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Antifungal effect of bioprocessed surplus bread as ingredient for bread-making: Identification of active compounds and impact on shelf-life

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ABSTRACT

Bread is one of the most consumed food products in the world and one of the most discarded, due to its intrinsic short shelf-life and susceptibility to mold spoilage. Additionally, bread waste is generated during production and distribution, leading to the disposal of bread otherwise still fit for consumption. To avoid generating huge amount of bread waste, strategies to enable its reutilization should be sought. In this study, surplus bread, still suitable for consumption, was bioprocessed with enzymes and fermented by selected lactic acid bacteria generating an ingredient with antifungal properties. Bread hydrolysate fermented by *Lactobacillus brevis* AM7 showed broad inhibitory spectrum against the fungal species tested and antifungal activity ranging from 20 to 70%. Nine antifungal peptides were identified via Liquid Chromatography-Electrospray Ionisation-Mass Spectra/Mass Spectra (nano-LC-ESI-MS/MS), having 10–17 amino acid residues and mass ranging from 1083.6 to 1980.7 Da, all of them encrypted in wheat proteins sequences. Bread hydrolysate fermented by *Lb. brevis* AM7, non-fermented bread hydrolysate and a slurry consisting of water-bread mixture were used as ingredients in bread making and compared to regular wheat bread. Breads containing the fermented hydrolysate (18 and 22% of the dough weight) showed the longest mold-free shelf-life compared to the other breads, lasting up to 10 days before mold appearance. Additionally, the fermented hydrolysate was the least detrimental on bread quality, emphasizing the positive impact and potential of the studied biotechnology.

1. Introduction

Bread is one of the most consumed food products in the world with an estimated production of approximately 100 million tons per year (Melikoglu & Webb, 2013). At the same time, bread is also one of the most discarded food at industrial and household level. Bread and baked goods waste in general arise at different levels of the food chain, during processing, distribution and consumption. At the bakery level, waste can derive from excess or defective products or from specific operations, e.g. during the removal of the external layer of soft bread when producing sliced bread for sandwiches. During distribution, baked good waste originates due to the intrinsic short-shelf-life of bread, governed by two distinct factors: bread staling and microbial spoilage. Bread staling is a complex phenomenon due to multiple mechanisms including retrogradation of amylopectin and migration of water from the gluten network to starch and amylopectin with consequent general loss of bread quality (Gray & Bemiller, 2003). Although stale bread is still fit for consumption, this loss in texture and flavor decreases consumers' acceptance and leads to bread being discarded. If bread staling only results in poor sensory quality, microbial spoilage of bread makes it unfit for consumption. Thus, strategies to avoid bread waste should have a diversified approach: on one hand stale bread can still be utilized for consumption and should be recycled rather than discarded as waste; on the other hand, microbial spoilage of bread should be avoided or delayed as much as possible, to reduce its disposal. The main cause of microbial spoilage of bread are molds of different genera, among which *Penicillium* and *Aspergillus* spp. are the most commonly found (Legan, 1993). Mold spoilage of bread mostly occurs due to post-production

Abbreviations: LAB, lactic acid bacteria; CFU, colony forming unit; d.w, dough weight; SV, specific volume.

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contamination, therefore a series of preventive measures together with the use of antifungal ingredients (i.e. preservatives) are the most common strategies adopted by the bakery industry. Generally, the use of chemical preservatives such as weak acids and their salts, particularly salts of propionic and sorbic acids, is very common in bred making. Their use in different bakery products is regulated within EU and, depending on the type of breads, they can be added up to 0.1–0.3% (w/w). However, the use of chemical preservatives and other type of additives in the food industry has been going under a deep revision, also due to the growing consumers' appreciation of more natural foods and concomitant negative perception of artificial additives (Asioli et al., 2017). To satisfy this request, the food industry response has been to provide more "clean label" food products and to communicate the absence of certain types of artificial ingredients (Katz & Williams, 2011). Besides, the addition of chemical preservatives like sorbate or propionate not only can impact the sensory properties of the bread but has also been suspected of causing an increase of the mold resistance towards these compounds (Levinskaite, 2012; Stratford et al., 2013; Suhr & Nielsen, 2004). For all these reasons, the research on antifungal compounds, and particularly those of natural origin, has been carried out very intensively in the last 15 years (as reviewed by Axel et al., 2017). The range of these natural compounds is very broad, from plant-derived extract or essential oils to the use of starter cultures with a protective effect. In the latter case, fermentation technology has led to the discovery of several functional metabolites in the landscape of natural preservatives. Microorganisms like lactic acid bacteria (LAB) and yeasts have been used to ferment several food matrices, originating an array of antifungal metabolites. These fermented substrates, mostly based on plants, have then been used as functional ingredients to prolong the mold-free shelf life of bread (Axel et al., 2017). In the bakery industry, sourdough technology is one of the most utilized methods to substitute chemical preservatives and to guarantee that "clean label" status, thanks to the formation *in situ* of antimicrobial metabolites. During LAB fermentation, several antifungal compounds can be generated, such as different types of acids (e.g. acetic and propionic acid, hydroxy derivatives of fatty acids, phenyl lactic acid) and protein-type compounds like cyclic dipeptides or other small molecular weight compounds, as reviewed by Crowley et al. (2013). This study describes the use of bioprocessing (i.e. enzymatic hydrolysis followed by LAB fermentation) to obtain an ingredient with antifungal properties for bread manufacture. Bread hydrolysate was fermented by different LAB strains, including *Lactobacillus brevis* AM7, previously shown to release antifungal peptides during wheat flour fermentation (Coda et al., 2008). The utilization of surplus bread to produce an ingredient with functional properties for new bread production is a strategy that aims at reducing bread disposal. In this way bread still fit for consumption can be reutilized for bread making, transforming it into an ingredient able to prolong bread shelf-life, thus decreasing the overall amount of bread discarded. Here, we describe the setting up of bread bioprocessing procedure, the antifungal properties and the identification of the active compounds generated. Finally, the impact of the bioprocessed bread on bread quality and shelf life have been assessed.

