFR was found to be particularly rich in valine. Among the peptides identified in the purified fractions, two had hydrophobic ratio higher than 50 % and other three between 31 and 37 % (Table 2). AAVSHVQQVFR was also the only cationic peptide with a net charge of +1.25, an experimental mass of 1240.7 Da. Indeed, a certain hydrophobicity is necessary for the penetration and binding of peptides into the bilayer of the cell membrane; in general, up to 50 % of the residues should be hydrophobic (Thery et al., 2019; Struyfs et al., 2021). Positive net charges are often associated with a strong interaction with the negatively charged cell membrane of microbes (Laverty et al., 2011). According to the APD3 peptide analysis tool (Wang et al., 2016), three of the peptides identified in fermented faba bean extract (ELAFPGSAQEVDTLLENQK, LSPGDVLVIPAGYPVAIK and SDQDNPFVFVFESNR), may form alpha helices and may have 3 to 4 residues on the same hydrophobic surface thus interacting with membranes. Indeed, antimicrobial peptides, known for their sequence diversity and wide range of secondary structures, can be distinguished in four group based on their conformation *i*) α-helical peptides, *ii*) β-sheet peptides with disulfide bridges, *iii*) cyclic peptides, and *iv*) residue-rich peptides (Struyfs et al., 2021). Moreover, all the peptide sequences identified in this study showed a similarity percentage ranging from 30 to 42 % with antimicrobial peptides and bacteriocins already described. Peptides identified in fermented faba bean extract were reported in the NCBI database to be encrypted in vicilin and legumin type-B proteins. Although little can be found in the literature about legumin type-B, the antifungal properties of chickpea legumin-like peptides (Heymich et al., 2021), as well as vicilin from cottonseed (He et al., 2021) or from a mixture of faba bean, pea, and lentils (Rizzello et al., 2017) were previously reported as deriving from protein hydrolysis with commercial enzymes. Thus, these results suggest that proteins and bioactive peptides originating during fermentation with *Lv. brevis* AM7 are part of a greater network of compounds, originally playing a big role in plant physiology, which can also have technological relevance when faba bean is used as food ingredient.

To assess the antifungal effect in food processing conditions, fermented faba bean flour was used as ingredient in breadmaking. Composite-breads were manufactured with untreated and fermented

faba bean dough substituted at the 30 % of the dough weight (17 % flour weight) and compared to a bread containing calcium propionate, a commonly used preservative. Based on recipe formulation, and on the protein content of the flour (ca. 30 %) the faba bean breads in this study could be also claimed as "source of proteins", according to the current EC Regulation on nutrition and health claims on food products since 16 % of the energy value of the food is provided by protein. When untreated faba bean flour was used, bread showed lower specific volume (up to 20 %) and higher hardness (up to 27 %) compared to WB and cpWB. This is a common drawback of legume-containing breads, rendering the incorporation of high amount of faba bean particularly challenging in leavened products, mostly due to the lack of gluten and high fiber content, thus limiting the fortification below 15 % of wheat flour (Verni et al., 2019). These negative effects were mitigated when faba bean fermented with *Lv. brevis* AM7 was used as ingredient restoring partly the volume (17 % lower than WB) and almost as soft as WB. These results differ from our previous study, in which faba bean flour fermented with a *Pediococcus pentosaceus* strain was used as sourdough, possibly due to the higher acidity and substitution level of faba bean flour (30 % on flour weight) used (Coda et al., 2017b). A preliminary sensory assessment indicated that fermentation possibly enhanced the performance of faba bean composite bread compared to its native counterpart, while beany attributes were moderately perceived (supplementary material, Table 4S). Concerning the antifungal properties, faba bean sourdough delayed the appearance of molds on breads. Overall, microbial shelf-life was 2–5 days longer in fFWB compared to the control breads (WB and FWB), lasting at least 8–10 days for loaves and slices, respectively. This behaviour was similar to cpWB, and comparable to our previous study in which the use of bread hydrolysate fermented by *Lv. brevis* AM7 as ingredient for wheat bread extended the mold-free shelf life up to 10 days (Nionelli et al., 2020). Although further trials to define the complete spectrum of activity and the effectiveness under industrial conditions are needed, these results proved that, besides nutritional benefits, fermented faba bean can be used to produce clean label foods by replacing chemical preservatives. This approach has several advantages since not only it can improve the nutritional balance of a staple food like bread, but also contributes to decrease bread wastage.

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Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2023.110403.

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