2. Material and methods

2.1. Materials

The wheat bread (Sinuhe bakery, Finland) used for this study had the following chemical composition: carbohydrates, 46.1%, of which sugar 0.5%; dietary fibers 3.2%; protein, 9.3%; fat 1.7%, salt 1.5%. Upon reception from the bakery, bread was stored at −20 °C and grated before hydrolysis. The wheat flour used for baking was purchased from Helsingin Mylly, Finland and had the following characteristics: moisture content 14.8%; protein content 13%; ash content 0,64–0,71%. The carbohydrases: amyloglucosidase (Grindamyl plusweet G) and alpha-amylase (Grindamyl A 140 000, fungal) and the protease Corolase 7089 (AB Enzymes) were used to prepare the bread hydrolysate.

2.2. Microorganisms and cultivation conditions

LAB used to ferment the bread hydrolysate were selected based on their capacity to delay mold growth during different antifungal tests (data not shown), namely: *Leuconostoc mesenteroides* I21, *Pediococcus pentosaceus* I02 (Verni et al., 2017) and *Lactobacillus brevis* AM7 (Coda et al., 2008). The strains were routinely cultivated in MRS Broth (de Man, Rogosa & Sharpe Broth, LABM, UK) for 24 h at 30 °C. The eight mold strains belonging to the genera *Penicillium*, *Eurotium* and *Aspergillus* used for the antifungal assay are presented in Table 1. The molds were propagated on potato dextrose agar (PDA, Oxoid) at 25 °C for seven days before use.

2.3. Bread hydrolysis and fermentation

Bread hydrolysate was obtained by mixing bread and distilled water (1:2 ratio) and enzymes. In details, 0.25% of amyloglucosidase and 0.05% of alpha-amylase (on wheat bread basis) and 300 nKat/g (on bread protein basis) of protease were first suspended in water, and then mixed with the bread. Bread and water mixtures (herein referred to as "slurries") containing the enzymes were incubated at 50 °C with 120 rpm shaking for 24 h. After hydrolysis, pH was adjusted to 6.5 with 1 M NaOH. Afterwards, to inactivate the enzymes, the bread hydrolysates were treated at 90 °C in stirring conditions for 20–35 min, depending on the volume, and cooled at 30 °C before inoculation. The LAB inoculum was prepared by harvesting the cells (centrifugation at 10.000 rpm for 10 min) and washing with sterile phosphate buffer saline (0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, pH 7.4, Sigma-Aldrich, Saint Louis, USA), following by centrifugation. The supernatant was discarded, and the cells re-suspended in 4 mL of distilled water and mixed with the bread hydrolysate, targeting an initial LAB cell density of 6–7 Log CFU/g of hydrolysate. Afterwards the hydrolysates were incubated at 30 °C for 24 h. Non-fermented bread hydrolysate and the non-fermented bread hydrolysate obtained without protease addition were also prepared and used as controls.

2.4. Microbial growth and acidification

Microbial growth was estimated at the beginning and at the end of bread hydrolysate fermentation by plate counts. In detail, hydrolysate samples (10 g) were homogenized with 90 mL of sterile saline solution (0.9% NaCl) in a Stomacher 400 lab blender (Seward Medical, London).

Table 1
Fungal species used for the antifungal assays and their origin.

Fungi	Source of isolation	Origin
Penicillium roqueforti DPPMA1	Bread, Italy	Department of Plant, Soil and Food Science, University of Bari
Penicillium roqueforti P1	Bread, Finland	Department of Food and Nutrition, University of Helsinki
Penicillium crustosum P2	Bread, Finland	Department of Food and Nutrition, University of Helsinki
Penicillium commune P3	bread, Finland	Department of Food and Nutrition, University of Helsinki
Penicillium paneum CBS 101032	Rye bread, Denmark	Culture Collection of Centraalbureau voor Schimmelcultures (Utrecht, Holland)
Penicillium albocoremium CBS 109582	Cake factory, Denmark	Culture Collection of Centraalbureau voor Schimmelcultures, Utrecht, Holland
Aspergillus niger P4	Bread, Finland	Department of Food and Nutrition, University of Helsinki
Eurotium herbarioum CBS 117336	Chocolate cake, Netherlands	Culture Collection of Centraalbureau voor Schimmelcultures, Utrecht, Holland

Serial dilutions were made and enumeration of different microbial groups was performed. Enumeration of LAB was performed on MRS agar supplemented with cycloheximide (0.1 g/L) (LABM, UK) incubated at 30 °C for 48 h in microaerophilic conditions. Enterobacteriaceae were enumerated on VRBGA (violet red bile glucose agar, LABM), and incubated at 37 °C for 24 h. *Bacillus cereus* was enumerated on PEMBA (*B. cereus* medium, LABM) supplemented with polymyxin B and egg yolk emulsion (LabM), incubated at 30 °C for 24 h. Yeasts and molds were enumerated on Malt Extract agar (LABM) supplemented with chloramphenicol (0.1 g/L) and incubated at 25 °C for at least 48 h.

The pH was measured with a pH-meter (Model HI 99161, Hanna Instruments, Woonsocket, RI, USA). Total titratable acidity (TTA) was measured on aliquots of 10 g of hydrolysate re-suspended in 90 mL of distilled water, with an automatic titrator (EasyPlus Titration, Mettler Toledo, Columbus, OH, USA) and expressed as the amount of 0.1N NaOH (hereafter reported as "mL" in the text) necessary to reach a final pH of 8.3.

2.5. Water/salt-soluble extracts, organic acids and free sugars analysis

Before organic acid and sugar analysis, water/salt-soluble extracts from the bread hydrolysates were obtained according to Weiss et al. (1993). The extracted hydrolysates were centrifuged at 11 000 rpm for 20 min at 4 °C and supernatant were sterilized by filtration through 0.22 μm sterile filters. Sterile extracts were used for organic acid analysis or finally kept at −20 °C for further analyses. 1 mL of clear supernatant was collected and 1 mL of 5% perchloric acid was added and vortexed thoroughly. The mixture was kept in 4 °C overnight and then centrifuged (11 000 rpm, 15 min). 1 mL of clear supernatant was then syringe-filtered (Acrodisc GHP Minispike 45 μm, Pall Corporation, USA) into HPLC vials. The analysis was performed on a Hi-Plex H column (Agilent Technologies, Inc., CA, USA; 300 × 6.5 mm), with a Hi-Plex H guard column (Agilent; 50 × 7.7 mm). The HPLC system was equipped with a Waters 515 pump (Waters Corp., Milford, MA, USA), autosampler, ultraviolet (UV) detector (Waters 717), and refractive index detector (HP 1047A, HP, USA) using 10 mM H_2SO_4 as an isocratic mobile phase (0.5 mL/min, run time 40 min with 40 °C column temperature). UV-chromatogram was used to quantify organic acids and RI-chromatogram to quantify free sugars.

2.6. Antifungal assays

2.6.1. Hyphal radial growth rate assay

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 water-soluble extract 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 water extract)/mycelial growth under control conditions] * 100. The control conditions were represented by the non-fermented bread hydrolysate or the non-fermented bread hydrolysate obtained without protease addition.

2.6.2. Micro-titer plate assay

To assess the antifungal activity of the re-dissolved and purified fractions a micro-titer plate assay as previously proposed by Muhialdin et al. (2016), was used with some modifications. After growth for 7 days on PDA plates, conidia of *P. roqueforti* DPPMAF1, used as indicator, were harvested in sterile water containing 0.05% (vol/vol) Tween 80. Counts of the conidia in the suspension were carried out using a Petroff-Hausser counting chamber. Conidia were then resuspended in Malt Extract Broth (MEB, Oxoid Laboratories, Hampshire, United Kingdom) at the density of 10^4 conidia/mL and placed in 96 wells-plates. Each well was also complemented with 30% (vol/vol) of the purified fractions. MEB alone (CT1) and inoculated MEB (CT2) added with sterile water (30% vol/vol) instead of resuspended purified fractions, were included in the analysis and used as controls. The plates were incubated at 25 °C for 72 h. Fungal growth inhibition was determined by measuring the absorbance at 560 nm through an ELISA reader (Labomed, model UVD-2950, USA) and expressed as % of inhibition compared to CT2 (growth without purified fractions).

2.7. Proteolysis and heat stability of antifungal compounds

Water-soluble extracts were treated with trypsin (EC 3.4.21.4; Sigma Aldrich Co.) as described by Atanassova et al. (2003). Trypsin was dissolved in 0.25M Tris HCl, pH 8 (1%, wt/vol, final concentration). The supernatant and the buffered enzyme solution were mixed in a 1:1 ratio. After 5 h of incubation at 25 °C, the reaction was stopped by boiling the mixture for 3 min. After treatment, the pH of the solution was adjusted to 6.0 and the residual activity was determined by agar diffusion assay. Heat stability of the water-soluble extracts was assessed after heating for 5 min at 100 °C. Afterwards, the residual activity was determined by agar diffusion assay as previously described on the indicator molds *P. roqueforti* P1, *P. albocoremium* and *A. niger* P4.

2.8. Identification of antifungal compounds

2.8.1. Purification

The water/salt soluble extract from the fermented hydrolysate was first fractionated by ultra-filtration (Ultrafree-MC centrifugal filter units, Millipore) using membrane sizes of 50, 30, 10, and 3 kDa cut-off, following the manufacturer's instructions.

Fractions were tested for the antifungal activity on *P. roqueforti* DPPMAF1 as described in 2.6. An aliquot of the 3 kDa partially purified fraction (corresponding to 10 mg of peptides) was further automatically fractionated (32 fractions for each run) by Reversed-Phase Fast Performance Liquid Chromatography (RP-FPLC), using a Resource RPC column and an ÄKTA FPLC equipment, with the UV detector operating at 214 nm (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Fractions were separated as described in Rizzello et al. (2011). Solvents were removed from collected fractions by freeze drying. Fractions were re-dissolved in 600 μL sterile water and assayed for the antifungal activity and the peptide concentration.

Proteins and peptides concentration in the extract and purified fractions was determined by the Bradford (Bradford, 1976) and *o*-phthaldialdehyde (OPA) methods (Church et al., 1983), respectively.

2.8.2. Identification of antifungal peptides

Purified fractions having the highest antifungal activity on the indicator mold were subjected to nano-Liquid Chromatography-Electrospray Ionisation-Mass Spectra/Mass Spectra (nano-LC-ESI-MS/MS), aiming at identifying peptides. A Finnigan LCQ Deca XP Max ion trap mass spectrometer (ThermoElectron) through the nano-ESI interface was used. According to manufacturer's instrument settings for nano-LC-ESI-MSMS analyses, MS spectra were automatically taken by Xcalibur software (ThermoElectron), in positive ion mode. MS/MS spectra were processed using the BioWorks 3.2 software (ThermoElectron) generating peak lists suitable for database searches. Peptides were identified using MS/MS ion search of Mascot search engine (Matrix Science, London, England) and NCBInr protein database (National Centre for Biotechnology Information, Bethesda, USA). For identification of peptides the following parameters were considered: enzyme: "none"; 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.

2.9. Baking test and mold-free shelf life assessment

The effects of the bioprocessed bread ingredient on mold-free shelf life of bread was determined with baking trials. Bread recipes are shown in Table 2. Seven types of breads were produced, in the bakery facility of the University of Helsinki, including a wheat control bread (WBC), breads containing bread slurry only at two different substitution levels (SB18 and SB22 for the substitution levels of the slurry at 18 or 22% of the dough weight), hydrolysate containing breads, either non fermented (control hydrolysate, CHB18 or 22) or fermented with AM7 (AM7B18 or 22) at the same substitution levels as above. For bread making, a straight dough process was used as described in Wang et al. (2018). A total of ten breads per type were obtained and used for subsequent analysis. After 1 h of cooling, the loaves were weighed, packed into plastic bags and stored at room temperature for texture and profile analysis or antifungal observations or at −20 °C for pH and TTA measurements. The specific volume (SV) of the breads was measured from three breads during the day after baking with BreadVolscan laser scanner (Backaldrin, Austria). Texture profile analysis was performed with texture analyzer (TA-XT2i, Stable Micro Systems Ltd., UK) according to Wang et al. (2019). Crumb hardness (g) was measured after storage at room temperature for 1 and 4 days. The pH and TTA were measured from breads crumb as described by Wang et al. (2018). For antifungal observations, bread loaves (two per type) and bread slices (three per each loaf) were packed singly in polyethylene bags and stored at room temperature until the breads were molded. Loaves and slices were observed daily for the appearance of mold colonies. The molds growth was estimated by visual observation and approximately quantified as % of spoiled surface on the total surface (whole loaf or slice).

2.10. Statistical analysis

All the experiments were performed in duplicate and each analysis was repeated two or three times. Statistical analysis was performed with SPSS Statistics 25 software (IBM Corp., NY, USA) by one-way analysis of variance (ANOVA) and Tukey's *post hoc* test using p-value < 0.05.

Table 2
Breads recipes. WBC: wheat bread control; SB: breads containing the slurry (mixture of bread and water), CHB: control (not fermented) hydrolysate bread; AM7: bread containing hydrolysate fermented by *Lb. brevis* AM7. The numbers 18 and 22 refer to the addition level 18 or 22% of the above ingredients to the bread, calculated on dough weight (d. w.).

Breads	WBC	SB18[a]	SB22[a]	CHB18/ AM7B18[b]	HB22/ AM7B22[b]
Ingredients (g)					
Flour	1500	1449.8	1438.8	1449.8	1438.8
Water	873	520.2	443	520.2	443
Hydrolysate				480	585
Bread slurry		480	585		
Salt	22.5		22.5	22.5	22.5
Sugar	30	75	75		
Yeast	75	75	75	75	75
Fat	90	90	90	90	90

[a] Bread slurry contains: 26.5% dry matter (of which 15.9% glucose), 73.5% water.

[b] Hydrolysate contains: 15.9% glucose, 10.5 other dry matter and 73.5% water. The hydrolysate was used as such or fermented with *Lb. brevis* AM7.

3. Results

3.1. Bread hydrolysate fermentation

The fermentation parameters related to microbial growth and acidification (pH, TTA and organic acids) of control and fermented hydrolysates are reported in Table 3. Before inoculum, the hydrolysate had pH of 6.5 and TTA of 2.8 mL and the presence of the microbial groups considered was not detected at the lowest dilution considered. Following the initial inoculum of the LAB starter of ca. 6.5 Log CFU/g, in all the fermented hydrolysates, the presumptive LAB cell density after fermentation was higher than 9 Log CFU/g, corresponding to an increase of approximately 3 log cycles compared to the initial inoculum. No microbial group other than LAB was found in the fermented hydrolysates. After 24 h of fermentation, the bread hydrolysate showed a pH drop of 2.2–2.7 units, and a pH range of ca. 3.8–4.3. These values were also reflected in the TTA, ranging from 7.8 to 11.2 mL, with bread hydrolysate fermented by *Ln. mesenteroides* I21 being the least acidic. The amount of lactic and acetic acid in the fermented hydrolysate was in line with the above data. Lactic acid was found at higher concentrations than acetic acid and particularly in the bread hydrolysates fermented by *Lb. brevis* AM7 and *P. pentosaceus* I02, ca. 86 mmol/kg of hydrolysate, while nearly half the amount of lactic acid 42.8 ± 3.2 mmol/kg of hydrolysate was detected for BH fermented by *Ln. mesenteroides* I21. Acetic acid was found in very low concentration (1.1–1.8 mmol/kg of hydrolysate) for all the fermented hydrolysates. The bread contained originally small amount of fermentable sugars, i.e. fructose and maltose, 0.8% and 3.0% of the dry weight, respectively. After hydrolysis, the glucose content was approximately 15% (w/w), while the amount of glucose in the fermented hydrolysates was approximately 15.2–15.3% (w/w), with no significant differences between the samples (data not shown). No other monosaccharides were detected.

3.2. Antifungal assays

Antifungal effect of the fermented bread hydrolysate was observed for all the LAB tested to a different extent on the different molds (Table 4). Generally, the least inhibited mold was *A. niger* P4 showing inhibition rates of approximately 20%. On the contrary, except for *P. paneum* CBS 101032, *Penicillium* spp. and *E. herbariourum* CBS 117336 were more susceptible to the fermented bread hydrolysate. In particular, the hydrolysate fermented by *Lb. brevis* AM7 showed the best antifungal activity especially on *P. roqueforti* P1, *E. herbariorum* CBS 117336 and *P. albocoremium* CBS 109582 (70–80% of inhibition). The results above refer to the use of the bread hydrolysate containing protease. To assess whether protease addition during bread hydrolysis had an impact on the antifungal effect observed, bread hydrolysate containing protease was assessed against the bread hydrolysate without protease. The antifungal effect of protease addition alone was not found or very low (<8%) for all the molds tested and it was ca. 20% on *P. albocoremium* CBS 109582 (data not shown). The hydrolysate fermented by *Lb. brevis* AM7 showing the highest antifungal effect was then selected for further experiments.

3.3. Trypsin digestion and heat stability

The water-soluble extract of the bread hydrolysate non fermented and fermented by *Lb. brevis* AM7 was subjected to trypsin digestion, to verify the potential protein nature of the compounds involved in the antifungal activity. The inhibitory activity of the supernatant was totally lost after treatment with trypsin as observed on *A. niger* P4 and *P. roqueforti* P1 for both the fermented and control hydrolysates, while the inhibition on *P. albocoremium* CBS 109582 decreased to 13.3% (Fig. 1). After heat treatment, 10–30% of the inhibitory activity was retained. This outcome supported the hypothesis of the protein-type nature of the antifungal compounds.

Table 3
Microbial growth and acidification parameters of bread hydrolysates fermented by the selected lactic acid bacteria starters[a]. BHAM7: bread hydrolysates fermented by *Lb. brevis* AM7; BHI21: bread hydrolysate fermented by *Ln. mesenteroides* I21; BHI02 bread hydrolysate fermented by *P. pentosaceus* I02.

	LAB cell density Log CFU/g		Acidification parameters			
Sample	time 0	time 24 h	pH 24 h	TTA (mL NaOH 0.1N)	Lactic acid (mmol/kg)	Acetic acid (mmol/kg)
Ct hydrolysate[b]	–	–	6.5 ± 0.1[a]	2.8 ± 0.35[a]	6.1 ± 0.3[a]	0.8 ± 0.06 [a]
BHAM7	6.7 ± 0.15	9.5 ± 0.0	3.76 ± 0.01[c]	11.2 ± 0.05[c]	86.6 ± 8.6[c]	1.1 ± 0.06 [b]
BHI21	6.3 ± 0.2	9.45 ± 0.05	4.29 ± 0.05[b]	7.8 ± 0.35[b]	42.8 ± 3.2[b]	1.1 ± 0.05 [b]
BHI02	6.5 ± 0.1	9.25 ± 0.2	3.84 ± 0.03[c]	10.1 ± 0.2[c]	86.04 ± 2.1[c]	1.8 ± 0.10 [c]

[a] Values in the same column with different letters (a–c) are significantly different ($p < 0.05$).
[b] Ct hydrolysate refers to the hydrolysate after heat treatment and stored or used/analyzed after its preparation; no fermentation was carried out for this sample.

Table 4
Inhibitory spectrum of the extract of bread hydrolysate fermented by the selected LAB (25%, vol/vol, on PDA medium), as determined by hyphal radial growth rate of fungi, after 7 days of incubation at 25 °C[a]. BHAM7: bread hydrolysates fermented by *Lb. brevis* AM7; BHI21: bread hydrolysate fermented by *Ln. mesenteroides* I21; BHI02 bread hydrolysate fermented by *P. pentosaceus* I02.

% of Inhibition[b]								
Sample	*P. roqueforti DPPMA1*	*P. roqueforti P1*	*P. crustosum*	*P. commune*	*P. paneum*	*P. albocoremium*	*E. herbariorum*	*A. niger*
BHAM7	19.1 ± 4.4[a]	76.9 ± 2.6[a]	66.0 ± 11.3 [a]	28.8 ± 1.4[a]	18.8 ± 1.9[a]	72.6 ± 3.6[a]	72.5 ± 2.5[a]	23.5 ± 0.6[a]
BHI21	24.3 ± 0.7[a]	34.6 ± 1.3[b]	36.8 ± 2.8 [ab]	22.6 ± 8.9[a]	13.0 ± 7.8[a]	32.1 ± 3.6[b]	38.8 ± 1.3[b]	18.7 ± 4.2[a]
BHI02	9.6 ± 0.7[a]	21.8 ± 1.3[c]	22.6 ± 1.9 [b]	11.0 ± 1.4[a]	11.7 ± 0.0[a]	17.9 ± 1.2[b]	43.8 ± 1.2[b]	0.6 ± 0.6[b]

% Inhibition = [(mycelial growth under control conditions - mycelial growth in the presence of water/salt soluble extract)/mycelial growth under control conditions] x 100.
[a] Values in the same column with different letters (a–c) are significantly different ($p < 0.05$).
[b] Each datum point is the mean of at least four measurements of a growing colony. The percentage of growth inhibition was calculated from mean values as.

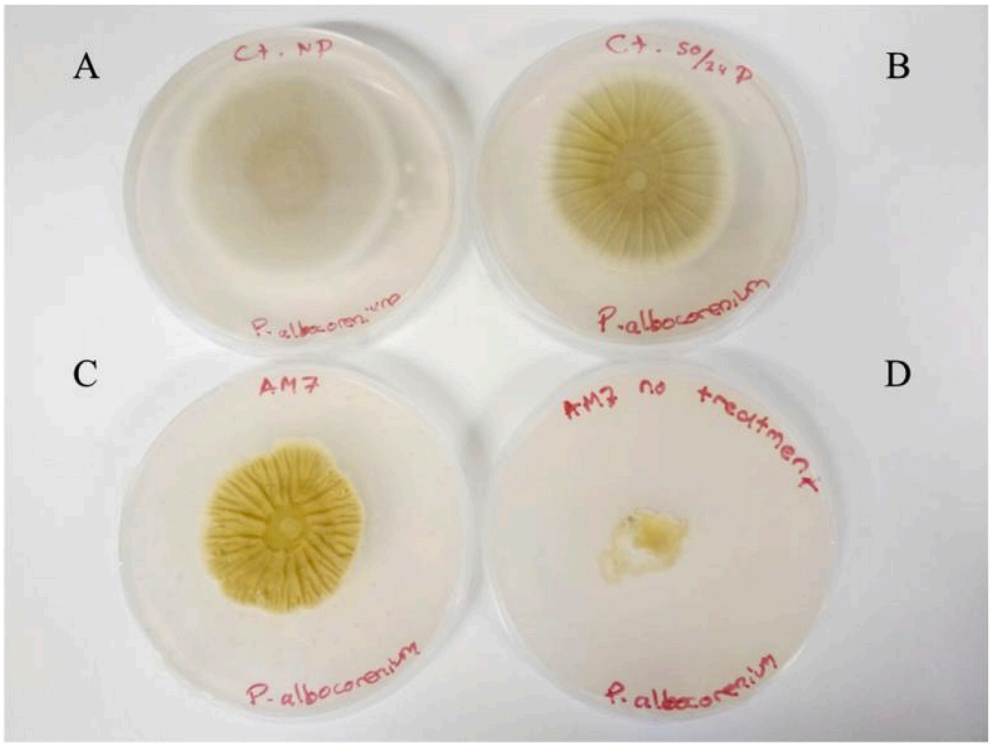

Fig. 1. Antifungal activity of the bread hydrolysates as determined by hyphal radial growth rate assay on the indicator mold *P. albocoremium* CBS 109582. A: water soluble extract of the bread slurry; B: water soluble extract of the bread hydrolysate before fermentation and after trypsin treatment; C: bread hydrolysate fermented by *Lb. brevis* AM7 after trypsin treatment; D: bread hydrolysate fermented by *Lb. brevis* AM7 without trypsin treatment.

3.4. Purification and identification of antifungal compounds

The water/salt-soluble extract had a protein concentration of 4.3 ± 0.2 mg/mL, while peptides were 8.2 ± 0.4 mg/mL. OPA method, here used for the determination of the peptide concentration, is largely used as index of the proteolysis degree. The extracts obtained from the non-fermented bread hydrolysate prepared without protease addition and from the non-fermented bread hydrolysate were respectively characterized by peptides concentration of 2.1 ± 0.2 and 6.2 ± 0.1 mg/mL. The active water/salt-soluble extract was fractionated by ultra-filtration. All the fractions (corresponding to the permeate at cut-off 50, 30, 10, and 3 kDa) caused the 70% inhibition of *P. roqueforti* DPPMAF1 growth. Based

on these results, it was hypothesized that active compounds were overall characterized by molecular masses lower than 3 kDa. The fraction with a molecular mass of <3 kDa (peptide concentration of 4.0 ± 0.2 mg/mL) was further fractionated by reversed-phase liquid chromatography in thirty-two fractions, five of which (A-E) having inhibitory activity on the *P. roqueforti* DPPMAF1. Active fractions showed peptide concentration ranging from 2.10 ± 0.05 to 3.02 ± 0.06 mg/mL).

The highest inhibitory activity was observed for fractions B and E (47 and 55%, respectively), eluted at 12 and 35% of the acetonitrile gradient, while fractions A, C and D (eluted at 8, 20, and 25% of the acetonitrile gradient) showed a weak activity (Fig. 2).

Peptides of the fractions B and E were identified by nano-LC–ESI–MS-MS, followed by MS-MS and ion search with the Mascot search engine (Table 5). Nine peptides, having 10–17 amino acid residues, mass from 1083.6 to 1980.7 Da, and hydrophobic ratio from 25 to 63%, were identified. All the sequences were identified as fragments of native wheat proteins (NCBI accession numbers are reported in Table 5).

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

Compared to WBC (volume 4.26 ± 0.15 g/mL), the addition of either slurry or hydrolysate, fermented or not, significantly decreased the specific volume of the breads. In particular, the decrease was higher when slurry was added, independently of the substitution level. The addition of 18% w/w of hydrolysate whether fermented or not decreased the specific volume of 8–10%, with no significant difference between the two conditions. When the substitution level raised to 22%, the specific volume of hydrolysate containing breads decreased further of 11–15%, and it resulted up to 20% lower when slurry was added. The trend observed for the specific volume reflected on hardness after 1 and 4 days. WBC hardness was 4.26 ± 0.15 and 130.1 ± 14.8 after 1 and 4 days, respectively. Breads containing slurry were the hardest while breads containing hydrolysate at both substitution level had similar hardness, except for 18% AM7B, resulting softer than the other substituted breads (175.1 and 345.0 g for day 1 and 4, respectively). WBC as well as breads containing slurry had pH and TTA ranging from 5.4 to 5.6 and 3.1–3.7 mL, respectively, showing that when the content of slurry was higher, a slightly but signifcantly lower TTA was found. Similar results were observed for bread containing 18% of hydrolysate. The most acidic breads were those containing the fermented hydrolysates, and particularly at 22% substitution level (pH 4.8, TTA 5.7 mL). The specific volume and TPA parameters and acidification of the breads are reported in Table 1S in supplementary material.

Mold contamination, deriving from the pilot bakery environment, appeared after 4 days of storage, visible as dots (3 mm) first on the slices of wheat bread control (WBC) and then after 4.5–5 days, also on slices containing hydrolysate and slurry (22%). After 6 days, slices of control breads (wheat bread, slurry and hydrolysates) showed varying signs of fungal contamination, corresponding to approximately 5% of the total surface. Gradually, throughout the observation period, these bread slices and loaves kept spoiling, showing 20–50% of molded surface. Signs of mold contamination appeared after 9 days on one loaf of the bread containing 18% d.w. of the hydrolysate fermented by *Lb. brevis* AM7 (AM7B18). The first sign of fungal appearance on the loaf containing 22% of fermented hydrolysate (AM7B22) occurred after 10 days. From this time onward, the loaves and slices of these latter breads continued to spoil. In Fig. 3 the trend of bread spoilage is reported at 2 days intervals throughout 10 days of daily observations.

4. Discussion

Mold contamination is the most common cause of bread loss worldwide. This phenomenon, in addition to the staling process makes bread one of the most discarded food at household and industry level. The research for natural substitutes of the commonly used organic acids preservatives has intensified in the past 15 years. To fulfill the growing requests for more natural food products, the antifungal effects has been sought in compounds of natural origin, such as those deriving from plants or obtained via bioprocessing with microbes and/or enzymes. These methods, defining the concept of biopreservation (Stiles, 1996), have become an increasingly important field of research. The raw material chosen in this study was “surplus” bread unsold from the previous day, still fit for consumption therefore not presenting any microbial contamination or safety risk, or physical alteration. Our aim was to obtain an antifungal ingredient through bioprocessing of bread and assess its functionality in bread making. Upon treatment with amyloglucosidase and α-amylase, an increase in glucose content up to 15% (w/w) was detected (i.e. 55% of the bread dry weight). At the same time, peptides concentration increased of ca. 3 times as consequence of the protease addition.

The enzymatic hydrolysis of the bread was followed by heat treatment to inactivate the enzymes, generating a glucose-rich, sterile

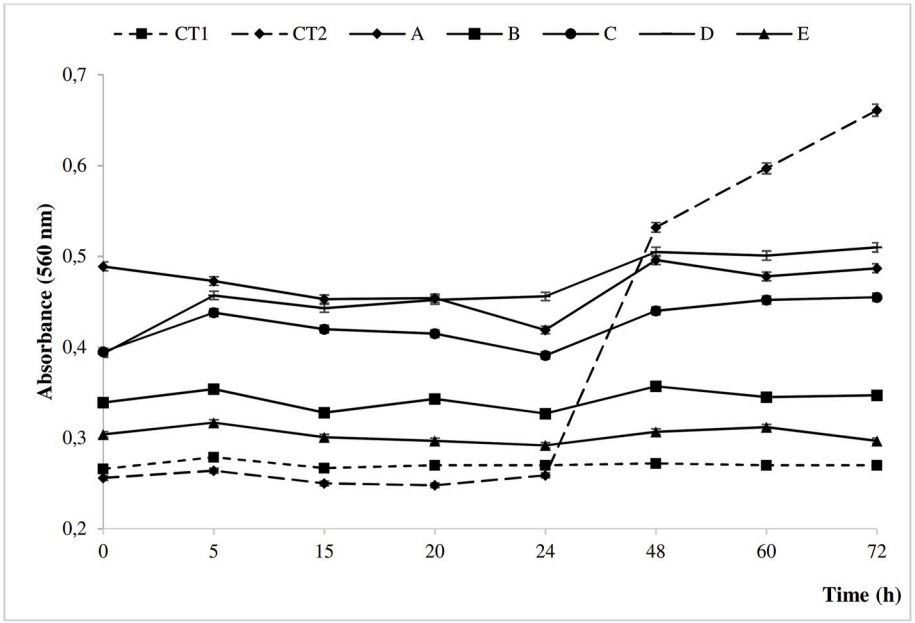

Fig. 2. Inhibitory activity of the five active partially purified peptide fractions (A–E) obtained from the bread hydrolysate fermented by *Lb. brevis* AM7 towards *P. roqueforti* DPPMAF1. CT1, not inoculated Malt Extract Broth (MEB) added with 30% (vol/vol) of sterile water; CT2, MEB inoculated with 10^4 conidia/mL and added with 30% (vol/vol) of sterile water; A, B, C, D, and E: MEB inoculated with 10^4 conidia/mL and added with 30% (vol/vol) of the fractions A, B, C, D, and E, respectively. Incubation was carried out at 25 °C.

Table 5
List of peptides identified in partially purified peptide fractions B and E obtained from the bread hydrolysate fermented by *Lb. brevis* AM7 through RP-RPLC.

Fraction	Sequence	Experimental mass (Da)	Calculated mass (Da)	Δmass (Da)	Length	Net charge	Hydrophobic ratio (%)	NCBI Accession number* (Protein)	Fragment
B	NIQVDPSGQVQ	1183.5833	1183.5833	0.0000	11	−1	27	P08453.1 (γ-gliadin)	20–30
	EGEGVIVLLR	1083.6282	1083.6288	0.0006	10	−1	50	B8AL97.1 (Globulin-like)	122–131
E	LTAASITAVCR	1161.6175	1161.6176	0.0001	11	1	63	P01085.1 (α-amylase inhibitor)	90–100
	DCCQQLADINNEWCR	1980.7927	1980.7877	0.0050	15	−2	46	ABF93411.1 (α-amylase inhibitor)	71–85
	DVAGGGGAQQCPVETK	1572.7193	1572.7202	0.0009	16	−1	31	P33432.2 (Puroindoline-A)	29–44
	DYVLQQTCGTFTPGSK	1800.8404	1800.8353	0.0051	16	0	25	AQT26482.1 (α-amylase/	45–60
	SGNVGESGLIDLPGCPR	1726.8316	1726.8308	0.0008	17	−1	29	trypsin inhibitor)	116–132
	QQCCGELANIPQQCR	1860.8077	1860.8029	0.0048	15	0	40		66–80
	QQCCQPLAQISEQAR	1815.8397	1815.8356	0.0041	15	0	40	P0CZ09.1 (Avenin-like)	71–85

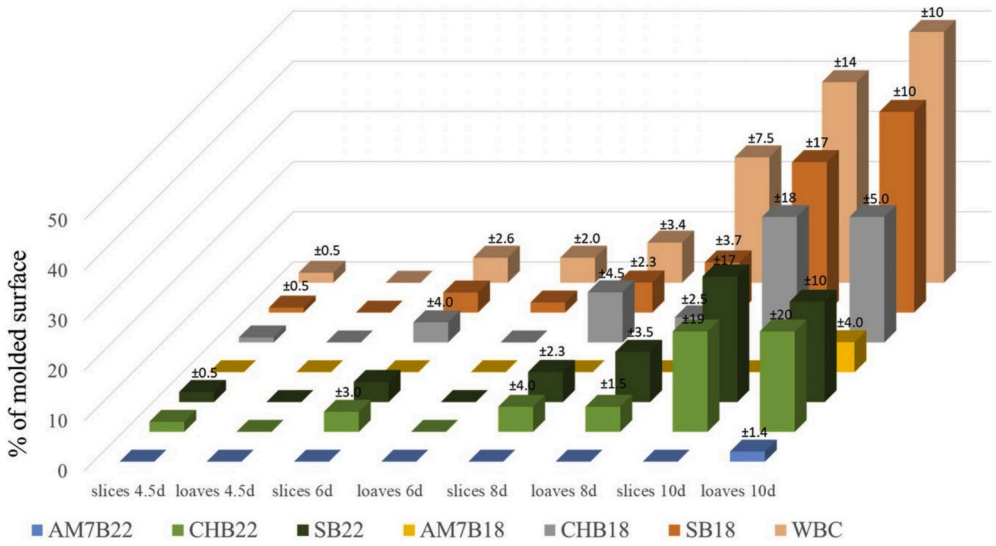

Fig. 3. Percentage of molded surface of breads slices and loaves during 10 days of storage. WBC: wheat bread control; SB: breads containing the slurry (mixture of bread and water); CHB: control (not fermented) hydrolysate bread; AM7B: bread containing hydrolysate fermented by *Lb. brevis* AM7. The numbers 18 and 22 refer to the addition level 18 or 22% of the above ingredients in the bread, calculated on dough weight (d.w.). Standard deviation values > 0 are reported on the columns.

substrate in which all the LAB starters grew approximately 3 log cycles reaching cell densities in line with a sourdough environment (Gobbetti, 1998). However, the LAB strains showed different production of lactic acid and very low production of acetic acid. Among the possible reasons for the very low formation of acetic acid in the obligate heterofermentative species *Lb. brevis* and *Ln. pseudomesenteroides* could be the absence of external electron acceptors, e.g. fructose being the most typical of sourdough/cereal based systems (Gobbetti et al., 2005). The high osmotic conditions due to the glucose content of the hydrolyzed bread, nearly 8 times higher than that in the LAB cultivation substrate, did not inhibit the LAB metabolic activity. However, the low acidification capacity of *Ln. pseudomesenteroides* in the conditions of this study can indicate a relatively low tolerance to acidic conditions. Generally, LAB have shown large variability in stress tolerance and different adaptive responses, many of them being species and strain specific (Le Marrec, 2011; Zotta et al., 2009). The glucose content of the hydrolysate was not changed at the end of fermentation. This led to hypothesize that the carbohydrases were not completely inactivated during the heat treatment, thus kept releasing small amount of glucose during LAB fermentation process. As consequence, the amount of glucose consumed could not be quantified (data not shown). All the fermented hydrolysates were able to inhibit mold growth to some extent, showing higher inhibitory activity on *Penicillium* spp. and *E. herbariorium* than on *A. niger*, with the bread hydrolysate fermented by *Lb. brevis* AM7 showing the highest growth inhibition. This result aligns with previous findings, when the same LAB starter was used for sourdough fermentation and showed the strongest antifungal effect among several LAB (Coda et al., 2008). Since the content of acetic acid was very low and lactic acid amount was not enough to justify the overall antifungal effect (Lind et al., 2005), the presence of other antifungal compounds was hypothesized. First, the extract was subjected to trypsin digestion to verify the presence of proteins or peptides with potential antifungal properties. The marked decrease of the inhibitory activity of the fermented hydrolysate confirmed this hypothesis. The purification of the bread hydrolysate through sequential steps including ultra-filtration, FP- and nano-LC allowed the identification of a mixture of potentially active peptides. According to the literature (for a review see Rizzello et al., 2016) the antimicrobial activity of the protein hydrolysates is commonly due to the synergistic action of different peptides. Antifungal peptides are generally characterized by a large structural and sequence diversity, although some similar features have been reported, such as the small size and the presence of cationic and hydrophobic residues (Thery et al., 2019; Wang, 2015). Among the peptides identified in the two active purified fractions (Table 5), LTAASITAVCR was the only cationic sequence (net charge of +1, experimental mass of 1161.6 Da and total hydrophobic charge of 63%). The cationic nature of the peptide might facilitate the binding to the negative-charged compounds of the cell wall of the fungi (Laverty et al., 2011, McPhee and Hancock, 2005). All peptides identified have hydrophobic ratio higher than 25%, due to the presence of alanine (Ala, A), valine (Val, V), leucine (Leu, L), isoleucine (Ile, I), proline (Pro, P), phenylalanine (Phe, F), and cysteine (Cys, C). Hydrophobicity allows the binding to and the penetration into the

bilayer of the cell membrane (Thery et al., 2019), thus inducing cellular lysis (Muhialdin et al., 2016). As previously reported for many plant-derived antifungal peptides, all peptides identified in the most active fraction obtained from the fermented bread hydrolysate (E) contained Cys residues (Thery et al., 2019). The peptide QQCCQPLAQISEQAR, with neutral net charge, 40% hydrophobicity and experimental mass of 1815.8 Da, contains two sequential Cys residues. According to the Antimicrobial Peptide Calculator and Predictor-APD3 (Wang et al., 2016) it might form defensin-like β-structure or helical structures containing disulfide-bond. Moreover, this peptide is encrypted in an avenin-like protein which has already been reported, in the native form, as effective against fungi (Zhang et al., 2018). The anionic peptide DVAGGGGAQQCPVETK, having molecular mass of 1572.7 Da and showing 31% of hydrophobicity ratio, is encrypted in Puroindoline-A protein, a component already recognized as part of the barrier defense system of wheat (Nawrot et al., 2014). The activity of the hydrolysis products of these proteins was never reported before, although an increased activity of antimicrobial proteins due to a moderate proteolysis was largely observed before in different food matrices (Rizzello et al., 2016). DCCQQLADINNEWCR and LTAASITAVCR show four total hydrophobic residues on the same surface, hence the peptides may form α-helix. Similar feature was found for peptide EGEGVIVLLR, presenting three hydrophobic residues on the same surface. Linear peptides forming α-helix can interact with membranes, thus expressing their antimicrobial potential (Wang, 2015). Moreover, the formation of α-helix is considered a specific structure of antifungal peptides (Rogozhin et al., 2018). The combined action of protease and fermentation with *Lb. brevis* AM7 allowed the release of bioactive sequences encrypted within the protein originating from the wheat flour, with the bacterium proteolytic activity playing a pivotal role.

The bread hydrolysate fermented by *Lb. brevis* AM7 delayed fungal appearance when added to wheat bread. The effects of bread slurry addition on bread quality were recently investigated (Immonen et al., 2020). Consistently with those results, in our study, with increasing addition of the slurry or bioprocessed bread to the bread dough, the technological properties of the new bread decreased, although not in the same way. The addition of untreated slurry had the highest detrimental impact on bread volume and hardness, showing a decrease of bread volume up to 20% and an almost two-fold hardness value, especially at the highest addition level (22%). These effects were mitigated when bioprocessed bread hydrolysate was added to the bread, leading to a volume decrease of 10% for the hydrolysate addition or of 8–15% when fermented hydrolysate was added. This might be due to several phenomena occurring during wheat bread baking, including starch gelatinization and consequent modification of its functionality, and the denaturation of wheat proteins and consequent loss of structural capacity (Ortolan et al., 2015; Immonen et al., 2020). Since the use of a mere mixture of bread and water as strategy for bread recycling poses obvious challenges, it might be necessary to find solutions to restore at least partly the quality. In this study, the use of 18% d.w. of bread hydrolysate fermented by *Lb. brevis* AM7 delayed fungal growth of 3–4 days compared to control bread, while the addition of 22% d.w. of this fermented hydrolysate extended the shelf life of 1–2 extra days, leading to an overall duration of at least 10 days. This ingredient exerted an antifungal effect during bread baking which clearly increased proportionally to its addition to the bread recipe. Generally, the fermented hydrolysate showed a positive effect on the bread quality if compared with the impact of the addition of bread slurry alone. In this process about 70 g of bread are recycled into 1 kg loaf (7% of d.w.). Considering that on average 5–10% of bakery production goes to waste, this technology allows significant bread recycling potentially performed at the bakery site, leading to environmental and economic advantage. These results represent a proof of concept of a possible reutilization of surplus bread by obtaining an ingredient with antifungal functionality responding to "clean label" requirements. Clearly, optimization of the hydrolysis conditions and/or of the fermentation process could be sought to further enhance the antifungal effect and mitigate the impact on bread quality.

Declaration of competing interest

None.

CRediT authorship contribution statement

L. Nionelli: Investigation, Writing - original draft, Methodology. **Y. Wang:** Investigation. **E. Pontonio:** Investigation, Visualization. **M. Immonen:** Investigation. **C.G. Rizzello:** Writing - original draft, Validation. **H.N. Maina:** Validation, Funding acquisition. **K. Katina:** Conceptualization, Funding acquisition. **R. Coda:** Supervision, Conceptualization, Writing - original draft, Writing - review & editing, Funding acquisition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2020.107437.

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